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Short title: Avian mycobacteriosis in raptors in Majorca.

Abstract

Avian mycobacteriosis is a chronic, infectious disease caused by different species of mycobacteria, usually belonging to the *Mycobacterium avium* complex (MAC). From 2004 to 2007, 589 raptors brought dead or sick to a wildlife rehabilitation centre in Majorca (Balearic Islands, Spain) were necropsied. The birds belonged to 12 different species, chiefly common kestrel (*Falco tinnunculus*) (n=297), scops owl (*Otus scops*) (n=109), barn owl (*Tyto alba*) (n=75), long-eared owl (*Asio otus*) (n=58), peregrine falcon (*Falco peregrinus*) (n=27), and booted eagle (*Hieraaetus pennatus*) (n=13). Gross lesions compatible with mycobacteriosis were observed in 14 birds (2.4%) found in several locations in Majorca. They were 12 kestrels (prevalence in this species=4.0%), one long-eared owl (1.7%) and one scops owl (0.9%), all the birds presenting white-yellowish nodules from pinpoint size to 1 cm in diameter in diverse organs, mainly in liver, spleen and intestine. Affected organs were subjected to bacteriology and molecular identification by PCR and, in all the cases, infection with *Mycobacterium avium* subsp. *avium* was confirmed. The observed prevalences are similar to those previously observed in Holland, though the actual prevalence detected in this study is likely to be higher than reported because only birds with gross lesions were subjected to culture. Further molecular characterization with a set of six MIRU-VNTR loci was used to sub-typify the isolates in order to show the existence of possible epidemiological links. Six different genotypes were found, which points to infection from multiple focuses. No temporal or geographical aggregation of the cases was observed associated to the presence of positive birds or to the different VNTR allelic profiles. The most feasible origin might be water or food sources, though the reservoir of mycobacteria remains unknown.

Introduction

Avian mycobacteriosis (AM) is a chronic, infectious disease caused by *Mycobacterium avium* complex (MAC) organisms (Thoen, 1997; Thorel *et al.*, 1997), although other mycobacteria can produce mycobacteriosis in birds (Hoop *et al.*, 1996; Tell *et al.*, 2001). All avian species are susceptible to infection by the MAC, although some bird orders, such as Galliformes, Columbiformes or Gruiformes may be more susceptible to the disease (Friend, 1999). AM is a relatively common disease in domestic flocks (González *et al.*, 2002; Bougiouklis *et al.*, 2005), or in captive wild birds (Singbeil *et al.*, 1993; Marco *et al.*, 2000; Dvorska *et al.*, 2007). This is mainly due to the persistence of the organism once established, its contagiousness and its ability to survive in the environment (Thorel *et al.*, 1997). Nevertheless, reports of AM in free-living wild birds are not uncommon (Smit *et al.*, 1987; Millán *et al.*, 2004; Gerhold & Fischer, 2005).

Captive raptorial birds are not free of suffering from AM (van Nie *et al.*, 1982). However, in free-living raptors, cases are only sporadically reported. Kaliner and Cooper (1973) detected a case of AM in an African fish eagle (*Haliaeetus vocifer*). In North America, single cases of AM were reported in two red-tailed hawks (*Buteo jamaicensis*) (Emerson *et al.*, 1970; Sykes, 1982) and in two American bald eagles (*Haliaeetus leucocephalus*) (Hoenerhoff *et al.*, 2004; Heatley *et al.*, 2007). In Europe, reports include those of two common kestrels (*Falco tinnunculus*) (Wilson 1960; Bucke & Mawdesley-Thomas 1974) and a barn owl (*Tyto alba*) (Wilson 1960). Smit *et al.* (1987) isolated *M. avium* from multiple dead-found raptor species, both diurnal and nocturnal. The only reported case in Spain was detected in a Eurasian goshawk (*Accipiter gentilis*) from southern peninsular Spain (Aranaz *et al.*, 1997). In the present article we report the

prevalence of AM in free-living raptors that were brought to a wildlife rehabilitation centre at Majorca Island, Spain.

