

Serological evidence of exposure to zoonotic tick-borne bacteria in pheasants (*Phasianus colchicus*)

Valentina Virginia Ebani¹, Fabrizio Bertelloni¹, Paolo Mani¹

¹ Department of Veterinary Science, University of Pisa

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Abstract

Introduction and objective. Previous studies on tick-borne pathogens in the avian population have focused mainly on the detection of the agents in ticks collected from birds, but data about the presence of tick-borne bacteria in these animals are scant. The aim of the presented study was to verify the exposure to some zoonotic tick-borne bacteria, in particular, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, and the *Rickettsia* species of the Spotted Fever Group (SFG), in pheasants (*Phasianus colchicus*) living in a central Italy area, characterized by conditions favourable for the diffusion of the ticks.

Materials and method. Blood serum samples from 276 farm-reared pheasants were examined by indirect immunofluorescence antibody test to detect antibodies against the 4 reported pathogens.

Results. A total of 124 (44.92%) birds resulted as seropositive: in particular 3 (1.08%) to *C. burnetii*, 31 (11.23%) to *A. phagocytophilum*, 46 (16.67%) to *B. burgdorferi* s.l., 49 (17.75%) to SFG *Rickettsia* spp. antigens. Three pheasants resulted positive both to *A. phagocytophilum* and *B. burgdorferi* s.l. antigens, whereas 2 – both to *B. burgdorferi* s.l. and SFG *Rickettsia* spp. antigens. The antibody titers varied from 1:40–1:320.

Conclusions. The obtained results show that the pheasants had developed antibodies to the investigated tick-borne agents. For this reason, they seem to be involved in the epidemiology of the studied tick-borne bacteria.

Key words

pheasant (*Phasianus colchicus*); *Anaplasma phagocytophilum*; *Borrelia burgdorferi* s.l.; *Coxiella burnetii*; SFG *Rickettsia* spp.; IFAT

INTRODUCTION

The common pheasant (*Phasianus colchicus*) is a bird of the Phasianidae family. It is one of the most hunted birds worldwide; for this purpose, it has been introduced in many regions, and is also common on game farms where it is commercially bred [1]. In central Italy, several pheasant farms are indeed spread throughout the region, located in rural areas, rich in vegetation where wild animals and ticks are often present. Wild mammals are demonstrated to be reservoirs of tick-borne pathogens, and often the cause of disease in domestic animals and humans [2].

Wild birds are often carriers of infected ticks [3], in particular, the most common hard tick species associated with avian hosts in various European Countries is *Ixodes ricinus*, which is known to be a vector of several important zoonotic bacteria [4].

Previous studies on tick-borne pathogens in the avian population have focused mainly on the detection of the agents in ticks collected from birds. A recent molecular study has been carried out on ticks collected from migratory birds in Italy, which found that they are often carriers of ticks, in particular *Hyalomma marginatum*, infected by *Rickettsia* sp, *B. burgdorferi* s.l., *C. burnetii* and *Babesia microti* [5]. However, data about the detection of tick-borne bacteria in birds are scant [4, 6, 7, 8], and in particular, there is no

data available on the prevalence of these pathogens among birds in Italy.

OBJECTIVE

The aim of the presented study was to verify the exposure to some zoonotic tick-borne bacteria, in particular, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, and *Rickettsia* species of the Spotted Fever Group (SFG), in pheasants in an area of central Italy characterized by conditions favourable for the diffusion of ticks, because of the abundant vegetation and presence of wild animals.

MATERIALS AND METHOD

Study area. The birds lived in an area of Tuscany (43° 43'N, 10° 20'E, at sea level), characterized by a mild climate and abundant vegetation, mainly composed of pines (*Pinus pinea* and *Pinus pinaster*), oak (*Quercus ilex*), ash (*Fraxinus* sp.), alder (*Alnus* sp.) and poplar (*Populus alba*). *Ixodes ricinus* is the most common hard tick species found in this area, but *Dermacentor marginatus*, *Haemaphysalis punctata*, *Rhipicephalus* sp. and *Hyalomma* sp. are also present [9]. Several different bird species live usually in this area, as well as various large and small mammals, such as fallow deer (*Dama dama*), wild boar (*Sus scrofa*), red fox (*Vulpes vulpes*), red squirrel (*Sciurus vulgaris*), common rabbit (*Oryctolagus cuniculus*), European badger (*Meles meles*), hedgehog (*Erinaceus europaeus*), and different small rodent species.

Address for correspondence: Valentina Virginia Ebani, Department of Veterinary Science, University of Pisa
E-mail: valentina.virginia.ebani@unipi.it

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Farm animals, in particular cattle and horses employed for trekking, are present.

Samples. Blood serum samples from 276 pheasants (*Phasianus colchicus*), kept at -20°C in the seroteca of the Avian Pathology Section of the Department of Veterinary Science at the University of Pisa, were tested by indirect immunofluorescence antibody test (IFAT) for the presence of antibodies to *A. phagocytophilum*, *B. burgdorferi* s.l., *C. burnetii*, and SFG *Rickettsia* spp.

