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Serological and molecular diagnosis of paratuberculosis in dairy cattle

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Abstract: In this study, the presence of paratuberculosis was investigated by serological and molecular methods in herds of dairy cattle. Blood, milk, and stool samples of 147 cows aged 2 years old or older with chronic diarrhea were collected. A California mastitis test (CMT) was performed on milk samples. Indirect paratuberculosis enzyme-linked immunosorbent assay (ELISA) test was used for serological investigation. Polymerase chain reaction (PCR) was utilized for molecular identification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from milk and stool samples. Immunomagnetic separation (IMS) method was used for milk and fecal samples. Eighteen (12.24%), 44 (29.93%), 36 (24.49%), 28 (19.05%), and 21 (14.29%) of 147 milk samples were negative, suspicious, CMT (+), CMT (++), and CMT (+++) by CMT, respectively. According to ELISA results, 18 (12.24%) serum samples were positive. In PCR of milk and stool samples, MAP DNA was detected in 20 (13.61%) and 42 (28.57%) of samples, respectively. In IMS PCR assays of the same samples, positivity was not detected. In this study, paratuberculosis was found at high rates in Kayseri. In conclusion, it was detected that mastitis symptoms in paratuberculosis were subclinical and not always observed, and the use of diagnostic laboratory methods may be an important aid in revealing diseases.

Key words: Cattle, enzyme-linked immunosorbent assay, immunomagnetic separation, paratuberculosis, polymerase chain reaction

1. Introduction

Paratuberculosis (Johne's disease), found mainly in cattle, sheep and goats, camels, buffalo, antelope, and deer as well as many domestic and wild animals, is an infectious and contagious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (1).

MAP, an acido-resistant bacterium previously known as *Mycobacterium paratuberculosis* and *M. johnei*, is a member of the *M. avium-Mycobacterium intracellular* complex. MAP is known as an intracellular, gram-positive, bacterium with thick, short rods (1). It is biochemically weak, does not have any exotoxin, and gives a positive reaction to avian tuberculin (1).

The bacteriological examination of stools for paratuberculosis is a helpful diagnostic method for determining healthy animals found in apparently infected herds. Nowadays, the most reliable method for the diagnosis of paratuberculosis from live animals is the fecal culture method (1). In addition, by using polymerase chain reaction (PCR) and real-time PCR methods, diagnosis can be performed faster (2).

Animals with paratuberculosis shed viable MAP agents, especially in their milk. MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products. The current methods of milk pasteurization are not sufficient to kill all MAP cells present in milk, and MAP has been cultured from raw and pasteurized milk and isolated from cheese. The presence of MAP in milk samples can be detected via culture, PCR, immunomagnetic separation (IMS), and enzyme-linked immunosorbent assay (ELISA) (3–5). In the diagnosis of subclinical paratuberculosis infections, 4 serological methods, including complement fixation, agar gel immunodiffusion, and 2 different ELISA methods were utilized. In addition, different ELISA methods have been used by various authors (6,7).

For the diagnosis of paratuberculosis from clinical samples and typing of agents, epidemiological studies are ongoing with the purpose of finding a reliable and speedy molecular method. For this purpose, IS*900* restriction fragment length polymorphism (RFLP), multiplex PCR typing, real-time PCR, immunomagnetic separation-PCR (IMS-PCR), variable number tandem repeat (VNTR)-mycobacteria interspersed repetitive units (MIRU) typing, nested PCR, and pulsed-field gel electrophoresis (PFGE) were used by some authors (2,5,7–9).

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In this study, our aim was to investigate the presence of paratuberculosis by using various identification methods in blood, milk, and stool samples from the herds of dairy cattle in Kayseri, Turkey.

2. Materials and methods

2.1. Samples

In the central area and the surrounding districts of Kayseri, in the herds of dairy cattle companies, symptoms including diarrhea, recession, mastitis, milk reduction, and discoloration were detected in 147 cows, aged 2 years and over, from which blood, milk, and feces samples were collected. The cattle were all diarrheic. The ages of the animals were recorded as evidence of history.