Material and Methods

In the period 2004-2007, 589 free-living raptors were submitted for necropsy at the "Cofib" rehabilitation centre at Majorca (Table 1). When gross lesions compatible with mycobacteriosis (e.g. granuloma) were identified, tissue samples of the affected organs were frozen at -20°C and analyzed for the detection and identification of mycobacterias. Tissues from each animal were homogenized with sterile distilled water and decontaminated with 0.35% hexadecylpyridinium chloride (Sigma Aldrich Chemie GmbH, Buch, Switzerland) for 30 min (Corner and Trajstman, 1988), centrifuged at 1,068 x g for 30 min and cultured onto Colestos, 0.2% (wt/vol) pyruvate-enriched Löwenstein-Jensen media (bioMèrieux España and Biomedics, Madrid, Spain) and Middlebrook 7H10 (Biocult Laboratorios, Madrid, Spain) at 37°C. Isolates were identified by staining for acid-alcohol fastness, a multiplex PCR amplification targeting the *Mycobacterium* specific 16S rRNA fragment (Boddinghaus *et al.*, 1990; Wilton and Cousins, 1992), and PCRs aimed at specific insertion sequences IS*901* and IS*1245* (Kunze *et al.*, 1992; Guerrero *et al.*, 1995).

Recent studies have shown the usefulness of tandem repeats or repetitive units to characterize members of MAC (Bull *et al.*, 2003; Thibault *et al.*, 2007; Mobius *et al.*, 2008). Included within these tandem repeats are the variable number tandem repeats (VNTR), which are repetitive units dispersed throughout the genome with a length between 10-100 bp (Supply *et al.*, 2000); and also the mycobacterial interspersed repetitive units (MIRU), small interspersed repetitive elements described by Supply *et al.* (1997) in

the genome of *M. tuberculosis*. Isolates of *M a. avium* were sub-typified by MIRU-VNTR with six specific loci previously published: MIRU-1, MIRU-2, MIRU-3, MIRU-4 (Bull *et al.* 2003), VNTR-25 and VNTR-32 (Thibault *et al.* 2007; Mobius *et al.* 2008), using the same specific primers described (Table 2).

PCR mix contained 5 µl of sample DNA, 1x standard reaction buffer with 1.5 mM MgCl$_2$, 200 µM deoxynucleoside triphosphate (Biotools, B&M Labs S.A, Madrid, Spain), forward and reverse primers (Roche Diagnostics S.L., St. Cugat del Vallés, Spain) (0.4 µM), 10% of DMSO (Sigma Aldrich Chemie GmbH) and 2.5 U of Hot-Start-Taq-Polymerase (Qiagen GmbH, Hilden, Germany), in a final volume of 50 µl. For MIRU-3, primer concentrations were three times higher than for the rest of the loci (Mobius *et al.* 2008).

The amplification reactions for these MIRU-VNTR loci were: denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 1 min, extension at 72°C for 2 min, with a final cycle of extension at 72°C for 10 min; conditions reported by Frothingham and Meeker-O'Connell (1998) with slight modifications. In the case of MIRU-3, MIRU-4 and VNTR-32 the same recommended melting temperatures (Mobius *et al.* 2008; Thibault *et al.* 2007) were used.

Finally, PCR amplicons were separated in a 2.5% agarose gel, stained with SYBR safe (Invitrogen, product no. 531478) and using 100 bp molecular ladder (Biotools B&M Labs, Madrid, Spain) to detect the variation in the number of repeats for each of the locus.

Chi^2 test was used to analyze differences in the observed prevalence among bird species and years.

Results

Overall, 2.4% of the necropsied raptors presented gross lesions compatible with AM. Tissue samples from all the birds with lesions were cultured and then *M. avium* subsp. *avium* was identified by PCR amplification of the insertion sequences IS*901* and IS*1245*. Lesions were observed in three species: 12 of 297 common kestrels (4.0%), one of 58 long-eared owls (*Asio otus*) (1.7%) and one of 109 scops owls (*Otus scops*) (0.9%). No lesions were observed in the other species analyzed (Table 1). The kestrels were 9 adult birds (5 males, 4 females) and 3 sub-adults between 1 and 2 years-old (2 males, 1 female); the long-eared owl was an adult female; and the scops owl was a sub-adult female. The affected birds arrived throughout the year, dead or sick, from multiple locations in Majorca (Figure 1). Non-affected raptors also arrived from all around the island, including from the locations were infected birds were found.

Infected birds included road-killed birds (n=5) as well as debilitated birds (n=9). Of the latter, one bird suffered from gunshot wounds while the others presented no traumatic lesions. The latter birds arrived as a rule emaciated but did not present other clinical signs. With the exception of one kestrel that survived for eight months after arrival, the other affected birds died within few days (usually 1-2 days, mean= 11.8 ±16.9 hours). Another kestrel was euthanatized two months after arrival for humane reasons. On necropsy, all the birds were cachectic and presented white-yellowish nodules from pinpoint size to 1 cm in diameter in diverse organs, chiefly in liver, spleen and intestine (Table 3 and Figure 2). Lesions did not markedly differ among species. No other signs were observed in the infected birds, with the exception of one kestrel in which oral lesions of trichomoniasis confirmed by microscopy were observed.

