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Original Article

Frequency of *Mycobacterium avium* subsp. *Paratuberculosis* in milk, meat, ileum, and feces of cattle in Tabriz, Iran

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Summary

Mycobacterium avium subsp. *Paratuberculosis* is the causative agent for Johne's disease, which is a chronic and virulent disease, usually leading to incurable chronic enteritis. The bacterium afflicts domestic and wild ruminants. Milk and feces are important sources for the bacterial presence as well as pathogenic factors for the dissemination of infection. The human beings can be affected by raw and pasteurized milk, meat, and the environment. The aim of this study was to identify and determine the mycobacterium avium paratuberculosis prevalence in milk, feces, ileocecal area, and meat of slaughtered cattle in the Tabriz slaughterhouse. For the present study, 30 dairy cows two years of age or older were randomly selected in the Tabriz slaughterhouse. Milk and fecal sampling, and ileocecal and meat sampling, were conducted in several stages before and after slaughtering, respectively. The DNA was extracted from 120 samples at first, and then PCR was performed using IS900 specific primers. The obtained PCR products were electrophoresed on the agarose gel. Considering the results, the rate of mycobacterium infection was 26.7% in milk, 33.3% in feces, and 6.7% in ileocecum, and the infection rate was negative in meat. With regard to the procured results of the present study, the identification of mycobacterium avium subsp. paratuberculosis using the PCR method is a valuable test in herds. Since the present study showed a high infection rate in the district and considering the probable association between the bacterium and Crohn's disease in humans, the need for further attention deems necessary.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, PCR, Tabriz

Introduction

John's disease or paratuberculosis is a chronic disease that usually leads to incurable chronic enteritis and affects domestic and wild ruminants; hence, it is economically attended. As a chronic granulomatous intestinal disease, John's disease may encompass a wide range of ruminants. The disease is commonly transferred through the mouth and feces, as well as milk and infected placenta (Berghaus et al., 2005, Collins et al., 2005). Given the damages imposed by John's disease on livestock industries and human health, it should be highly considered, and correspondingly,

tremendous efforts should be put into eradicating it. *Mycobacterium avium paratuberculosis* (MAP) is excreted in milk or feces of the infected cattle (with or without clinical symptoms) and thus infects their bedding, feed, water and finally renders the dissemination of infection throughout the herd (Sweeney et al., 1992; Leroy et al., 2008). The MAP is considered a zoonotic microorganism between animals and humans in Crohn's disease and even in idiopathic inflammatory bowel disease (IIBD) and ulcerative colitis (Green et al., 2021). Resistance in the environment, long incubation period, unknown pathogenesis, MAP residues in immune cells, and cross-reactivity among nearby subspecies are the challenges facing the zoonotic concerns of MAP (Adhikari, 2020).

John's disease has a global distribution and is rapidly and increasingly spreading to some countries (Quinn et al., 2002; Radostits et al., 2007). The MAP is able to survive in the environment for one year. It should be reminded that bacterium could be excreted in the milk or transmitted at mating; however, calves become infected during the initial days of their life through eating the germs found in the feces of infected cattle. The clinical symptoms of the disease rarely appear before two years of age. Moreover, not all infected livestock manifest the clinical symptoms, and some of them, as subclinical carriers, constantly excrete the germ in their feces till the end of their life (Pillai and Jayarao, 2002; Alonso et al., 2009; Degado et al., 2011;Fatahi et al., 2011; Okura et al., 2011;). It is estimated that the financial damage inflicted on MAP-infected herds is approximately 1% of the gross milk income, equivalent to 33 USD per year per cow. The MAP damage in the United States is estimated at 198 million USD annually, 75 million USD in Germany, 56 million USD in France, 54 million USD in New Zealand, and between 17 million and 28 million USD in Canada (Richards et al., 2021).

