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# Detection of *Borrelia burgdorferi* sensu lato and *Chlamydophila psittaci* in Throat and Cloacal Swabs from Birds Migrating through Slovakia

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**ABSTRACT.** We have screened 91 migratory birds representing 32 species during the autumn of 2003 for the presence of the zoonotic pathogens *Borrelia* and *Chlamydophila*. Using polymerase chain reaction (PCR), *B. burgdorferi* sensu stricto was detected in cloacal swabs and, in two causes, also in throat swabs in 8 individuals (8.7 %) representing 7 birds species; *B. garinii* and *B. afzelii* were not detected. *C. psittaci* was detected only in cloacal swabs; 6 birds (6.6 %) from four species were found to be positive. The PCR products were sequenced and the sequences were compared phylogenetically with the gene sequences of 14 *Chlamydophila* strains retrieved from nucleotide databases; although the sequenced DNA was only 110 bp long, all obtained sequences created a new cluster with sublines branching from a position close to the periphery of the genus. All tested samples appear distinct within the known species and were most similar to *C. felis* or *C. abortis*.

Wild migratory birds can be infected by a number of pathogenic microorganisms that are transmissible also to humans. In Europe, the migratory routes of birds are more diverse than in North America (Olsen *et al.* 1995). Slovakia is a country that is crossed by some dominant European North–South and East–West birds' migratory routes. In Europe, birds are considered to play an important role as distributors of several pathogenic biological agents and it is clear that they contribute to the global spread of emerging infectious diseases (*e.g.*, Lyme borreliosis, chlamydial infections, influenza, cholera, salmonelosis, tuberculosis, coxeliosis, ornithosis, *etc.*) either as a reservoir host or by dispersing infected arthropod vectors.

Lyme borreliosis is a multiphase multisystem disease characterized by dermatological, musculoskeletal, and neurological manifestations. The etiological agent, *Borrelia burdorferi* sensu lato (s.l.), has been identified in a variety of tick species across the globe (Steere 1989). In Europe, *B. burgdorferi* s.l. has been isolated from *Ixodes ricinu*s, *I. persulcatus*, *I. uriae*, *I. hexagonus*, *Culex* spp., birds, shrews, voles, mice, hares, and humans (Gern and Humair 2002; Halouzka *et al.* 1998; Hubálek 2004; Olsen *et al*. 1993; Kurtenbach *et al*. 1998; Schwarzová and Čižnár 2004; Rudenko *et al*. 2005). Of the 10 genospecies of *B. burdorferi* s.l., 4 (*B. burdorferi* sensu stricto, *B. garinii*, *B.afzelii*, and *B. valaisiana*) are know to occur in North-Western Europe. Some European *Borrelia* strains genetically resembled the North American strains which suggests that *Borrelia* species may share a common ancestry (Postic *et al.* 1999). Studies about *Borrelia* intraspecific heterogeneity revealed that *B. garinii* was phenotypically the most heterogeneous genospecies (Gern and Humair 2002).

The fact that the same type of Lyme disease exists in both the Northern and the Southern Hemisphere shows that birds participate in the natural circulation of *Borrelia* spirochetes (Gern and Humair 2002; Poupon *et al*. 2006). The relatively high prevalence of *Borrelia*-infected *I. ricinus* larvae collected from migratory birds plays a role as spirochetal reservoirs infective to ticks. Infected ticks have been collected from various bird species in almost all European countries (Humair *et al.* 1993; Olsen *et al*. 1995; Hubálek *et al*. 1996; Gern and Humair 2002; Hubálek 2004; Hanincová *et al*. 2003), especially from thrushes (*Turdus* spp.), blackbirds (*Turdus merula*), robins (*Erithracus rubecula*) and pheasants (*Phasianus colchicus*), as well as in colonial seabirds (Olsen *et al*. 1993; Kurtenbach *et al.* 1998; Humair *et al.* 1998; Poupon *et al*. 2006). Certain birds are less suitable as hosts for the ticks, or fail to infect feeding ticks, even after extensive exposure to infected ticks (Matuschka *et al*. 1991).

