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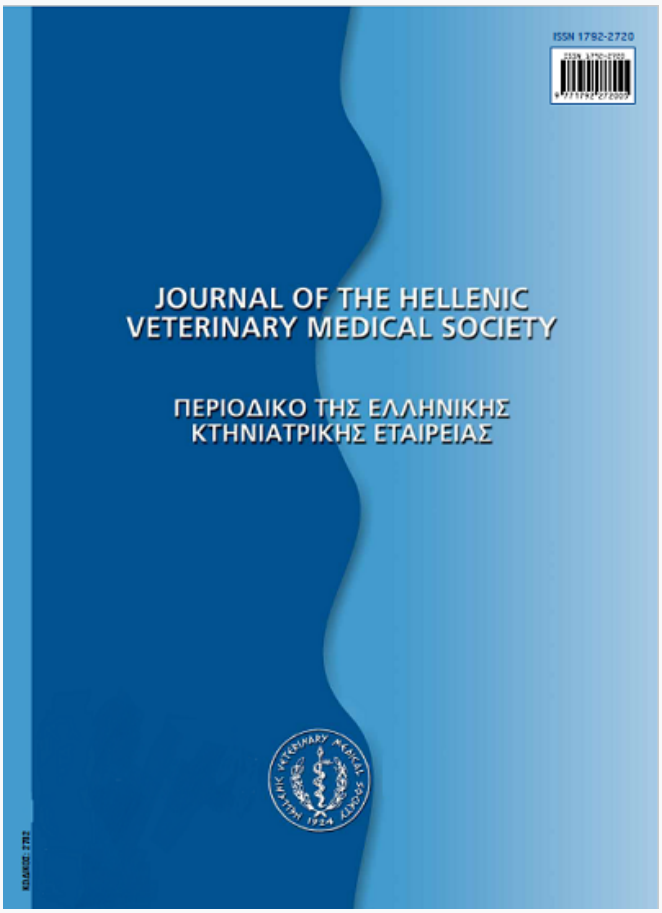

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Research article
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Seroprevalence of *Mycobacterium avium* Complex in Wild Mammals in the Iberian Peninsula

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ABSTRACT. A retrospective serologic survey was conducted for antibodies against MAC in a random sample of 623 free-ranging wild mammals killed on roads and by hunters, or found dead in east-central Portugal. Animals were tested for antibodies to *Mycobacterium avium* complex with a commercial enzyme linked assay.

The seroprevalence of *Mycobacterium avium* complex infection was 4.7% (n=29; CI 95%: 25.4 - 32.7%). Antibodies against MAC were detected in 4 out of 11 animal species included in this study, consisted of 1/42 red fox *(Vulpes vulpes)* (2.4%; CI 95%: 0.0-4.0%), 1/6 Eurasian otter *(Lutra lutra)* (16.7%), 1/3 European badger *(Meles meles)* (33.3%), and 26/109 wild boar (*Sus scrofa)* (23.9%; CI 95%: 17.8-34.2%). Infection was found in three taxonomic families: 2.4% (CI 95%: 0.0-4.0%) in Canidae, 16.7% (CI 95%: 0.0-37.8%) in Mustelidae, and 23.9% (CI 95%: 17.8-34.2%) in Suidae. No positive sera were found in the common genet, Egyptian mongoose, beech marten, hedgehog, wild rabbit, red deer or fallow deer.

Results of the present study indicate that antibodies against MAC were present in wild carnivores and wild boars in Iberian Peninsula. According to the test sensitivity and specificity claimed by the manufacturer, the true prevalence *Mycobacterium avium* complex infection among wild mammals in the Iberian Peninsula was calculated to be between 10.7% and 13.6%.

Keywords: *ELISA, Mycobacterium avium complex, seroprevalence, wild mammals*

INTRODUCTION

The members of *Mycobacterium* spp., comprising the *Mycobacterium avium* complex (MAC) consist a very interesting group in terms of ecology, differing in virulence and ecology and are the most frequently isolated nontuberculous mycobacteria (Mackenzie et al., 2009). The MAC comprises slow-growing mycobacteria that are ubiquitous in the environment (soil and water) and have a wide source range, causing disease in various mammals and birds (Mackenzie et al., 2009).

Mycobacterium avium complex has been divided into the subspecies *M. avium, M. paratuberculosis, M. silvaticum* and *M. hominissuis* (Mijs et al.,

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Table 1. Results of serologic survey of MAC infection in free-ranging wild animals from Portugal. Results are presented by species and taxonomic family.