The MAP is an intracellular pathogen, and cell-mediated immune responses are the main causes of gastrointestinal lesions. On the other hand, Crohn's disease is an inflammatory disease that affects the digestive system. The etiology of this disease is not well understood, and various factors such as bacteria and genetics or some factors stimulating the immune system are involved in the emergence of this disease. Polymerase Chain Reaction (PCR) has been clearly recognized as a method for rapid molecular detection. The MAP possesses specific loci termed IS900, which acts as a fingerprint for this pathogen. Moreover, pathogenic mycobacteria are very strict germs and hardly grow outside of the human body and even it takes more than weeks or months for it to grow in the medium; hence, PCR is preferred to other detection methods. The use of PCR and other novel culture techniques has reinforced the association between MAP and Crohn's disease and that the etiology of Crohn's disease is not unrelated to MAP (Quinn et al., 2002; Bernstein et al., 2004; Radostits et al., 2007; Behr and Kapur, 2008). The present study intended to identify the MAP in milk, meat, feces, and ileocecal of cattle in the Tabriz slaughterhouse.

Materials and methods

Sampling

In the present study, 30 cows older than two years were randomly identified in the Tabriz industrial slaughterhouse, and 10 mL milk and 1 g fecal samples before slaughtering and 1 g ileocecal and 1 g neck muscle meat samples after slaughtering were collected from each cow in several stages. In total, 120 were collected. They were transferred (near to ice and in sterile packaging) to the laboratory of the Department of Food Hygiene, College of Veterinary, Islamic Azad University, Tabriz Branch, Tabriz, Iran.

DNA extraction and PCR performance

DNA was extracted from the samples using Tab DNA Kit protocol, and polymerase chain reaction (PCR) was performed according to the IS900 protocol described by Stabel et al. (2002) with some modifications. The standard samples of bacterial DNA were purchased from Razi Vaccine and Serum Research Institute and were used as the control samples. To perform PCR, The sequence of specific primers (Fara Pajouh, Iran) used for detection of MAP (Möbius et al., 2008) were as follows:

IS900 Primer Forward:
5'-CCGCTAATTGAGAGATGCGATTGG-3'
IS900 Primer Reverse:
5'-AATCAACTCCAGCAGCGCGGCCTCG-3'
The products were electrophoresed on 1% agarose gel (Nioton and Graham, 2002).

Statistical analysis

SPSS software version 20 was used for statistical analysis. Chi-square and Fisher's exact test were used to analyze the data.

Results

Among the 30 cattle used for this study, 17 (56.7%) were under five years of age, and 13 (43.3 %) were older than five years. Among 30 milk samples, the presence of MAP in 8 samples (26.7%) was detected by PCR, whereas it was not detectable in 22 samples (73.3%) (Table 1). In cows younger than 5 years, MAP was not isolated from the milk samples of 15 cows (68.2 %), and in those older than 5 years, MAP was not isolated from 7 (31.8 %) milk samples. In other words, in cattle younger than 5 years, MAP was isolated from 2 (25 %) milk samples, and in milk samples of older-than-five-year cattle, MAP was isolated from 6 (75 %) samples.

The results of Fisher's exact test indicated a significant difference in the number of MAP isolated from milk samples of cattle based on their age. It means the number of MAP isolated from the cattle older than 5 years was more than their number in another group of age ($\chi^2 = 4.45$, $p < 0.05$).

Among 30 fecal samples, the presence of MAP in 10 samples (33.3 %) was detected by PCR, wherease it was not detectable in 20 samples (66.7 %) (Table 2). It could be discerned that in samples of cattle younger than 5 years, MAP was not isolated from the fecal samples of 12 (60%) cattle, and in cattle older than 5 years, MAP was not isolated from 8 (40%) fecal samples. In other words, in cattle younger than 5 years, MAP was isolated from 5 (25 %) fecal samples by PCR, and in fecal samples of cattle older-than-five-year, MAP was isolated from 5 (50%) samples. The results of Fisher's exact test indicated that there is no significant difference in the number of MAP isolated from fecal samples of cattle based on their age ($\chi^2 = 0.271$, $p > 0.05$).

