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Investigating the Prevalence of *Mycobacterium avium Subspecies Paratuberculosis* (MAP) in Industrial Dairy herds using Ziehl-Neelsen Staining, Culture, and PCR in Mashhad, Iran

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ABSTRACT

Mycobacterium avium subspecies paratuberculosis (MAP) is the causative agent of Johne's disease in wild and domestic ruminants. Clinically, infected cattle show emaciation symptoms, diarrhea, and death. Asymptomatic subclinical cases can intermittently shed MAP through feces and milk and infect other cattle animals, thus increasing the risk of infection. The purpose of the present study was to detect the prevalence of this disease in dairy cattle. For this purpose, 348 samples were randomly collected from 15 cattle farms. All fecal samples were subjected to ZN staining and PCR of nucleotide sequences related to specific MAP gene fragments (IS900, F57) and were cultured in Harold's egg yolk culture medium after being disinfected with solution (0.0% HPC75). PCR testing of 116 fecal samples (33.3%, CI:95% 28.3-38.2), ZN staining of 23 samples (6.6%, CI: 95% 4-9.2), and fecal sample culture of only 15 samples (4.3%, CI:95% 2.3-6.3,) were infected with MAP. Comparison of test results showed poor agreement (kappa statistic: 0.19) between ZN staining and PCR results and poor agreement (kappa statistic: 0.13) between PCR test and fecal culture. This study highlights the advantages of PCR for detecting MAP in subclinically infected cattle compared to ZN staining and fecal culture. Therefore, it can be used for early detection and control of MAP in cattle and at-risk populations.

Keywords

Johne's disease, Mycobacterium avium subspecies paratuberculosis, Ziehl-Neelsen, Iran

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Abbreviations

MAP: Mycobacterium avium subsp. paratuberculosis
ZN: Ziehl-Neelsen staining
JD: Johne's disease
CI: Confidence interval

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Introduction

Johne's disease (JD) is granulomatous enteritis that affects a wide range of domestic and wild animals worldwide [1,2]. In 1895, this disease was first detected in the German state of Oldenburg [3]. Infected animals include cattle, sheep, goats, deer, camels, and wild ruminants [4]. Ingestion of feces, milk, and colostrum contaminated with MAP is the predominant mechanism of MAP transmission in dairy cattle [2]. The pathogenesis of MAP infection has recently been further studied, showing that after MAP enters the small intestine, it invades subepithelial macrophages, interferes with the formation of phagolysosomes, and remains in the phagosome, triggering humoral immune responses. Finally, the disease course changes from the preclinical stages to the period of increased bacterial shedding and the development of obvious clinical symptoms [3]. The infection's livestock and herd performance consequences have been widely studied. Most studies have shown that cows with a positive MAP contamination test have a 2-17% reduction in milk production compared to cattle with a negative test result. Reduced fertility and adverse effects on the reproduction of infected animals can also be another important complication of infected animals [5]. MAP also weakens the livestock's immune system, predisposing animals to develop clinical mastitis or other infectious diseases [6]. One of the problems in controlling MAP infection is that some infected cows may not show clinical symptoms if they are producing enough milk because they frequently shed MAP in the feces before showing symptoms, indirectly contributing to the spread of infection [7]. Due to the long incubation period of JD, clinical symptoms such as resistant watery diarrhea, weight loss, and reduced milk production are usually observed in cows over 2 years of age [8]. Many theories have been proposed regarding the potential of MAP to be involved in human Crohn's disease and infections in non-ruminant animals such as dogs and rabbits [9]. Humans become infected by consuming contaminated milk or dairy products because MAP bacilli survive in pasteurized milk [10]. Usually, no effective measures are taken to control and eradicate this disease, which imposes significant economic losses to the animal husbandry industry and the production of dairy and meat products worldwide [11, 12]. Since there is no cure for JD, herd health control strategies are based on detecting infected hosts and their subsequent elimination to prevent transmission of infection to healthy cattle, which imposes a financial burden on herd management [6, 13]. Due to the slow growth of MAP and the lack of sensitive tests, it is very difficult to detect subclinically infected cattle and diagnose the disease's early stages [10, 14]. It is often impossible to distinguish between infected animals with only one diagnostic method due to their false positive and negative responses [15]. Fecal shedding of MAP organisms can be intermittent. Contamination and low fecal content of microorganisms can reduce culture results by 50% [16]. Therefore, although the culture of samples is the "gold standard," it cannot be used as a single test to accurately detect all infected animals in some herds [17]. Molecular methods based on bacterial DNA and detection of specific sequences in the genome allow hard-to-grow bacteria such as MAP to be detected in samples quickly [15]. MAP-infected animals can be detected directly through fecal PCR and detection of the IS900 or F57 fragment [18]. Comparing the results obtained in similar studies show that the relationship between different diagnostic tests and their combination can improve MAP diagnosis at the herd level [15]. Therefore, it seems that the presence of specific and sensitive diagnostic tools and a better understanding of the prevalence of JD in different parts of the world is necessary to develop control and eradication programs disease [14]. The present study aimed to estimate the amount of MAP contamination in cattle farms of the region using three laboratory detection methods. Comparing the results of these three methods can help to choose the best MAP diagnostic method to obtain accurate results in epidemiological studies and disease control in dairy cattle.