The family *Chlamydiaceae* comprises intracellular Gram-negative bacteria, which are responsible for serious chlamydial infections of both humans and animals. The family *Chlamydiaceae* is classified into two genera: (*i*) genus *Chlamydia* with the species *Chlamydia trachomatis*, *Chlamydia suis* and *Chlamydia*

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*muridarum* and (*ii*) genus *Chlamydophila* with the species *Chlamydophila abortus*, *Chlamydophila caviae*, *Chlamydophila felis*, *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* (Laroucau *et al*. 2001). Each species has a limited host range and is usually associated with specific disease syndromes including respiratory infections (caused by *C. pneumoniae*), urogenital or sexually transmitted infection of reproductive organs (*C. trachomatis*), and psittacosis (*C. psittaci*). In animals, *C. psittaci* and *C. pecorum* cause a variety of important diseases, including abortion, pneumonia, enteritis, encephalomyelitis or conjunctivitis (Madico *et al*. 2000; Takahashi *et al.* 1997).

The aim of this study was to investigate if wild birds resting in Slovakia during their autumn migration carry serious zoonotic pathogens transmissible to humans. This is the first study of *Borrelia* and *Chlamydophila* pathogens isolated from crop and cloacal swabs of migratory birds in Slovakia. A better understanding of avian migration patterns and infectious diseases of birds would be useful in helping to predict future outbreaks of infections due to emerging zoonotic pathogens.

## MATERIAL AND METHODS

*Study site.* The birds were captured on two comparative localities in Eastern Slovakia, Senianské and Perin-Chymské ponds in October and November 2003. Senianské ponds are located in the orographical unit 'Vychodoslovenska' plane near Senné, Inacovce and Blatné Remety villages in DFS 7298 and 7398 squares, with geographic location N 48´ 42″, E 22´ 02″. This habitat (National Park) is one of the most significant localities for breeding and resting water birds in Slovakia. The area with the system of active ponds is ≈703 $hm^2$ large. The vegetation is dominated by *Typha latifolia*. Chymské ponds are located in the aerographical unit Košická fold in DFS 7492 and the geographic location N 49´ 28″, E 21´ 12″ near the villages: Chym and Vyšný Lanec. The biggest pond is about 58 $hm^2$, the central one 36 $hm^2$ and the smallest pond 6 $hm^2$ large. *Phragmites communis* and *Typha latifolia* dominate amongst the vegetation at the boundary (Danko 1997).

*Collection of samples from birds.* Birds were caught in nets at ground level of the sewed ponds, in nightfall and during the night. Netted birds were carefully removed, identified to species, ringed, the cloacal and throat samples were collected and the birds were released (Betáková *et al*. 2005). The birds were not harmed or killed. Monitoring and collection of ticks attached to the captured birds was not performed.

*DNA extraction.* Each swab (cloacal and throat) was individually extracted in 3 mL of phosphate-buffered saline (pH 7.2) and 100 µL of the extract was used for purification of DNA by using the DNeasy Mini kit (*Qiagen*) following manufacturer's recommended protocol; extracted DNA was stored at –20 °C.

*Detection of* Borrelia *and* Chlamydophila *by PCR.* One µL of the DNA extract was amplified in 20 µl of PCR reaction using 2× PCR Master Mix (*Merck Life Science*). Four different sets of primers specific to *Borrelia* and *Chlamydophila* were used for each PCR reaction; primer set specific for each of four *Borrelia* species was designed by Kurtenbach *et al.* (1998). *B. burgdorferi* s.l. specific PCR product was 650 bp, *B. garinii* 380 bp, and *B. afzelii* 200 bp. Oligonucleotide primers specific for *C. psittaci*: CPS100 and CPS101 were described by Madico *et al*. (2000) and the predicted size of the PCR product was 111 bp.

PCR was performed in 35 cycles (94 °C 30 s – denaturation, 55 °C 30 s – annealing, 72 °C 1 min – extension); specific products were identified after agarose (2 %)-gel electrophoresis by ethidium bromide staining.

*Phylogenetic tree construction.* The PCR products were sequenced and the sequences were compared with those available in *GenBank*. The phylogenic relationships of the sequences of nucleotides of chlamydial 16S rRNAs were compared using the program on the web site: **`http://www.ebi.ac.uk/clustalw/index.html`**.

