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Evidence of Borna disease virus genome detection in French domestic animals and in foxes (*Vulpes vulpes*)

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Borna disease virus (BDV) is an enveloped, non-segmented negative-stranded RNA virus which belongs to the *Bornaviridae* family. BDV is an aetiological agent of encephalitis in horses, sheep and several other vertebrate species. In order to extend our knowledge about the presence of BDV in France, a study based on BDV RNA detection by RT–nested-PCR was done with 196 animal tissues: 171 brain samples collected from different animal species (75 horses, 59 foxes, 31 cattle, 4 dogs, 1 sheep, 1 roe deer) and 25 horse blood samples. An RNA internal standard molecule was constructed and was co-amplified with the test template. This study reports the first detection of BDV RNA in France in 10 brain samples collected from horses, foxes and cattle, and from 14 horse blood samples. Detection of the BDV genome in the brains of six red foxes is the first evidence of BDV infection in this species.

Borna disease (BD) is characterized by a disseminated non-purulent meningoencephalomyelitis with infiltration of mononuclear cells and a predilection for the grey matter of the cerebral hemispheres and the brain stem (Richt *et al.*, 1997). It owes its name to the town of Borna in Saxony, Germany (Dürrwald & Ludwig, 1997; Ludwig & Bode, 2000). Until recently, BD was recognized as a sporadically occurring encephalopathy that affected horses and sheep in southeastern Germany (Gonzalez-Dunia, 1997). Its aetiological agent, Borna disease virus (BDV), has been recently characterized as an enveloped, non-segmented, negative-stranded RNA virus (de la Torre, 1994; Cubitt & de la Torre, 1994). BDV is now considered as the prototype genus of a new family, *Bornaviridae*, within the order *Mononegavirales* (Lipkin *et al.*, 1990; Van de Woude *et al.*, 1990; Cubitt & de la Torre, 1994). BDV replicates at lower levels than most known viruses (Ludwig *et al.*, 1988; Richt *et al.*, 1992), is not cytolytic, and persists in the nervous system despite an immune response.

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Originally described as a disease of horse and sheep (Metzler *et al.*, 1979; Dürrwald & Ludwig, 1997; Berg *et al.*, 1999; Bahmani *et al.*, 1996; Hagiwara *et al.*, 1997), BD has also been described in ostriches (Malkinson *et al.*, 1995), dogs (Weissenböck *et al.*, 1998), cattle (Nicolau & Galloway, 1928; Matthias, 1954; Caplazi *et al.*, 1994; Bode *et al.*, 1994; Hagiwara *et al.*, 1996), sheep, cats (Lundgren *et al.*, 1995), goats, monkeys, rabbits, deer, alpacas and llamas (Metzler *et al.*, 1978; Stitz *et al.*, 1980; Rott & Becht, 1995) and recently in lynx (Degiorgis *et al.*, 2000). Furthermore, BDV RNAs were found in human brains (de la Torre *et al.*, 1996), which supports the hypothesis that BDV is probably a zoonotic worldwide pathogen (Dürrwald & Ludwig, 1997). Extensive epizootiological studies in horses have shown that although BD is rare, it occurs all over Germany, extending beyond the classic disease endemic regions (Richt *et al.*, 1997). The geographical distribution of BDV is still uncertain, but infections have been reported in Central and Northern Europe, USA, Japan, Iran and Israel (Herzog *et al.*, 1997). Recent reports of asymptomatic naturally infected animals suggest that the virus may be even more widespread than previously thought (Kao *et al.*, 1993; Nakamura *et al.*, 1995). However, this apparent geographical restriction may reflect a lack of reliable methods and reagents for diagnosis of infection or failure to consider the possibility of BDV infection (Ludwig & Bode, 2000). Until this year, no data concerning the presence of BDV in France was available, but two recent studies reported for the first time the detection of BDV antibodies in French animals (Galabru *et al.*, 2000; Boucher *et al.*, 1999). To extend our knowledge about the epidemiology of BDV in France, the RT–nested-PCR method was used to detect BDV RNA in 196 biological samples of various animal species (Sorg & Metzler, 1995; Sauder & de la Torre, 1998).

