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SHORT PAPER
Wild birds as a possible natural reservoir of Borna disease virus

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SUMMARY

The natural reservoir of Borna disease virus (BDV) is unknown. In this paper, we show that mallards (*Anas platyrhyncos*) and jackdaws (*Corvus monedula*) can be subclinically infected carriers of this virus. From faecal samples collected at a bird pond, we were able to amplify fragments of the BDV p24 and p40 genes. Following cloning and sequencing, a phylogenetic analysis revealed that these birds carry strains of BDV closely related to but distinct from the reference strains BDV V and He/80. To our knowledge, this is the first confirmed finding of BDV in wild birds.

Borna disease virus (BDV)† is a neurotropic, non-segmented RNA virus of negative polarity and so far the only member of the family *Bornaviridae* within the order *Mononegavirales* [1, 2]. The natural host spectrum of BDV includes many different species, for instance horses, sheep, cattle, cats and ostriches [3–7]. In most naturally infected species, BDV causes a severe neurological syndrome called Borna disease (BD), morphologically manifested as a non-suppurative encephalomyelitis with predilection for the limbic system, the basal ganglia and the brain stem [8]. However, serological surveys indicate that clinically manifest BD is in fact a rare event and that a significant proportion of animals are subclinically infected [9–16].

The broad host spectrum of BDV has raised the question whether this agent may also affect humans. Findings of BDV-specific antibodies, BDV antigen and BDV RNA in peripheral blood mononuclear cells (PBMCs) of patients with affective disorders and schizophrenia suggest a link between BDV and certain human neuropsychiatric diseases [17–21]. In addition, isolation of BDV from the PBMCs of patients with psychiatric disorders has been reported [22, 23]. Although evidence for a direct aetiological role of BDV in human diseases has so far not been presented, it is important to investigate the epidemiology of BDV. Its wide host range, as well as the high sequence homology between isolates from different animal species [24, 25] suggests the possibility that BD may be a zoonosis.

The epidemiology of BDV is largely unknown. Several observations indicate the existence of a wildlife reservoir, for instance the fact that BD shows a seasonal occurrence (spring and early summer) and that the prevalence of BD is higher in some years than in others [26]. In Sweden, BDV-infection in association with clinical neurological signs and non-suppurative encephalomyelitis has been documented in domestic cats [3, 5, 27] and in a free-ranging lynx

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† The nucleotide sequence data reported in this article have been deposited in the GenBank database under accession no. AF232700 (mallard sample B, p24), AF232701 (mallard sample A, p24), AF232702 (mallard sample B, p40), AF232703 (mallard sample A, p40) and AF233071 (jackdaw, p40).

[28]. Risk factors for feline BD in Sweden were identified as access outdoors in a rural/woodland environment, and hunting mice [29]. The latter finding suggests the possibility that rodents may act as virus carriers. However, cases of BD sometimes appear in cats with no hunting opportunities, and no access outdoors except for the balcony (A.-L. Berg, unpublished observations). We therefore considered an alternative wildlife source of BDV: feral birds.

The role of wild birds as reservoirs of viruses in nature is well established. Various species of wild birds, especially waterfowl such as mallards, constitute an important reservoir of avian influenza viruses. The virus is shed with secretions from the respiratory tract and the conjunctiva, and in faeces [30]. Wild birds also play an important part in the epidemiology of arboviruses such as Eastern and Western equine encephalitis virus. Accordingly, we hypothesized that birds may function as subclinically infected carriers of BDV, spreading the virus for instance via faeces (thus explaining how cats only going outdoors to balconies may get infected).

As a first step, we collected six faecal samples from a bird pond in Uppsala, Sweden. This area was chosen because cases of feline BD occur there regularly. Identification of faecal samples as belonging to a particular species of bird was performed by an ornithologist (see Acknowledgements). Five samples were from mallards (*Anas platyrhyncos*) and one from a black headed gull (*Larus ridibundus*). All samples were collected in Falcon tubes and stored at −20 °C. After thawing, faecal samples were suspended in 1:1 (vol/vol) in 0·01 M phosphate buffered saline (PBS) and homogenized by vortexing. Insoluble components were pelleted for 5 min at 10000 ***g*** and the supernatant stored at −20 °C awaiting further analysis.