## RESULTS

*Collection of samples.* The water birds were predominantly *Passeriformes*, *Charadriiformes*, *Anseriformes*, and *Gruiformes*. In total, 91 birds representing 32 species were netted and examined for the pathogen presentation. Fifty-nine birds representing 25 species were caught on Perin-Chymské ponds (Table I) and 32 birds belonging to 15 species were caught on Senianské ponds (Table II). Some species were specific only for one locality.

*Detection of* Borrelia *in birds.* The samples from throat and cloacal swabs were analyzed by PCR with three sets of primers specific to *B. burgdorferi* s.l., *B. garinii*, and *B. afzeli*, respectively. *B. burgdorferi* s.s. was the only species detected in the samples collected from the birds. According to 650-bp PCR product

(Fig. 1A) all our DNA had a 99–100-% sequence similarity to *B. burdorferi* s.s. Spirochetal DNA was detected in 8 individuals (8.7 % out of 91 tested birds) representing 7 birds species (Table I). *B. burgdorferi* s.s. was found in throat swabs of 3 bird species, individually in European robin, Eurasian jay, and song trush. Cloacal swabs were found positive in 7 cases: in mallard, European robin, Eurasian jay, water rail, jack snipe, ruff, and song thrush. The samples obtained from throat and cloaca were found positive only in two birds, *viz*. in European robin and Eurasian jay. When the localities were compared, bird species resting on Senianské ponds were *Borrelia* negative (*data not shown*).

**Table I.** *Borrelia* prevalence in migratory and free-living birds in Perin-Chymské ponds[a]

| Family | Species | | Total[b] | Throat[c] | Cloaca[c] |
|---|---|---|---|---|---|
| *Passeriformes* | *Acrocephalus palustris* | marsh warbler | 1 | 0 | 0 |
| | *Carduelis carduelis* | European goldfinch | 2 | 0 | 0 |
| | *Erithacus rubecula* | European robin | 7 | **1** | **1** |
| | *Fringilla montifringilla* | brambling | 3 | 0 | 0 |
| | *Garrulus glandarius* | Eurasian jay | 1 | **1** | **1** |
| | *Sturnus vulgaris* | common starling | 1 | 0 | 0 |
| | *Turdus merula* | common blackbird | 3 | 0 | 0 |
| | *Turdus philomelos* | song thrush | 2 | 1 | 1 |
| *Coraciiformes* | *Alcedo athis* | common kingfisher | 2 | 0 | 0 |
| *Anseriformes* | *Anas platyrhynchos* | mallard | 1 | 0 | 1 |
| *Gruiformes* | *Rallus aquaticus* | water rail | 2 | 0 | 1 |
| *Charadriiformes* | *Calidris alpina* | dunlin | 2 | 0 | 0 |
| | *Gallinago gallinago* | common snipe | 2 | 0 | 0 |
| | *Lymnocryptes minutus* | jack snipe | 1 | 0 | 1 |
| | *Philomachus pugnax* | ruff | 2 | 0 | 1 |
| Total | | | 32 | 3 | 7 |

[a]All samples were negative for *Chlamydophila.* [b]Total number of animals.
[c]Number of positive samples; the cloacal and throat samples detected positive in the same birds are **bold**.

