Schweizer Archiv für Tierheilkunde
M. Büker et al., Band 155, Heft 7, Juli 2013, 411–416
DOI 10.1024/0036-7281/a000483

First detection of *Borrelia burgdorferi*-antibodies in free-living birds of prey from Eastern Westphalia, Germany

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Summary

Borrelia (B.) burgdorferi sensu lato, the causative agent of Lyme disease, is the most important arthropod-borne zoonosis-pathogen in the Northern hemisphere. Besides small mammals, birds, primarily *Passeriformes* and sea birds, play an important role in the transmission, distribution and maintenance of this disease. Previous studies on birds have focused mainly on the detection of *Borrelia*-infected ticks. However, the presence or absence of an infected tick cannot be taken as an indicator of the infective status of the avian host; to date this area of research has not been explored. In this study, serological analyses of blood collected from free-living birds of prey (n = 29) at the rehabilitation centre in Eastern Westphalia, Germany, highlights that birds of prey are also susceptible to *B. burgdorferi* and react immunologically to an infection. Increased antibody-levels could be found by using a modified Indirect Immunofluorescent-testing in two common buzzards, *Buteo buteo*, and two eagle owls, *Bubo bubo*. Further research regarding the serological diagnostics of *B. burgdorferi* within the avian host is required. In the future, it should be taken into account that birds of prey can be reservoirs for *B. burgdorferi*, as well as carriers of infected ticks; although at present their epidemiological importance is still to be confirmed.

Keywords: *Borrelia burgdorferi*, Lyme disease, vector, antibody, bird of prey

Erster Nachweis von *Borrelia burgdorferi*-Antikörpern bei freilebenden Greifvögeln in Ostwestfalen-Lippe, Deutschland

Borrelia (B.) burgdorferi sensu lato, der Erreger der Lyme-Borreliose, ist das bedeutendste vektorassoziierte Zoonose-Pathogen der nördlichen Hemisphäre. Neben Säugetieren spielen auch Vögel, vor allem Sperlingsvögel (*Passeriformes*) und Meeresvögel, eine wichtige Rolle bei der Übertragung, Verbreitung und Aufrechterhaltung dieser Krankheit. Bisherige Untersuchungen an Vögeln haben sich hauptsächlich mit dem Nachweis Borrelien-infizierter Zecken befasst. Dabei wurde deutlich, dass nicht jede Vogelspezies gleichermassen für alle *Borrelia* spp. empfänglich ist; Rückschlüsse auf eine mögliche Infektion des aviären Wirtes sind hierbei nicht möglich. In dieser Studie wurden serologische Untersuchungen an wildlebenden Greifvögeln (n = 29) in Ostwestfalen-Lippe, Nordrhein-Westfalen, Deutschland, durchgeführt. Die Ergebnisse machen deutlich, dass auch Greifvögel und Eulen empfänglich für *B. burgdorferi* sind. Es konnten erhöhte Antikörpertiter mittels indirektem Immunfluoreszenz-Antikörper-Test bei Mäusebussard, *Buteo buteo*, und Europäischem Uhu, *Bubo bubo*, festgestellt werden. Obwohl bisher davon ausgegangen wird, dass Vögel keine klinischen Symptome einer Borreliose ausbilden, sprechen einige wenige Studien sowie diese Arbeit dafür, dass eventuell doch ein mildes Krankheitsbild auftreten kann. Vor allem Diarrhoe und lokale Hautveränderungen sind möglich. Weitere Untersuchungen, auch bezüglich der serologischen Diagnostik der Lyme-Borreliose bei Vögeln sind notwendig. Zukünftig sollte berücksichtigt werden, dass auch Greifvögel als Vektoren für *B. burgdorferi* in Frage kommen.