In order to detect BDV-specific nucleic acid, a reverse transcriptase–polymerase chain reaction (RT–PCR) assay was performed. To minimize the contamination risk, all steps in the RT–PCR procedure including extraction of RNA were performed in laboratory facilities where no previous handling of BDV or BDV-positive PCR products had occurred. Reagents were unopened before the start of this study.

Total RNA was extracted from the supernatant fraction using the Trizol reagent (Life Technologies). One μg of total RNA was reverse-transcribed to cDNA in a final volume of 20 μl by Superscript II (Life Technologies), using oligo $d(T)_{12-18}$ (Pharmacia Biotech) as a primer. The cDNA was amplified by a nested PCR using two sets of primers specific for a fragment of the BDV p24 gene: for the first round of PCR 5′-TGA CCC AAC CAG TAG ACC A-3′ at nt 1387–1405 and 5′-GTC CCA TTC ATC CGT TGT C-3′ at nt 1865–1847; for the second round of PCR 5′-TCA GAC CCA GAC CAG CGA A-3′ at nt 1443–1461 and 5′-AGC TGG GGA TAA ATG CGC G-3′ at nt 1834–1816.

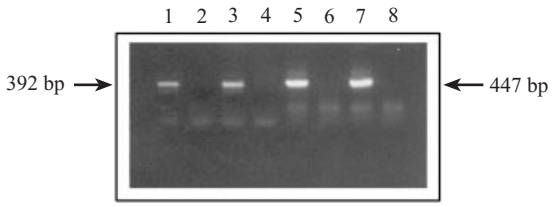

Fig. 1. Gel showing amplification of 392 bp (BDV p24) and 447 bp (BDV p40) fragments following nested RT–PCR. Lanes: 1, mallard sample A, 2, water control, 3, mallard sample B, 4, water control, 5, mallard sample A, 6, water control, 7, mallard sample B, 8, water control. Other bird samples, which were negative, are not shown.

In addition, a nested PCR was carried out with two sets of primers specific for a fragment of the BDV p40 gene: for the first round of PCR, 5′-GCC TTG TGT TTC TAT GTT TGC TAA TCC-3′ at nt 277–303 and 5′-CAT TGT GGG GTT TTC CTT CTT ACT CC-3′ at nt 1004–979; for the second round of PCR 5′-TCT CCT CTA TCT TCA GCC ATT GTT GC-3′ at nt 451–476 and 5′-CCA GAA ACG GGA ACA GGT CAG CAT-3′ at nt 903–880.

PCR amplifications were carried out in 25 μl volumes containing 1 μl of the cDNA. Reaction conditions consisted of 50 mM KC1, 10 mM Tris–HCl pH 9·0, 1% Triton X-100, 3·5 mM M_gCl_2, 2·5 mM each dNTP, 0·5 μM each primer and 0·5 U Taq polymerase (Promega). The samples were processed through 35 cycles (first round) and 25 cycles (second round) of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. Positive and negative controls were included in each analysis. Ten μl of each reaction was analysed by electrophoresis in a 2% agarose gel in the presence of 0·5 μg/ml ethidium bromide. The quality of each RNA sample was controlled by amplification of β-actin RNA (not shown).

From two mallard samples (A and B), fragments of the expected sizes 392 bp (p24) and 447 bp (p40) were visible after the second round of PCR (Fig. 1). The remaining three mallard samples, as well as the gull sample, were negative. The amplified PCR fragments were purified using the Wizard PCR purification kit (Promega), and cloned into the pGEM-T vector

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Table 1. *Mallard BDV p24, sample A, compared to mallard sample B, the reference strains He/80 and BDV V, and some p24 sequences from various hosts (cat, lynx, horse, human)*