The origin of the blood and brain samples is described in Table 1. Some of the blood samples were collected from French horses which had previously been diagnosed as BDV seropositive (Galabru *et al.*, 2000). The other blood samples were obtained from horses showing nervous disorders or in good health. Horse brains were sampled from autopsies done at the AFSSA Dozulé laboratory (France). Bovine brains were sampled from cattle with nervous disorders and were received from the AFSSA Lyon laboratory (France). All the BDV positive samples were confirmed as BSE negative. Red fox

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Table 1. Origin of the blood and brain samples tested for BDV

Samples	Animal species	Origin
171 Brain samples	72 Horses and 3 foals	67 Horses, AFSSA Dozulé 3 Foals and 5 horses, DVL Caen* Randomly selected samples from horses with no specific nervous disorders
	59 Red foxes	40, DVL Caen* (*Neospora caninum* negative) 19, AFSSA Nancy (rabies negative)
	31 Cattle	30, AFSSA Lyon (BSE negative) 1, DVL Caen*
	4 Dogs	3, AFSSA Nancy (rabies negative) 1, DVL Caen*
	1 Roe deer	AFSSA Nancy (rabies negative)
	1 Sheep	AFSSA Nancy (rabies negative)
25 Blood samples	25 Horses	Sampled by practitioners 22 Samples: most horses had nervous disorders, among which 3 horses (nos 6, 7 and 8) were resampled after a 6-month interval 3 Samples: horses previously detected BDV seropositive (Galabru *et al.*, 2000)

* Diagnostic Veterinary Laboratory (Frank Duncombe Laboratory, Calvados, France).

brain tissues were received from the Caen Diagnostic Veterinary Laboratory (France) after negative PCR results for *Neospora caninum* detection. Other red fox brain tissues were received from the AFSSA Nancy laboratory (France) after negative results for rabies virus detection.

RNA detection by RT–nested-PCR was carried out using specific primers of the two BDV targets described by Sauder *et al.* (1996): the p40 gene, encoding the nucleoprotein, and the p24 gene, encoding the polymerase co-factor phosphoprotein. To avoid the risks of false-negative results, two internal standard molecules, named 'mimic-p24' and 'mimic-p40' were developed for RT–nested-PCR (Ballagi-Pordany & Belak, 1996; Legay *et al.*, 2000). The internal standard molecules were produced by PCR (Ballagi-Pordany & Belak, 1996) from a cDNA fragment of the serotype 3 African horse sickness virus (an *Orbivirus*) partial segment 2 (Sailleau *et al.*, 2000) with specific 'mimic primers' (Legay *et al.*, 2000). It is then easy to distinguish the 'mimics' from the templates by agarose gel electrophoresis. Such internal standard molecules have been developed to ensure the amplification efficiency. No competition was detected between the mimics and the two BDV targets (Ballagi-Pordany & Belak, 1996; Legay *et al.*, 2000). RNA extraction and RT–nested-PCR are described in detail in Legay *et al.* (2000).

The RT–nested-PCR was estimated to detect around 140 copies of BDV RNA with the p40 primers and 14 copies of BDV RNA with the p24 primers (Legay *et al.*, 2000). To prevent possible contamination of the samples with BDV amplicons, tissue preparation, RNA extraction and cDNA amplification were carried out in separate rooms following strict rules of separation of pre- and post-PCR environments (Belak & Ballagi-Pordany, 1993).

Seven p40 and five p24 PCR products were directly sequenced in both directions by direct automated sequencing (Genome Express). In order to avoid laboratory contamination, PCR products were not cloned. The sequences were analysed using the Megalign program of the Lasergene software (DNAstar). These sequences were aligned and compared with those of the reference strains described by Cubitt & de la Torre (1994) (strain He/80, accession number L27077), Briese *et al.* (1994) (strain V, accession number U04608) and Nowotny *et al.* (2000) (accession number AF136236). The only laboratory strain handled in our laboratory is the BDV He/80 strain.

Because of the high asymptomatic carriage of BDV in various animal species (Dürrwald & Ludwig, 1997), we did not focus our study on animals with neurological disorders. In order to confirm the first set of RT–nested-PCR results, total RNA was extracted from new aliquots of the same samples and was subjected to RT–nested-PCR with the p40 and p24 primers (the results are summarized in Table 2).

