Seroprevalence of Paratuberculosis in Cattle, Sheep and Goats in Burdur, Southwestern Turkey

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ABSTRACT

In this study we aimed to investigate the presence of antibodies to *Mycobacterium avium* subsp. *paratuberculosis* by ELISA in blood samples obtained from animals of at least 2 years of age selected by random sampling method in cattle, sheep and goat herds in Burdur province in Southwestern Turkey and to determine the apparent and true individual-animal, within-herd and between-herd seroprevalences of paratuberculosis. For this purpose, a total of 450 blood serum samples were collected from 150 cattle, 150 sheep and 150 goats, and the individual-animal, within-herd and between-herd seroprevalences of the disease were determined by using a commercial ELISA kit. The apparent and true seroprevalences based on the individual-animal, within-herd and between-herd were calculated as 8% - 8.9%, 17.1% - 20.4%, 46.7% - 57.8% in cattle; 48% - 100%, 48% - 100%, 100% - 100% in sheep; and 24% - 35.9%, 25.7% - 38.6%, 93.3% -100% in goats, respectively. The distribution of disease according to cattle, sheep and goat breeds was not statistically significant, although an increase in the infection rate was recorded with increase in age of the animals and size of the herd ($P>0.05$). In conclusion, this study indicated that in cattle and especially sheep/goat paratuberculosis were widespread in Burdur province and many measures will be required to reduce the rate of infected animal and/or herds.

Keywords: Paratuberculosis; Cattle; Sheep; Goat; ELISA; Seroprevalence.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causal agent of paratuberculosis (Johne's disease), a chronic enteric granulomatous disease affecting cattle, sheep and goats (1-3). Paratuberculosis is recognized worldwide as one of the most economically important food animal disease due to the examination and treatment costs, loss of milk production and reduced slaughter value (2-4). Various assays for the diagnosis of *Map* infections have been established, including Ziehl-Neelsen staining of fecal samples, histopathological examinations, detection of *Map*-antibodies in blood, serum and milk, intradermal Johnin test or, alternatively, *in vitro* assays for cytokines, PCR-amplification, and bacteriological culture from affected tissues, feces and milk. Although bacteriological culture method is accepted as a golden standard for diagnosis of paratuberculosis, it has been stated that this method can be insufficient to diagnose non-shedding or transiently shedding animals in the early phase of infection (3-6). ELISA is the most commonly used serological test for diagnosis of *Map* infection and have several advantages such as ease to perform, allowing objective evaluation of results, testing many samples at the same time, and being cheaper than other tests (3-7). The specificity of ELISA is increased by *Mycobacterium phlei* absorption of sera (3, 4). It has been reported that the absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step and this procedure eliminates nonspecific cross-reacting antibodies (3).

Before designing a control and prevention program for paratuberculosis (such as hygiene and husbandry measures, test-and-cull strategies and vaccination), both herd and ani-

mal-level prevalences of *Map* infection should be established. Although several regional surveys for cattle (8-12) and ovine (13) have been conducted to estimate the seroprevalence of *Map* infection in Turkey, there is no currently available eradication strategy for the paratuberculosis. On the other hand, to date there has never been any report of seroprevalence of paratuberculosis for goat herds in Turkey. In our previous bovine seroprevalence study (10), seropositive samples were found as 6.2% in Burdur province and we hypothesized that small ruminants would probably also be infected. The purpose of the present study was to estimate the individual-animal, within-herd and between-herd prevalence of paratuberculosis in cattle and small ruminant in Burdur province of Turkey, using a commercially available absorbed ELISA kit.

MATERIALS AND METHODS

Animals and samples

This study was conducted on dairy cattle, sheep and goat herds in the Burdur province, located in southwest of Turkey. The area is the crossing point of the Aegean, Central Anatolia and Mediterranean parts of Turkey. Burdur territories are located between 36°-53° and 37°-50° north latitudes and 29°-24° and 30°-53° longitudes. The area is 6 883 km^2 and mean altitude is approximately 1000 m above sea level. Burdur has a continental Mediterranean climate with cold, snowy winters and very hot, long and dry summers.

Two years and older female animals whose owners agreed to participate in the study randomly selected without consideration of herd size and breed (This study was approved by the Local Ethical Committee of Animal Experiment of Mehmet Akif Ersoy University with protocol no. MAKU-HADYEK/2014-83).