Results

This study tested 348 cow fecal samples for MAP contamination using different techniques. According to PCR, this disease has a prevalence rate of 33.3% (95% CI: 28.2-38.2), a culture prevalence rate of 4.3% (95% CI: 2.3-6.3), and a ZN staining prevalence rate of 6.6% (95% CI: 4-9.2). Three different prevalence rates of MAP infection were detected using three other diagnostic methods. The degree of agreement between the tests results was measured using the Kappa statistic. After analyzing the results of these three tests, it can be concluded that there is a poor agreement (kappa statistic: 0.19) between the results of ZN staining and PCR, as well as a poor agreement (kappa statistic: 0.13) between the results of the PCR test and fecal culture. First, the appearance of the culture medium was investigated by examining colony growth, followed by direct microscopic observation to obtain these results. Figure 1 shows the positive electrophoresis results of PCR products of IS900 and F57 MAP genes in 413-bp and 704-bp samples, respectively. Cows were diagnosed positive in 100% of cases of herds 1, 2, 4, 95% of cases of herd 11, 68% of cases of herd 7, and 10% of the cases of the herd 9. All tests were negative in the other 9 herds.

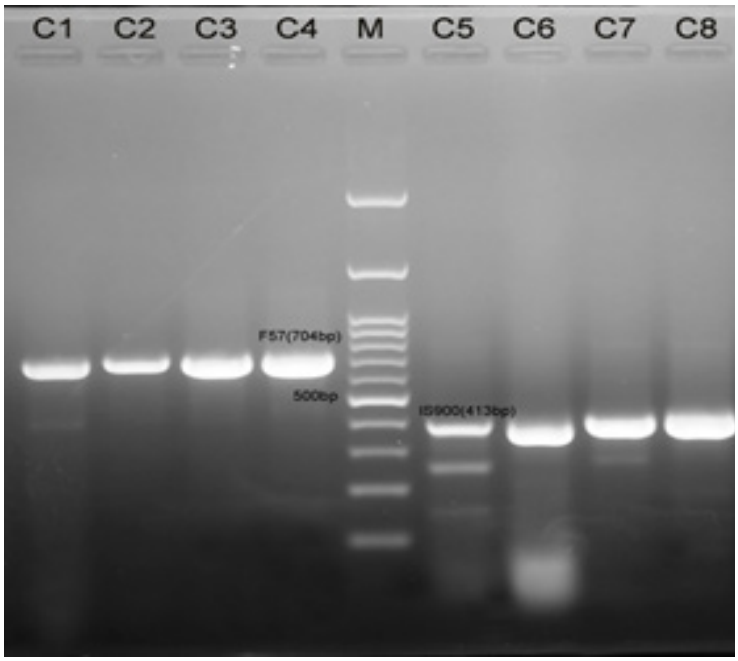

Fig 1.
Confirmation of positive culture results using PCR F57 and IS900 (C1: culture positive F57 PCR; C2 and C3: feces samples positive F57 PCR; C4: positive control (F57); M: 100-bp DNA ladder as the molecular size marker; C5: positive control (IS900); C6 and C7: feces samples positive IS900 PCR; C8: culture positive IS900 PCR).

Table 1 shows the total number of positive animals for each herd in the three tests (based on the number of samples per herd).

Table 1.
The number and proportion of positive PCR results on the feces and feces culture in 15 herds are based on the number of samples taken in each herd (Herd-Level prevalence and total).

