Molecular screening of *Nocardia* spp. in wild mammals: a cross-sectional study

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Abstract

Nocardiosis is an emerging zoonosis caused by *Nocardia* spp. bacteria. The disease is linked to infections in immunocompromised individuals, and is potentially lethal when it turns systemic and is left untreated. The development of reliable and valid diagnostic methods is crucial to the identification of these pathogenic agents, particularly in wild mammals, which are potential reservoirs. The aim of this study was to determine the molecular prevalence of *Nocardia* spp. in wild mammals in Portugal. A sample of 114 wild mammals belonging to the orders *Canidae, Cervidae, Erinaceidae, Herpestidae, Leporidae, Mustelidae, Viverridae*, and *Suidae* were studied. Tissue samples (*n*=206) were collected from different organs. The molecular prevalence of *Nocardia* in wild mammals was 55.3% (95% CI: 45.7-64.6%). *Nocardia* spp. were detected in nine of ten species studied: 83.3% (95% CI: 62.2-100%) in Egyptian mongoose (*Herpestes ichneumon*), 63.2% (95% CI: 41.5-89.9%) in red fox (*Vulpes vulpes*), 45.9% (95% CI: 29.8-62.0%) in red deer (*Cervus elaphus*), 44.4% (95% CI: 28.2-60.6%) in wild boar (*Sus scrofa*), 3/3 beech martens (*Martes foina*), 2/2 Eurasian otters (*Lutra lutra*), 1/1 European badger (*Meles meles*), 1/1 genet (*Genetta genetta*), and 1/2 hedgehog (*Erinaceus europeaeus*). Of the 206 tissue samples studied by molecular techniques, *Nocardia* spp. were detected in 37.4% (95% CI: 36.7-38.1%), with a predominance in mesenteric lymph nodes (13.1%; 95% CI: 12.4-13.9%) and kidneys (9.2%; 95% CI: 8.5-9.9%). These results provide new insight into the prevalence of *Nocardia* in wild mammals and highlight the need for surveillance of wildlife as a potential reservoir of these emergent pathogens.

Key words: *Nocardia spp.; PCR, Portugal, wild mammals*

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Introduction

Nocardiosis is an emergent granulomatous zoonosis that until the early 21st century was considered a rare opportunistic disease and only a clinical curiosity (Corti and Villafane-Fioti, 2003; Rodrigues et al., 2009; Li et al., 2020). In fact, nocardiosis typically manifests as an opportunistic disease, with 60% of affected patients having immunosuppressive conditions, though infection may also develop in immunocompetent patients. The most common clinical presentation in immunocompetent patients includes superficial cutaneous disease, lymphocutaneous disease, mycetomas, and eye infections that may occur after traumatic inoculation (Ambrosioni et al., 2010; Baio et al., 2013).

Nocardia spp. are Gram-positive filamentous rods, and members of the family Nocardiaceae (together with the genus *Rhodococcus*). They are difficult to isolate in culture due to their fastidious growth (3–5 days). In humans, they can cause pulmonary infection, most commonly, primary cutaneous infection and dissemination to other sites as the brain or other internal organs, thus becoming systemic and potentially fatal (Abreu et al., 2015; Conville et al., 2017).

The increase of cases of infection by *Nocardia* spp. in domestic or wild animals included in consumption, and in humans themselves, has contributed to the status of this emerging zoonosis (Condas et al., 2015; Matos et al., 2015; Eroksuz et al., 2016; Díaz-Santana et al., 2022). Currently, there are more than 100 described species of *Nocardia*, with 30 of these being important to humans (Brown-Elliott et al., 2006; Betrán et al., 2016; Lebeaux et al., 2019). The use of molecular techniques, such as amplification by polymerase chain reaction (PCR) of the gene encoding RNA of ribosome subunit 16 (Torres et al., 1996; Kandi, 2015) has allowed new species to be identified or existing species to be differentiated (Conville et al., 2000). These different species have already been found in domestic animals (Biberstein et al., 1985; Emeruwa, 1986; Maldonado et al., 2004; Ribeiro et al., 2008; Mund et al., 2021; Yoon et al., 2022) and in wildlife (Gezuele, 1972; Kinde et al., 1992; Vemireddi et al., 2007; Domenis et al., 2009; Matos et al., 2015; Ito et al., 2021).