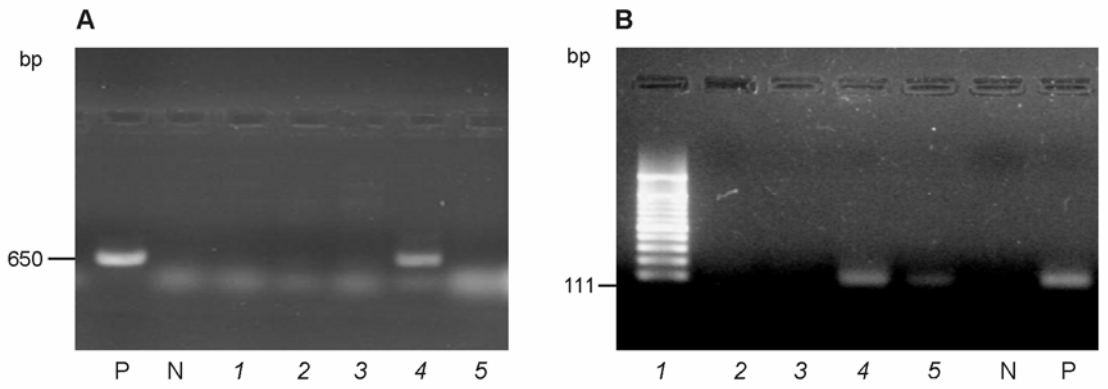


**Fig. 1.** Representative PCR of *B. burdorferi* s.s. (**A**) and *C. psittaci* (**B**). **A**: *1–5* – samples; **B**: *1* – molecular size standards (O'RangeRuler$^{TM}$ 200 bp DNA ladder, 200–3000 bp – 200-bp increments; *Fermentas*), *2–5* – samples; P – positive control, N – negative control.

*Detection of* Chlamydophila *in birds.* Screening of throat and cloacal samples by PCR (Fig. 1B) gave positive results in 6 birds (Table II). *Chlamydophila* was not detected in the samples obtained from the birds captured on Perin-Chymské ponds (*data not shown*). A 111-bp PCR products were sequenced and the sequences of nucleotides of chlamydial 16S rRNAs were compared with those from *GenBank*.

*Phylogenies of chlamydial 16S rRNAs.* Close relatedness of each species of the genus *Chlamydophila* was apparent in all species (Fig. 2). Sequences of *C. psittaci* (D85713), *C. psittaci* VS1 (AF481052), *C. psittaci* VS225 (AF481049), *C. psittaci* Daruma (AF481048), *C. pneumoniae* J138 (BA000008), *C. pneumoniae* (AF347606), *C. pneumoniae* IOL207 (Z49874), *C. pneumoniae* CWL029 (AE001303), *C. abortus* EBA (U76710), *C. abortus* S26/3 (CR848038), *C. felis* (U68457), *C. felis* FP (U68458), *C. pecorum* (U68434),

and *C. caviae* (U68451) were obtained from *GenBank*. The small sequences obtained from bird samples created a new cluster with sublines branching from a position close to the periphery of the genus. All tested samples appeared distinct within the known species and were most similar to *C. felis* or *C. abortis*. The sam-

**Table II.** *Chlamydophila* prevalence in migratory and free-living birds in Senianské ponds[a]

| Family | Species | | Total[b] | *Chlamydophila*[c] |
|---|---|---|---|---|
| *Passeriformes* | *Acrocephalus schoenobaenus* | sedgewarbler | 3 | 0 |
| | *Anthus cervinus* | red-throated pipit | 1 | 0 |
| | *Anthus pratensis* | meadow pipit | 1 | 0 |
| | *Delichon urbica* | house martin | 2 | 0 |
| | *Emberiza schoeniclus* | reed bunting | 7 | 1 |
| | *Hirundo rustica* | barn swallow | 3 | 0 |
| | *Lanius collurio* | red-backed shrike | 1 | 0 |
| | *Motacilla alba* | white wagtail | 2 | 0 |
| | *Phylloscopus collybita* | common choffchaff | 5 | 0 |
| | *Phylloscopus trochilus* | willow warbler | 1 | 0 |
| | *Sturnus vulgaris* | common starling | 2 | 0 |
| *Coraciiformes* | *Alcedo athis* | common kingfisher | 1 | 0 |
| *Anseriformes* | *Anas crecca* | common teal | 2 | 0 |
| *Gruiformes* | *Rallus aquaticus* | water rail | 1 | 0 |
| *Charadriiformes* | *Calidris alpina* | dunlin | 2 | 0 |
| | *Gallinago gallinago* | common snipe | 11 | 3 |
| | *Charadrius dubius* | little ringed plover | 2 | 0 |
| | *Charadrius hiaticula* | great ringed plover | 3 | 0 |
| | *Lanius collurio* | red-backed shrike | 1 | 0 |
| | *Larus ridibundus* | black-headed gull | 1 | 0 |
| | *Lymnocryptes minutus* | jack snipe | 2 | 0 |
| | *Pluvialis squatarola* | grey plover | 3 | 0 |
| | *Tringa glareola* | wood sandpiper | 1 | 1 |
| *Strigiformes* | *Asio otus* | long-eared owl | 1 | 1 |
| Total | | | 59 | 6 |

[a]All samples were negative for *Borrelia.* [b]Total number of animals. [c]Number of positive samples.

