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NEWCASTLE DISEASE VIRUS AND *CHLAMYDIA PSITTACI* IN FREE-LIVING RAPTORS FROM EASTERN GERMANY

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ABSTRACT: Organ samples from free-living raptors from the federal states of Berlin and Brandenburg in eastern Germany were tested for Newcastle disease virus (NDV; $n=331$) and *Chlamydia psittaci* ($n=39$) by polymerase chain reaction (PCR). In 18 individuals NDV nucleic acids were detected. These samples originated from barn owls (*Tyto alba*; $n=15$, 28%), tawny owl (*Strix aluco*; $n=1$, 5%), common buzzard (*Buteo buteo*, $n=1$, 1%), and European kestrel (*Falco tinnunculus*; $n=1$, 4%). In 29 (74%) of 39 samples *C. psittaci* was detected. *Chlamydia psittaci* is common in free-living birds of prey in the investigated area.

Key words: *Chlamydia psittaci,* Germany, Newcastle disease virus, polymerase chain reaction, raptors.

INTRODUCTION

Newcastle disease (ND) and chlamydiosis are present in Germany. Two hundred and nine ND and 1,111 chlamydiosis outbreaks were reported from Germany between 1994 and 1997 (OIE, pers. comm.). Newcastle disease is caused by an avian paramyxovirus-1 (NDV) within the family *Paramyxoviridae* that has an intracerebral pathogenicity index in 1 day-old chicks (*Gallus gallus*) of 0.7 and greater or in which multiple basic amino acids have been demonstrated at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein (Alexander, 2000). *Chlamydia psittaci,* the etiological agent of chlamydiosis, is a highly infectious, obligate, intracellular bacterium. Both pathogens affect a wide range of avian hosts and have been isolated from captive and free-living birds of prey (Kaleta and Baldauf, 1988; Gerbermann and Korbel, 1993). Both diseases, but especially chlamydiosis can be a risk for personnel working with wildlife (Lüthgen, 1978; Anonymous, 1998a). Therefore, epizootiologic studies of NDV and *C. psittaci* in free-living birds of prey are of considerable importance. As many migratory birds also European raptors may facilitate rapid spread of infections across countries, especially those species that congregate before, during or after migration. Moreover, ND or chlamydiosis might be transferred by translocation of birds of prey and could also be spread during flying of falconry birds (Forbes, 1997).

For numerous reasons many raptor species have become threatened in Europe (Gerlach, 1974; Anonymous, 1998b). Thus, detailed knowledge of raptor diseases is of considerable importance (Sander, 1995), especially since wild birds of prey are increasingly kept in raptor centers for conservation or rehabilitation purposes and reintroduction programs are conducted (e.g., in Brandenburg state with peregrine falcons [*Falco peregrinus;* Sömmer, 2000]). In this context, investigations of relevant diseases in free-living birds of prey are important.

Our objective was to determine occurrence of NDV and *C. psittaci* in free-living birds of prey in the states of Berlin and Brandenburg in eastern Germany using polymerase chain reaction (PCR). Moreover, we were interested to know whether free-living birds of prey could serve as reservoirs of these pathogens and to what extent rare raptor species such as peregrine falcon, osprey (*Pandion haliaetus*), and

TABLE 1. Polymerase chain reaction positive raptors to Newcastle diseases virus and *Chlamydia psittaci.*

Species	NDV[a]	*C. psittaci*[b]
Common buzzard (*Buteo buteo*)	1/105[c]	4/5
Barn owl (*Tyto alba*)	15/54	3/5
Eurasian sparrowhawk (*Accipter nisus*)	0/45	2/3
Goshawk (*Accipiter gentilis*)	0/28	3/4
Eurasian kestrel (*Falco tinnunculus*)	1/24	6/6
Tawny owl (*Strix aluco*)	1/21	3/4
White-tailed sea eagle (*Haliaeetus albicilla*)	0/16	2/2
Long-eared owl (*Asio otus*)	0/8	0/2
Osprey (*Pandion haliaetus*)	0/7	0/1
Red kite (*Milvus milvus*)	0/7	1/1
Peregrine falcon (*Falco peregrinus*)	0/4	2/2
Eagle owl (*Bubo bubo*)	0/3	0/1
Tengmalm's owl (*Aegolius funenereus*)	0/3	nd[d]
Black kite (*Milvus migrans*)	0/2	1/1
Northern rough legged buzzard (*Buteo lagopus*)	0/2	1/1
Honey buzzard (*Pernis apivorus*)	0/1	1/1
Marsh harrier (*Circus aeruginosus*)	0/1	nd
Total	18/331	29/39