The sample size required was estimated using seroprevalence data from a previous study done in same area, which resulted in a cow-level apparent prevalence of 6.2% (10). The sample size of animals was determined using an expected prevalence of 10%, a confidence level of 95% and error of 5%. This yielded a number of 138 samples from each of the animal species needed for this study (14). However a total of 150 cattle, 150 sheep and 150 goats were sampled to increase precision. Blood samples were collected from 15 different herds including at least 10 animals from each animal species between October 2014 and February 2015. Blood samples were collected from the jugular vein in 10 ml vacutainer tubes (BD Vacutainer, Plymouth, UK) and were transported immediately with refrigerant to the laboratory. Sera were separated by centrifugation and frozen at -80°C until tested.

The herds consisted of a single animal species and their managements were small holder livestock as family farms. Although the herds were separate, in most of farms the pasture was common for cattle, sheep and goats. The age of animals, the breed and herd size were recorded at the time of blood collecting. The age of animals and herd sizes in the study population are presented in Table 1. Animals in the study belonged to the following breeds: Holstein-Friesian (n=144), Simmental (n=3) and mixed breed (n=3) cattle; Merino (n=64), semi-fat-tailed (Sakiz, n=34) and mixed breed (n=52) sheep; and Hair Goat (n=107), Saanen (n=17) and mixed breed (n=26) goats. None of the animals tested had been vaccinated against paratuberculosis. No herds were reported to have had clinical signs of *Map* or other infections by farmers.

Table 1: The age of animals and herd sizes in the study population

Animal	Age-group (years)				Herd size			
	2	3	4	≥5	10-19	20-49	50-99	≥100
Cattle (n=150)	42	31	26	51	3	5	5	2
Sheep (n=150)	11	23	19	97	0	2	4	9
Goat (n=150)	25	27	24	74	0	2	7	6

Serological testing

A commercial ELISA kit (Parachek®2, Prionics AG, Zurich, Switzerland) based on antibody detection were used to test all sera according to the manufacturer's instructions. The samples were diluted and incubated in a dilution of buffer containing a *M. phlei* extract in order to eliminate the cross reactions. After incubation, the negative, positive controls and the samples were placed in microplate wells. Positive and negative control sera provided by the manufacturer were run in duplicate on each plate. Plates were read at 450 nm with an automated plate reader. The mean optical density (OD) values of positive and negative control sera were checked for validation of the test. The mean corrected value of positive control was greater than 0.500 and was also found to be greater than 5 times the corrected value of negative controls. For each sample, percent positivity (% P) was calculated by using the following equation: Sample % P= (OD test serum – Mean OD negative control serum / Mean OD positive control serum – Mean OD negative control serum). Results of serum samples which were above or equal to the cut-off

Table 2: Seroprevalences of paratuberculosis for cattle, sheep and goats in Burdur province

Prevalence type	Number tested	Number positive for *Map*	Apparent prevalence		True prevalence	
			Estimate (%)	95% CI	Estimate (%)	95% CI
Cattle						
Individual	150	12	8	4.6-13.5	8.9	3.4-14.4
Within-herd	70	12	17.1	10.1-27.6	20.4	9.3-31.6
Between-herd	15	7	46.7	24.8-69.9	57.8	25.8-89.8
Sheep						
Individual	150	72	48	40.2-55.9	100	105.4-148.6
Within-herd	150	72	48	40.2-55.9	100	105.4-148.6
Between-herd	15	15	100	79.6-100	100	267.6
Goat						
Individual	150	36	24	17.9-31.4	35.9	25.3-46.6
Within-herd	140	36	25.7	19.2-33.5	38.6	27.3-49.9
Between-herd	15	14	93.3	70.2-98.8	100	124.5-164.0

of 15% P were considered as positive. The samples which were below the cut-off of 15% P were considered as negative.

Data used in the analysis

Case definitions: A cattle, sheep or goat was defined as seropositive when serum was positive for *Map* antibodies on the Parachek®2 ELISA test. A herd was considered as positive for *Map* infection when at least one animal was seropositive for *Map* (14).

