Communication

Molecular and Serological Screening Support the Lack of *Coxiella burnetii* Circulation in Wild Birds of Portugal

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Simple Summary: Birds are a natural reservoir of *C. burnetii* and play an important role in its epidemiological cycle. *Coxiella burnetii* is the aetiological agent of Q fever, a zoonotic infectious disease currently considered a priority disease for coordinated surveillance under the One Health approach. Although its impact on public health is limited, under certain epidemiological circumstances and for particular risk groups, it can have serious consequences and have a significant negative impact for people. It is therefore extremely valuable to know the presence and transmission potential of the disease in wild birds in a given area. In this study, all the avian samples tested negative for the presence of *C. burnetii*, both in terms of the presence of antibodies against the agent and the presence of its DNA. To the best of our knowledge, this is the first epidemiological study of *C. burnetii* in wild birds in Portugal, carried out with samples from birds entering wildlife recovery centres.

Abstract: *Coxiella burnetii* is a highly infectious zoonotic pathogenic bacterium that has a major economic impact in the livestock industry throughout the world and causes unpredictable outbreaks in humans worldwide. Although it is known that birds are potential reservoirs of *C. burnetii*, their role in the epidemiological cycle of the pathogen has not been fully verified. Due to its non-specific symptoms and clinical signs, it is certainly an underdiagnosed disease. The objective of this study was to obtain more information on *C. burnetii* prevalence in wild birds in Portugal. Blood, plasma, and other tissue samples were obtained from wild birds admitted at wildlife rehabilitation centres in Portugal in the scope of passive surveillance. Antibodies specific to *C. burnetii* were screened using a

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commercial enzyme-linked immunosorbent assay according to the manufacturer's instructions. Evidence of *C. burnetii* infection was sought based on the detection of bacterial DNA. No positive results were found, either in terms of antibodies to *C. burnetii* or molecular biology. These serological findings do not indicate the endemic circulation of *C. burnetii* in wild birds, which can be considered relevant information. However, a more complete and serialized approach over time is necessary to be able to make real inferences about the endemicity of the pathogen in the country and its dispersion among wild avian populations. qPCR results were also negative, a finding suggesting that this host population may not play a significant role in the transmission dynamics of *C. burnetii*. Given the importance of wild bird species as natural reservoirs of this zoonotic bacterium, we consider these data useful for multidisciplinary work in the prevention and control of Q fever, following a One Health approach.

Keywords: avian; genotyping; One Health; public health; Q fever; PCR; reservoirs; seroprevalence; zoonosis

1. Introduction

Wild birds can be affected by microorganisms that pose a threat to their state of health, as well as being transmissible to mammals, including humans. The role of birds in the epidemiological cycle of *C. burnetii* has been reported since the 1950s and, more recently, several serological and molecular studies have been conducted in avian populations [1,2]. The excretion of the pathogen in avian droppings and the presence of antibodies to *C. burnetii* in avian individuals can be considered indirect evidence that birds are a natural reservoir of the pathogen [3]. As ticks are recognized vectors of *C. burnetii*, birds contribute to this agent dispersal by carrying infected ticks, a fact especially relevant when it comes to migratory species that travel long distances [2].

Coxiella burnetii is an obligate intracellular Gram-negative bacterium, replicating in eukaryotic cells. This agent has a biphasic developmental cycle, with two distinguishable morphologic forms: the small cell variant (SCV) and the large cell variant (LCV). LCV is the replicating form, being resistant to lysosomal enzymes and low pH. SCV is a stationary nonreplicating form, stable in the environment without a host, and highly resistant to chemical, heat, and desiccation stresses, among others. *C. burnetti* is the etiological agent of Q fever, a worldwide (with the exception of New Zealand)-spread zoonosis of public health concern [4,5]. Q fever is a disease that represents a severe threat for the health of humans and other mammals, causing severe economic losses on ruminant farms [6].

The main reservoirs of Q fever are domestic ruminants, but many other animals have been reported to shed the pathogen, namely wild ruminants [7], dogs, cats [8], marine mammals [9], ticks [10,11], and birds [12]. Bacteria can be found in birth products, urine, feces, and milk of infected animals [13]. The main mode of transmission to humans is most frequently through the inhalation of aerosolized bacteria that are spread in the environment, specially at the time of delivery or abortion [14]. Moreover, transmission can occur by the consumption of contaminated food or water or direct contact with infected animals or other contaminated material [5]. An aggravating factor is that, once it has contaminated an environment, it can infect a human or an animal with a very low infective dose [15]. The combination of these characteristics—its widespread availability, natural potential for aerosolized use, and environmental stability, and the possibility of producing large amounts of infectious material—makes it a biological terrorist agent (category B) [16]. In addition to several outbreaks that have been reported in Europe [17], the occurrence of

Q fever in humans based on clinical diagnoses is certainly underestimated, because the disease manifestations are very unspecific and are reminiscent of flu symptoms. Infection during pregnancy has the added risk of resulting in a miscarriage, stillbirth, or other related complications. Less than 5% of the infected people may develop a chronic infection, which often results in endocarditis, and can be fatal if not treated properly and in time [18].