Isolate	Nucleic acid identity (%)	Amino acid identity (%)	Position*	Change
Mallard B	98·5	96·9	14	Asp → Asn
			85	Asp → Asn
			117	Met → Lys
			125	Pro → Leu
He/80	97·4	99·2	14	Asp → Asn
BDV V	98·2	99·2	see He/80	see He/80
Cat 0·436/92†	98·0	98·5	14	Asp → Asn
			92	Met → Thr
Lynx‡	99·0	97·7	14	Asp → Asn
			35	Glu → Gln
			102	Val → Glu
Horse #1§	96·9	97·7	14	Asp → Asn
			46	Arg → His
			92	Met → Thr
RW98**	95·9	99·2	see He/80	see He/80

* Position refers to the mallard sample A sequence, GenBank AF232701.
† Isolate from Swedish cat, GenBank AF201073.
‡ Isolate from Swedish lynx, GenBank AF200463.
§ Isolate from Swedish horse, GenBank AF203970.
** Human isolate (ref. 23).

(Promega). From each bird, 2–3 clones were sequenced on both strands, using an automatic fluorescence-activated DNA sequencer. Sequence data were analysed using the MegAlign program of the DNA Star software package (DNA Star Inc.). A comparison was made between the bird sequences and the well-characterized BDV reference strains He/80 and BDV V. Sequences obtained from other naturally infected animal species, as well as a recent human isolate [23], were included in the analysis.

The bird sequences were found to be closely related to previously reported BDV isolates (Tables 1, 2). Ten single-base differences were found in the mallard sample A p24 sequence compared to He/80, and five compared to BDV V. The majority of these changes were silent, resulting in only one amino acid substitution (Table 1). A slightly higher divergence was found between mallard p24 and corresponding p24 sequences obtained from other naturally infected animal species (Table 1). Interestingly, the highest divergence was found between the two mallard samples (A and B): four amino acid substitutions in p24, and seven in p40 (Tables 1, 2).

Due to our findings of BDV RNA in the faeces of mallards, we performed a second sampling from various bird species. Faecal samples were once again collected from the same bird pond in Uppsala, and were identified by an ornithologist: one was from a gull, two from mallards, two from pigeons (*Columba livia*), two from jackdaws (*Corvus monedula*), two from starlings (*Sturnus vulgaris*) and one from a tree sparrow (*Passer montanus*). In addition, eight faecal samples were obtained from various small birds while ringing. The samples were processed as described above, and subjected to nested RT–PCR for the BDV p24 and p40 genes.

No positive PCR products were obtained with the p24 primers. However, one sample from a jackdaw yielded a 447 bp (p40) fragment after the second round of PCR (not shown). Phylogenetic analysis showed that the jackdaw p40 sequence was closely related to, but distinct from, the mallard sequences. Seven amino acid substitutions were found in the jackdaw sample compared to the mallard sample A p40 sequence (Table 2).

The results of the present study indicate the existence of a new potentially important reservoir of BDV: wild birds. Previous studies have shown domestic fowl chicks to be highly susceptible to experimental BDV infection, while adult fowl and pigeons seem to be more resistant [7]. Little is known, however, about the occurrence and significance of

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Table 2. *Mallard BDV p40, sample A, compared to mallard sample B, jackdaw sample, and the reference strains He/80 and BDV V*

Isolate	Nucleic acid identity (%)	Amino acid identity (%)	Position*	Change
Mallard B	98·0	94·6	29	Lys → Arg
			72	Leu → Phe
			77	Leu → Pro
			81	Leu → Phe
			86	Lys → Ile
			87	Gly → Glu
			130	Val → Asp
Jackdaw	98·2	94·6	46	Glu → Lys
			59	Ala → Val
			72	Leu → Phe
			81	Leu → Phe
			87	Gly → Glu
			130	Val → Asp
			141	Asp → Asn
BDV V	98·4	96·6	72	Leu → Phe
			81	Leu → Phe
			87	Gly → Glu
			130	Val → Asp
He/80	95·7	95·9	72	Leu → Phe
			81	Leu → Phe
			87	Gly → Glu
			130	Val → Asp
			141	Asp → Glu

* Position refers to the mallard sample A sequence, GenBank AF232703.