Calculation of apparent and true prevalence: The apparent prevalence of *Map* infection at individual-animal and between-herd were calculated, respectively, as the number of serologically positive animals (cattle, sheep or goats) among the total number of animals tested, and the number of serologically positive herds among the total number of herds tested. The within-herd apparent prevalence was calculated only in serologically *Map* positive herds, as the number of serologically *Map* positive animals among the total number of animals tested in that herd (14). The 95% confidence intervals (CI) for apparent prevalences were estimated using the Wilson binominal approximation method as described (15). The true prevalence of *Map* infection at individual-animal, within-herd and between-herd were calculated by the application of the Rogan-Gladen correction (16). In all calculations, the apparent sensitivity of the ELISA kit for detecting *Map* antibodies in cattle, sheep and goat sera were assumed to be 80%, 38% (range 38-44%) and 65% (range 65-88%), respectively, and specificity was assumed to be 99% as reported by the kit manufacturer.

Statistical analysis: To compare the seropositivity results to age, breed and herd size, Pearson Chi-squared (χ^2) test was used. Statistical significance was defined at $P \leq 0.05$. All data analyses were done with SPSS 22.0 for Windows (17).

RESULTS

A total of 12 cattle, 72 sheep and 36 goats were found to be seropositive for *Map*. Seven cattle, 15 sheep and 14 goat herds among herds tested were positive for *Map* antibodies. The apparent and true seroprevalences based on the individual-animal, within-herd and between-herd were shown in Table 2.

Regarding age-groups, the highest value of *Map* seropositivity was found in animals older than 5 years of age, but there was no significant difference in seropositivity results among the age-groups ($P>0.05$). The number of seropositive and seronegative animals in the study population according to age was presented in Table 3. The distribution of *Map* seropositivity rate according to cattle, sheep and goat breeds was not statistically significant ($P>0.05$) (Table 4). Cattle herds of 20-49 head, sheep herds of ≥100 head and goat herds of 50-99 head had the higher seropositivity. Although the infected herds were relatively larger, there was no significant difference in seropositivity results among the cattle and goat herd size groups ($P>0.05$). In sheep herds, the calculation was not done because all herds were detected seropositive. Table 5 summarizes the number of seropositive and seronegative herds according to herd size.

Table 3: The number of *Map* seropositive and seronegative animals according to age groups in years

Animal	Age-groups (years)									
	2		3		4		≥5		Total	
	P	N	P	N	P	N	P	N	P	N
Cattle	3	39	3	28	0	26	6	45	12	138
Sheep	5	6	9	14	9	10	49	48	72	78
Goat	3	22	6	21	7	17	20	54	36	114
Total	11	67	18	63	16	53	75	147	120	330

P: *Map* positive, N: *Map* negative

Table 4: The distribution of *Map* seropositive and seronegative animals according to breeds

Animal	Breeds	Positive		Negative	
		n	%	n	%
Cattle	Holstein-Friesian (n: 144)	12	8.3	132	91.7
	Simmental (n: 3)	0	0	3	100
	Mixed breed (n: 3)	0	0	3	100
	Total (n: 150)	12	8	138	92
Sheep	Merino (n: 64)	28	43.8	36	56.2
	Sakiz (n: 34)	15	44.1	19	55.9
	Mixed breed (n: 52)	29	55.8	23	44.2
	Total (n: 150)	72	48	78	52
Goat	Hair Goat (n: 107)	28	26.2	79	73.8
	Saanen (n: 17)	4	23.5	13	76.5
	Mixed breed (n: 26)	4	15.4	22	84.6
	Total (n: 150)	36	24	114	76

Table 5: The number of *Map* seropositive and seronegative herds according to herd size

Herds	Herd size groups							
	10-19		20-49		50-99		≥100	
	P	N	P	N	P	N	P	N
Cattle (n: 15)	0	3	3	2	2	3	2	0
Sheep (n: 15)	0	0	2	0	4	0	9	0
Goat (n: 15)	0	0	2	0	7	0	5	1
Total	0	3	7	2	13	3	16	1