Q fever is endemic in northern Spain [14,19] and in Portugal, and is a mandatory notifiable disease for national public health authorities [20]. Nevertheless, the knowledge about its occurrence and distribution in the country remains scarce [21], making further serological and epidemiological studies necessary to better understand how common *C. burnetii* infections are at global and regional levels. Some studies from Europe have previously reported the presence of *C. burnetii* in wild birds, namely in Italy [12,22,23], Slovakia [24], and Spain [25]. Coxiellosis has also been described in both domestic and wild birds in Japan [26] and North America [27]. To the best of our knowledge, there are no reports of *C. burnetii* in wild birds in Portugal. In the present study, we aimed to assess the seropositivity and molecular detection of *C. burnetii* in wild birds admitted at wildlife rehabilitation centres in Portugal and assess their potential in the dissemination of the agent.

2. Materials and Methods

In Portugal, there is a national network of wildlife rehabilitation centres (WRCs), which aims to receive and treat injured or debilitated wild fauna, with the aim of returning the animals to their natural habitat. All wildlife admitted to the WRC are given an identification number, and the species, carrier, date, location of each animal, and probable cause of admission are documented, as well as its final destination.

A total of 92 birds, belonging to 27 different species, that were admitted at the WRC of the Veterinary Teaching Hospital of UTAD (CRAS-HVUTAD) between 2020 and 2024 were sampled. Information on each individual was recorded. Birds' health status was assessed through a complete physical examination and diagnostic procedures as necessary. Blood samples (0.3–1.0 mL) were collected from the ulnar vein, metatarsal vein, or jugular vein, according to the anatomy of each species and veterinarian's preference, and transferred into heparin-lithium tubes. Samples were centrifuged at 2000 rpm for 10 min. Plasma was separated and stored at −20 °C until a further serological analysis.

Samples were taken from wild birds admitted to 3 WRCs in mainland Portugal: CRAS-HVUTAD, WRC of Santo André (CRASSA), and Biological Park of Gaia (PBG). A total of 74 blood samples were collected and stored in EDTA tubes, frozen at −20 °C. Additionally, 29 organ samples (mixed) were collected from dead birds during necropsy (from which no blood had been drawn). A mix of brain, cardiac muscle, kidney, liver, lung, and spleen samples was collected from each individual and frozen at −20 °C until a further analysis. Both total blood and organs were used for a qPCR assay.

Plasma and whole blood samples used were left over from collections carried out for other diagnostic purposes, so the individuals sampled were not the same for the two tests. Organs were only collected from birds that had been necropsied for diagnostic interest of the WRC.

2.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples were tested using an indirect ELISA for the detection of antibodies directed against *C. burnetii* (ID Screen® Q fever Indirect Multi-species; ID.vet Innovative Diagnostic, Grabels, France), according to the manufacturer's instructions. Results were expressed as a percentage (S/P%), calculated using the following formula: SP% = [(sample optical density [OD] − mean OD of negative controls)/(mean OD of positive controls −

mean OD of negative controls)] × 100. The result threshold values were set as suggested by the manufacturer and the samples were classified as follows: samples with S/P ≤ 40% were considered negative; samples with S/P > 40% and ≤50% were considered doubtful; samples with S/P between >50% and ≤80% were considered positive; and samples with S/P > 80% were considered strongly positive. Any bird that tested positive for *C. burnetii* antibodies was considered infected.

2.2. DNA Extraction

DNA extraction from blood and organs was performed by adding a mixture of 420 µL of a lysis buffer and 25 µL proteinase K solution and incubated at 37 °C for 10 min. After, incubation samples were briefly vortexed and centrifuged for 2 min at 6000× *g*. After centrifugation, 140 µL of the supernatant was used for DNA extraction and purification with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The extraction was automated using the QIAcube® platform (Qiagen). The purified DNA was then stored at −80 °C in RNase-free water.