Regarding MIRU-VNTR typing, only MIRU-3 and MIRU-4 showed allelic variation among the *M. a. avium* isolates tested with five and two allelic variants

respectively. The combination of loci allowed the differentiation into six different genotypes, where allelic profile number 1 (1-8-1-5-9-5) and 5 (1-8-1-5-7-5) represented 42.86% and 28.57% of the isolates tested respectively (Table 4). The two isolates from the scops owl and the long-eared owl were both clustered into the most common allelic profile no.1, which was shared with four isolates from kestrel.

No differences were observed in the prevalence of AM lesions among years. Prevalence of AM lesions was higher in common kestrel than in barn owl, though differences were only residually significant (Chi^2=3.13, p=0.07).

Discussion

Other than the single goshawk reported by Aranaz *et al.* (1997), this is the first report of widespread AM in raptorial birds from Spain. As mentioned above, AM in free-living raptors has rarely been diagnosed. In the Netherlands, Smit *et al.* (1987) isolated *M. avium* from, among other raptors, 17/450 (3.8%) of common kestrels, and in 3/313 (0.96%) of long-eared owls. Those prevalences are similar to these reported in the present article. As mentioned before, a case of AM was also reported in one common kestrel from UK (Wilson, 1960). As far as we know, AM was not previously diagnosed in a scops owl. However, it is worth to note that the prevalence of mycobacteriosis was likely to be higher than reported here, because only tissue samples from birds with gross lesions were subjected to culture.

Affected birds were mostly adults. This is in accordance with most of the previous reports of AM in raptors (Sykes, 1982), or in other wild birds (Millán *et al.*, 2004). Older animals are more commonly affected by mycobacteriosis because of the long incubation

period and accumulated risk of exposure (Thoen, 1997). According to Thorel *et al.* (1997), affected birds die within two months or may survive for six months depending on the extent of disease.

On necropsy, macroscopic lesions were seen in the liver, spleen and intestine of all the affected raptors, which is the typical feature of AM in birds (Skyes, 1982; Thorel *et al.*, 1997; Millán *et al.*, 2004; Saggese *et al.*, 2008). According to the distribution of the lesions, the affected birds probably contracted the infection by ingestion. However, due to the presence of lesion in the lungs in one third of the cases, an inhalation route should not be excluded (Kaliner & Cooper, 1973).

The source of the infection remains unknown. The hypothesis of a nosocomial infection can be ruled out due to the affected birds arrived dead or survived for a short period. Smit *et al.* (1987) proposed that kestrels and other aggressive raptors may contract AM through injuries during fights, and even isolated *M. avium* from some local injuries. However, in the present case no infected injuries were observed in any of these birds, though subcutaneous granulomas were detected in seven birds that may be derived from old injuries. In any case, the most feasible origin of the mycobacterias might be water or food sources. Therefore, feeding ecology may explain the differences in prevalence among species. Very little is known about the diet of the three affected species in Majorca. In the Mediterranean part of the peninsular Spain, the common kestrel feeds mainly upon lizards (*Lacerta* spp.), which are no present on Majorca, passerines, several species of insects and micromammals (Valverde, 1967; Veiga, 1985). For the long-eared owl, the most important prey species are Murinae, followed by Arvicolinae (not present in Balearic Islands) and passerine birds (Corral *et al.*; 1979), although this species depends less on rodents in Mediterranean regions (García & Cervera, 2001). However, the barn owl also feeds mainly on rodents in Balearic Islands (Sommer *et al.*, 2005), and no individual out of 75

necropsied was apparently affected. If rodents were a major source of mycobacteria, this would indicate a species specific difference in susceptibility, with barn owls being less susceptible. However, several cases of AM have been reported in the species (Wilson, 1960; Bucke & Mawdesley-Thomas, 1974). Thus, differences may be related to prey accessibility more than to susceptibility.

According to Friend (1999), AM is most commonly seen in scavenger species. Thus, an alternative hypothesis of the infections is that the affected birds contracted AM through the scavenging of carcasses of diseased red-legged partridges or pigeons (*Columba* spp.), which consitute too large a prey for the affected raptors (Calderón, 1977). Due to the intense, often illegal predator control carried out in Spanish hunting estates, sick animals are not removed from the environment by carnivores, developing disseminated AM and even dying by the disease, as has been sporadically observed in partridges (Millán *et al.*, 2004).