2.2. California mastitis test (CMT)

The CMT was performed according to the procedure reported by Middleton et al. (10). The mastitis conditions of the milk samples were considered as negative, suspicious, (+), (++), and (+++) according to the formation of a gelatinous layer and fluidity (10).

2.3. Serological analysis

The *Mycobacterium paratuberculosis* Antibody Test Kit (Institut Pourquier, France) was utilized according to the manufacturer's directions. Test results were read at 650 nm in an ELISA reader (BioTek ELx808, USA).

2.4. Immunomagnetic separation (IMS)

The IMS was used for pure identification of MAP from milk and feces and PCR applications. Biotinylated MptD (GKNHHHQHHRPQ) peptide was used for coating the beads utilized in IMS (Kinexus Bioinformatics Corporation, Canada). In the IMS process, streptavidin-coated beads (Dynal Myone Streptavidin Beads, Invitrogen) were used. These beads were then coated with MptD peptide. The IMS process was carried out in 4 steps, as reported by Grant et al. (3) and Khare et al. (11).

2.5. Molecular analysis

The MAP (RSKK 647) strain was obtained from the Veterinary Control Center Research Institute. The Tuberculosis-Paratuberculosis and Glanders Diagnostic Laboratory was used for the optimization of PCR and for the positive control.

Fermentas and ENZA extraction kits were used for DNA extraction from milk and feces. Extraction procedures were conducted according to the recommendations of the manufacturer. For the amplification of MAP from the milk and stool samples, IS*900* P90 and P91 primers were used (Table 1) (11).

In the investigation of MAP agents from the milk and stool samples of the cattle, PCR was carried out. The PCR method was used at 2 different stages of the study; first it was carried out on stool and milk samples directly, and then it was performed after IMS application. In the PCR reaction, a total volume of 50 µL of mixture was included with 10X PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100), 250 µM dNTP mix, 2 U Taq DNA polymerase enzyme, and 20 pmol P90 and P91 primers. After the addition of 5 µL of target DNA to the prepared mix, amplification was performed as follows: initial denaturation at 94 °C for 1 min and 30 amplification cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The amplified products were resolved in 1.5% (w/v) Tris-acetate-EDTA (TAE) agarose gel and the band patterns were analyzed in a gel documentation system (Vilber Lourmat) at 90 V for 1 h. The 400-bp band was evaluated as positive for PCR (12).

Table 1. Primers used in MAP PCR.

Primers	Sequence (5' - 3')	Base pairs
P90	GAAGGGTGTTCGGGGCCGTC	400 bp
P91	GAGGTCGATCGCCCACGTGAC	400 bp

2.6. Statistical analysis

The compatibility of the results between the ELISA and PCR methods used in the study were evaluated by Cohen's kappa test. Pearson's chi-square test was used to determine whether there was a statistically significant relationship between the ages of the animals that the samples were taken from and paratuberculosis infection incidence (13).

3. Results

The ages of 147 paratuberculosis-suspected animals aged 2 and over were recorded as evidence of history. These animals were divided into 4 groups in terms of ages as 2–4, 5–7, 8–10, and 11 and over (Table 2).

Eighteen (12.24%), 44 (29.93%), 36 (24.49%), 28 (19.05%), and 21 (14.29%) of the 147 milk samples examined by the California mastitis test were detected as negative, suspicious, CMT (+), CMT (++), and CMT (+++), respectively (Table 3). There were no mastitis symptoms observed in animals with negative, suspicious, or (+) scores.

Table 2. Age distribution of cattle suspected of paratuberculosis.

Age groups	Number of animals
2–4	54
5–7	68
8–10	22
11 and over	3
Total	147

Table 3. The distributions of CMT, ELISA, and PCR results.

CMT scores	CMT results (%)	ELISA			PCR			
					Milk		Stool	
		N	S	P	N (%)	P (%)	N (%)	P (%)
Negative	18 (12.24)	16	2	-	18 (100)	-	13 (72.22)	5 (27.78)
Suspicious	44 (29.93)	41	3	-	44 (100)	-	38 (86.36)	6 (13.64)
+	36 (24.49)	31	3	2	33 (91.67)	3 (8.33)	30 (83.33)	6 (16.67)
++	28 (19.05)	16	6	6	22 (78.57)	6 (21.43)	15 (53.57)	13 (46.43)
+++	21 (14.29)	9	2	10	10 (47.61)	11 (52.38)	9 (42.86)	12 (57.14)
Total	147	113	16	18	127	20	105	42

N: Negative, S: Suspicious, P: Positive.