2.3. Real-Time Polymerase Chain Reaction (qPCR) and Sanger Sequencing

The detection of *C. burnetii* was performed with the set of primers Trans 3 F/Trans4R, targeting the CB IS1111 element and amplifying a fragment of 243 bp, as previously described by Capuano et al. [28]. PCR reactions were carried out using Bio-rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A total of 20 µL reaction volume contained 0.4 µM of each primer, the 10 µL Xpert Fast SYBR 2X mastermix (GRiSP®, Porto, Portugal), 6.5 µL of RNase-free water, and 2 µL of sample DNA, following the manufacturer's instructions. The criterion used to verify a specific positive reaction was a melting curve (defined as the peak of the curve) of 88.1 °C. If positive by qPCR, further genetic characterization was attempted. Briefly, amplicons that matched the expected melting temperature were purified using the GRS PCR and Gel Band Purification Kit (GRiSP®) and subsequently sequenced via the Sanger dideoxy sequencing method by the company STABvida (Monte da Caparica, Portugal).

2.4. Statistical Analysis

The statistical analysis was carried out using descriptive statistics using Microsoft Excel™ 2016. Totals and percentages of each variable are presented in the Results Section.

3. Results

3.1. Serology

All 92 samples were negative for *C. burnetii* antibodies (the seroprevalence of *C. burnetii* infection was 0%). Bird species tested belonged to nine different orders and 12 distinct families (Table 1). Common Buzzard (*Buteo buteo*, *n* = 12), Tawny Owl (*Strix aluco*, *n* = 10), and White Stork (*Ciconia ciconia*, *n* = 9) were the most represented species.

Regarding spatial distribution, the birds were rescued in 27 municipalities from the North and Centre regions of the country (NUTS II) and from eight different subregions, NUTS III (Table 2). It was not possible to find out the location of 24 birds, because this information was not recorded at the WRC.

Table 1. Birds tested for *Coxiella burnetii* antibodies through serological assay (ELISA).

Order	*n* (%)	Family	*n* (%)	Species *	*n* (%)
Accipitriformes	4 (47.8)	Accipitridae	44 (47.8)	Northern Goshawk, *Accipiter gentilis*	8 (8.7)
				Sparrowhawk, *Accipiter nisus*	4 (4.3)
				Cinereous Vulture, *Aegypius monachus*	2 (2.2)
				Common Buzzard, *Buteo buteo*	12 (13.0)
				Montagu's Harrier, *Circus pygargus*	2 (2.2)
				Griffon Vulture, *Gyps fulvus*	7 (7.6)
				Booted Eagle, *Hieraaetus pennatus*	2 (2.2)
				Black Kite, *Milvus migrans*	2 (2.2)
				Red Kite, *Milvus milvus*	5 (5.4)
Apodiformes	1 (1.1)	Apodidae	1 (1.1)	Pallid Swift, *Apus pallidus*	1 (1.1)
Ciconiiformes	9 (9.8)	Ciconiidae	9 (9.8)	White Stork, *Ciconia ciconia*	9 (9.8)
Columbiformes	4 (4.3)	Columbidae	4 (4.3)	Rock Pigeon, *Columba livia*	2 (2.2)
				Common Woodpigeon, *Columba palumbus*	1 (1.1)
				Eurasian Collared Dove, *Streptopelia decaocto*	1 (1.1)
Coraciiformes	1 (1.1)	Alcedinidae	1 (1.1)	Common Kingfisher, *Alcedo atthis*	1 (1.1)
Falconiformes	6 (6.5)	Falconidae	6 (6.5)	Peregrin Falcon, *Falco peregrinus*	3 (3.3)
				Common Kestrel, *Falco tinnunculus*	3 (3.3)
Passeriformes	5 (5.4)	Corvidae	2 (2.2)	Carrion Crow, *Corvus corone*	1 (1.1)
				Eurasian Jay, *Garrulus glandarius*	1 (1.1)
		Fringillidae	1 (1.1)	European Greenfinch, *Chloris chloris*	1 (1.1)
		Hirundinidae	2 (2.2)	Western House Martin, *Delichon urbicum*	2 (2.2)

Table 1. *Cont.*

Order	*n* (%)	Family	*n* (%)	Species *	*n* (%)
Pelecaniformes	2 (2.2)	Ardeidae	2 (2.2)	Grey Heron, *Ardea cinereal*	2 (2.2)
Strigiformes	20 (21.7)	Strigidae	14 (15.2)	Little Owl, *Athene noctua*	1 (1.1)
				Short-eared Owl, *Asio flammeus*	1 (1.1)
				Eurasian Eagle Owl, *Bubo bubo*	2 (2.2)
				Tawny Owl, *Strix aluco*	10 (10.9)
		Tytonidae	6 (6.5)	Barn Owl, *Tyto alba*	6 (6.5)
Total	92 (100)		92 (100)		92 (100)

* The species names are organized in alphabetical order of the scientific name within each order.