[a] Newcastle disease virus.
[b] *Chlamydia psittaci.*
[c] Number of positive reactors/number of samples tested.
[d] nd=not determined, insufficient volume available.

white-tailed sea eagle (*Haliaeetus albicilla*) are possibly threatened by these agents.

MATERIALS AND METHODS

A total of 342 lung and brain samples and 153 spleen samples from free-living birds of prey was collected between 1994 and 1997. The samples originated from Berlin (52°30′N, 13°20′E) and Brandenburg (51°35′–53°45′N, 11°60′–14°70′E) in eastern Germany and came from various raptor species (Table 1) that were submitted to the federal veterinary investigation center in Berlin. Information on case history and pathologic findings was only available in 46 cases. All samples were stored at −80 C until examined.

Brain and lung from 331 raptors were tested for NDV nucleic acids by a modification of the PCR described by Stäuber et al. (1995). Due to logistic reasons, brain and lung samples were not tested separately. At first, total RNA was isolated from lung and brain samples (30–50 mg) using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA was stored at −80 C. Oligonucleotide primers were NCD3 and NCD4 (Stäuber et al., 1995). Reverse transcription (RT) was performed in a total of 25.2 μl containing 30–300 ng RNA in 14.75 μl, 200 U M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA), 1.25 U RNase inhibitor (Promega), 3 mM $MgCl_2$, 2 mM of each dNTP (Promega), and 120 pmol NCD3 sense primer, 50 mM Tris-HCl (pH 8.3), and 75mM KCl. After denaturation (95 C, 5 min) of the sense primer NCD3 and RNA, remaining components of the RT mixture were added. The RT was performed for 30 min at 37 C followed by 15 min at 42 C. Finally, the RT was heat inactivated for 5 min at 99 C. The cDNA was stored at 4 C until further use. Following RT a PCR (50 μl) was performed under the following conditions: 10 μl cDNA (from the 25.2 μl RT-reaction), 1 U AmpliTaq DNA polymerase (Perkin Elmer, Langen, Germany), 5 mM $MgCl_2$ (Perkin Elmer), 0.5 mM of each dNTP (Promega), 50 pmol NCD3, 50 pmol of the antisense primer NCD4, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. After an initial denaturation period (90 sec at 94 C), DNA was amplified throughout 35 cycles (94 C, 30 sec; 51 C, 30 sec; 72 C, 60 sec). The PCR was completed with a terminal elongation phase (10 min, 72 C). The expected length of the amplified DNA fragment was 310 bp. Five μl of the amplicons were analysed in 2% agarose gels in tris-acetate EDTA buffer. Amplified DNA was size-evaluated with a 100 bp DNA ladder 323–1S (New England Biolabs, Schwalbach, Germany). In order to identify PCR amplicons as NDV fragment direct sequencing was performed (Sanger et al., 1977) using BigDye

TABLE 2. Primer sequences for the detection of *Chlamydia.*

Primer	Specifity	Nucleotide sequence[a]
201CHOMP	*Chlamydia* spp.	5′-GGI GCW GMI TTC CAA TAY GCI CAR TC-3′
CHOMP 336	*Chlamydia* spp.	5′-CAA GMT TTT CTG GAY TTM AWY TTG TT-3′
TRACH 269	*C. trachomatis*	5′-ACC ATT TAA CTC CAA TGT ARG GAG TG-3′
PNEUM 268	*C. pneumoniae*	5′-GTA CTC CAA TGT ATG GCA CTA AAG A-3′
218 PSITT	*C. psittaci*	5′-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3′
204 PECOR	*C. pecorum*	5′-CCA ATA YGC ACA ATC KAA ACC TCG C-3′

[a] Degenerated nucleotides: K=(G, T) M=(A, C) R=(A, G) W=(A, T) Y=(C, T) I=(Inosin).