Among 30 ileocecal samples, the presence of MAP in 2 samples (6.7 %) was detected, and 28 samples (93.3 %) were free from the MAP. In the samples of cattle younger than 5 years, MAP was not isolated from the ileocecal samples of 17 (60.7 %) cattle and in cattle older than 5 years of age, MAP was not isolated from 11 (39.3 %) ileocecal samples. Moreover, in cattle older than 5 years, MAP was isolated from 2 (100 %) ileocecal samples by PCR. The results of Fisher's exact test showed that there is no significant difference in the number of MAP isolated from ileocecal samples of cattle based on their age ($\chi^2 = 2.802$, $p > 0.05$).

In the case of meat samples, the presence of MAP in all 30 samples (100%) was not detectable. In the samples of cattle younger than 5 years, MAP was not isolated from the meat samples of 17 (56.7 %) cattle and in cattle older than 5 years, MAP was not isolated from 13 (43.3 %) meat samples.

Table 1. Comparison of the number of MAP isolated by PCR from milk samples of cattle (grouped by age)

PCR of milk samples		**Age**		**Total**	χ^2	***P-value***
		< 5 years	**> 5 years**			
Negative	**Frequency**	15	7	22	4.45	0.049
	%	68.2	31.8	100		
Positive	**Frequency**	2	6	8		
	%	25	75	100		
Total	**Frequency**	17	13	30		
	%	56.7	43.3	100		

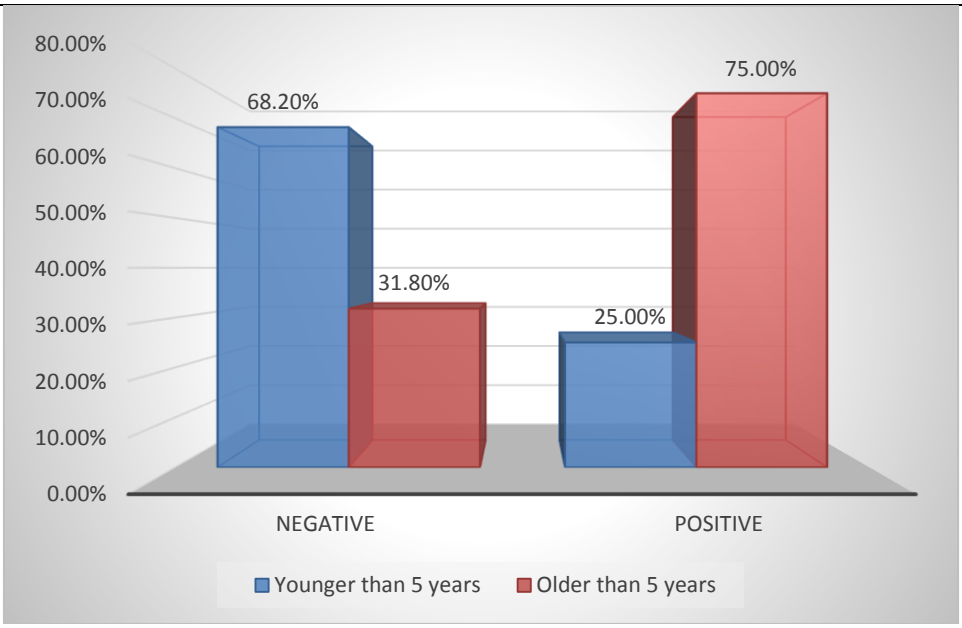

Fig 1. Comparison of the number of MAP isolated by PCR from milk samples of cattle (grouped by age).

Table 2. Comparison of the number of MAP isolated by PCR from fecal samples of cattle grouped by age

PCR of fecal samples		Age		Total	χ^2	*p-value*
		< 5 years	> 5 years			
Negative	**Frequency**	12	8	20	0.271	0.705
	%	60	40	100		
Positive	**Frequency**	5	5	10		
	%	50	50	100		
Total	**Frequency**	17	13	30		
	%	56.7	43.3	100		