The number of reported cases of nocardiosis has gradually increased worldwide. In North America, according to the Center for Disease Control and Prevention (CDC), between 500 and 1000 new cases of *Nocardia* spp. infection occur annually (Bafghi et al., 2014).

To the best of the authors' knowledge, in the Iberian Peninsula, *Nocardia* spp. have only been found in wild boar (*Sus scrofa*) (Matos et al., 2015), with no cases of nocardiosis reported from red fox (*Vulpes vulpes*), Egyptian mongoose (*Herpestes ichneumon*), European badger (*Meles meles*), Eurasian otter (*Lutra lutra*), red deer (*Cervus elaphus*), genet (*Genetta genetta*) or hedgehog (*Erinaceus europaeus*).

Determination of *Nocardia* prevalence in wild populations can provide new insight into the likelihood of pathogen transmission. The aim of this study was to determine the molecular prevalence of *Nocardia* spp. in wild mammals in Portugal.

Material and Methods

Population and samples

A cross-sectional study was conducted to estimate the molecular prevalence of *Nocardia* spp. in samples from wild mammals in Portugal.

Between 2010 and 2015, during the hunting season, a molecular study of *Nocardia* spp. was performed on 206 tissue samples from 114 randomly obtained free-ranging wild mammals found dead (on roads or otherwise) or hunted in the municipalities of Idanha-a-Nova (39°55′11″North, 7°14′12″West) and Penamacor (40°10′8″North, 7°10′14″West), district of Castelo Branco, east-central Portugal.

The examined 114 wild mammals represented 10 different species belonging to eight taxonomic families (Table 1). Samples from ungulates were obtained immediately after hunting, while the carcasses of the other wild mammals were transported to the Laboratory of Histopathology of the University of Trás-os-Montes and Alto Douro, Portugal, where they were subject to a standard necropsy procedure prior to sampling. Collection site, date, and state of carcass preservation according to gross inspection were recorded.

Histopathology and culture

At necropsy, samples of the mesenteric lymph nodes, mediastinal lymph nodes, retropharyngeal lymph nodes, kidneys, lungs, bladder, tonsils, ileocecal valves, brain, and liver were collected for molecular and histopathological analyses. Samples for PCR were frozen at -20ºC before processing. Samples for histopathology were fixed in 10% neutral buffered formalin, routinely processed for paraffin embedding and stained with haematoxylin & eosin and by the Ziehl–Neelsen method for acid-fast bacteria (AFB). Cultures were performed with tissue samples according to established microbiological protocols (Saubolle and Sussland 2003). Briefly, material was inoculated onto Columbia blood agar plates, and incubated at 35°C for 48 h.

DNA extraction and PCR amplification

Genomic DNA was extracted from all samples using the DNeasy Blood and Tissue kit (Qiagen®, Hilden, Germany). NG1 (5′ACCGACCACAAGGGG3′) and NG2 (GGTTGTAACCTCTTCGA) primers were used for PCR amplification of a 16S ribosomal RNA (rRNA) gene segment specific for the genus *Nocardia* (Laurent et al., 1999). PCR was performed in a 20 μL volume using the Taq Master Mix Bioron® (2x), 1 μL each primer (10 μM) and 30 ng DNA. Positive and negative controls were tested in each PCR cycle. *Nocardia* DNA was used as the positive control and sterile water as the negative control. Amplification was performed in a thermocycler programmed as follows: 10 min for the initial denaturation step at 94ºC, 30 cycles (94ºC for 60 s, 52 ºC for 20 s, 72 ºC for 60 s) and a 10 min final extension step at 72ºC. Amplification was evaluated by means of 1% agarose gel electrophoresis and the presence of a 596 bp fragment (Laurent et al., 1999) was regarded as a positive result. Gel visualisation and image capture were performed with the Molecular Imager Gel DocTM XR equipment associated with the Image LabTM software (Bio-Rad, Hercules, CA, USA).