BDV infection in free-living birds. BDV was identified as the likely causative agent in farmed ostriches with a paralytic disease [6] in Israel, a country with no recorded cases of clinically manifest BD in other animal species. Seropositive horses have, however, been identified in three areas of Israel. Interestingly, two of these areas are said to be favoured routes of migratory birds [31]. Apart from the findings in farmed ostriches, there are no previous reports on the existence of BDV in naturally infected birds. Although it has been suggested that the causative agent of the so-called Near-Eastern equine encephalomyelitis, which also could be isolated from the brains of wild birds [32], may have been identical with BDV, there is no formal proof of this.

It should be noted that the present BDV bird sequences showed a certain intraspecies divergence, especially in the p40 gene. This is in contrast to previous BDV isolates from naturally infected animals, which all show a remarkably high sequence conservation [24, 25]. However, in a recent paper Nowotny and co-workers reported the isolation of a new subtype of BDV from an Austrian horse [33]. The nucleotide sequence of this new strain differed from the reference strains by more than 15%, causing it to be undetectable by the standard BDV RT–PCR assay. It is therefore possible that as yet unknown BDV subtypes may exist, and that several variants may circulate in the avian population.

From a geographical point of view, both mallards and jackdaws are common in areas where BDV infection, with or without clinical disease, is known to exist in horses. Mallards are frequently found in Europe, central Asia and in the coniferous forest area of North America. Jackdaws breed in Europe, North Africa and Asia. The exact role of wild birds in the epidemiology of BDV is unknown. It may be that they function as intermediary reservoir hosts, the virus being transmitted to mammals by ticks or other arthropods. A similar model was recently proposed for the spirochaete *Borrelia burgdorferi*, which was shown to be carried in the blood by redwing thrushs (*Turdus iliacus*) [34].

Alternatively, mammals may be infected directly through contact with contaminated feed and water, or through predation. The finding of BDV in faecal

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samples from mallards and a jackdaw highlight once more wild birds as reservoirs of potentially zoonotic agents, and provides yet another piece of the widely unknown BDV epidemiology puzzle.