Herd (number of samples)	Ziehl-Neelsen stain	feces PCR		feces culture	feces culture PCR
		IS900	F5		
1(41)	15(36.6%)	41(100%)	16(39.02%)	11(26.8%)	11(26.8%)
2(21)	4(19.05)	21(100%)	(0%)	2(9.5%)	2(9.5%)
3(23)	(0%)	(0%)	(0%)	(0%)	(0%)
4(15)	1(6.6%)	15(100%)	2(13.3%)	(0%)	(0%)
5(30)	(0%)	(0%)	(0%)	(0%)	(0%)
6(22)	(0%)	(0%)	(0%)	(0%)	(0%)
7(67)	(0%)	17(25.4%)	(0%)	(0%)	(0%)
8(20)	3(15%)	(0%)	(0%)	2(10%)	2(10%)
9(28)	(0%)	(0%)	(0%)	(0%)	(0%)
10(17)	(0%)	(0%)	3(17.6%)	(0%)	(0%)
11(20)	(0%)	19(95%)	10(50%)	(0%)	(0%)
12(17)	(0%)	(0%)	(0%)	(0%)	(0%)
13(7)	(0%)	(0%)	(0%)	(0%)	(0%)
14(10)	(0%)	(0%)	(0%)	(0%)	(0%)
15(10)	(0%)	(0%)	(0%)	(0%)	(0%)
Total 348	**23(6.6%)**	**113**	**31**	**15(4.3%)**	**15(4.3%)**
		116(33.3%)			

Discussion

This study was carried out using random sampling of cows and the method of direct microscopic observation, culture and molecular detection to understand the contamination status of dairy cattle better. A total of 348 samples were tested, and 136 samples (39.08%) of those tested were positive for at least one of the three tests. Based on the results, only 23 (6.6%) of 116 (33.3%) cows with positive fecal PCR results for one or both genes (IS900, F57) had positive ZN staining. Mahmoudi et al. (2018) sampled 150 fecal samples from apparently healthy dairy cows from industrial cattle farms in Hamadan City and investigated them for the presence of MAP using ZN staining and molecular methods. The positive results for ZN staining and Nested PCR method were 5.33% and 30.23%, respectively [19]. In another study on 200 samples of rectal mucus and serum of cows slaughtered in the Ahvaz slaughterhouse, Zarei et al. (2016) investigated the prevalence of MAP using three staining methods: ZN, molecular, and ELISA. The results showed that the prevalence of MAP in PCR, ZN staining, and ELISA was 13.5%, 4%, and 3%, respectively [20]. The results of the ZN staining test in the two above studies are consistent with the present research results. MAP can be directly observed in samples using (ZN) staining, which is based on the resistance of mycobacteria to acid decolorization by acid alcohol after fuchsin staining. The results are qualitative. However, this method is fast, simple, and inexpensive, with low sensitivity and specificity in milk, colostrum, and fecal samples [21]. The possibility of false negative results increases with the observation of clinical symptoms such as diarrhea in cattle because the MAP number of the samples is low in the lower fecal concentration. Besides, subclinically infected animals also shed large amounts of bacteria in their feces. Due to the possibility of obtaining false positive results from other environmental mycobacteria, this method has low sensitivity and specificity. Herd contamination can be estimated and detected using direct microscopic observation [22]. Fecal culture, the most accurate test for confirming paratuberculosis, is another test in this unique study. The results showed that only 15 (4.3%) out of 348 cultured samples were positive. A study was conducted by Seyedeyin et al. (2010) on the feces of Holstein-Friesian cows with and without clinical symptoms to determine the prevalence of JD in Razavi Khorasan. In this study, 16 and 103 fecal samples were taken from sick and healthy animals, and two different methods analyzed the results. Culture results were positive in 11.7% of asymptomatic samples and 81.3% of clinical samples [21]. In a study by Sultani (2018) in Kerman, the contamination rate was 15.1% in 212 animal fecal samples without clinical symptoms [22]. A low positive culture test rate may be due to several reasons, including:

The need for highly selective culture media, chemical decontamination of the sample (which kills some microbes and reduces cell viability), and long culture durations are just some of the limitations of using culture as a routine diagnostic method [24]. Also, the culture depends on the number of bacteria in the sample. When there are fewer bacteria in the inoculum, culture is more likely to become negative [13]. When investigating the culture results, the optimal contamination rate should be considered. As mentioned, many cells are destroyed during disinfection, or fecal shedding may occur intermittently or rarely. Finally, the only method to detect live MAP is the only culture, although PCR and bacterial DNA can detect it more effectively [15]. Since the sensitivity of MAP bacterial culture is low and the level of MAP shedding in colostrum, milk, and feces in the subclinical phase is below the minimum detection limit, negative results should be interpreted cautiously. Bacterial culture is mainly used to detect live MAP and is considered a diagnostic reference [15, 19, 25]. Based on host infection response, the fecal PCR test had the highest positive results in this study (N=116 cases, 33.3). Of these, 8.91% and 32.47% occurred in F57 and IS900 genes, respectively. IS900 gene was positive in 85 samples, both genes were positive in 28 samples, and the F57 gene was positive in 3 samples. Due to the resistance of the mycobacterial cell wall to lysis and high concentrations of inhibitors in fecal content, PCR-based MAP detection is limited to small numbers of fecal MAP bacilli and has poor DNA recovery [26]. The 14-20 copy of the IS900 sequence in the MAP genome is a suitable target for MAP detection. On the other hand, due to the detection of this sequence in other mycobacteria, its uniqueness for MAP detection has been questioned. Since F57 has fewer copies than alternative genes such as IS900, it can be specifically used as a target gene in PCR to detect MAP in feces, which may be sensitive. The IS900 gene is still a good candidate for PCR, but caution should be exercised when interpreting the results of a diagnostic test based on IS900 PCR alone. Based on IS900 analysis of fecal and milk samples from several regions, the prevalence of this disease is reported as follows:

In a study by Nasiri et al. (2012) in the suburbs of Mashhad, the IS900 gene of MAP using the PCR method was detected in 243 fecal samples (44%) and 56 suspicious raw milk samples (18%) [27]. In another study based on the IS900 Nested PCR method, Seyedeyn et al. (2010) reported the prevalence of MAP in the fecal samples of clinical and subclinical animals as

87.5% and 9.7% in Razavi Khorasan, respectively [21]. In addition, Haghkhah et al. (2008) reported that the prevalence of MAP in the bulk milk of dairy cows in Fars province was 0.11% [28]. The accuracy and sensitivity of this test are excellent due to the similarity and results between the above studies and the present study. However, there are advantages and disadvantages to molecular diagnosis of MAP, including the following:

In situations where bacterial isolation and mass culture are impossible due to long incubation periods and other culture requirements, useful genetic markers F57 and IS900 may be used in vitro [29]. In particular, the F57 sequence seems to be a highly discriminating biological factor that has so far only been detected in MAP. Therefore, detecting this specific MAP gene sequence provides a new technique for finding this organism [30]. As a result, the PCR-based detection of MAP F57 in the sample indicates it is infectious.

Eight of the fifteen herds sampled had no detectable MAP by any of the methods used in the present study. Suppose the infected animals are in the non-infectious phase and the shedding phase has not yet started. In that case, the negative test results in some cattle do not necessarily mean that the animals in the herd are not infected. Although clinical symptoms in any herd are essential as a screening symptom for JD, accurate and sensitive tests are needed both to detect asymptomatic subclinical animals and monitor the health and welfare of the herd. One of the challenges facing this process is the limited sensitivity of the available diagnostic tests to detect the subclinical stages of MAP infection.

Based on the results of the present study, direct fecal PCR testing yielded better results than other tests for rapid MAP diagnosis. Considering the lack of appropriate conclusions in ZN staining tests and fecal culture results in epidemiological studies, the simultaneous use of PCR tests is necessary for achieving accurate and reliable results. Knowledge of the disease prevalence in the target population is the first step for its diagnosis and prevention. The present study was carried out as an epidemiological component of the JD in the suburbs of Mashhad. Eliminating cattle diseases, especially common zoonotic diseases, is one of the ideals and necessities of this industry. The studied area is one of the country's strategic milk and meat production economies.

Further studies on the epidemiology of MAP in herds using advanced laboratory diagnostic techniques are needed to address this major issue. To achieve this important goal, combining the results of this study with other results of previous studies can be a great help. Using current common diagnostic methods and minimal human error, attempts were made to report the exact prevalence rate of the disease in the studied region.

Materials & Methods

Geographic Location

This study was conducted on several industrial cattle farms in the suburbs of Mashhad. The city of Mashhad is located in northeast Iran with a longitude of 59 °and 35' and a latitude of 36 ° and 20'.

Herd selection

Based on geographical distribution and herd management cooperation, 15 herds were selected for this study. The number of samples in each herd was calculated proportionally to the total sample volume and the herd size.
Size formula: n = P * N, where, P = proportion, N = total sample size

Sampling

A total of 348 cows were randomly selected from the study population, and fecal samples were taken from their rectums. Also, the following formula was used to obtain the required sample size. n=Z2×P-exp (1-Pexp)/d2
Z = standard deviation for the given confidence interval for 95% CI, if α = 0.05, Z α=1.96, p = expected proportion = 15% [21, 27, 31] and d = Margin of error (accuracy) = 5
All fecal samples were collected in closed containers and immediately sent to the laboratory. All fecal samples taken by the ZN method were first stained and examined under a direct microscope.