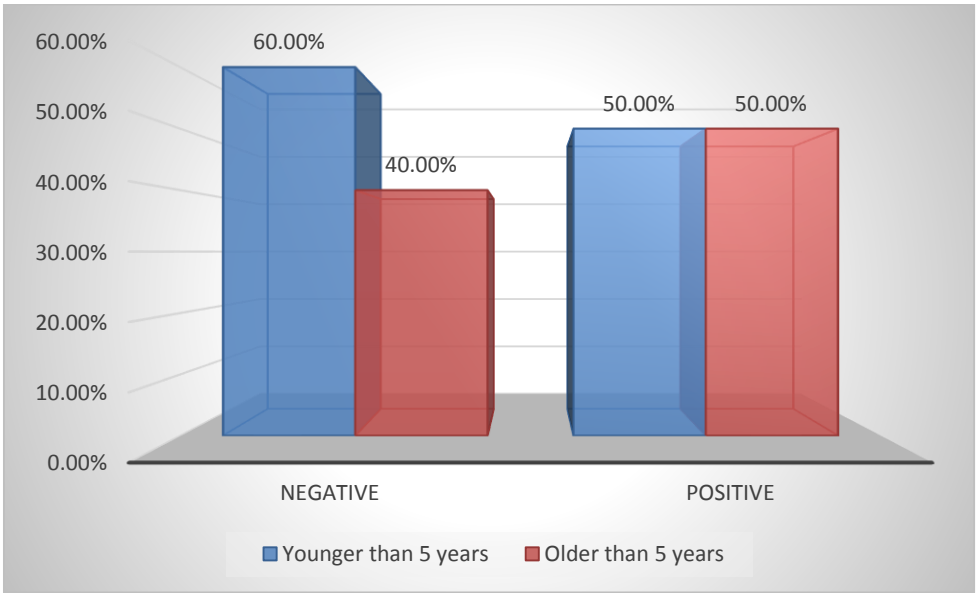

Fig. 2. Comparison of the number of MAP isolated by PCR from fecal samples of cattle (grouped by age).

Table 3. Comparison of the number of MAP isolated by PCR from ileocecal samples of cattle (grouped by age).

PCR of ileocecal samples		Age		Total	χ^2	*p-value*
		< 5 years	>5 years			
Negative	**Frequency**	17	11	28	2.802	0.179
	%	60.7	39.3	100		
Positive	**Frequency**	0	2	2		
	%	0	100	100		
Total	**Frequency**	17	13	30		
	%	56.7	43.3	100		

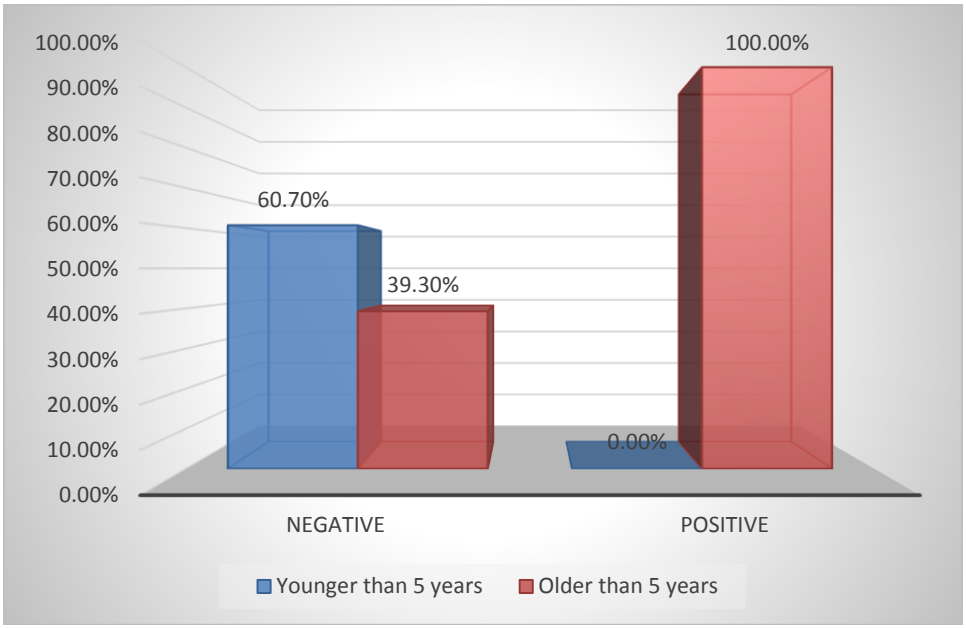

Fig. 3. Comparison of the number of MAP isolated by PCR from ileocecal samples of cattle (grouped by age).