Schlüsselwörter: *Borrelia burgdorferi*, Lyme Krankheit, Vektor, Antikörper, Greifvogel

Introduction

The importance of the Lyme borreliosis, a tick-borne disease caused by the bacterium *Borrelia (B.) burgdorferi* sensu lato, is constantly increasing. It is the most frequent arthropod-borne disease in the Northern hemisphere today (Lindgren and Jaenson, 2006; Hubálek, 2009; Nau et al., 2009; Anonymous, 2010; Marconi and Earnhart, 2010). Clinical disease of borreliosis occurs not only in humans but also in other mammals including pets and wild animals (Burgess et al., 1987; Parker and White, 1992; Gern et al., 1998; Butler et al., 2005). Since the first detection of *B. burgdorferi* in 1982, many efforts have been made to clarify the complex epidemiology of Lyme disease.

Numerous vectors and susceptible animals have been described (Nakao et al., 1994; Gern et al., 1998; Humair and Gern, 2000; Peisman, 2002; Gern, 2008; Eisen et al., 2009; Földvári et al., 2009). Furthermore, it seems that several bird species, especially *Passeriformes* and sea birds, may act as competent reservoirs for *B. burgdorferi* and are capable of transmitting the spirochete to host-seeking ticks (Olsen, 2007). However, no information about the role and susceptibility of raptors to *B. borreliosis* exist until now.

The aim of the present study was to determine the prevalence of *B. burgdorferi* in free-living birds of prey. Over the winter of 2010, blood sampling was conducted at the Adlerwarte Berlebeck Bird of Prey shelter station, located in the mountainous area of the Teutoburger Wald near Detmold, county of Lippe, Eastern North Rhine-Westphalia (NRW), Germany. The Adlerwarte Berlebeck is a municipal institution of the city of Detmold and has an officially recognised native animal haven for birds of prey since 1975. Serum samples were screened for the presence of IgG-antibodies against *B. burgdorferi* sensu lato using indirect immunofluorescence.

Animals, Material and Methods

From September 2010 to April 2011, blood samples (n = 29) were taken in accordance with the Agency of Nature, Environment and Consumer Protection of North Rhine – Westphalia, Recklinghausen, Germany (licence number: 8.87-51.05.20.10.207), from animals submitted to the shelter. In addition, a clinical examination of each animal as well as a detailed anamnesis (especially place of origin or place of finding) was carried out; further examinations were done according to the clinical signs, when possible. Single blood samples were collected from the brachial vein (*V. ulnaris*) or the metatarsoplantar vein (*V. metatarsalis plantaris superficialis*). Following centrifugation, the sera were stored at –25 °C until analysis.

Indirect immunofluorescence (IFT) on avian serum samples

Indirect immunofluorescence (IFT) was used to screen for the presence of IgG-antibodies against *B. burgdorferi* sensu lato within the collected serum samples, the methodology was performed in accordance with the manufacturer's instructions (MegaScreen® FLUOBORRELIA dog ad us. vet., MegaCor Diagnostik GmbH, Hörbranz, Austria). Four serial dilutions were prepared (titres: 1:16, 1:32, 1:64, and 1:128) in PBS; if necessary, two more serial dilutions were performed (titres: 1:256, 1:512).

One drop of anti-chicken antibody – conjugate (Goat-Anti-Chicken IgG, AbD Serotec, Oxford, Great Britain) at a dilution in PBS of 1 in 200 was added. Additionally, a parallel indirect immunofluorescence testing with protein A – conjugate from *Staphylococcus aureus* was performed as described above (Protein A soluble, Sigma Aldrich Chemie GmbH, Munich, Germany). Protein A is a highly stable cell surface receptor produced by several strains of *Staphylococcus aureus*. It is capable of binding to the Fc portion of the immunoglobulin, especially IgGs. This conjugate is often used to detect immunoglobulins in various immunochemical assays. Because this protein binds IgGs from a large number of species, it was used in the second test in order to increase the sensitivity of the diagnostic approach. The dilution of this conjugate was 1:250.

Positive control wells were coated with *B. burgdorferi* – positive equine serum and anti-horse antibody – conjugate and conjugate A respectively. For negative control, blood samples from 20 laying hens (26 and 8 weeks old, each with ten animals) were taken. The chickens were housed indoor and had no contact to ticks or even outdoor exposure before testing. All slides were examined immediately on a fluorescence microscope with 400 x magnification and filter system for fluorescein isothiocyanate (maximum excitation wavelight 490 nm, mean emission wavelight 530 nm).