Table 2. Geographical distribution of birds sampled.

NUTS II	*n* (%)	NUTS III	*n* (%)
North	64 (69.6)	Alto Tâmega	6 (6.5)
		Área Metropolitana do Porto	3 (3.3)
		Ave	2 (2.2)
		Cávado	6 (6.5)
		Douro	35 (38.0)
		Tâmega e Sousa	1 (1.1)
		Terras de Trás-os-Montes	11 (12.0)
Centre	4 (4.3)	Beiras e Serra da Estrela	4 (4.3)
Unknown	24 (26.1)	Unknown	24 (26.1)
Total	92 (100)		92 (100)

3.2. qPCR

A total of 103 samples were tested by qPCR for *C. burnetii* (Table 3), with 2 of the samples yielding results compatible with the presence of the bacterium in the assay. They were subjected to sequencing, to confirm the identity of the detected pathogen, but bidirectional sequences retrieved from both samples produced non-specific results.

Table 3. List of birds tested for *Coxiella burnetii* qPCR.

Species *	*N*
Northern Goshawk, *Accipiter gentilis*	3
Sparrowhawk, *Accipiter nisus*	3
Greylag Goose, *Anser anser*	1
Pallid Swift, *Apus pallidus*	1
Common Swift, *Apus apus*	2
Grey Heron, *Ardea cinereal*	1
Little Owl, *Athene noctua*	1
Eurasian Eagle Owl, *Bubo bubo*	3
Common Buzzard, *Buteo buteo*	5
White Stork, *Ciconia Ciconia*	13
Montagu's Harrier, *Circus pygargus*	2
Rock Pigeon, *Columba livia*	4
Common Woodpigeon, *Columba palumbus*	3
Carrion Crow, *Corvus corone*	1
Western House Martin, *Delichon urbicum*	1
Little Egret, *Egretta garzetta*	1
European Robin, *Erithacus rubecula*	1
Eurasian Jay, *Garrulus glandarius*	2
Griffon Vulture, *Gyps fulvus*	6
Booted Eagle, *Hieaaretus pennatus*	2
Lesser Black-backed Gull, *Larus fuscus*	5

Table 3. *Cont.*

Species *	*N*
Yellow-legged Gull, *Larus michahellis*	25
European Bee-eater, *Merops apiaster*	2
Black Kite, *Milvus migrans*	3
House Sparrow, *Passer domesticus*	2
Eurasian Collared Dove, *Streptopelia decaocto*	2
Tawny Owl, *Strix aluco*	2
Blackbird, *Turdus merula*	3
Song Thrush, *Turdus philomelos*	1
Eurasian Hoopoe, *Upupa epops*	1
Barn Owl, *Tyto alba*	1
Total	103

* The species names are organized in alphabetical order of the scientific name.

From CRASSA (*n* = 46) and PBG (*n* = 7), only blood samples were analyzed; and from CRAS-HVUTAD, blood (*n* = 21) and mixes of organs (*n* = 20) were included. Regarding age, 30 birds were adults, 34 juveniles, 18 nestlings or fledglings, 10 subadults, and 11 had no age determined at the time of admission. Most of the birds sampled were found in the North (*n* = 48) and Alentejo (*n* = 43), followed by the Centre (*n* = 2), Setúbal Peninsula (*n* = 2), and Greater Lisbon (*n* = 1) regions. The locations of seven birds were not included in the WRC databases. Since no positive results were found, the species' migratory behaviour and the breeding season in Portugal were not considered.

4. Discussion

Birds are known to carry a variety of potentially zoonotic pathogens, either as a reservoir host or through the dispersal of infected arthropods [29]. Bacterial, parasitic, and viral zoonotic agents have been reported to be isolated from birds—*Chlamydia* spp., *Escherichia coli*, *Cryptosporidium* spp. and *Giardia* spp., and Influenza A and West Nile virus are only a few examples [23,30,31]. So far, few studies have been carried out on the distribution and occurrence of *C. burnetii* infection in wildlife. The present study provides new epidemiological data on *C. burnetii* in wild birds in Portugal. No positive results were detected for the presence of *C. burnetii*, neither in serology testing nor in the molecular biology assay.