Regarding the molecular characterization by MIRU-VNTR, it discriminated the *M. a. avium* isolates into six different genotypes. From the loci included for the analysis, MIRU-3 yielded the highest number of allelic variants in comparison with the rest of the loci. MIRU-3 is located between a two-component regulatory system SenX3-RegX3. Former studies found the presence of a lower number of TRs at this locus in the *M. bovis* (BCG) and *M. avium* subspecies *paratuberculosis* (316F) vaccine strains in comparison to the rest of the strains they tested (Bull *et al.* 2003; Magdalena *et al.* 1998). Probably differences in pathogenesis and progression of the disease have a relationship with the variation of TR units at this particular locus. To our knowledge, this is the first analysis of *M. a. avium* isolates from animals using MIRU-VNTR typing; therefore we cannot further assess our results in comparison to other populations. However, available information from literature shows that the genotypes isolated from raptors described in this report would be

related to that described for the *M. a. avium* reference strain ATCC 25291 (1-7-1-5-5-5) (Bull et al., 2003; Inagaki et al., 2009). Further studies concerning this issue would be necessary.

In the light of the cases presented in this article, AM must be considered a factor in the conservation of raptors in Majorca and the rest of the Balearic Islands. An undetected source is acting as a reservoir of mycobacteria that is currently affecting raptors and potentially may affect other protected species, livestock, or humans. As the infected raptors were retrieved in several points of the Majorca Island, and six different genotypes were found, there may be multiple infection focuses. However, though the affected species are territorial, could have become infected in other locations different from where they were found. In addition, some of the affected birds could be migrant individuals and may have become infected in remote locations. Studies to detect the different reservoirs of this infection in Majorca are currently being performed.

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Figure Legends

Figure 1. *Location of the cases and the distribution of VNTR allelic profiles of avian tuberculosis in raptors in Majorca Island (Spain).*

Figure 2. *Avian tuberculosis granulomas in a scops owl (A) and two common kestrels (B, C). A: in liver (box), lung (arrowhead), spleen (dotted arrow) and intestine (arrow); B: in liver (arrow) and subcutaneous tissue (arrowhead); C: in lung.*

Table 1. *Species of raptor necropsied per year and prevalence (in %) of avian mycobacteriosis in a wildlife rehabilitation centre in Majorca Island (Spain).*

	2004		2005		2006		2007		Total	
	n (pos.)	prev.	n (pos.)	prev.	n (pos.)	prev.	n (pos.)	prev.	n (pos.)	prev. (95% C.I.)
Accipitriformes										
Hieraaetus pennatus	2	-	7	-	5	-	0	-	13	0 (0-19)
Accipiter nisus	0	-	1	-	1	-	2	-	4	0 (0-53)
Circus pygargus	0	-	0	-	0	-	1	-	1	0 (0-95)
Circus aeruginosus	0	-	1	-	1	-	0	-	2	0 (0-77)
Pandion haliaetus	0	-	1	-	0	-	0	-	1	0 (0-95)
Aegypius monachus	0	-	0	-	1	-	0	-	1	0 (0-95)
Falconiformes										
Falco peregrinus	5	-	8	-	10	-	4	-	27	0 (0-14)
Falco eleonorae	0	-	1	-	0	-	0	-	1	0 (0-95)
Falco tinnunculus	47 (2)	4.2	89 (2)	2.2	87 (6)	6.9	74 (2)	2.7	297 (12)	4.0 (3-7)
Strigiformes										
Tyto alba	15	-	19	-	21	-	20	-	75	0 (0-5)
Asio otus	10	-	12	-	12 (1)	8.3	24	-	58 (1)	1.7 (0-9)
Otus scops	29	-	40	-	26	-	14 (1)	7.1	109 (1)	0.9 (0-4)

**Table 2**.*List of primers and melting temperatures [Tm (^{o}C)] for the MIRU-VNTR loci used in this study.*

VNTR-MIRU	Reference	Primer F (5'-3')	Primer R (5'-3')	Tm
MIRU-1	Bull *et al.* 2003	cgcggacttgatggtctc	ccgttgtccaggtggagt	58
MIRU-2	Bull *et al.* 2003	gaacgaagatcctgggactg	cgacgacgaacacctcaac	58
MIRU-3	Bull *et al.* 2003	acattcaccctgtccattcc	cctccttacggagcaggaa	52
MIRU-4	Bull *et al.* 2003	cgttcagcctgtgcatgg	caagtcgtcacgggcaac	53
25	Thibault *et al.* 2007	gtcaagggatcggcgagg	tggacttgagcacggtcat	58
32	Thibault *et al.* 2007	ccacagggtttttggtgaag	ggaaatccaacagcaaggac	55

Table 3. *Locations of the granulomas due to avian mycobacteriosis observed in 14 raptors in Majorca Island (Spain). Some individuals*

presented lesions in multiple organs.