Results

During the testing period, 29 blood samples from 10 different species were taken and analysed (Tab. 1). Most frequently analysed species were common buzzards, *Buteo buteo*, (11 samples), barn owls, *Tyto alba*, and kestrels, *Falco tinnunculus*, (each with 4 samples), eagle owls, *Bubo bubo*, (3 samples) and red kites, *Milvus milvus*, (2 samples). All samples were taken from birds found in Eastern Westphalia, with the exception of one sample (peregrine falcon, *Falco peregrinus*), which originated from bordering Northern Hesse. Titres above ≥ 1:64 were considered positive according to Burgess (1989). Four birds showed antibody-titres of ≥ 1:64, of which three birds had elevated antibody titres to *B. burgdorferi* of 1:128 and 1:256 (Fig. 1). Four birds revealed slight fluorescent reactions at

titres between 1:16 and 1:64, whereas 21 birds were clearly negative tested. The prevalence of *B. burgdorferi* sensu lato positive serum was 13.8 %. All negative control birds showed titres of < 1:16. All IFT-tests performed in parallel using conjugate A showed no fluorescence. The positive equine serum samples reacted positive with the conjugate and also negative with the protein A – conjugate. No ticks were detected on any bird during the sampling period.

Two common buzzards serologically tested positive showed diarrhoea (Tab. 1). The parasitological faecal examinations (direct smear and flotation process) were negative in both cases. A bacteriological faecal examination was not performed because the diarrhoea was self-limiting during care at the sanctuary.

Discussion

One challenge in the planning and execution of this study was to ensure a sufficient number of birds of prey were included within the sampling period. Till now, similar experiments – mainly on *Passeriformes* – were often carried out by means of Japanese nets. Studies using sea birds are often possible without problems due to the absence of fear towards humans in some species – especially in isolated areas. However, birds of prey cannot be caught in nets due to their size, the low population density and their behaviour (Mayer, 1961). In addition, birds of prey are subject to special legal protections in Germany that forbid such capture methods.

Table 1: Overview of serologic testing for *Borrelia burgdorferi* sensu lato in free-living birds of prey in Eastern North Rhine-Westphalia, Germany, submitted to a rehabilitation centre.

Species	Sampling date	Location	(Clinical) findings	Borrelia-titre (IgG)*
Goshawk, *Accipiter gentilis*	24-01-2011	Detmold	Male, juvenile	< 1:16
Eagle owl, *Bubo bubo*	13-09-2010	Blomberg		1:128
	13-09-2010	Detmold		1:256
	10-01-2011	Leopoldstal	Mild diarrhoea	< 1:16
Common buzzard, *Buteo buteo*	06-09-2010	Augustdorf		< 1:16
	06-09-2010	Kalletal		< 1:16
	06-09-2010	Lemgo		< 1:16
	06-09-2010	Dörentrup	Mild diarrhoea	1:128
	06-09-2010	Vlotho	Mild diarrhoea	1:32
	08-11-2010	Blomberg		< 1:16
	07-01-2011	Verl		< 1:16
	10-01-2011	Bad Salzuflen	Haematoma at the left wing	< 1:16
	28-02-2011	Verl		< 1:16
	14-03-2011	Detmold		1:64
	14-03-2011	Detmold		< 1:16
Marsh harrier, *Circus aeruginosus*	27-09-2010	Paderborn		< 1:16
Peregrine falcon, *Falco peregrinus*	13-09-2010	Breuna (Hesse)	Male	1:16
Kestrel, *Falco tinnunculus*	11-10-2010	Detmold	Male	< 1:16
	11-10-2010	Kalletal	Female	< 1:16
	24-11-2010	Lage	Male, mild headtrauma	< 1:16
	30-04-2011	Detmold	Female	< 1:16
Red kite, *Milvus milvus*	06-09-2010	Güterlsoh		< 1:16
	06-09-2010	Extertal		1:32
Honey buzzard, *Pernis apivorus*	27-09-2010	Lage		1:16
Tawny owl, *Strix aluco*	13-09-2010	Detmold		< 1:16
Barn owl, *Tyto alba*	13-09-2010	Hiddenhausen		< 1:16
	13-09-2010	Bad Driburg		< 1:16
	13-09-2010	Nieheim		< 1:16
	14-10-2010	Vlotho	Euthanasied because of comminuted fracture of radius and ulna, left wing	< 1:16