Data analysis

For this study, an animal was regarded as infected if *Nocardia* was detected by PCR in at least one tissue. Molecular prevalence of *Nocardia* spp. was described as absolute frequencies, percentages and 95% confidence intervals. The chi-square test compared molecular positivity values between demographic variables. A $p < 0.05$ was considered as statistically significant. Data analysis was performed using SPSS® 25.

Results

Based on PCR, 63 out of the 114 wild mammals were found infected with *Nocardia* spp. The molecular prevalence of *Nocardia* spp. in animals was 55.3% (95% CI: 45.7-64.6%). *Nocardia* spp. was detected in nine of the ten species examined (Table 1): 12/19 red foxes (*V. vulpes*) (63.2%; 95% CI: 41.5-84.9%), 3/3 beech martens (*Martes foina*) (100%), 2/2 Eurasian otters (*L. lutra*) (100%), 1/1 European badger (*M. meles*) (100%), 1/1 genet (*G. genetta*) (100%), 10/12 Egyptian mongooses (*H. ichneumon*) (83.3%; 95% CI: 62.2-100%), 1/2 hedgehogs (*E. europaeus*) (50.0%; 95% CI: 1.3-98.7%), 16/36 wild boar (*S. scrofa*) (44.4%; 95% CI: 28.2-60.6%), 17/37 red deer (*C. elaphus*) (45.9%; 29.8-62.0%). Infection was found in seven taxonomic families: *Canidae* (63.2% positive), *Cervidae* (55.3%), *Erinaceidae* (50.0%), *Herpestidae* (83.3%), *Mustelidae* (100%), *Suidae* (44.4%), and *Viverridae* (100%). No positive tissues were found from wild rabbits (*Oryctolagus cuniculus*). *Nocardia* spp. was not confirmed in culture.

Of the 206 tissue samples studied by PCR, *Nocardia* spp. were detected in 77 tissues (37.4%; 95% CI: 36.7-38.1%), mainly in mesenteric lymph nodes (13.1%; 95% CI: 12.4-13.9%) and kidneys (9.2%; 95% CI: 8.5–9.9%). In addition, the microorganism was detected in other tissues such as lungs (5.3%; 95% CI: 4.6-5.9%), bladder (2.4%; 95% CI: 1.7-3.1%), mediastinal lymph nodes (2.4%; 95% CI: 1.7-3.1%), tonsils (1.9%; 95% CI: 1.2-2.6%), retropharyngeal lymph nodes (0.97%; 95% CI: 0.3-1.7%), ileocecal valves (0.97%; 95% CI: 0.3-1.7%), brain (0.49%; 95% CI: 0.2-1.2%) and liver (0.49%; 95% CI: 0.2-1.2%). The differences were found to be statistically significant (P=0.019) (Table 2).

Table 1. Distribution of *Nocardia* spp. by taxonomic families and species in wild animals collected and examined between 2010 and 2015

Family/species		Total *N*	Total positive *n*	Prevalence %	95% CI
Canidae (*n*=19)	Red fox (*Vulpes vulpes*)	19	12	63.2	41.5 – 84.9
Cervidae (*n*=37)	Red deer (*Cervus elaphus*)	37	17	45.9	29.8 – 62.0
Erinaceidae (*n*=2)	Hedgehog (*Erinaceus europaeus*)	2	1	–	1.3–98.7
Herpestidae (*n*=12)	Egyptian mongoose (*Herpestes ichneumon*)	12	10	83.3	62.2 - 100
Leporidae (*n*=1)	Wild rabbit (*Oryctolagus cuniculus*)	1	0	–	–
Mustelidae (*n*=6)	Beech marten (*Martes foina*)	3	3	–	–
	Eurasian otter (*Lutra lutra*)	2	2	–	–
	European badger (*Meles meles*)	1	1	–	–

Suidae (*n*=36)	Wild boar (*Sus scrofa*)	36	16	44.4	28.2 – 60.6
Viverridae (*n*=1)	Genet (*Genetta genetta*)	1	1	–	–
Total		**114**	**63**	**55.3**	**45.7 - 64.6**

Table 2. Distribution of positive PCR results in 206 tissue samples from 114 wild mammals.