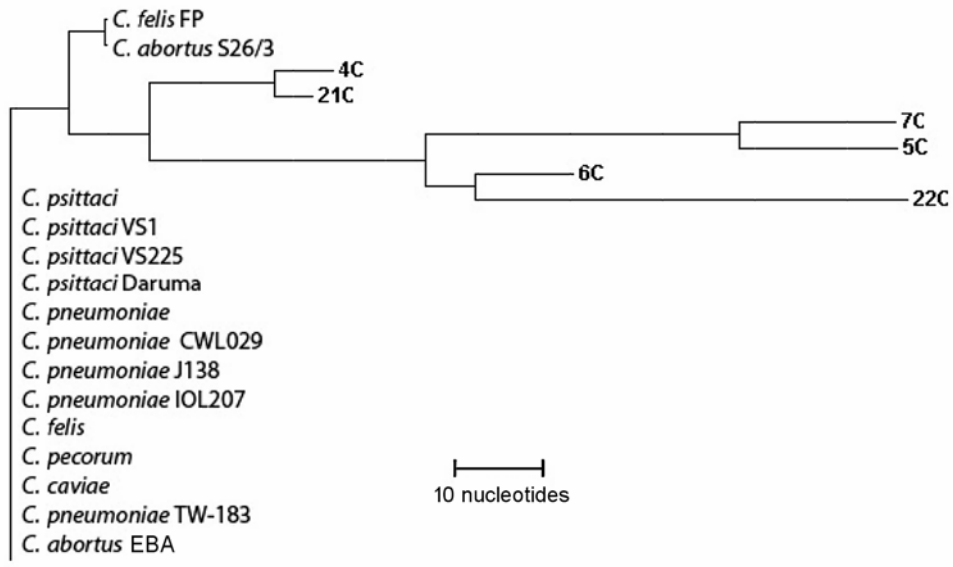


**Fig. 2.** Phylogenetic analysis based on a comparison of the 16S rRNA sequences (110 bp) of *Chlamydophila* species (obtained by the maximum-likelihood method); sequences of strains *C. psittaci*, *C. pneumoniae*, *C. abortus*, *C. felis*, *C. pecorum* and *C. caviae* were obtained from *GenBank*.

ple 22C, whose evolutionary distance was 1.68 per 0.1 kb, exhibited the deepest branch in the clade. Deep branches were also observed in samples 5C and 7C, whose evolutionary distance values with other samples in the clade were 0.60727 and 0.60570 per 0.1 kb, respectively. The sample 6C, whose evolutionary distance was 0.38 per 0.1 kb, was related with sample 22C. On the other hand, samples 4C and 21C were similar to each other and formed a specific cluster (evolutionary distance values in the clade were 0.22 and 0.14, respectively).

## DISCUSSION

*Borrelia* infection was found only in the birds captured on Perin-Chymské ponds and *Chlamydophila* was found only in the birds captured on Senianské ponds. However, the birds captured on Perin-Chymské ponds belonged to species that were not captured on Senianské ponds and *vice versa.* We do not have enough representative individuals from the species of the birds captured on each pond, and thereby we are not able to compare the prevalence of one or the other pathogen in these two localities. These throat and cloaca samples were also tested for the presence of influenza virus. Double infection with *C. psittaci* and the influenza virus was detected in the cloaca sample of the owl (Betáková *et al*. 2005).