The seroprevalence analysis of this pathogen in wildlife in Portugal was recently described by Pires et al. [21] in Wild Boar (*Sus scrofa*) and Red Deer (*Cervus elaphus*). Cumbassá et al. [32] accessed the presence of the agent in wild mammal carnivores and in Griffon Vultures (*Gyps fulvus*), of which none of the latter tested positive in that study. Focusing only on birds, *C. burnetii* DNA has been detected in different avian species across Europe [3,12,33–35]. *C. burnetii* has been detected in birds from the families Accipitridae, Anatidae, Ardeidae, Caprimulgidae, Ciconiidae, Columbidae, Falconidae, Gruidae, Laridae, Phasianidae, Phoenicopteridae, Rallidae, Recurvirostridae, Scolopacidae, Strigidae, Tytonidae, and Upupidae in Cyprus [33,34]. Accipitridae and Ciconiidae have already tested positive for this bacterium in Spain [14,25]. In some birds, *C. burnetii* was detectable in the serum, proving that they can experience bacteremia [36]. DNA of *C. burnetii* was detected in Griffon Vultures and Black Kites (*Milvus migrans*) in northern Spain, showing that this agent was present in nature and could take a part in the sylvatic cycle of Q fever [25]. Among wild birds, only one White Stork (*Ciconia ciconia*) (1/218; 0.46%) tested positive [8]. These low prevalence values are in line with what was seen in this study, but it was even

lower in this most recent case. With regard to the samples tested in this study, we always have to consider that the birds might have no longer been in a period of bacteraemia when the blood was taken.

Coxiellosis associated with fatal disease in birds has already been detected by a molecular analysis [37] and Coxiella-like bacteria have been reported by isolation in the liver, spleen, and cardiac tissues of birds by PCR and bacterial 16S rRNA gene sequences [38–40]. The organs collected for the analysis in this study are in line with others that have already been tested and shown lesions in birds [41].

Different species of ticks have been identified on wild birds from Portugal [42,43]. Although it has been suggested that migratory birds act as efficient vehicles in the dispersal of ixodid ticks infected with *C. burnetii* [3], and despite that the key role of migratory birds in the dispersal of ticks and tick-borne pathogens between continents is proven [44,45], pathogenic *C. burnetii* was not identified in any of the ticks collected in a recent study carried out in Italy [46]. We would also add that no ticks were detected on any of the birds sampled in this study, according to their medical records.

Infected wild birds can shed *Coxiella* through feces [3], thus contributing to infection spread to humans and other animals. It is currently unknown if birds can suffer from subclinical infections, but the period of fecal shedding could be prolonged if that is the case [36]. In the present study, we can probably say that the risk of transmission through wild birds in the northern part of Portugal is low for the time being. The impact of *C. burnetti* on the population health of some wild species is poorly understood, and its consequences in terms of the conservation of populations remain unknown. Just like with livestock, reproductive failure related to Q fever has been reported in free-roaming wildlife [7].

Considering the current epidemiological situation of Q fever in Europe, *C. burnetii* has been recognized as an important zoonotic pathogen of public health concern, with Q fever being included in the European Food Safety Authority's top 10 list as a priority disease for coordinated surveillance under a One Health approach [47]. In Portugal, the notification of human Q fever cases is mandatory since 1999, but the surveillance mechanisms remain passive. Some difficulties arise with regard to Q fever surveillance. The disease does not involve typical clinical signs in animals, and seroconversion does not always equal pathogen isolation. In addition, Western European countries need to involve many different species for the surveillance for Q fever [47]. It has been pointed out that serology can fail to diagnose acute Q fever, due to the length of time it takes for antibodies against *C. burnetii* to appear after the onset of the disease [48]. qPCR is considered a valuable tool to complement early diagnoses of acute disease. qPCR is a quantitative method that is used to simultaneously amplify and quantify a target DNA. The availability of *C. burnetii* whole-genome sequence data has enabled major developments in molecular typing systems [49].

Wildlife disease surveillance can be a useful and complementary component of human and animal disease surveillance, monitoring, prevention, and control programmes, as well as conservation efforts. The detection of pathogens and diseases and analysis and communication of results are critical components for a successful protection of wild birds and other wildlife [50]. Analyzing the general panorama of infections in protected species, as in this study, can be relevant for timely and multidisciplinary conservation measures.

5. Conclusions

In conclusion, the absence of *C. burnetii* detection in the population studied indicates that there is no infection in these wild birds tested in Portugal. Further studies are needed to better access the *C. burnetti* circulation status and to estimate with greater certainty the prevalence of this agent in this group of animals, which is still poorly studied. Mon-

itoring programmes are necessary and very useful to provide a broader perspective of the epidemiology of Q fever in avian wildlife (but not only) in Portugal. These are an important component of integrative surveillance strategies, essential to reduce the risk of the transmission of *C. burnetii* to sympatric species, including humans. Although *C. burnetii* has been found in many avian species, but not in this work, it has not always been correlated with outbreaks of Q fever. However, prophylaxis on farms must take domestic and wild birds into account.

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