Table 4. Comparison of the number of MAP isolated by PCR from meat samples of cattle (grouped by age).

PCR of ileocecal samples		Age		Total	χ^2	*p-value*
		< 5 years	>5 years			
Negative	**Frequency**	17	13	30	-	-
	%	56.7	43.3	100		
Positive	**Frequency**	0	0	0		
	%	0	0	0		
Total	**Frequency**	17	13	30		
	%	56.7	43.3	100		

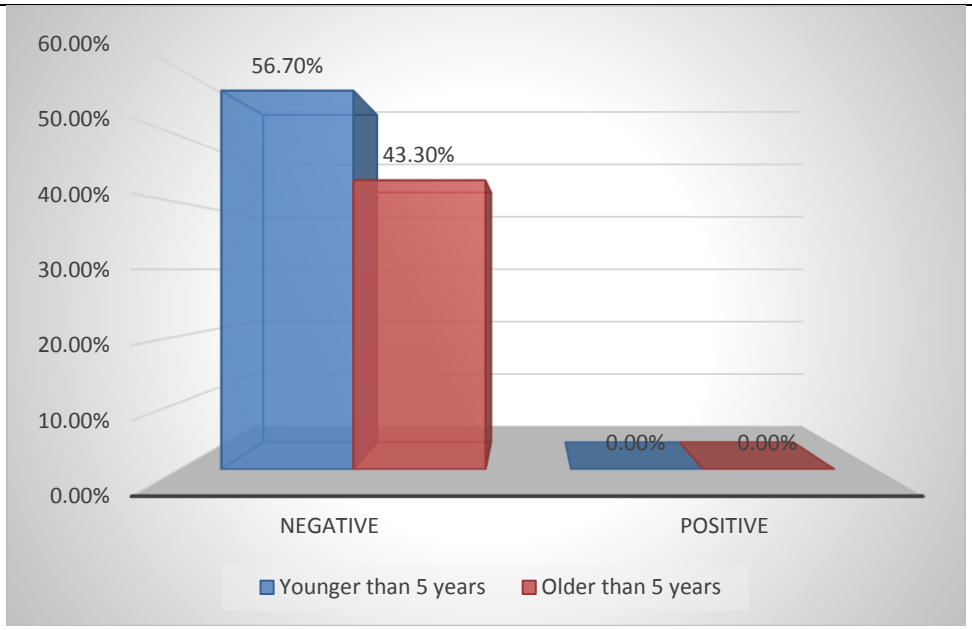

Fig. 4. Comparison of the number of MAP isolated by PCR from meat samples of cattle (grouped by age).

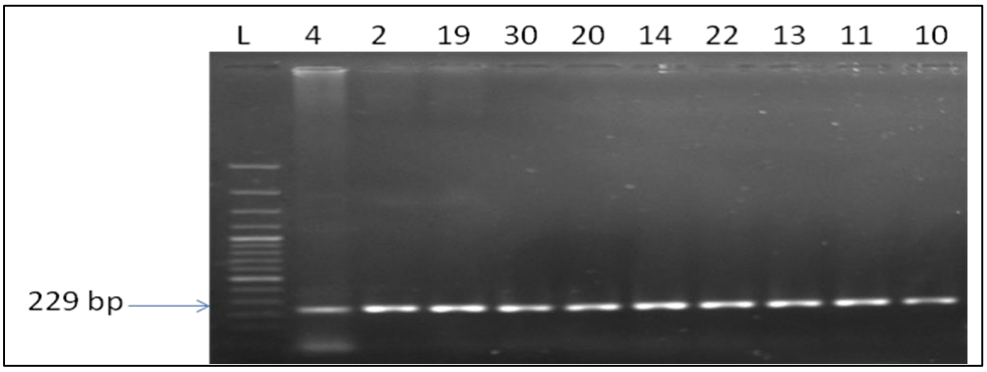

Fig. 5. Gel electrophoresis of IS900 PCR product from fecal, milk, and ileocecal samples.