Family	Species	Total collected *n*	Total Positive *n*	Antibody prevalence %	95% CI[a]
Canidae	Red fox (*Vulpes vulpes*)	42	1	2.4	25.4 - 32.7
Viverridae	Common genet (*Genetta genetta*)	1	0	0.0	—
	Egyptian mongoose (*Herpestes ichneumon*)	16	0	0.0	—
Mustelidae	Eurasian otter (*Lutra lutra*)	6	1	16.7	—
	European badger (*Meles meles*)	3	1	33.3	—
	Beech martem (*Martes foina*)	3	0	0.0	—
Erinaceidae	Hedgehog (*Erinaceus europaeus*)	2	0	0.0	—
Leporidae	European wild rabbits (*Oryctolagus cuniculus*)	3	0	0.0	—
Suidae	Wild boar (*Sus scrofa*)	109	26	23.9	17.8-34.2
Cervidae	Red deer (*Cervus elaphus*)	435	0	0.0	—
	Fallow deer (*Dama dama*)	3	0	0.0	—

aNot calculated for small samples

2002). The known host range of MAC includes ruminant and nonruminant wildlife. In wild ruminants, the MAC infections have been documented worldwide (Biet et al., 2005; Glawischnig et al., 2006). Although numerous wild species in the Canidae, Mustelidae and Viverridae are susceptible to function as agents of MAC complex-transmission (Matos et al., *2014*), the epizootiology of the infection is poorly understood. Infection with MAC species in wild boar has also been previously demonstrated using molecular and microbiological methods (Santos et al., 2009; Muñoz-Mendoza et al., 2013). MAC agents have also been isolated from kangaroos, macaques and mandrills (Biet et al., 2005). Presently the impact of MAC in wild mammals as well as on other indigenous wildlife species is largely unknown (Funk et al., 2001; Santos et al., 2009).

Determination of mycobacteria prevalence in wild

populations can provide new insights into likelihood of disease transmission. Information on potential pathogen exposure is necessary for monitoring the health of free-ranging wildlife populations (Munson and Karesh, 2002). Serological surveys are widely applied to study the presence and distribution of infectious diseases in wild animals (Curi et al., 2006; Fiorello et al., 2007).

The aim of this study was to investigate the occurrence of MAC antibodies in wild mammals killed on roads, found dead or killed by hunters in Portugal, to provide information that could be used to determine a prevalence and distribution of MAC in the Iberian Peninsula.

MATERIALS AND METHODS

Sampling

Between 2009 and 2013, a serologic survey for MAC was performed on serum samples from randomly selected free-ranging wild mammals found dead (killed on roads or otherwise) or hunted in Idanha-a-Nova (39°55′11″North, 7°14′12″West) and Penamacor (40°10′8″North, 7°10′14″West) in Castelo Branco, east-central Portugal.

A total of 623 wild mammals, representing 11 different species belonging to seven families, were examined (Table 1).

The state of carcass preservation according to the gross inspection, date and collection site was recorded. Information regarding age, sex, body condition and location of capture when available, was used to describe the distribution of seropositive individuals.

Samples from ungulates were obtained after hunting and the other wild mammal carcasses were transported to the pathology laboratory, and were subjected to a standard necropsy procedure prior to sample.

The blood samples were obtained from the thoracic cavity or the heart of the animals. In some cases blood was drawn from thawed carcasses and allowed to clot. Clotted blood was centrifuged at 2.000 *g* for 20 minutes and serum was collected and frozen at -20ºC. The samples were partly haemolysed.

Antibodies detection

All samples were tested for antibodies against MAC with an indirect enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ID Screen®, *Mycobacterium avium* Indirect Multi-Species, ID.vet, Innovate Diagnostics). The specific ELISA test is used to measure serum antibodies against *Mycobacterium avium* complex using an absorption step to remove non-specific antibody. On each 96-well plate, 92 serum samples were tested in single wells. The negative and positive control samples provided by the manufacturer were run in duplicate (first 4 wells). Results were expressed with the mean sample to positive ratio (S:P-ratio)=[OD_{450} of sample-OD_{450} negative contro/ [OD_{450} of positive control-OD_{450} negative control].

Following the manufacturer's instructions, readings equal to or below 40% of the positive control serum OD were considered as negative, readings equal to or greater than 50% as positive, and readings between 40 and 50% were scored as doubtful. Doubtful results were run in duplicate wells, with the same protocol. According to the manufacturer data and preliminary studies, utilized ELISA has a sensitivity between 34.48% and 44%, and a specificity of 100%.

Data Analysis

Antibody prevalence among foxes and wild boars and differences in prevalence among demographic categories in wild boar were compared with a chi-square test ($\chi 2$) (alpha<0.05) for statistical significance. The correlations between positivity and host factors such as gender, age, body condition and presence or absence of gross lesions was only analysed for wild boars because of the small number of other mammal species samples. True prevalence was calculated by the application of the Rogan–Gladen correction (Rogan and Gladen, 1978).