During 2007–2008, blood samples from the brachial vein of 151 females and 125 males, all 8–12 weeks old, were collected during periodical health monitoring of the birds. The pheasants lived fenced-in, without direct contact with other birds or mammals. All of them were mainly bred for releasing in restocking areas, and in a few cases, they were sold for personal meat consumption and introduction in hobbyist small farms.

Serological tests. The tests were carried out on 4 types of commercial IFAT slides prepared with *A. phagocytophilum*, *B. burgdorferi* s.l., *C. burnetii* and *R. conorii* (SFG) antigens (Fuller Laboratories Fullerton, California, USA), respectively. Blood sera were diluted 1:40 in phosphate buffered saline (PBS), and incubated on wells of the slides in a humidified chamber at 37°C for 30 min. The slides were rinsed 3 times in PBS containing 0.4% Tween 80 (Sigma-Aldrich, St. Louis, Missouri, USA), once in distilled water, then air-dried. Each well of the slides was probed with fluorescein isothiocyanate-conjugated rabbit anti-Chicken IgG (Sigma-Aldrich) diluted 1:30 in Evans Blue (Sigma-Aldrich) solution and incubated at 37°C in a humid chamber for 30 min. The slides were washed and dried as described above and examined with a fluorescence microscope. Negative and positive controls were included in each test: PBS, instead of serum, probed with the anti-chicken IgG conjugate used as negative control; one human serum reactive for each agent (Fuller Laboratories) respectively, probed with anti-human IgG conjugate (Fuller Laboratories), was included as positive control.

Samples scored positive at 1:40 cut-off dilution were 2-fold serially diluted, from 1:80 to 1:320, to determine the endpoint titre. Scores from 1–4 were assigned to the intensity of specific fluorescence, the antibody titre was defined as the major dilution with a ≥ 2 score.

Positive reactions were observed with the human sera used as positive controls, whereas no fluorescent reactions were observed when PBS was included as the negative control.

Statistical analysis were carried out by χ^2 test ($P < 0.05$) to compare the results in relation to bird gender.

RESULTS

From the 276 blood serum samples tested, a total of 124 (44.92%), 56 (56/125, 44.8%) males and 68 (68/151, 45%) females, resulted seropositive. In particular, 31 (11.23%) pheasants showed antibodies to *A. phagocytophilum* antigen, 46 (16.67%) to *B. burgdorferi* s.l. antigen, 49 (17.75%) to *R. conorii* (SFG) antigen, and 3 (1.08%) to *C. burnetii* antigen. Three samples resulted positive both to *A. phagocytophilum* and *B. burgdorferi* s.l. antigens and 2 both to *B. burgdorferi* s.l. and SFG *Rickettsia* spp. antigens. The antibody titers varied from 1:40–1:320 (Tab. 1).

Table 1. Number of pheasants (*Phasianus colchicus*) resulted seropositive to the different agents according to antibody titer (cut-off 1:40)

Agents	No. positive pheasants at the given antibody titer				Total of positive pheasants (%*)
	1:40	1:80	1:160	1:320	
<i>A. phagocytophilum</i>	14	12	5	-	31 (11.23)
<i>B. burgdorferi</i> s.l.	15	18	9	4	46 (16.67)
SFG <i>Rickettsia</i> spp.	14	18	11	6	49 (17.75)
<i>C. burnetii</i>	3	-	-	-	3 (1.08)

* Percentage of positive animals, to each agent, among the 276 tested

No statistical differences were observed when comparing males and females, analyzing the results with χ^2 test ($P < 0.05$).

DISCUSSION

The test used in the presented study was IFAT which is largely employed for the diagnosis of tick-borne diseases in animals and humans. In particular, IFAT is considered the gold standard serological method for the diagnosis of rickettsioses and anaplasmosis [10], and it is often used for the detection of antibodies against *B. burgdorferi* s.l. [10] and *C. burnetii* [11].

A fluorescein isothiocyanate-conjugated rabbit anti-chicken IgG was used as secondary antibodies, because no commercial conjugate specific to detect pheasant immunoglobulins is available. IgG domains of different bird species are variable and might not present the same homology with chicken antibodies; thus, the secondary antibody used could have negatively influenced the results of IFAT [12]. However, the obtained results revealed the exposure of pheasants to the 4 tick-borne agents studied.

The lowest values of seroprevalence at low antibody titer was observed for *C. burnetii*, the agent of Q fever, a severe zoonotic disease [13]. Animals and humans usually acquire this bacterium through inhalation of contaminated aerosol and ingestion of milk and dairy products from infected ruminants. Infected animals shed coxiellae mainly in birth products, but also in faeces and urine [14]. More than 40 species of ticks can be naturally infected with *C. burnetii* during a blood meal on infected animals, and transmit the bacterium to other mammals during the next blood meal, or by the aerogenic spread of dried tick faecal excretions, maintaining *C. burnetii* in wildlife [15].

The low seroprevalence found in the tested pheasants is probably related to the absence of direct or indirect contact with livestock, which usually represent the main source of infection [14], but also to the moderate spreading of the pathogen in the tick population and probably in this geographic area. In fact, previous molecular surveys carried out in mammals and ticks in several areas of Tuscany, including the one under study, have found prevalences for *C. burnetii* of 5.1% in hunting dogs [16], 3.75% in horses [17] and 0% in ticks [9].

