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Serological detection of *Mycobacterium avium* subsp. *paratuberculosis* and analysis of risk factors in dairy farms of Fars province, southern Iran

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes a chronic, debilitating, progressive form of enteritis in ruminants, especially cattle. Given the substantial socioeconomic impact of this disease, regional studies are needed to clarify its epidemiology and identify associated risk factors. This study assessed the prevalence of paratuberculosis and the factors associated with its occurrence in dairy cattle herds in Fars province, Iran. The province's counties were categorized into northern, central, and southern sectors according to their geographic distribution. A total of 827 serum samples were collected from nine dairy farms across the three regions. The presence of antibodies against MAP were detected using a commercial ELISA kit. The findings revealed a herd-level prevalence of 44.44% (95% CI: 18.87–73.33%) and an animal-level prevalence of 4.84% (95% CI: 3.57–6.52%). Statistical analysis indicated that geographic region, age group, and farm production type were all significantly associated with MAP seropositivity ($p<0.05$). Older cattle exhibited a markedly higher likelihood of testing positive (OR = 3.823, 95% CI: 1.158–12.619, $p<0.05$), and animals raised in industrial production systems had significantly greater seroprevalence than those in traditional systems (OR = 6.423, 95% CI: 2.210–18.661, $p<0.001$). Overall, the results indicate that MAP infection is widespread among dairy cattle in southern Iran. These findings emphasize the necessity of identifying and removing infected animals and implementing effective management, sanitation, and hygiene measures to limit transmission and prevent further spread within and between herds.

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative microorganism responsible for paratuberculosis, commonly referred to as Johne's disease (JD). This is a chronic and progressive granulomatous enteritis that primarily affects ruminant species, most notably dairy cattle, along with numerous

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domestic and wild animals. The pathogen gains entry through the gastrointestinal tract and is capable of surviving in the host for several years before any clinical manifestations become evident. During this extended asymptomatic phase, infected animals may shed MAP into the environment even before the infection can be identified through diagnostic testing or clinical observation (1). Paratuberculosis leads to significant economic losses due to declines in milk production, impaired reproductive performance, early culling, and reduced value of culled animals in infected herds (2). *Mycobacterium avium* subsp. *paratuberculosis* has also been implicated as a potential contributor to Crohn's disease and multiple sclerosis in humans, although these associations remain disputed and are not universally accepted (3, 4). Because of this, early detection of MAP in breeding herds is crucial, especially considering that shedding of the organism begins well before any clinical signs become apparent and can facilitate transmission to other susceptible animals. Estimating infection prevalence at both the herd and individual-animal levels plays a critical role when policymakers and health authorities evaluate the relevance of an infection and determine which interventions should be implemented. Strategies may differ based on prevalence, for example, eradication efforts in low prevalence settings, control programs when prevalence is high, or surveillance activities when the pathogen appears to be absent. Furthermore, prevalence data are essential inputs for epidemiological models used to simulate disease spread, conduct risk assessments, and support evidence-based decision-making (5). Low herd-level prevalence (<1%) has been documented in only a limited number of countries such as Thailand, Norway, and Sweden. In contrast, many regions report herd-level prevalence's exceeding 20%, including Belgium, Canada, Chile, Denmark, and France, with estimates reaching approximately 85% in Denmark and 70% in the United States dairy industry (6-8). In Iran, a meta-analysis covering animal studies from 1999 to 2020 reported a MAP prevalence of 22.33% at the animal level in cattle populations (9). Given the substantial socioeconomic burden of this disease, further investigation is required across different geographic areas to clarify infection status and identify influential risk factors. Such information enables both governmental bodies and livestock owners to adopt appropriate management and control strategies for paratuberculosis. Fars province, home to roughly 0.4 million cattle—predominantly crossbred animals—is the leading region for cattle population in southern Iran and plays a major role in national milk and meat production (10). The present study examines the prevalence of paratuberculosis in dairy cattle herds and evaluates multiple risk factors affecting disease occurrence in Fars province, Iran.