The sera of 18 CMT-negative cattle were tested by ELISA. According to the test results, 2 (11.11%) and 16 (88.89%) of the sera were determined as suspicious and negative, respectively. In the PCR results of milk and feces samples belonging to the same cattle, while positivity was not detected in the milk samples, 5 (27.78%) stool samples were positive.

According to the ELISA results of the blood sera of 44 cattle evaluated as "suspicious" by the California mastitis test, 3 (6.82%) and 41 (93.18%) sera were found to be suspicious and negative, respectively. Six (13.64%) stool samples from these cattle were detected as positive with PCR, but positivity was not detected in the milk samples.

The blood sera of 36 cattle that were evaluated as (+) according to the CMT results of the milk samples were subjected to ELISA. At the end of the testing, 2 (5.56%), 3 (8.33%), and 31 (86.11%) of the serum samples were determined to be positive, suspicious, and negative, respectively. In PCR analysis of milk and stool samples, 3 (8.33%) milk and 6 (16.67%) stool samples were detected as positive.

The blood serum of 28 milk samples determined as (++) by CMT were subjected to ELISA. As a result of ELISA, 6 (21.43%), 6 (21.43%), and 16 (57.14%) of the sera were positive, suspicious, and negative, respectively. In PCR analysis of milk and stool samples belonging to the same cattle, 6 (21.43%) milk and 13 (46.43%) stool samples were found to be positive. According to the ELISA test results applied to the blood sera of 21 cattle whose CMT results were determined as (+++), 10 (47.62%), 2 (9.52%), and 9 (42.86%) of the sera were evaluated as positive, suspicious, and negative, respectively. In PCR analysis of milk and stool samples, 11 (52.38%) milk and 12 (57.14%) stool samples were detected as positive.

In the ELISA testing, 18 (12.24%), 16 (10.88%), and 113 (76.87%) serum samples were detected as positive, suspicious, and negative, respectively. In the PCR test of milk and stool samples, MAP DNA was detected in 20 (13.61%) and 42 (28.57%) of the samples, respectively. The distributions of CMT, ELISA, and PCR results are given in Table 4. In IMS-PCR analysis of milk and stool samples, positivity could not be determined.

In the PCR analysis of milk samples belonging to the same cattle, 11 out of 21 (52.38%) of the samples were positive. Twelve (57.14%) of the 21 analyzed stool samples were detected as positive.

In order to reveal the effects of the ages of animals on paratuberculosis infection, the age groups of 42 animals whose stool PCR results were positive were analyzed.

According to the test results, while 13 (24.07%) of the 54 cattle aged between 2 and 4 years were found to be positive, 41 (75.93%) of these animals were negative. Twenty-two (32.35%) of the 68 cattle aged between 5 and 7 years were positive and 46 (67.65%) of them were found to be negative. While 7 cattle (31.82%) with chronic diarrhea aged between 8 and 10 were positive, 15 (68.18%) were negative. In the evaluation of 3 cattle aged 11 and over, positivity was not found (Table 4).

MAP DNA was detected in milk and stool samples by PCR at rates of 20 (13.61%) and 42 (28.57%), respectively (Figure). In the IMS-PCR assays of the same samples, positivity was not observed.

The compatibilities between milk and fecal sample PCR and ELISA results and the results of stool PCR tests were evaluated. Accordingly, moderate agreement between the results obtained from the PCR results of milk and stool samples were detected (Cohen's kappa coefficient (κ): 0.565, $P < 0.001$). However, a high level of alignment was found between serum ELISA results and fecal PCR results

Table 4. The age group distribution of cattle with positive fecal PCR test results.