Discussion

The spread of John's disease in most countries of the world has increased as a result of the export of infected pure-bred cattle; as research in the Wisconsin state of the USA has shown that around 34% of dairy cows in this state are infected with MAP (Haghkhah et al., 2008). The diagnosis of the disease is considered very significant as most infected cattle do not reveal the obvious clinical symptoms of disease and are only emaciated and skinny. Yet, there are ruminants that are seemingly thin and scrawny and, in their necropsy, the intestine is thick and folded, but they are not infected with MAP. Therefore, the clinical symptoms cannot lonely be considered reliable (Quinn et al., 2002; Radostits et al., 2007). Several years have passed from the time that the presence of John's disease has been approved in Iran, and it is now imposing irreparable damages to our industrial livestock operations and animal production, regardless of the fact that many studies have been conducted around this disease (Garriodo et al., 2000).

In this study, the amount of PCR product was 229 bp in all positive samples. The humans could be infected with MAP by the consumption of raw and pasteurized milk, meat as well as the environment

(Grant et al., 2002; Donaghy et al., 2008); however, our study did not corroborate infection with the meat. It may be because of MAP detection technique, sampling methods, error in the experiment, or other factors. In general, the possibility of the presence of MAP is very low in meat, as MAP is an intracellular pathogen and is colonized within lymph nodes, and whenever it finds appropriate circumstances, it comes into action. Such suitable conditions could be found in milk rather than in meat (Radostits et al., 2007).

Despite numerous studies in Iran, the accurate rate of disease prevalence is not clear. A similar study by Haghkhah et al. (2008) conducted in Shiraz province of Iran corroborated our results as of 110 dairy cows studied, 23% represented disease presence. In the study of Anzabi et al. (2005), 25 out of 80 cattle that were susceptible to John's disease were infected with MAP as detected by PCR.

Our study also showed that the rate of infection was increased in older cows. Our study was not consistent with the research conducted in Tabriz city of Iran in which the ileocecal valve was asserted as a suitable organ for search on the presence of MAP (Anzadi et al., 2005). Our study did not certify the ileocecal valve as a suitable organ for detecting the MAP; the reason may be relevant to the lower ability of the DNA IS900 detection method in comparison with ISH and Direct *In Situ* PCR method in tissue (Delgado et al., 2011) or that the disease is not yet recognized as a chronic disease. Sampling from the herds, which are not infected with MAP is one of the factors can cause underestimating the prevalence of disease in the herds, while it is possible that the disease is more prevalent (Haghkhah et al., 2008).

A study in herds of MAP-infected sheep showed that serum ELISA alone and in combination with fecal PCR is the best reference for individual identification of infected sheep. qPCR IS900 in milk has been identified as the most sensitive and specific diagnostic test for monitoring MAP and animals with specific clinical signs. Milk qPCR and ELISA analysis also showed that MAP positivity in sheep has a seasonal pattern (Hosseiniporgham et al., 2020). In a study by Nebbia (2003) in Italy, sheep were serologically tested at first. Then milk samples of serologically positive and negative sheep were examined by PCR. The results showed that 9 out of 17 seropositive and 4 out of 14 seronegative sheep showed the presence of MAP in their milk samples. An Indian study on water buffalos showed that out of 20 cases with clinical symptoms of disease, 14 cases were reported by PCR as infected, and only 6 cases showed positive culture results (Sivakumar, 2004). In another research in India, the sensitivity of different methods to detect of John's disease was comparatively investigated. Of the investigated detection methods of John's test, tissue culture, fecal culture, tissue PCR, and ELISA, the tissue PCR method has the second most convenient sensitivity after tissue culture. In another investigation in India in 2007, three methods of milk ELISA, milk culture, and milk PCR were used and conducted on local cattle. Of these methods, milk PCR represented the best result. In that study, the milk residue and skim milk were used as sources of bacteria for DNA extraction. The use of milk residue (with 62% positive result) was comparatively preferred to the use of skim milk with a 50% positive result (Sharma, 2007).