P: *Map* positive, N: *Map* negative

DISCUSSION

Paratuberculosis is recognized worldwide as one of the most economically important animal disease affecting cattle, sheep and goats (1-3, 18). The gold standard for diagnosing paratuberculosis is the identification of the microorganism by bacterial culture (3, 4). However, this test does not allow accurate identification of truly infected animals because of lack of shedding in early phases, intermittent shedding or limitations of culture protocols (14). Nielsen (7) suggested that occurrence of antibodies was a good indicator of progression of *Map* infection especially for non-shedding and transient shedding animals. The serum ELISA is at present the most sensitive and specific test and is also the most widely used technique in view of its low cost and easy automation (3, 4, 19). Several researchers (10, 13, 20-24) have pointed out that the absorbed ELISA should be permit epidemiological studies and be a useful tool in the management and control of *Map* infection in cattle, sheep and goats. Therefore, a commercially available absorbed ELISA kit was used in this study and serum samples before testing for specific antibodies to *Map* were diluted with a tampon including *M. phlei* into the kit to remove cross-reactivity to environmental bacteria. Collins *et al.* (20) have stated that the absorbed ELISA sensitivity was low relative to the sensitivity of many other diagnostic tests because the sensitivity was calculated on the basis of the results of testing individual animals, and with the herd sampling strategy used, the ability of the test to detect infected herds is much higher. In present study, it was aimed to evaluate of the individual-animal together with within-herd and between-herd prevalences and therefore, blood sera were randomly collected from 15 different herds including at least 10 animals from each animal species.

It has been reported that clinical signs may develop in cattle, sheep and goats of two or more years of age and that the *Map* antibodies increased with age, as expected since the humoral response increases as the infection progresses or greater chance of exposure to infection with age (7, 25-27). Nielsen and Toft (18) have been also stated that ELISAs will usually not detect infected animals less than 2 years of age, as these have not developed antibodies to *Map*. In this study, 10 animals aged 2 years and above from each herd were tested and the rate of *Map* seropositivity in cattle, sheep and goat was found as 8%, 48% and 24%, respectively. Coelho *et al.* (25) reported that an association between paratuberculosis clinical signs and seroprevalence in sheep was found and all positive animals were in an advanced phase of the disease. In this study, no cattle, sheep or goat herds considered seropositive were reported to have had clinical signs of *Map* infection by farmers. This result is consistent with previous reports (3, 4, 20, 26) showing that clinical observation is not sufficient for early detection of *Map* infection and the majority of infected animals in a herd may be subclinically infected due to the long incubation period.

The prevalence for the Wisconsin dairy herds in the United States was found to be 7.29% of cows and 50% of herds with one or more cows positive, and the true cow-level and herd-level prevalence were calculated as 4.79% and 34%, respectively (20). Reported apparent and true prevalences in European countries have been found to vary widely (21-23, 28-31). It has been explained that herd-level prevalence can vary from country to country in the range from very low (i.e. almost 0% in Finland and Sweden) to very high (e.g. >50% in Denmark and The Netherlands) (19). In this study, the apparent and true seroprevalence based on the individual-animal, within-herd and between-herd in cattle were calculated as 8%-8.9%, 17.1%-20.4%, 46.7%-57.8%; respectively. We propose that the differences in prevalence values may have arisen from the climate, nutrition, age, geographical variations, housing conditions, the frequency of purchasing animal and the use of different diagnostic methods (10, 18, 23, 32, 33). Nevertheless, the seroprevalence values observed in this study are very similar to that reported in Germany, Spain, the Netherlands, Italy, Denmark and Holland (19, 21, 22, 29, 31). This finding also supports the results in a review of literature on current herd prevalence estimates of Nielsen and Toft (6) which stated that the true prevalence among cattle in Europe appeared to be approximately 20% and was at least 3-5% in several countries, and herd prevalence guesstimates appeared to be more than 50%.

There are a few studies on seroprevalence of bovine paratuberculosis in Turkey (8-11). Reported seroprevalences have included an apparent individual-animal prevalence of 2.7% in central Anatolia region (11), and apparent individual-animal prevalence of 6.2% and apparent herd prevalence of 58.3% in Burdur province (10), and apparent individual-animal prevalence of 3.5% and apparent herd prevalence of 41.6% in Kars province (9). Although the apparent individual prevalence for cattle was stated to be 4.6% in across the Turkey (8), Nielsen and Toft (18) noticed that an apparent prevalence of 4.6% could be converted to a true prevalence of 20% taking into account the sensitivity of the ELISA test used in that study.