Age group (years)	Number of positive cattle (%)	Number of negative cattle (%)	Total number of cattle
2-4	13 (24.07)	41 (75.93)	54
5-7	22 (32.35)	46 (67.65)	68
8-10	7 (31.82)	15 (68.18)	22
11 and older	0 (0)	3 (100)	3
Total	42	105	147

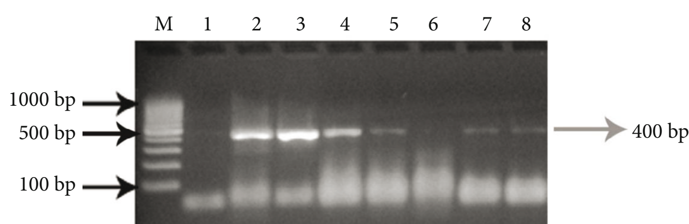

Figure. Gel image of PCR products (1.5% agarose gel). M: Marker (100-bp DNA ladder), 1: Negative control (sterile distilled water); 2: Positive control [*Mycobacterium avium* subsp. *paratuberculosis* (RSKK 647)]; 3–5, 7, 8: Positive samples; 6: Negative sample.

(κ: 0.783, P < 0.001) (Table 5). The effects of the cattle's age and the incidence of paratuberculosis infection were not statistically significant (P > 0.05) (Table 6).

4. Discussion

Paratuberculosis is an infection characterized by chronic granulomatous inflammatory changes in the intestines of ruminants and is still prevalent in many parts of the world. The clinical findings detected in paratuberculosis are similar to those of many other diseases (1,14). A limited number of studies are available for the evaluation of mastitis resulting from paratuberculosis (15,16). While McNab et al. (17) reported an increase in somatic cell counts in animals with paratuberculosis, DeLisle et al. (18) reported a decrease in the number of cells.

In our study, the milk samples of 147 cattle aged 2 years and over with chronic diarrhea were investigated for mastitis using the CMT. Although paratuberculosis was observed in these animals, the results for mastitis were negative. MAP may be the primary factor in mastitis, or it may be caused by secondary agents. In particular, understanding of the pathophysiological of subclinical mastitis in paratuberculosis is not sufficient at present; there is therefore a need for further research (14).

Humoral antibodies that develop against paratuberculosis agents can be detected by ELISA. Although an immune response has been seen at a relatively late stage of infection, the response emerged before clinical symptoms arose. While in the stools of animals containing limited numbers of agents positivity was detected at 15% by ELISA, this rate was approximately 87% in animals showing clinical symptoms. The reason for this is the low sensitivity of the culture and the inability of ELISA to identify early positivity. Donat et al. (19) reported that, in the diagnosis of paratuberculosis, the effectiveness of ELISA testing is low. In seroprevalence studies using ELISA, the lowest rate of 0.4% was reported from Slovenia and the highest rate of 24.1% was reported from Germany (20). Positivity was detected in 89 (4.6%) of 1950 serum samples (6) and in 424 (12.4%) of 10,280 samples in cattle (21), while 3.48% of 3961 cattle over 2 years of age were found to be positive in terms of seroprevalence in Poland (22). In Colombia, Fernandez-Silva et al. (23) determined positivity at a rate of 10.1%, while in Turkey, Makav and Gokce (24) found a 3.5% prevalence rate for subclinical paratuberculosis in the Kars region, and in Burdur Province a rate of 6.2% was reported by Ozturk et al. (25). In the present study, in Kayseri Province, 12.24% and 10.88% of 147 blood samples taken from paratuberculosis-suspected animals were detected as positive and suspicious by ELISA, respectively. The obtained serum positivity value was higher than those found by other researchers. The fact that the samples were taken from animals over 2 years old suspected of paratuberculosis is the reason for the high value.

Table 5. Statistical assessment of milk PCR with stool PCR and serum ELISA with stool PCR results.

			Stool PCR						Stool PCR		
			-	+	Total				-	+	Total
Milk PCR	-	Count	105	22	127	Serum ELISA	-	Count	105	8	113
		Milk PCR %	82.7%	17.3%				Serum ELISA %	92.9%	7.1%	
		Stool PCR %	100.0%	52.4%				Stool PCR %	100.0%	30.8%	
	+	Count	0	20	20		+	Count	0	18	18
		Milk PCR %	0%	100.0%				Serum ELISA %	0%	100.0%	
		Stool PCR %	0%	47.6%				Stool PCR %	0%	69.2%	
	Total	Count	105	42	147		Total	Count	105	26	131

Cohen's kappa coefficient (κ): 0.565 (P < 0.001).
Cohen's kappa coefficient (κ): 0.783 (P < 0.001).