Materials and Methods

Study Design

A cross-sectional study employing cluster random sampling was carried out in Fars province, southern Iran. This province spans latitudes 27°3′ to 31°40′ N and longitudes 50°36′ to 55°35′ E, covering an approximate area of 133,000 km². The region receives an average annual rainfall of around 230 mm and includes 29 administrative counties. Based on its geomorphological features, Fars province is divided into three major zones. The northern zone incorporates areas in the north, northwest, and west, characterized by relatively mild and cold winters. The central zone experiences a moderate climate, with winter precipitation and hot, dry summers. The southern zone, stretching from the south to the southeast, is distinguished by extremely hot summers (11). For this study, the counties of Fars province were categorized into northern, central, and southern regions according to their geographic location. Three dairy farms were then randomly selected from each region, resulting in a total of nine farms. Sampling was performed once during the warm season (July–August 2021), and all cows present on each farm were included.

Sample Size

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The study population consisted of cattle herds, while individual cattle served as the sampling units. Altogether, nine dairy farms were included. At the beginning of each visit, researchers explained the study objectives and procedures to the farm owners, and verbal consent was obtained. Although the required sample size was calculated to be 384 animals based on the following standard formula (12), the final sample number was intentionally increased to more than double (827 samples) to enhance the reliability of the results.

$$n = \frac{(1.96)^2 \times EP \times (1 - EP)}{d^2}$$

In this equation, *n* denotes the necessary sample size; *d* represents the desired precision, set at 0.05 with a 95% confidence level; and *EP* indicates the expected prevalence. For this study, an assumed prevalence of 50% was used due to limited data regarding the seroprevalence of the targeted pathogens in southern Iran. None of the farms included in the study utilized vaccination against MAP.

Samples and antibodies evaluation

Blood samples were collected from the coccygeal vein using sterile vacuum tubes without anticoagulant (VACUETTE®, Greiner Bio-One GmbH, Kremsmünster, Austria). Each tube was labeled and transported to the laboratory under chilled conditions. Additional information, including age (categorized as young animals between 6 months and 2 years, and adults older than 2 years), sex (male or female), and production system (traditional or industrial), was documented for all sampled animals. Serum was obtained by centrifuging the blood at 3000 rpm for 10 min and subsequently stored in microtubes at –20°C until further analysis. Detection of antibodies against MAP was performed using a commercially available ELISA kit produced by the Razi Vaccine and Serum Research Institute (Karaj, Iran). Following the manufacturer's instructions, dilution buffer was prepared, sample sera were diluted in a designated dilution plate, and kit-provided reference sera were diluted separately. Both samples and control sera were dispensed into the wells of an antigen-coated microtiter plate and incubated at 21°C for 1 h. After incubation, the wells were rinsed with washing solution, and the plate underwent three consecutive wash cycles. A diluted conjugate solution was then added to all wells, followed by another incubation period and a second washing step. The enzymatic reaction was developed using a chromogenic substrate for 10 min. The reaction was terminated by adding 1 M phosphoric acid, and optical density (OD) values were measured at 450 nm.

The percentage of sample-to-positive ratio (S/P) was calculated using the formula:

$$\mathrm{S/P} = \frac{\text{OD of sample} - \text{mean OD of negative controls}}{\text{mean OD of positive controls} - \text{mean OD of negative controls}} \times 100$$

Samples with an S/P value greater than 15% were classified as MAP-positive according to the kit manufacturer's instructions. Specificity and sensitivity of the ELISA kit were recorded as 99% and 86%, respectively. Although the presence of a single seropositive animal may be sufficient to classify a small herd as positive, in the present study, a herd was classified as positive only when at least two seropositive animals were identified.

Statistical analysis

All statistical examinations were performed using SPSS software (version 22). Descriptive analyses were conducted to determine seroprevalence at both the animal and herd levels. To evaluate the relationship between MAP seropositivity and various factors, including geographic region, age category, sex, and farming system, a chi-square test was applied at the individual-animal level. Logistic regression modeling was then employed to assess the influence of potential risk factors on seroprevalence outcomes. The magnitude of association between independent variables and MAP serostatus was expressed using odds ratios (OR) along with their corresponding 95% confidence intervals (CI).

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Results

A total of 827 cattle were sampled, representing the entire study population. The herd-level seroprevalence of MAP was calculated to be 44.44%, and herds located in the central region exhibited approximately twice the likelihood of infection compared with herds in the northern or southern regions (Table 1).