RESULTS

The seroprevalence of *Mycobacterium avium* complex infection was 4.7% (n=29; CI 95%: 25.4 - 32.7%). Antibodies against MAC were detected

in four out of 11 studied species: 1/42 red fox (*Vulpes vulpes*) (2.4%; CI 95%: 0.0-4.0%), 1/6 European otter (*Lutra lutra*) (16.7%), 1/3 badger (*Meles meles*) (33.3%), and 26/109 wild boar (*Sus scrofa*) (23.9%; CI 95%: 17.8-34.2%). Infection was found in three taxonomic families: 2.4% (CI 95%: 0.0-4.0%) in Canidae, 16.7% (CI 95%: 0.0-37.8%) in Mustelidae, and 23.9% (CI 95%: 17.8-34.2%) in Suidae. No positive sera were found in common genet, Egyptian mongoose, beech marten, hedgehog, wild rabbit, red deer or fallow deer. Seroprevalence of MAC was significantly higher (p=0.002) in wild boar (96.3%) than in red foxes (3.7%) but the small sample sizes prevented further analysis. When the estimated prevalence was adjusted for the sensitivity of 34.5% and 44%, and for the specificity of 100% claimed by the manufacturer and determined by preliminary studies (Lesceu and Pourquier, 2011; Eisenberg et al., 2012), the expected true prevalence of *Mycobacterium avium* complex infection among wild mammals in Iberian Peninsula was calculated to be 13.6% (95% CI: 10.9-16.3) for a sensitivity of 34.48%, and 10.7% (95% CI: 8.3-13.1) for a sensitivity of 44%.

Serologic reactivity data for all species examined are presented in Table 1.

Demographic data were unavailable for many samples but could be compared to wild boar. There were no statistical differences by age, gender, body condition or presence of gross lesions (Table 2).

Table 2. Prevalence of MAC antibody according to age class, gender, body condition and presence of gross lesions in wild boar.

Characteristic	Total collected *n*	Total Positive *n*	Antibody prevalence %	95% CI
Age class[a]				
Adult	46	14	30.4	3.9-24.0
Juvenile	29	6	20.7	0.0-14.6
Yearling	34	6	17.6	0.0-13.9
Gender[b]				
Female	55	16	29.1	6.3- 25.7
Male	54	10	18.5	2.0-18.0
Body condition[c]				
Good	70	15	21.4	6.6- 23.4
Moderate or Bad	39	11	28.2	1.2-20.8
Presence of gross lesions[d]				
Yes	70	20	28.6	10.6-29.4
No	39	6	15.4	0.0-13.5

n = number of wild boars identified in each characteristic
aPrevalence antibody to MAC not significant between different age classes (p= 0.372; chi-square test)
bPrevalence antibody to MAC not significant between different gender (p= 0.195; chi-square test)
cPrevalence antibody to MAC not significant between different body condition (p= 0.426; chi-square test)
dPrevalence antibody to MAC not significant between presence or absence of gross lesions (p= 0.426; chi-square test)

DISCUSSION

The present study represents the largest systematic serosurvey for MAC in wild mammals in Portugal, to date. Reports of *Mycobacterium avium* subsp. *paratuberculosis* serologically positive wild mammals in Iberian Peninsula include red deer (*Cervus elaphus*) (Reyes-García et al., 2008), European wild rabbit (*Oryctolagus cuniculus*) (Maio et al., 2011), and wild boar (*Sus scrofa*) (Boadella et al., 2011), but there was no previous report of MAC seropositivity in red foxes, Eurasian otter and European badger, and this study reports for the first time the presence of MAC antibodies in these species in the Iberian Peninsula.

Because serological methods, particularly ELISA, have not been widely used to assess the diagnosis of wild mammals MAC infection, it is difficult to compare the results obtained in this survey with the results presented in other studies. In this study apparent prevalence has been weighted to adjust to sensitivity of 34.5% and 44% and specificity of 100%, respectively, claimed by the manufacturer and determined by few preliminary studies (Lesceu and Pourquier, 2011; Eisenberg et al., 2012).

Evidence of MAC antibodies in Canidae, Mustelidae and Suidae families suggests that MAC can infect animals in multiple taxonomic groups, and confirms that infection in non-ruminant wildlife can also occur in Portugal.

Seven of the 11 wild mammal species studied were negative. The different sample size of wildlife species analyzed should also be taken into account; no evidence of infection was detected in the common genet, hedgehog, wild rabbit and fallow deer, but in these species only a few (1 to 3) animals were examined, therefore is not possible to make any conclusion about the role of these species in the epizootiology of MAC.

Although, the results indicate evidence of infection in the Eurasian otter and European badger, in this study the small sample sizes for these species caused an inability to calculate their prevalence estimates.

For some species the small sample sizes obtained were a reflection of their restricted range and low relative abundance, for example the Eurasian otter (IUCN, 2013).