Ecological studies on Lyme borreliosis conducted in Europe showed that some mammalian and bird species frequently infected by *I. ricinus* transmit infection to ticks. Birds like pheasants (*Phasianus colchicus*), *Turdus* sp. and migrating birds may act as amplifying reservoirs for *B. burgdorferi* s.l. (Olsén *et al.* 1995; Hanincová *et al*. 2003; Humair *et al*. 1998; Kurtenbach *et al.* 1998). Little is known about the development of *B. burgdorferi* s.l. in the host. *Borrelia* has been isolated and/or detected in various organs or tissues, such as skin, blood, joints, internal organs (spleen, heart, brain, liver, urinary bladder, kidneys, and nervous system) in various vertebrate hosts (Demaerschalck *et al*. 1995; Kurtenbach *et al.* 1998; Olsen *et al.* 1993; Gern and Humair 2002). However, evidence about throat and cloacal samples infected with *Borrelia* species is very rare and, thus, it is not fully possible to compare our data with other findings. An experimental inoculation of mallard ducks (*A. platyrhynchos*) with *Borrelia* spirochetes showed that the ducks remained asymptomatic but the spirochete was recoverable from the blood for 7 d after inoculation and from the cloaca content for 3–4 weeks after inoculation (Burgess 1989).

According to Hubálek (2004) among pathogens carried by migratory birds *B. afzelii* seems to be one of the most abundant genospecies in continental Europe. Some songbird species, seabirds, and pheasants are reservoir hosts of *B. garinii* and *B. valaisiana* but not of *B. afzelii* (Kurtenbach *et al.* 2002) but these genospecies were not detected in southern England (Kurtenbach *et al.* 1998). Although 19 % of the rodents harbored *B. burgdorferi* s.s. and/or *B. garinii* in internal organs, only *B. burgdorferi* s.s. was transmitted by the rodents to ticks. Strains of *B. burgdorferi* s.s. appear to be much less specialized than strains of the other genospecies.

We showed that the migratory and free living birds in Slovakia were infected only with *B. burgdorferi* s.s. However, we did not perform PCR with primers specific for *B. valaisiana.* It would be of a great interest to include new, more specific primers for *B. burgdorferi* s.l. which in the future should allow to determine all species.

For the chlamydial infection diagnosis, cloacal swab extracts seem to provide a suitable material. Wild ducks and other bird species represent also a significant reservoir of ornithosis and can spread the *C. psittaci* pathogens by direct contact or aerosol to vertebrates. Throat infection with *C. psittaci* was not observed. Chlamydial organisms are labile but can remain infectious for several months if protected by organic debris (egg, litter and feces). Some infected birds can appear healthy and shed the organism intermittently, activated by, *e.g*., stress factors (Greco *et al.* 2005; Heddema *et al*. 2006).

According to the phylogram, within the cluster of *C. psittaci* strains, several sublines were branched at positions that were relatively close to each other. The phylogenetic analysis based on 16S rRNA sequences indicates that the following groups exist within the species *C. psittaci*: (*i*) a feline pneumonitis strain, (*ii*) ovine enzootic abortion strains, (*iii*) strains related to human psittacosis, (*iv*) other strains, which can probably be sub-divided into several subgroups, such as *C. pecorum* and *C. caviae* (Takahashi *et al*. 1997). The limited number of strains used requires that the detection should be complemented with additional analysis for more precise identification; otherwise the results cannot be compared with the findings of other authors. (At least four groups of strains exist within the species *C. psittaci*, *C. trachomatis* has been divided into three biotypes and 15 serovars and the genealogical characteristics of the newly classified species, *C. pecorum*, are also distinct; Takahashi *et al.* 1997.)

This is the first study of *Borrelia* and *Chlamydophila* pathogens isolated from throat and cloacal swabs of migratory and free living birds in Slovakia. The majority of studies have focused on detection and

characterization of *Borrelia* in ticks. Our data show that it is possible to detect *Borrelia* also in throat swabs of birds. Specific association was shown between *Borrelia* species and reservoir hosts; the birds are mainly associated with *B. valaisiana* and *B. garinii* and these strains ought to be the subjects for future studies. The birds as the main natural reservoir of *C. psittaci* (*cf.* Everett *at al.* 1999) should be considered a probable source of animal and human infections. A more sensitive and reliable test for specific identification of *Chlamydia* strains would considerably facilitate relevant epidemiological studies.

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