The apparent individual prevalence of *Map* infection found in this study of 8% was similar to a previous estimated seroprevalence of 6.2% of randomly sampled cows from a serological study done 5 years ago in Burdur province (10). While the herd prevalence estimate (46.7%) was relatively stable (9, 10), the apparent individual prevalence (8%) was higher than those reported by other studies in Turkey (8, 9, 11). The results obtained in this study are difficult to compare with those of other studies, because of differences in the diagnostic test used, the study population, the sampling technique for both herds and cattle and the herd management (5, 18, 20, 23, 32, 33). However, in the study region, cattle herds were with closer contact between sheep and goat herds and this may have increased the opportunities for transmission to cattle herd. In addition, the reason for this may be related to the introduction of new animals into the cattle herd from different origins, because the prevalence values can vary by frequency of buying new animals from outside (5, 20, 33).

Nielsen and Toft (18) explained that the between-herd prevalence estimates for sheep and goats in European countries were higher than 20%, based only on estimates from Switzerland and Spain. A study in Slovenia suggests that the individual-animal and herd seroprevalence of *Map* infection in small ruminants is 3.5% and 11.6%, respectively (23). The apparent animal prevalence for sheep was found as 3.7% in Northeast of Portugal (25) and 3.3% in Serbia (34). The apparent animal and within-herd prevalences for goats were found as 2.9% and 5.9% in France (35). The animal, within-herd and between-herd apparent prevalence estimates reported in a Missouri (USA) study of goat herds were 1.9%, 2% and 36%, respectively (24). Although the presence of *Map* infection in goats and sheep has been known for a long time in Turkey (36, 37), only one previous study has been carried out to estimate its seroprevalence in sheep (13). In that study conducted in Kars sheep herds, the apparent individual-animal, within-herd and between-herd prevalences were calculated as 6.2%, 10.2% and 57.7%, respectively. At individual-animal, within-herd and between-herd levels, true prevalence values from the apparent prevalence by using the Rogan-Gladen method were estimated to be 8.3%, 14.6% and 90%, respectively. In this study, we also used Rogan-Gladen method for estimating the true prevalence and the true prevalence at individual-animal level was calculated as 100% for sheep and 35.9% for goats. The results of this study showed that *Map* infection is widespread in sheep and goat herds in Burdur province of Turkey (within-herd true prevalence for sheep and goats estimated to be 100% and 38.6%, respectively). The prevalence of *Map* in small ruminants found in this study is substantially higher than the other countries (23-25) and also Kars region of Turkey (13). The reason for this may be related to regional differences, frequency of purchasing animal, sampling methods and the

use of different ELISA kits (10, 18, 23, 24, 32, 33). Mixed housing and mixed-grazing of small and large ruminants is common in Burdur province. So we considered that the high prevalence values in sheep and goats may have originated from the contact of herds in villages close to each other, the use of the common pastures and also failure to take protective measures.

In this study, the animals aged 2 years and above were tested. Prevalence increased with age as reported the other studies (7, 10, 22, 25-27, 38), but there was no significant difference in seropositivity results among the age-groups. This can be explained with the absence of the equal number sampling in age-groups or lack of the great difference between the groups in terms of age. In contrast to previous studies which found that paratuberculosis is more prevalent in milk animals than in meat animals (5, 21, 23, 27, 28, 30), in this study no significant differences were found among breeds of cattle, sheep and goats. Therefore, we thought that it would be possibly related to the other herd factors such as the high within-herd *Map* prevalence and frequent mobility between herds of dairy animals rather than genetic or breed susceptibilities as reported by other researchers (24, 25).

The present study results indicated that the rate of seropositive animals was increased with increasing herd size. This is similar to previous studies (5, 10, 20, 25, 27, 33) which state that the seroprevalence of *Map* increases with herd size. This is not surprising because a higher density of animals may be increased the potential expose to contaminated feces. In sheep herds of the present study, the statistical analysis was not done because all of 15 herds were detected seropositive. But, there was no significant difference in seropositivity results among the cattle and goat herd size groups. This may be due to sampling carried out without considering herd size (18, 24).

In conclusion, *Map* infection is widespread in cattle, especially sheep and goats in Burdur and many measures are needed to reduce the percentage of infected herds. Reliable management practices together with test and culling eradication strategies in cattle, a vaccination approach in sheep and goats should be considered in herds with high within-herd prevalence.

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