Tissue examined	PCR positives *n* (%)	CI 95%
Mesenteric lymph nodes	27 (13.1%)	12.4–13.9
Mediastinal lymph nodes	5 (2.4%)	1.7–3.1
Retropharyngeal lymph nodes	2 (0.97%)	0.3–1.7
Bladder	5 (2.4%)	1.7–3.1
Kidneys	19 (9.2%)	8.5–9.9
Tonsils	4 (1.9%)	1.2–2.6
Lungs	11 (5.3%)	4.6–5.9
Ileocecal valves	2 (0.97%)	0.3–1.7
Brain	1 (0.49%)	0.2–1.2
Liver	1 (0.49%)	0.2–1.2
Total	**77 (37.4%)**	**36.7–38.1**

Histological lesions consistent with a diagnosis of *Norcadia* spp. infection were detected in 65 animals (57.0%; 95% CI: 56.1-57.9%). These results are presented in Table 3. These lesions consisted of abscesses (1/114; 0.9%; 95% CI: 0.0-1.8%), granulomas with caseo- calcareous features (21/114; 18.4%; 95% CI: 17.5-19.3%), dermatitis (1/114; 0.9%; 95% CI: 0.0-1.8%), lymphadenopathy (2/114; 1.8%; 95% CI: 0.21-6.19%), purulent lymphadenitis (39/114; 34.2%; 95% CI: 25.6-43.7%) and orchitis (1/114; 0.9%; 95% CI: 0.0-1.8. From the animals that also rendered positive results in PCR, abscesses were observed in one animal, granulomas with caseous calcification in seven animals, dermatitis in one animal, lymphadenopathy in one animal, orchitis in one animal, and purulent lymphadenitis in 16 animals. Granulomatous lymphadenitis was consistent with tuberculosis-like lesions, with multifocal lesions centred by caseous and/or liquefied material or showing calcification at the centre of the lesion, followed by inflammatory infiltrate and a thin capsule of surrounding fibrous tissue. Gram-stained smears from those lesions revealed Gram-positive short filaments, coccoid forms, and branching rods in all samples. In the Ziehl-Neelsen stain, samples were partially acid fast.

Regarding healthy animals, 32.5% of animals without visible lesions had a positive PCR result. Regarding animals with gross lesions, 22.8% had positive results (Table 3).

Table 3. Comparison of PCR results with gross findings in 114 wild mammals.

	PCR Positive	PCR Negative	Total
Lesions	26 (22.8%)	39 (34.2%)	65 (57.0%)
No lesions	37 (32.5%)	12 (10.5%)	49 (43.0%)
Total	**63 (55.3%)**	**51 (44.7%)**	**114 (100%)**

Table 4. Distribution of PCR results by gross findings in 65 wild mammals.

Lesions (*n*=65)	PCR positive *n* (%)	PCR negative *n* (%)
Abscess	1	0
Caseo-calcareous granuloma	7 (33.3%)	14 (66.7%)
Dermatitis	1	0
Lymphadenopathy	1 (50.0%)	1 (50.0%)
Purulent lymphadenitis	16 (41.0%)	23 (59.0%)
Orchitis	0	1

Table 5. Frequency of pathological findings falling into different PCR test result categories.