One explanation for the negative results in red deer (n=435) might be that the animals tested in our study probably were in the preclinical stage of infection, and the antibody to MAC was simply not present. *Mycobacterium avium* complex in red deer in early infection is characterized by a strong cell-mediated immune response, while advanced stages with progressive lesions are associated with a humoral, antibody-producing immune response (Fawcett et al., 1995; Woodbury et al., 2008). Another explanation for the poor performance of ELISA in Cervidae may be that the protocol had not been optimized for use in these animals.

In Europe, MAC infection, particularly *Mycobacterium avium* subsp. *paratuberculosis* has been reported in wild ruminants such as red deer (*Cervus elaphus*) (Moravkova et al., 2008; Robino et al., 2008; Matos et al., 2013), fallow deer (*Dama dama*) (Marco et al., 2002) and roe deer (*Capreolus capreolus*) (Robino et al., 2008). This may indicate either a true lack of exposure to disease agents or a failure of the assay to detect animal antibodies in cervid animals despite reported cases of paratuberculosis in these animals in this region.

The high proportion of seropositive wild boar might indicate a high infection rate within the wild boar population studied. Antibody prevalence in wild boars suggests an endemic situation, with the mycobacteria continuously circulating in these populations.

There was no difference in prevalence of antibodies to MAC between adult and immature wild boar. Probably the lack of increased prevalence of infection in young animals reflects the importance of vertical transmission and may be an important component of new infections in Portuguese wild boars.

Our results confirm previous exposures to MAC in carnivore populations in Portugal (Matos et al., *2014*). The assay used in this study was not validated for carnivores, but it seems unlikely that the assay would recognize antibodies in red foxes but not in closely related carnivores. Some species may be less susceptible to infection with MAC or highly susceptible to fatal disease and unlikely to survive and produce antibodies.

The possible role of wild carnivores in the epidemiologic cycle of MAC in wildlife needs to be ascertained. The source of infection is unknown, but carnivores can be infected and develop antibodies by ingesting mycobacteria by consuming meat containing mycobacteria, and the natural diet of red foxes includes small mammals such as voles and rabbits, and birds. Wild carnivores can also be infected after scavenging infected wild ruminants (Beard et al., 2001; Tanner et al., 2006; Kidawa and Kowalczyk, 2011), and MAC has previously been demonstrated in the Iberian Peninsula (Balseiro et al., 2011). Moreover, wild canids should be regarded as a potential source of infection for other species (Curi et al., 2006).

This increased risk, compared to non-carnivores, alters the expected antibody prevalence of MAC in carnivores compared with herbivores such as red deer and wild rabbits. Our data suggests that red foxes in the Iberian Peninsula may have been infected through predation or scavenging of species other than red deer, such as wild boar.

The use of serology limits us to discussing the exposure and not the disease, and little is known of the clinical significance of MAC infection in free-ranging red foxes, wild boars, Eurasian otters or European badgers. No suggestive clinical signs of MAC infection or tuberculosis were verified.

The significance of MAC seropositive animals is unknown. Seroconversion of identified species has not been reported. The presence of MAC antibodies in carnivores may be used as a marker of contact with wild boars.

Antibody prevalence rates reported in this study may underestimate the true prevalence because the serum from decomposed carcasses might have reduced sensitivity (Santos et al., 2009), though previous studies have shown that antibodies can be detected in body fluids from decomposed carcasses up to 11 days post-mortem (albeit at decreasing titers) and are valuable for serologic testing (Tryland et al., 2006; Santos et al., 2009). In this study, correlation between positivity and putrefaction was not analysed because of the small number of carnivores.

Serological surveys are useful for active surveillance and as a diagnostic tool, however, they are influenced by the density and geographical distribution of species and the time of year (Jakubek et al., 2012). Availability of serum samples from wild animals is often limited by lack of opportunities to collect large numbers of samples from several sites, thus the collection of sera at hunter-check stations is an expedient approach. Haemolysis and dilution of samples are, however, potential disadvantages of this sample method (van der Leek et al., 1993).

CONCLUDING REMARKS

Results of the present study indicate that antibodies against MAC are present in wild carnivores and wild boars in Iberian Peninsula. Although based on a limited sample, these data suggest widespread MAC exposure among free-ranging Iberian wild mammals in Portugal, especially red foxes and wild boars. There is an urgent need for a risk assessment study to elucidate the importance of MAC infection in wild animals.

There is also a public health concern, and natural reservoirs should be investigated in order to formulate control plans. Diagnostic tools such as molecular methods validated for wild species are also necessary to explain the role of wildlife in the infection process. In future, other studies should be done to identify and characterize the causative microorganisms involved in the infection, creating a better understanding of the natural history and epizootiology of these infections in free-ranging mammals.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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