The rate of infection differs in different regions of Iran as well as in other countries, as recent studies have shown that around 49% of dairy cows in American herds were infected with MAP (Pillai, 2002). Another study in the UK in the 1980s represented the rate of infection of pasteurized milk by PCR around 30% (Millar, 1996). The development of molecular detection techniques has increasingly emphasized the importance and value of PCR technique in detecting diseases. This statement has even been certified for PCR in detecting John's disease, as in several studies, the use of PCR has detected more positive results in samples in comparison with other techniques, and the high sensitivity and good features of this technique have also been proved in detecting John's disease. As an example, in a study in 1997, in testing the milk samples from 72 dairy cows, 60% represented positive results by PCR

technique, while these samples showed only 30% positive result through bacterial culture (Stevenson, 1997). Singh et al. (2007) investigated the sensitivity of milk-ELISA, fecal IS900 PCR, fecal culture, and milk culture in sheep and goats and they indicated that the sensitivity of fecal culture, milk culture, milk-ELISA, and fecal PCR were 84.6%, 96.1%, 88.4%, and 23%, respectively. In another study, Wells et al. (2006) assessed the prevalence of MAP by fecal PCR and milk-ELISA in 1808 samples obtained from dairy cows. The samples represented 23% and 25.7% positive results in fecal PCR and milk-ELISA, respectively. It is worth emphasizing that the volume of samples is also of importance, as the volume of samples in this study was more than that in other works comparatively. Furthermore, the presence of anti-metabolites such as calcium ions in milk could also inhibit the PCR reaction (Millar et al., 1996; Englund et al., 1999; Singh et al., 2007).

In the current study, we applied the IS900 sequence of MAP genome for the molecular detection of the bacterium. This sequence has been used as an efficient sequence for primer design in different investigations. Moreover, in some studies, other sequences of the genome of this bacterium, particularly IS901, have been utilized; however, the IS900 sequence is yet proposed as a standard method (Bhide et al., 2005; Tripathi et al., 2005; Sharma et al., 2007).

Despite the fact that John's disease renders great commercial losses, such as reduction in milk and meat production, mortalities and losses, deletion of adult cattle, and giving birth to calves besides health problems, there is no definite recognized method for detection of John's disease in Iran. It is expected that the use of novel molecular methods and broad studies in the future will provide us with definite identification and correspondingly eradication of this disease. Therefore, considering that MAP controlling programs in Iran are arbitrary and the disease is considered a serious problem in dairy herds, the development of advanced controlling programs deems necessary (Haghkhah et al., 200). Hence, in comparison with previous studies performed by serologic tests and culture methods, detection of MAP with PCR is considered as a valuable test in MAP detection in herds.

The results of the present study represented the high rate of infection in this region, and regarding the probable association between this bacterium and Crohn's disease in humans, more attention to it seems required. This bacterium is sensitive to thermal treatments. The intracellular property of the bacterium also causes further survival of it, particularly in the milk pasteurization process; hence, it should be greatly considered by researchers working in public health programs (Tabatabayi and Firouzi, 2001; Anzabi, 2005). A study was conducted to examine the attitudes of dairy producers and veterinarians about Johne's disease. Both groups cited physical resources such as time, money, infrastructure, and the dairy producer's priority and the practicality of Johne's disease control recommendations as key barriers to successful disease control. Both groups offered external incentives such as insurance, penalties, and regulations, and internal incentives such as liability to motivate producers (Beaver et al., 2017).

Conclusion

Different manifestations and specific signs of John's disease, particularly the long incubation period and insufficient awareness about the risks of its subclinical forms, have rendered the dairy farmers pay less attention to the importance of Johne's disease. Hence, to control the disease, it would be of great assistance if dairy farmers become more knowledgeable on the disease, besides holding detection training programs for leaders of veterinary diagnostic laboratories to get them acquainted with novel detection methods.

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Ethics approval

Not applicable.

Conflict of interest statement

The authors declare no conflicts of interest.

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