With regard to age distribution, while Ozturk et al. (25), reported the prevalence in 465 cattle aged over 2 years as 6.2%, the seroprevalence was shown to range between 3.6% and 19.73% in different age groups and, in particular, prevalence was found to be highest in the 3-year-old age group. In our study, in the investigation of 42 animals whose fecal PCR test results were positive, positivity rates of 13 (24.07%), 22 (32.35%), and 7 (31.82%) were found in the 2–4, 5–7, and 8–10 age groups, respectively. In the examination of 3 cattle aged 11 and over, positivity was not found. It was also determined that paratuberculosis was seen more frequently in cattle aged 5–7 years. In some studies in cattle, paratuberculosis was usually observed in those 2 years old and over (25,26). However, in several studies, positivity was detected in different age groups (15,23). These differences are thought to be caused by the geographical distribution of MAP agents, the conditions regarding the nutrition of cattle, the features of animal productivity, etc.

Table 6. The statistical evaluation of age groups of infection.

			Stool PCR		Total
			-	+	
Ages	2–4	Count	41	13	54
		%	75.9%	24.1%	
	5–7	Count	46	22	68
		%	67.6%	32.4%	
	8+	Count	18	7	25
		%	72.0%	28.0%	
Total		Count	105	42	147
		%	71.4%	28.6%	

Pearson chi-square: 1.016, P > 0.05.

The examination of fecal samples by PCR has been reported to be useful in early diagnosis because of its high specificity and sensitivity (27). In a study conducted in France, positivity was detected in 77 stool samples of 1041 cattle by the molecular method (28). In India, in PCR analysis of the samples, positivity was found to be around 90% (29). In our study, MAP DNA was detected by PCR in 13.61% and 28.57% of 147 tested milk and fecal samples, respectively.

MAP agents continue to be excreted with the feces and could be defined by the diagnostic methods. Despite the positive results obtained from stools, the cause of negative and doubtful results from the serum samples of the same animals is thought to be due to the humoral response not being active enough in the early stages of the infection. In cattle with aseptic Johne's disease, in 50 mL of milk only about 2–8 CFU titer MAP agents are found. This extremely low amount reduces the chance of isolating the agent in milk. The IMS method has been developed as an alternative method in order to eliminate the difficulties encountered in the first isolation of MAP. Thus, heterogeneous bacteria can be eliminated, and MAP may be isolated purely in milk (3). In our study, paramagnetic beads coated with phage-originated MptD peptide were used in the IMS method for the analysis of milk and fecal samples. However, samples detected as positive by conventional PCR were not found to be positive by IMS-PCR. This is thought to be due to the effectiveness of the peptides used in the studies. In a study conducted in the Czech Republic, positivity was detected in the feces of animals at a rate of 10% and the milk samples of the same animals were also positive at a rate of 19.7%. These findings suggest that in infected dairy cows subclinical agents can be found in raw milk (30). In various countries around the world, positivity ranging from 2.8% to 35% has been reported in cow milk (31). In our study, in 13.61% of 20 milk samples, the agents were found by using the PCR method.

In this study, the compatibilities between the PCR and ELISA results found in the investigation of agents in milk and fecal samples and the results of stool PCR tests were evaluated. Accordingly, moderate agreement between the results obtained from the PCR of milk and stool samples was detected. However, a high level of alignment was found between serum ELISA results and fecal PCR results.

When comparing the 3 methods used in the study, PCR was concluded to be the most sensitive method in terms of blood, milk, and stool samples tested. Despite the positivity in the feces samples, the causes of negative and suspicious results from serum samples depended on the humoral response, thought to be insufficiently active in the early stages of the infection.

In conclusion, in the late stages of infection in animals that also have fecal excretion of agents, the spreading of MAP agents in milk showed that milk is a significant risk factor for public health. In Turkey, studies regarding paratuberculosis are limited. It was considered that using a combination of more than one method to obtain accurate results in the diagnosis of paratuberculosis may be useful.

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