Fluorescence Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Sequencing primers were NCD3 and NCD4 for the complementary strands. For pathotypic characterization (Seal et al., 1995), amplicons were cloned into pGemT vector (Promega) prior to sequencing. The sequence fragments were separated using the 310 C Automatic Sequencer (Applied Biosystems) equipped with Data Collection® V. 1.02 Software (Applied Biosystems). Sequence analysis was performed using the Sequencing Analysis® V.3.0. program (Applied Biosystems).

Sufficient spleen and lung samples were available to test 39 birds for *C. psittaci* by a modified version of the nested PCR procedure described by Kaltenböck et al. (1997) based on the *omp*1 gene of chlamydiae. This nested PCR only allows statements to classical *Chlamydia* species, in this case *C. psittaci.* DNA was isolated from approximately 25 mg of each organ sample using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction. DNA in the final eluate of 200 µl was precipitated with 0.6 volumes of isopropanol. After centrifugation (10 min at 12,000×G) and air drying the precipitate was redissolved in 20 µl of PBS (10 mM $Na_2 HPO_4$, 10 mM $NaH_2 PO_4$, 0.145 mM NaCl; pH 7.0).

The first step was a genus-specific amplification using primers 201CHOMP and CHOMP336 (JenaBioScience, Jena, Germany). Each reaction mix contained 1 µl of sample DNA extract, 1 µl of dNTP mix (2 mM each), 5 µl of 10× *Taq* DNA buffer (Roche Diagnostics), 1 µl of each primer (20 pmol/µl), 0.2 µl *Taq* DNA polymerase (5 U/µl; Roche Diagnostics), and 40.8 µl water. Negative controls without sample DNA, and positive controls containing DNA extract of a reference strain were included in each series. The following temperature-time program was used: initial denaturation of 1 sec at 97 C, 50 cycles with 1 sec at 97 C, 60 sec at 50 C, and 60 sec at 72 C, final extention step of 60 sec at 72 C. For the second amplification we used species-specific primer combinations: 218PSITT/CHOMP336 (for *C. psittaci*), 204PECOR/CHOMP336 (for *C. pecorum*), 201CHOMP/PNEUM268 (for *C. pneumoniae*), 201CHOMP/TRACH269 (for *C. trachomatis*). All primer sequences are listed in Table 2. One µl of the genus-specific PCR product was used as template in the species-specific amplification. Polymerase chain reaction conditions were the same as above except that the number of cycles was reduced to 30. An amplicon size of 389–404 bp confirmed the presence of *C. psittaci* DNA. Finally, PCR products were analysed by a 1% agarose gel electrophoresis.

Species (n=22), age (n=4), sex, and the investigation year (1995–97) were regarded as variables potentially influencing the outcome of the PCR test. Regarding age determination a bird was classified as nestling, juvenile, subadult or adult according to the method described by Baker (1993). Chi-square tests were used to detect interdependencies between pairs of categorical or binary variables (Bortz et al., 1990). Adjusted standardized residuals in contingency tables were calculated to identify the categories responsible for significant chi-square values (Everitt, 1977). The significance level was set to α=0.05. All statistical calculations were performed using the SPSS version 9.0 software.

RESULTS

Of 331 birds of prey 18 were positive for NDV nucleic acids using PCR (Table 1). Amplicons were cloned, sequenced, and classified as being lentogenic (Fig. 2) according to Seal et al. (1995). Sixteen (18%) of 89 owls were NDV nucleic acid positive while only two (<1%) reactors were found among 242 diurnal birds of prey. With one exception the PCR-positive raptors came from different locations in Berlin and Brandenburg (Fig. 1) and many of the birds were emaciated and injured (Table 3).