Species	No visible lesions, PCR negative	No visible lesions, PCR positive	Visible lesions, PCR negative	Visible lesions, PCR positive
Red fox (*Vulpes vulpes*)	7	11	0	1
Beech marten (*Martes foina*)	0	3	0	0
Common genet (*Genetta genetta*)	0	1	0	0
Eurasian otter (*Lutra lutra*)	0	2	0	0
Egyptian mongoose (*Herpestes ichneumon*)	2	10	0	0
European badger (*Meles meles*)	0	1	0	0
Hedgehog (*Erinaceus europaeus*)	1	1	0	0
Wild rabbit (*Oryctolagus cuniculus*)	1	0	0	0
Wild boar (*Sus scrofa*)	1	6	19	10
Red deer (*Cervus elaphus*)	0	2	20	15
Total, *n* (%)	**12 (10.5%)**	**37 (32.5%)**	**39 (34.2%)**	**26 (22.8%)**

The occurrence of *Nocardia* spp. was more frequent in adult males (26.3%) and less frequent in juvenile females (6.1%). Most cases occurred in Idanha-a-Nova (47; 72.3%), Penamacor (17; 26.2%) and Castelo Branco (1; 1.5%).

As shown in Table 4, PCR was positive in 33.3% of animals with caseo-calcareous

granulomas and in 41% of the purulent lymphadenitis cases (Table 4).

A comparison of the frequency of pathological findings according to their PCR result in species is given in Table 5. The results show that regarding the animal species affected, the most frequent findings were in wild boar and red deer. Red foxes and Egyptian mongooses had the most frequent PCR positive without lesions.

Discussion

The number of cases of *Nocardia* spp. recorded in animals and humans, and its range of hosts, has increased due to an increased number of immunocompromised individuals worldwide (Barry et al., 2022; Palomba et al., 2022), the greater attention by the scientific community to this microorganism, and improvements in diagnostic techniques.

The present study provides information on the *Nocardia* spp. status of wild mammals in Portugal, confirming the presence of the pathogen(s) in these animal populations. Some previous studies revealed the presence of this genus in wild boar (Matos et al., 2015). Nevertheless, this is the first report of *Nocardia* spp. molecular detection in red fox, Egyptian mongoose, European badger, Eurasian otter, red deer, hedgehog, or genet, suggesting a large proportion of these are infected with or exposed to zoonotic *Nocardia* spp.

In this study, small sample sizes resulted in the inability to calculate prevalence estimates for some species. However, the results indicate evidence of infection in these species. Given the high proportion of positive samples, it seems that *Nocardia* infection is relatively common among wild mammals in Portugal. The population sample size of different wildlife species should also be taken into account. For some species, the small sizes obtained could reflect their restricted range and relatively low abundance, e.g., for the Eurasian otter (Ruiz-Olmo et al., 2011). No evidence of infection was detected in wild rabbits, although only two animals were examined. Therefore, it is not possible to clearly ascertain whether infection occurs in this species.

The fact that a high prevalence was found in species such as the Egyptian mongoose, red fox, wild boar and red deer suggests a possible association between transmission and the trophic hierarchy within the analysed territory. This hypothesis is presented as a result of the level these species occupy in the food chain, as carnivores, suggesting a potential infection by ingestion of infected animals or contact with infected carcasses. This hypothesis is reinforced by analysis of the affected organs. The most affected organs were lymph nodes, with special reference to mesenteric nodes (Ramiro-Puig et al., 2008). Positive results were obtained from organs such as kidneys, lungs, bladder, tonsils, ileocecal valve, brain, and liver. The presence of *Nocardia* in different organs is consistent with disseminated nocardiosis (Saubolle and Sussland, 2003; Li et al., 2022). Cerebral nocardiosis is a rare entity in humans, but has been reported (Sayer et al., 2022; Sher et al., 2022).

Histopathological analysis showed that most of the infected animals did not present any relevant post mortem lesions. Even in those with lesions, there was a predominance of purulent lymphadenitis, an unspecific response in an attempt to restore immunity. Reports of *Nocardia* infection in free-living terrestrial mammal species are rare (Matos et al., 2015). The majority of reports come from free-ranging aquatic organisms and cap-

tive teleost and shellfish, as well as from marine mammals (St. Leger et al., 2018; Díaz-Santana et al., 2022).