The seroprevalence values against the other agents, which are transmitted only by ticks, in particular ixodid ticks [18, 19, 20], were higher, about 18% of pheasants resulted positive to SFG *Rickettsia*. IFAT, even if considered the gold standard method for the serological diagnosis of rickettsiosis [21], is often not able to differentiate antibodies against the

species belonging to the Spotted Fever Group [18]. In Italy, several SFG rickettsiae are circulating, as demonstrated by molecular investigations on tick populations. In particular, *R. conorii*, *R. helvetica*, *R. massiliae*, *R. slovaca*, *R. monacensis*, *R. aeschlimannii*, *R. raoultii*, and *R. africae* have been detected [22, 23, 24, 25, 26, 27]. Thus, the observed seroprevalence, in some cases with relevant antibody titers, could be due to the exposure of pheasants to one of the SFG *Rickettsia* species.

Previous surveys carried out in Europe have found DNA of SFG rickettsiae in ticks collected from birds, underlining the role of migratory birds in the dispersal of tick-borne agents. In particular, recent molecular studies have detected *Rickettsia* spp., mainly *R. aeschlimannii* and *R. africae*, in ticks collected from migratory birds in European Mediterranean areas [5, 28]. However, the competence of birds to function as *Rickettsia* reservoir capable of transmitting and infecting ticks with rickettsiae is not yet completely understood [29]. A recent study has detected bacteraemia by *R. helvetica* in wild avian hosts in Hungary [4], but further data about *Rickettsia* spp. infections in birds are not available worldwide.

On the contrary, *B. burgdorferi* s.l., in particular the genospecies *B. garinii* and *B. valaisiana*, have long been associated with the avian population [30]. Previous investigations have found that the bird host competency for maintaining and transmitting *Borrelia* spirochetes varies in different bird species. For example, pheasants in the United Kingdom [31, 32] and blackbirds and song thrushes in Central Europe have been shown to be important reservoirs of *B. garinii* and *B. valaisiana* [30, 33, 34, 35]. Moreover, *B. turdii*, a genospecies first reported from Asia, was found in *I. ricinus* and *Ixodes frontalis* larvae feeding on blackbirds in Portugal [6].

The presented study detected 16.67% seroprevalence, with antibody titers ranging from 1:40–1:320, confirming that birds, in particular pheasants, may be infected by *B. burgdorferi* s.l. The study also detected 11.23% of *A. phagocytophilum* seropositive pheasants. Seroprevalence of 13.68% were previously detected in central Italy during the testing of a total of 2,455 wild and domestic animals: particularly in 46.26% of fallow deer, 46.15% of red deer, 16.89% – horses, 16.78% – cattle, 12.74% – sheep, 8.76% – dogs and 4.16% of goats resulted positive to *A. phagocytophilum* [36].

A. phagocytophilum was found by PCR in some avian species (*Fringilla coedus*, *Passer domesticus*, *Passer hispaniolensis*, *Turdus merula*, *Emberiza cia*, *Lanius senator*, *Pica pica*, *Aegithalos caudatus*) [37], but no data are available about *A. phagocytophilum* infection in pheasants. Previous investigations carried out in Europe have focused mainly on the detection of *A. phagocytophilum* in ticks collected from birds, and have shown that migrating birds may be important in the dispersal of *A. phagocytophilum* infected *I. ricinus* [38, 39].

A. phagocytophilum is currently considered as a single bacterial species. However, a recent study carried out on *A. phagocytophilum* DNA found in ticks and different vertebrates including birds, identified 4 ecotypes. In particular, ecotypes I, II and III were found in mammals, whereas ecotype IV resulted most likely from association with avian species. These results indicate that the host specialization of *A. phagocytophilum* may occur, and suggests that ecotype IV might be adapted to a life cycle involving exclusively birds and bird-specific vectors [40].

CONCLUSIONS

To the best of our knowledge, the presented study is the first serological survey that suggests the exposure of pheasants to zoonotic tick-borne pathogens in Italy. The obtained results are not too surprising, considering that the examined birds lived in a geographic area with conditions favouring the diffusion of ticks, and in which arthropod-borne infections have been previously detected in ticks and mammals [9, 26, 41, 42].

The pheasants tested in the current study did not have direct contact with mammals; however, they lived in aviaries enclosed by wire meshes, therefore, they could be reached by ticks disseminated in the environment from other wild animals, including birds.

Farm-reared pheasants are usually released to increase the wild population; however, this common practice may introduce new pathogens into the autochthonous population, as frequently established for parasites [1, 43]. Likewise, the introduction in a new habitat of pheasants infested by infected ticks and/or with active infection by arthropod-borne pathogens, could contribute to the spread of these agents among birds and mammals.

The results obtained do not demonstrate whether or not pheasants are reservoirs of the investigated bacteria, because the presence of antibodies does not necessarily correspond to an infection. However, more accurate studies are needed because *P. colchicus* seem to be involved in the epidemiology of some tick-borne bacteria.

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