Most positive samples originated from

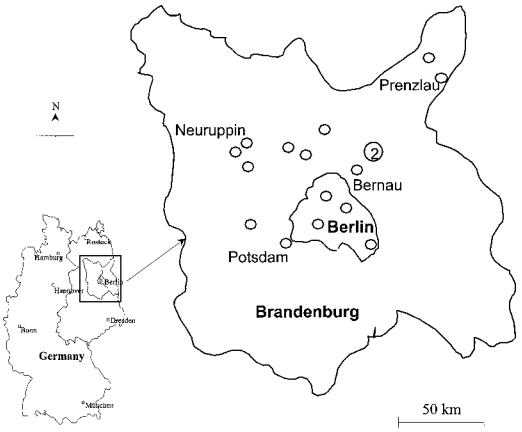

FIGURE 1. Distribution of raptors positive for NDV nucleic acids within study areas. ○ = one positive reactor; numbers indicate more than one finding.

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La Sota*  50  VNIYTSSQTG SIIVKLLPNL PKDKEACAKA PLDAYNRTLT TLLTPLGESI 99
b.o.1         .......... .......... .......... .......... .......D..
b.o.2         .......... .......... .......... .......... .......D..
b.o.3         .......... .......... .......... .......... .......D..

La Sota  100  RRIQESVTTS GGGRQGRLIG AIIGGVALGV ATAAQITAAA ALIQAKQNAA NIL 152
b.o.1         .......... .......... .......... .......... .......... ...
b.o.2         .......... .......... .......... .......... .......... ...
b.o.3         ..........._......... .......... .......... .......... ...
```

FIGURE 2. Predicted amino acid sequence alignment of the 310 bp NDV-F-protein fragment. Sequences of three representative barn owl samples (b.o.) are shown. The fusion protein cleavage site is underlined. Despite an amino acid change at position 97 (glutamic acid is substituted by aspartic acid), all samples were classified as lentogenic according to Seal et al. (1995). *Sequence from Seal et al. (1995).

barn owls (*Tyto alba,* 15 of 54; Table 1). Therefore, statistical analysis was restricted to this species. Age groups differed concerning the rate of NDV-positive samples ($P=0.044$, $n=51$) due to the above-average number of positive nestlings (four of five, standardized residual sr=2.6) compared to juvenile (one of ten), subadult (four of 13) and adult birds (six of 23). No difference was found between the three successive investigation years ($P=0.400$, $n=54$) and concerning sex ($P=0.312$, $n=40$).

Chlamydia psittaci DNA was detected in 29 (74%) of 39 tested raptors using PCR. Various raptor species were *C. psittaci* DNA positive (Table 1). Pathology of PCR reactors was available in five cases and varied from avian tuberculosis, lung edema, fractures, gout, blindness, and shock. None of the variables was found to be associated with the PCR result (species: $P=0.248$, $n=39$; sex: $P=0.433$, $n=33$; age: $P=0.802$, $n=36$; year: $P=0.361$, $n=39$).

DISCUSSION

Newcastle disease virus nucleic acid was detected in 18 of the tested birds. Most positive reactors were barn owls (28%); whereas only <1% of the tested diurnal birds of prey were NDV nucleic acid positive. This difference might be explained by the close proximity of barn owls to human settlements (Rutschke, 1983). The lentogenic strain La Sota identified in the barn owls is extensively used as live vaccine for chickens throughout Germany. Manvell et al. (1997) isolated a La Sota-like virus from a peregrine falcon. It remains unclear why barn owls have come into contact with the NDV-strain La Sota more often than other species. The distribution of PCR positive reactors within the study area does not indicate a common origin of infection (Fig. 1). The following chain of events is conceivable and might provide a plausible explanation: ND vaccine is excreted by chickens after vaccination. Mice (*Mus musculus*), which have almost free access to chicken houses might be infected or mechanical carriers of ND vaccine. The predominant prey of barn owls are mice. La Sota strain is considered to be apathogenic for chickens and for free-living birds (Seal et. al. 1995). However, information about the pathogenicity of NDV-strains is based on chickens and cannot necessarily be transferred to other species such as raptors (Gylsdorff and Grimm, 1998). In general, ND is known to be fatal in owls (Winteroll, 1976) whereas in diurnal birds of the family *Accipitridae,* a mild course with low mortality can be observed (Heidenreich, 1978; Forbes, 1997). Nevertheless, Winteroll (1976) demonstrated that owls which were vaccinated intranasally with La Sota strain reacted with protective antibody production. However, Schettler et al. (2001) did not