Nocardiosis due to nonspecific clinical manifestations can be misdiagnosed as tuberculosis or fungal lung disease (Pan et al., 2021). Given the limitations of conventional diagnostic techniques, PCR is an effective tool to diagnose *Nocardia* (Rouzaud et al., 2018).

Results suggest that *Nocardia* spp. circulate widely in the study area, which is a serious concern for wildlife. Our findings have also revealed a public health concern because professionals with frequent animal exposure, like veterinarians, biologists or hunters and other professional workers, who come into contact with these species can have an occupational risk of infection when handling infected tissues or samples (Garland-Lewis et al., 2017; Bournez et al., 2019). Consequently, our results indirectly suggest that the local human population may be exposed to infections with *Nocardia*, since hunted animals were killed for consumption, and the results of the present study need to be analysed in the scope of the One Health approach. Therefore, populations in contact with wild mammals should be instructed to prevent direct and indirect contact.

Conclusions

Despite some limitations, molecular diagnostic methods, such as PCR, are an accurate and rapid way of identifying pathogens, without the recurrent need to culture the bacteria. With this study, it was possible to successfully detect the presence of *Nocardia* spp. in different animal organs and samples. The results highlight the need for constant surveillance of this infectious agent, not only to prevent infections to humans or animals, but also to predict the dissemination and evolution of this agent in a poorly controlled and often neglected environment, as is the case of nature reserves and wild animals in these habitats.

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Molekularni probir *Nocardia* spp. u divljih sisavaca – presječna studija

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Nokardioza je emergentna zoonoza koju prouzroči bakterija *Nocardia* spp. Bolest je povezana s infekcijama u imunokompromiranih pojedinaca te je potencijalno smrtonosna ukoliko postane sistemska i ne liječi se. Razvoj pouzdane i valjane dijagnostičke metode je ključan za

identifikaciju ovih patogena, posebice u divljih sisavaca koji su potencijalni rezervoari među divljim životinjama. Cilj je ove studije bio odrediti molekularnu prevalenciju *Nocardia* spp. u divljih sisavaca u Portugalu. Istražili smo praktični uzorak od 114 divljih sisavaca iz porodice: *Canidae*, *Cervidae*, *Erinaceidae*, *Herpestidae*, *Leporidae*, *Mustelidae*, *Viverridae* i *Suidae*. Prikupljeni su uzorci tkiva (*n*=206) iz različitih organa. Molekularna prevalencija bakterije *Nocardia* u divljih sisavaca bila je 55,3 % (95% CI: 45,7-64,6 %). Nocardia spp. otkrivena je u devet od deset ispitanih vrsta: 83,3 % (95% CI: 62,2-100 %) u egipatskih mungosa (*Herpestes ichneumon*), 63,2 % (95% CI: 41,5-89,9 %) u crvenih lisica (*Vulpes vulpes*), 45,9 % (95% CI: 29,8-62,0 %) u običnih jelena (*Cervus elaphus*), 44,4 % (95% CI: 28,2-60,6 %) u divljih svinja (*Sus scrofa*), 3/3 kune bjelice (*Martes foina*), 2/2 europske vidre (*Lutra lutra*), 1/1 euroazijskog jazavca (*Meles meles*), 1/1 europske cibetke (*Genetta genetta*) te 1/2 ježa (*Erinaceus europeaeus*). Od 206 uzoraka tkiva ispitanih molekularnim tehnikama, bakterija *Nocardia* spp. otkrivena je u 37,4 % (95% CI: 36,7-38,1 %), s tim da je prevladavala u mezenteričnim limfnim čvorovima (13,1 %; 95% CI: 12,4-13,9 %) i bubrezima (9,2 %; 95% CI: 8,5-9,9 %). Ovi rezultati daju nove uvide u prevalenciju bakterije *Nocardia* u divljih sisavaca i naglašavaju potrebu za nadziranjem divljih životinja kao potencijalnih rezervoara ovih emergentnih patogena.

Ključne riječi: *Nocardia* spp., *PCR, Portugal, divlje životinje*