* values < 1:64 are considered negative

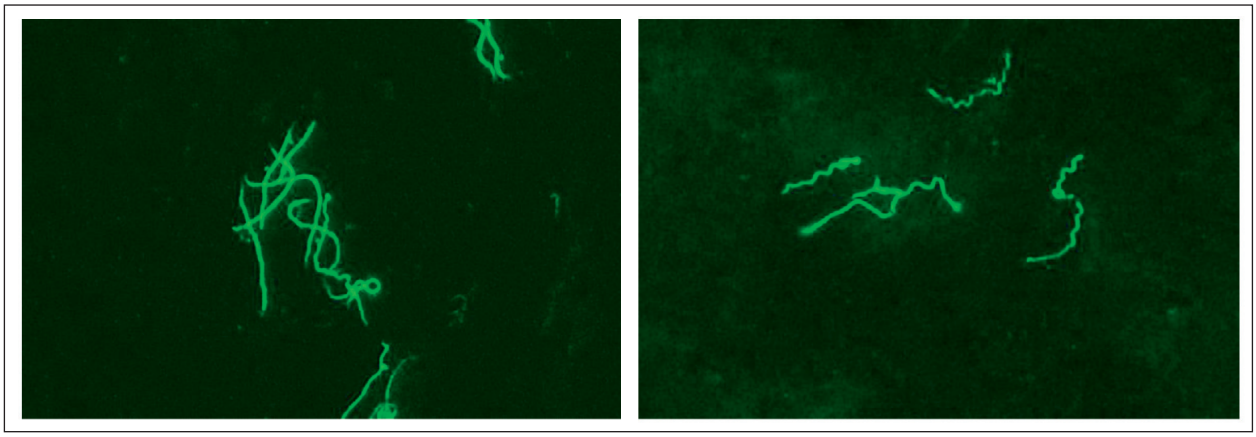

Figure 1: Microscopic pictures of positive indirect immunofluorescence testing (630x) for *Borrelia burgdorferi* sensu lato in the blood of two birds of prey: left: eagle owl, *Bubo bubo* (sample from 13-09-2010, Detmold), titre 1:256; right: common buzzard, *Buteo buteo* (sample from 06-09-2010, Dörentrup), titre 1:128.

A "standardised catch" of birds performed in other studies was not feasible. For these reasons, blood samples were randomly taken in this study from birds of prey found injured or weakened. It is generally assumed, that *B. burgdorferi* does not cause any weakness or clinical signs in birds (Olsen, 2007) and as such it is not suggested that these birds provide a biased collection beyond its opportunistic approach. For the same reason, any increased antibody levels would not correlate with the underlying disease.

Also, the relatively small sample quantity of 29 animals results from these circumstances; species are frequently only represented once (for example honey buzzard, peregrine falcon). The low occurrence of some species in NRW (honey buzzard < 350 breeding pairs; marsh harrier 110–120 pairs, eagle owl 180–200 pairs (Anonymous, 2009)) will have had some influence on the frequency with which these species were rescued. It cannot be answered in conclusion, whether these circumstances have an influence on the representation of the blood samples.

Till now, there is no standardised or even commercial test for the detection of *B. burgdorferi* in birds. The most frequently used test is the analyses of ticks found on the bird for *B. burgdorferi* via molecular means. Because this study was performed mainly during the winter, no ticks could be detected on the birds due to the little activity of ticks during the cold months. A problem in analysing sampled ticks from caught birds is that this approach does not allow a direct conclusion about an infection in the affected bird. Eventually, the infested bird carries the tick without getting infected itself by the spirochetes; a conclusion about the avian spirochetal compatibility is not necessarily possible.