Organ / Tissue	n	Locations
Liver	14	
Spleen	14	
Intestine	14	
Subcutaneous tissue	7	Neck (7), mandible (1)
Gizzard	7	
Lung	5	
Kidney	4	
Pancreas	1	
Muscle	3	Stifle (2), intercostal (2), pectoral (1), neck (1)
Substernal serosa	1	
Oral mucosa	1	Sublingual

Table 4. *Number or TRs and allelic variants observed in the Mycobacterium avium subspecies avium isolates with the combination of loci used.*

No. of *M. a. avium* isolates	Animal species	MIRU-VNTR locus						Allelic profile
		VNTR-25	VNTR-32	MIRU-1	MIRU-2	MIRU-3	MIRU-4	
n=6	*Falco tinnunculus* (n=4), *Asio otus* (n=1), *Otus scops* (n=1)	1	8	1	5	9	5	1
n=1	*Falco tinnunculus*	1	8	1	5	5	5	2
n=1	*Falco tinnunculus*	1	8	1	5	3	5	3
n=1	*Falco tinnunculus*	1	8	1	5	5	3	4
n=4	*Falco tinnunculus*	1	8	1	5	7	5	5
n=1	*Falco tinnunculus*	1	8	1	5	12	5	6
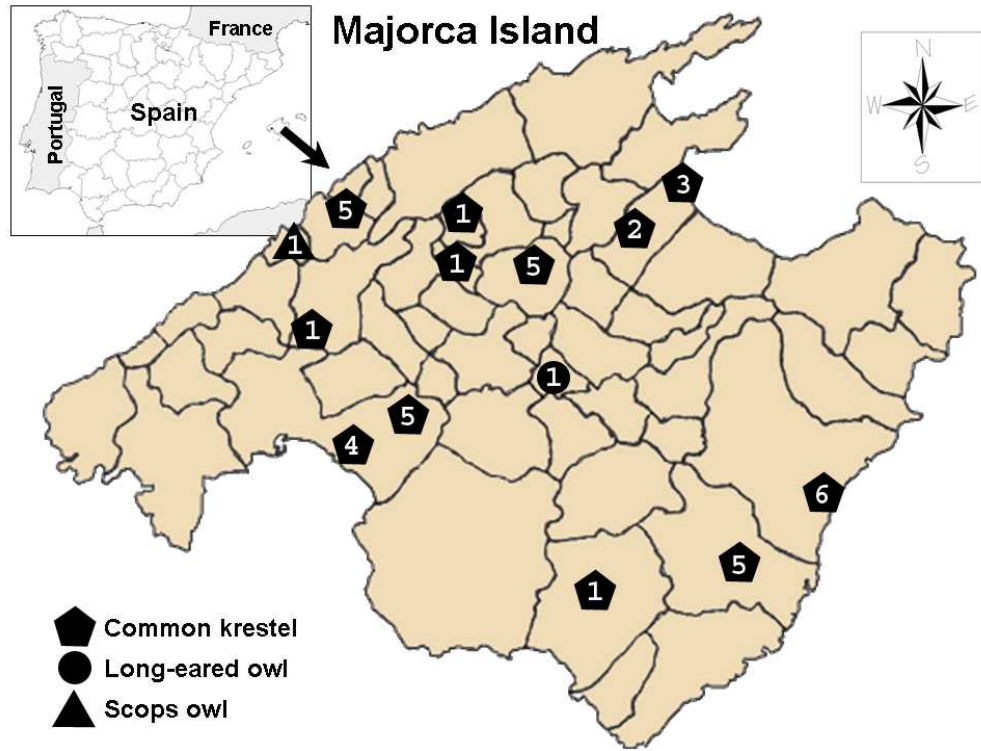
81x61mm (300 x 300 DPI)

Avian tuberculosis granulomas in a scops owl (A) and two common kestrels (B, C). A: in liver (box), lung (arrowhead), spleen (dotted arrow) and intestine (arrow); B: in liver (arrow) and subcutaneous tissue (arrowhead); C: in lung.
82x53mm (300 x 300 DPI)