Table 1. The regional distribution of studied farms and the number of cows and seroprevalence of MAP at farm-level

Geographic region	Farm	The number of cows		Serop†in farm (%)	Serop in region (%)
		Total	Positive		
Northern	1	90	0	0	33.33
	2	84	0	0	
	3	118	10	8.47	
Central	4	92	4	4.35	66.66
	5	111	12	10.81	
	6	70	0	0	
Southern	7	149	14	9.40	33.33
	8	22	0	0	
	9	91	0	0	
Total	9	827	40	-	44.44

† Seroprevalence

Among the 827 individual animals tested, 40 were seropositive, corresponding to an animal-level prevalence of 4.84% (95% CI: 3.57–6.52%). The outcomes of the Chi-square analyses at the individual-animal level are shown in Table 2. Geographic region, age category, and production system were all significantly associated with MAP seropositivity ($p<0.05$). In the logistic regression model, variables with $p\geq0.4$ were excluded, resulting in the removal of gender from the final analysis. Age and farming type remained significant predictors of infection (Table 3). Older cattle demonstrated a significantly higher likelihood of MAP seropositivity (OR = 3.823, 95% CI: 1.158–12.619, $p<0.05$). Additionally, animals raised in industrial farming systems exhibited a substantially greater probability of being MAP-positive compared with those from traditional systems (OR = 6.423, 95% CI: 2.210–18.661, $p<0.001$).

Table 2. Univariable analysis of associated variables for seropositivity at animal-level

Factor	The number of cows		Serop† (%)	$\chi2^{\dagger\dagger}$	p- value
	Total	Positive			
Geographic region				2.03	*0.362*
Northern	292	10	3.4		
Central	273	16	5.9		
Southern	262	14	5.3		
Gender				0.18	*0.675*
Male	68	4	5.9		
Female	759	36	4.7		
Age				5.42	*0.020*
< 2 years	186	3	1.6		
≥ 2 years	641	37	5.8		
Farming type				16.23	*0.001*
Industrial	492	36	7.3		

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Traditional	335	4	1.2
Total	827	40	4.8

† Seroprevalence ††Chi-square

Table 3. Logistic regression analysis of associated factors for MAP seropositivity at animal-level

Factor	OR†	95% CI††		*p- value*
		Lower	Upper	
Region				
Northern	1			
Central	1.118	0.484	2.579	*0.794*
Southern	1.063	0.453	2.494	*0.889*
Age				
< 2 years	1			
≥ 2 years	3.823	1.158	12.619	*0.028*
Farming type				
Industrial	6.423	2.210	18.661	*0.001*
Traditional	1			

† Odds ratio, †† Confidence interval

Discussion

Since no mycobacteria vaccination programs were implemented in the herds included in this study, the detected antibodies can reasonably be attributed to natural exposure to MAP. Prevalence studies contribute essential information such as determining the magnitude and progression of infection, identifying research priorities, guiding disease-control planning, and evaluating diagnostic tools capable of detecting subclinical cases. Such data play a central role in the development of veterinary public health strategies that mitigate production losses in livestock populations. Global assessments of MAP infection have revealed considerable variation across countries, with reported herd-level prevalence ranging from as low as 5% in Mexico to as high as 100% in Pakistan (13). The wide variation may be due from differences in diagnostic methods (ELISA, fecal culture, PCR, etc.), the number of herds and animals sampled, herd size, geographic location and management of farm. The herd-level prevalence observed in the current investigation (44.4%) is comparable to findings from dairy farms in Alberta, Canada, and the Yeongnam region of Korea, where serological surveys using ELISA reported herd prevalence rates of 40.0% and 41.7%, respectively (14, 15). Comprehensive epidemiological data for Iran remain limited (1). The herd-level seroprevalence identified in this study exceeded the value previously reported for Fars province (11%), which was based on molecular analysis of bulk tank milk samples (16). Paratuberculosis has been documented in nearly all regions of Iran, and a meta-analysis of studies conducted between March 1999 and October 2017 estimated an overall MAP prevalence of 22.33% (95% CI: 18.87–25.78) in the national cattle population (9). The relatively low animal-level seroprevalence found in the present study (4.4%) aligns with reports from Ahwaz (2%), Urmia (3.1%), Mazandaran (5.6%), and the Markazi and Alborz provinces (3.8%). However, this value was lower than prevalence estimates reported for industrial and semi-industrial cattle operations in Hamedan and Esfahan provinces, where MAP seropositivity reached approximately 32%. One possible explanation for the variability in reported seroprevalence rates is the diversity of diagnostic techniques used across studies. Diagnostic approaches for MAP infection generally fall into two categories. The first category targets direct detection of the pathogen, typically through bacterial culture or polymerase chain reaction (PCR). The second category focuses on measuring the host's immune response to MAP, using assays such as ELISA, agar gel immunodiffusion, or complement fixation tests—although the latter two are now used far less frequently compared with ELISA (8, 17). ELISA has become the preferred screening tool in MAP control and monitoring programs because it allows convenient sample