TABLE 3. Case history and pathologic findings of Newcastle disease nucleic acid positive raptors (n=18).

Species	Age	Sex	Case history	Pathologic findings
Common buzzard (*Buteo buteo*)	ad.[a]	f[b]	Traffic accident	Edema of lungs, hemorrhages of lungs and brain
Eurasian kestrel (*Falco tinnunculus*)	ad.	m[c]	Window crash	Cachexia, enteritis
Barn owl (*Tyto alba*)	immat.[d]	m	Found dead	Cachexia, sharp injury of the neck, edema of lungs
Barn owl	ad.	f	Unknown	Hemorrhages of brain, multiple fractures, rupture of liver
Barn owl	ad.	f	Found dead	Cachexia
Barn owl	immat.	f	Found dead	Emaciated, multiple fractures
Barn owl	ad.	f	Found dead	Rupture of liver, hypovolemic shock
Barn owl	immat.	m	Found dead	Hemorrhages of lungs and brain, pelvic fracture
Barn owl	ad.	f	Traffic accident	Hemorrhages of brain, blunt trauma
Barn owl	ad.	f	Traffic accident	Cachexia
Barn owl	nestl.[e]	m	Died shortly after found	Cachexia
Barn owl	nestl.	f	Found dead	Cachexia
Barn owl	nestl.	m	Found dead	Cachexia
Barn owl	nestl.	f	Found dead	Cachexia
Barn owl	nestl.	m	Died shortly after found	Cachexia
Barn owl	immat.	f	Found dead	Edema and hemorrhages of lungs, cachexia
Barn owl	immat.	f	Died shortly after found	Cachexia, hemorrhages of brain, skull trauma
Tawny owl (*Strix aluco*)	ad.	f	Found dead	Myocarditis, hepatitis

[a] Adult bird.
[b] Female.
[c] Male.
[d] Immature bird.
[e] Nestling bird.

detect NDV antibodies in 55 owls, among them 12 barn owls, from the same investigation area and periods, even though antibodies against NDV were found in 2% of free-living diurnal birds of prey.

The pathologic findings of the NDV nucleic acid positive raptors in the present study were nonspecific. However, NDV infected raptors often do not show any gross pathologic lesions (Chu et al., 1976). Therefore, it is unclear, whether the PCR-positive raptors died due to a NDV-infection or merely came into contact with NDV vaccine which might be apathogenic. Furthermore, it is not clear why significantly more nestling barn owls were PCR-positive.

This is the first extensive study of NDV in free-living birds of prey in eastern Germany. Even though no rare raptor species was positive for NDV (Table 1) a potential threat to these birds cannot be excluded.

Chlamydia psittaci DNA was detected in 74% of the 39 birds. These results correspond with investigations by Schettler et al. (2001) who detected antibodies against *C. psittaci* in 63% of free-living raptors from the same investigation area and period. Gerbermann and Korbel (1993) also detected *C. psittaci* antigen in 13% and antibodies in 85% of free-living birds of prey in southern Germany. Thus, *C. psittaci* appears to be common in free-living birds of prey in the study area. It remains unclear if the gross lesions found in five birds are correlated to an apparent *C. psittaci* infection. In general, adult birds often show an inapparent infection with *C. psittaci.* Nevertheless, the pathologic agent can still be shed. In young birds an acute, often fatal disease can be observed (Gerlach, 1994). It is possible that raptors may be reservoirs of *C. psittaci.* Staff handling raptors should be aware of this (Fowler et al., 1990; Anonymous, 1998a) and take appropriate precautions.

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