Bacteriological culture

After decontamination, 23 samples that were positive in terms of ZN staining were cultured individually in the culture medium. Other fecal samples (n=325) free of acid bacillus masses were cultured using the strategic pooling method. In the laboratory, fecal samples from 8 to 12 cows per pool were mixed with 2 g of feces from each cow [23, 32]. All fecal samples were disinfected according to the instructions. Three grams of pooled fecal sample was decontaminated with 0.75% (W/V) hexadecyl pyridinium chloride solution (0.75% HPC) for 24 hours, according to standard methods. This suspension was mixed manually by shaking and swirling and stood upright for 5 minutes at room temperature to allow the settling of large particles. Approximately 20 mL of the upper portion of the supernatant was transferred to another sterile 50 mL tube, where the entire suspension was stirred at 200 units/min for 30 min. The tubes were placed vertically in the dark for 24 hours at room temperature. Collected sterile fecal samples were centrifuged at 900 × g for 30 min, the supernatant was discarded and inoculated onto Herrold's egg yolk agar(HEYM) medium containing mycobactin J with 300 µl of the sterile pellet. The slants were incubated at 37°C for up to 20 weeks and investigated at 1-2 week intervals.

Polymerase Chain Reaction (PCR)

Genomic tests were performed using PCR detection methods, and detection of specific MAP IS900 and F57 markers. Only single fecal samples were used for PCR. DNA extraction was performed according to the method proposed by Soolingen et al. [33], and its purity was measured between 1.8-2 using a NanoDrop spectrophotometer. Primers, IS900f (5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'), IS900r (5'GGCGTTGAGGTCGATCGCCCACGTGAC-3') and F57f (5'-ACC GAA TGT TGTTGT CAC CG-3'), F57r (5'-GGA CAC CGA AGC ACA CTC TC-3') derived from IS900 and F57 sequences inserted in the DNA of MAP leads to the production

Table 2.
Sequence and product length of MAP primers used in the study

PCR primers	Nucleotide sequence	Product length(bp)	References
IS900f IS900r	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3' 5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3'	413	[27, 34]
F57f F57r	5'-ACC GAA TGT TGTTGT CAC CG-3' 5'-GGA CAC CGA AGC ACA CTC TC-3'	704	[29, 35]

Table 3.
Reaction conditions used in the study

Target	Thermal reaction condition
IS900	95°C for 5 min; 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; and 72°C for 5 min
F57	95°C for 5 min; 40 cycles of 95°C for 30 sec, 61°C for 30 sec, 72°C for 45 sec; and 72°C for 10 min

of 413bp and 704bp, respectively. The sequence of IS900 and F57 primers, as well as the size of their predicted PCR products, are summarized in Table 2. PCR was performed in a final volume of 20 µl for IS900. It contained a 5 µl sample of DNA mixed with 15 µl of master mix (AMPLIQON or SINACLON). Tubes were placed in a thermocycler (BIO-RAD; MJ Mini Gradient Thermal Cycler, USA), and the amplification was as follows:
One cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and final extension at 72 °C for 5 min. Secondary PCR was performed in a final volume of 12 µL for F57. It contained a 3 µl DNA sample and a 9 µl master mix. Tubes were then placed in a thermocycler, and amplification was as follows:
One cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 45 s and final extension at 72 °C for 10 min. The PCR conditions are summarized in Table 3.

Statistical analysis

Data were coded and entered into statistical software for each sampling from each cattle. All statistical analyzes were performed using SPSS ver. 22. Agreement between test combination (ZN staining-PCR tests and PCR tests - culture) were compared by kappa index test [36] (≤0.2, poor; 0.21–0.40, fair; 0.41–0.60,moderate; 0.61–0.80, good agreement; and >0.80, very good agreement).

Authors' Contributions

M.H. and G.R.M. conceived and planned the experiments. T.G.M. carried out the experiments. T.G.M. and M.H. contributed to sample preparation. T.G.M., M.H. and G.R.M. contributed to the interpretation of the results. M.H. took the lead in writing the manuscript. All authors provided critical feedback for analysis and manuscript.

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Competing Interests

The authors declare that they have no conflict of interest according to the work presented in this report.

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