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collection, high-throughput testing, and rapid processing times in the laboratory (18). In a study designed to compare the sensitivity and specificity of diagnostic methods for detecting MAP in dairy cattle, researchers reported that PCR exhibited lower sensitivity relative to ELISA. Considering the superior predictive values and overall accuracy of ELISA, coupled with the higher cost, longer processing time, and greater technical requirements associated with PCR, they concluded that ELISA is the more appropriate option for epidemiological surveillance and large-scale screening (19). Nevertheless, differences in sample material (feces, milk, serum, or tissue), animal species (dairy or beef cattle, sheep, goats, camels, deer, or wildlife), and inter-laboratory variability must be acknowledged when interpreting and comparing findings from different investigations. Risk factor analysis demonstrated that geographic region and gender did not show significant associations with MAP seropositivity, which is consistent with findings reported from China (20). By contrast, a study from Germany found a higher likelihood of MAP-positive farms in the southern part of the country, attributing the difference primarily to larger herd sizes in that region (21). The discrepancy between these findings may reflect differences in climatic conditions, herd size, farm management practices, production systems, and animal density among geographic regions. In the present study, the probability of infection increased significantly in cattle aged ≥2 years, with an approximately 3.82-fold elevation in risk compared with younger animals ($p<0.05$). Similar age-related trends have been described in other investigations from Middle Eastern regions (22, 23). Higher seroprevalence in older cattle is biologically plausible, reflecting MAP's prolonged incubation period of up to five years (24), age-associated development of anti-inflammatory immune responses dominated by IgG antibodies (25), and reduced ELISA sensitivity in young sub-clinically infected animals relative to clinically affected older cows (26). Farming system also emerged as a significant risk factor in the present analysis. Cattle kept in industrial operations had notably higher seroprevalence than those raised in traditional systems (OR = 6.423, $p<0.01$). Variations in MAP seroprevalence across farming types may reflect differences in herd size (20), farm-level hygiene and management practices (27), and biosecurity factors such as animal purchasing policies (26, 28). Larger and more densely stocked herds may facilitate the transmission and persistence of MAP by increasing opportunities for fecal contamination and animal-to-animal exposure. In addition, more frequent introduction, purchase, and movement of animals in intensive production systems may increase the risk of introducing MAP into herds and subsequently spreading infection within the farm. Additional management conditions such as pooling colostrum and rearing calves collectively in large groups may also enhance exposure of susceptible young animals to MAP. Furthermore, fecal contamination of feed and water can further facilitate within-herd transmission via the fecal–oral route (29).

Conclusion

The findings showed a prevalence of 44.44% and 4.84% at the herd and animal levels, respectively, indicating that MAP is circulating among dairy cattle in Fars province and emphasizing the need for targeted control strategies. Control of MAP should include regular herd-level screening, identification and removal of infected animals, quarantine and testing of newly introduced cattle, and immediate separation of calves from their dams after birth. Improving housing hygiene and manure management, preventing fecal contamination of feed and water, and avoiding pooled colostrum may also help reduce transmission within herds. Implementation of these measures could contribute to limiting the persistence and spread of MAP among dairy herds in the region.

Ethical approval

All relevant international, national, and institutional guidelines for the care and use of animals, including the protocol approved by the Deputy for Research and Technology of Razi Vaccine and Serum Research Institute, were followed.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Artificial Intelligence Statement

The authors declare that no artificial intelligence tools were used in the preparation of this manuscript.

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