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REFERENCES

1. Briese T, Schneemann A, Lewis AJ, et al. Genomic organization of Borna disease virus. Proc Natl Acad Sci USA 1994; **91**: 4362–7.
2. Cubitt B, Oldstone C, de la Torre JC. Sequence and genome organization of Borna disease virus. J Virol 1994; **68**: 1382–96.
3. Berg A-L, Berg M. A variant form of feline Borna disease. J Comp Path 1998; **119**: 323–31.
4. Bode L, Dürrwald R, Ludwig H. Borna virus infection in cattle associated with fatal neurological disease. Vet Rec 1994; **135**: 283–4.
5. Lundgren A-L, Zimmermann W, Bode L, et al. Staggering disease in cats: isolation and characterization of the feline Borna disease virus. J Gen Virol 1995; **76**: 2215–22.
6. Malkinson M, Weisman Y, Ashash E, Bode L, Ludwig H. Borna disease in ostriches. Vet Rec 1993; **133**: 304.
7. Rott R, Becht H. Natural and experimental Borna disease in animals. Curr Top Microbiol Immunol 1995; **190**: 17–30.
8. Gosztonyi G, Ludwig H. Borna disease – neuropathology and pathogenesis. Curr Top Microbiol Immunol 1995; **190**: 39–72.
9. Bahmani MK, Nowrouzian I, Nakaya T, et al. Varied prevalence of Borna disease virus infection in Arabic, thoroughbred and their cross-bred horses in Iran. Virus Res 1996; **45**: 1–13.
10. Berg A-L, Dörries R, Berg M. Borna disease virus infection in racing horses with behavioral and movement disorders. Arch Virol 1999; **144**: 547–59.
11. Herzog S, Frese K, Richt JA, Rott R. Ein Beitrag zur Epizootiologie der Bornaschen Krankheit beim Pferd. Wien Tierärztl Monatsschr 1994; **81**: 374–9.
12. Kao M, Hamir HN, Rupprecht CE, et al. Detection of antibodies to Borna disease virus in sera and cerebrospinal fluid of horses in the USA. Vet Rec 1993; **132**: 241–4.
13. Lange H, Herzog S, Herbst W, Schliesser T. Seroepidemiologische Untersuchungen zur Bornaschen Krankheit (Ansteckende Gehirn-Rückenmarkentzündung) der Pferde. Tierärztl Umschau 1987; **42**: 938–46.
14. Nakamura Y, Kishi M, Nakaya T, et al. Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells from healthy horses in Japan. Vaccine 1995; **13**: 1076–9.
15. Nakamura Y, Asahi S, Nakaya T, et al. Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells derived from domestic cats in Japan. J Clin Microbiol 1996; **34**: 188–91.
16. Richt JA, Herzog S, Haberzettl K, Rott R. Demonstration of Borna disease virus-specific RNA in secretions of naturally infected horses by the polymerase chain reaction. Med Microbiol Immunol 1993; **182**: 293–304.
17. Bode L. Human infections with Borna disease virus and potential pathogenic implications. Curr Top Microbiol Immunol 1995; **190**: 103–30.
18. Bode L, Zimmermann W, Ferszt R, Steinbach F, Ludwig H. Borna disease virus genome transcribed and expressed in psychiatric patients. Nature Med 1995; **1**: 232–6.
19. Kishi M, Nakaya T, Nakamura Y, et al. Demonstration of human Borna disease virus RNA in human peripheral blood mononuclear cells. FEBS Lett 1995; **364**: 293–7.
20. Rott R, Herzog S, Fleischer B, et al. Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science 1985; **228**: 755–6.
21. Sauder C, Müller A, Cubitt B, et al. Detection of Borna disease virus (BDV) antibodies and BDV RNA in psychiatric patients: evidence for high sequence conservation of human blood-derived BDV RNA. J Virol 1996; **70**: 7713–24.
22. Bode L, Dürrwald R, Rantam FA, Ferszt R, Ludwig H. First isolates of infectious human Borna disease virus from patients with mood disorders. Mol Psychiat 1996; **1**: 200–12.
23. Planz O, Rentzsch C, Batra A, et al. Pathogenesis of Borna disease virus: granulocyte fractions of psychiatric patients harbor infectious virus in the absence of antiviral antibodies. J Virol 1999; **73**: 6251–6.
24. Binz T, Lebelt J, Niemann H, Hagenau K. Sequence analysis of the p24 gene of Borna disease virus in naturally infected horse, donkey and sheep. Virus Res 1994; **34**: 281–9.
25. Schneider PA, Briese T, Zimmermann W, Ludwig H, Lipkin WI. Sequence conservation in field and experimental isolates of Borna disease virus. J Virol 1994; **68**: 63–8.
26 Dürrwald R. Die Natürliche Borna-Virus-Infektion der Einhufer und Schafe [dissertation]. Free University, Berlin, 1993.
27. Lundgren A-L. Feline non-suppurative meningoencephalomyelitis. A clinical and pathological study. J Comp Path 1992; **107**: 411–25.
28. Degiorgis M-P, Berg A-L, Hård af Segerstad C, Mörner T, Johansson M, Berg M. Borna disease in a free-ranging lynx (*Lynx lynx*). J Clin Microbiol 2000; **38**: 3087–91.
29. Berg A-L, Reid-Smith R, Larsson M, Bonnett B. Case

control study of feline Borna disease in Sweden. Vet Rec 1998; **142**: 715–7.
30. Webster RG. Influenza virus: transmission between species and relevance to emergence of the next human pandemic. Arch Virol Suppl 1997; **13**: 105–13.
31. Malkinson M, Weisman Y, Perl S, Ashash E, Teplitsky V, Pitlik S. Borna disease of livestock in Israel. In: Proceedings, XIth International Congress of Virology, 9–13 August 1999, Sydney, Australia.
32. Daubney R, Mahlau EA. Near-Eastern equine encephalomyelitis. Nature 1957; **179**: 584–5.
33. Nowotny N, Kolodziejek J, Jehle CO, Suchy A, Staeheli P, Schwemmle M. Isolation and characterization of a new subtype of Borna disease virus. J Virol 2000; **74**: 5655–8.
34. Gylfe Å, Bergström S, Lundström J, Olsen B. Reactivation of *Borrelia* infection in birds. Nature 2000; **403**: 724–5.