According to Olsen (2007) the direct detection of *B. burgdorferi* via cultivation from biopsies is the ultimate diagnostic confirmation. However, due to the difficult and protracted cultivation of spirochetes associated to the risk of contamination, this method was not performed in the current study. An alternative approach recommended in the literature is the detection of *B. burgdorferi* – antibodies (Isogai et al., 1994; Piesman et al., 1996; Staszewski et al., 2007); however, the relatively large blood volume required for this ELISA was not deemed appropriate given the nature of the studied population. The advantage of the IFT approach used in this study is primarily the good feasibility with published diagnostic parameters indicated that under experimental conditions – titres ≥ 1:64 were "positive"(Burgess, 1989). Interestingly, this value correlated also with positive *B. burgdorferi* – titres of dogs for which the used test originally was produced. Unfortunately, it was not possible to examine a positive avian control sample with the used IFT; despite intensive efforts, no positive control sample, e.g. from past experimental studies, could be found. To document the correct execution of the test, an equine positive control was done. Since four examined samples showed a clear increased antibody-titre, it can be assumed that the test works although false-positive results are conceivable. To get better prospects regarding the specificity of the used IFT, 20 blood samples from indoor-housed layers chickens were tested. All samples showed titres < 1:16, supporting a good specificity of this IFT. Interestingly, the testing series using conjugate A show no reaction at all; while this test is often used due to its species-independent reaction pattern mainly in public veterinary investigation laboratories in Germany; however, within the context of this investigation the application of this approach was not successfully reproduced within these avian samples. Altogether, due to the small number of samples and the lack of positive avian control samples, no statistical evaluation about specificity and sensitivity of the used IFT could be made. Interestingly, no previous serological field studies could be found giving information about specificity and sensitivity of their tests. Further examinations are necessary.

This is the first study identifying *B. burgdorferi* – antibodies in birds of prey. Previous, only one sparrow hawk, *Accipiter nisus*, from South Sweden was found with a

B. burgdorferi – positive tick (Olsen et al., 1995). As in the case of other migratory birds, migrating birds of prey (e.g. honey buzzard, marsh harrier, common buzzards from Scandinavia) are relevant for the distribution of infected ticks. Perhaps the different hunting strategies of different bird of prey and owl species also have an influence on the contact with the pathogen.

For example, common buzzards, which mostly hunt on the ground and also by foot, may be more likely to be in contact with ticks and therefore also with *B. burgdorferi*. A spirochetal transmission by direct contact with rodents or ticks attached on rodents or even small birds serving as prey is also conceivable. Furthermore, it is possible that nestlings could be exposed when the prey is transported back to a nest where there would be close contact between the birds and the infested animal carcass. To date there is no indication if an oral infection with *Borreliae* is possible, but the low pH of the gastric acid in birds of prey contradicts this theory (Heidenreich, 1995). A transmission via infectious faeces is possible (Gronesova et al., 2008) and may therefore play also a role in the distribution of *B. burgdorferi* in birds of prey; but it needs to be investigated if birds of prey excrete *B. burgdorferi* in the faeces.

The importance of birds of prey in Lyme disease may be underestimated due to current lack of research in this field. It is not possible to make major conclusions based on the individual results of this study and further research is clearly necessary. In the future, birds of prey should be highlighted as potential carriers of the Lyme disease; and while their epidemiological role is unclear, the risk that this poses predisposed persons (e.g. veterinarians, biologists, zookeepers, falconers, hunters) should be considered.

Conclusion

This study shows that free-living birds of prey respond immunologically to infections with *B. burgdorferi* and may therefore play a role in the transmission, maintenance and movement of borreliosis. It appears that Lyme disease in birds of prey seems to be underestimated due to the research to date. In general, this work suggests that these birds may play an important role in the epidemiology of Lyme disease.

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Received: 22 June 2012
Accepted: 15 August 2012