Among the 17 horse blood samples that tested positive in at least one RT–nested-PCR assay, eight and nine samples were confirmed to be positive using the p40 and p24 primers, respectively, after two or three assays. Nevertheless, only 4/17 blood samples were BDV positive with both the p40 and p24 primers. It must be noted that samples 6 and 25, 7 and 24, and 8 and 15 were obtained from only three animals, which were sampled twice, 6 months apart, and showed neurological disorders. Moreover, among the 26 brain samples positive in at least one RT–nested-PCR assay, only 10 samples were

Table 2. Summary of the RT–nested-PCR positive samples

Boldface indicates animals showing BDV-like clinical signs. Shading indicates that the sample was positive with both p40 and p24 primers. Blood sample pairs 8 and 15, 6 and 25, and 7 and 24 were collected from three horses (two samples per animal, taken 6 months apart).

Animal species	Sample tissue	Sample no.	Clinical signs or post-mortem lesions	PCR p24	PCR p40	Sequence p24/p40 (accession no.)
Cattle	**Brain**	**175**	**Nervous disorder**	−	+	**p40 (AF374606)**
		115	Unknown†	−	+	
		189	Unknown†	+	−	p24 (398110)
Red fox	Brain	198	Unknown†	ND	+	p40 (AF374604)
		204	Unknown†	ND	+	p40 (AF374605)
		210	Unknown†	+	−	p24 (AF374596)
		214	Unknown†	+	−	
Horse	**Brain**	**63**	**Wobbler syndrome, spinal ataxia**	+	+	**p40 (AF374603)**
Brain	66		Natural death	−	+	
Brain	99		Chronic lymphangitis and laminitis, euthanasia	+	−	
	3		Good health	+	+	
	4*		No clinical signs	+	+	p24 (AF374597)
	6, 25		**Ataxia, head trembling**	+	+	**p24 (AF374598), p40 (AF374602)**
	7, 24		**Ataxia, head trembling**	+	−	**p24 (AF374599)**
	8, 15		**Cutaneous hyperaesthesia, painful head, intermittent lameness of the hindlimbs**	−	+	
	16		**Lame (neurological origin), cutaneous hyperaesthesia**	−	+	**p40 (AF374600)**
Blood	17		Cutaneous hyperaesthesia, lameness of the forelimbs	−	+	
	18		Intermittent lameness of the forelimbs	+	−	p40 (AF374601)
	19		**Behavioural abnormalities, increased libido**	+	−	
	20		**Nervous disorder**	−	+	
	21		**Weakness**	+	−	
	22		**Hyper-reactivity**	−	+	
	26		Good health	+	−	
	30		Good health	+	−	

* BDV seropositive (Galabru *et al.*, 2000).
† Negative for *Neospora caninum*.
ND, Not determined.

confirmed positive after another PCR assay with the p40 or p24 primers, while only one was detected positive with both the p24 and p40 primers (no. 63). Confirming BDV positive PCR results, either in the same or a different laboratory, is generally problematical (Staeheli *et al.*, 2000). Vahlenkamp *et al.* (2000) underlined the need for a sensitive, reliable and reproductive assay to confidently detect BDV, in particular in human samples. Because of the controversy about BDV detection, we considered samples to be BDV positive only if they were regularly positive after two or three RNA extractions and RT–nested-PCR with the p40 or p24 primers (Table 2). For some samples, the clinical signs, the serological status and the nucleotide sequence analysis were also taken into account (Table 1).

Some positive brain samples were coded and sent to the Vienna and the Giessen laboratories (numbers 63, 66, 99 and 175). The PCR and the antigen detection (by immunohistochemistry) were negative in both laboratories. These discrepancies between the French laboratory and the Austrian and German laboratory results may be due to differences in the sensitivity of the detection methods and the fact that no RNA mimic was used in the second set of tests. Furthermore, the p24 and p40 antigen and genome distribution are very heterogeneous in brain tissue (Gonzalez-Dunia *et al.*, 1997; Nakamura *et al.*, 2000). As an illustration, in our laboratory, only five brain samples among 13 which were positive on one occasion with the p40 primers were positive in two or three aliquots. We cannot rule out the fact that storage and transport conditions may have resulted in partial or total degradation of viral RNA (Sailleau *et al.*, 1997).

To confirm the specificity of the PCR assays, BDV p40 and p24 cDNA amplified from horse, fox and bovine brain samples

Table 3. Percentage divergence between BDV nucleotide sequences

(*a*) p24 alignment

	Fox brain 213	Fox brain 189	Blood 4	Blood 6	Blood 7	He/80	Strain V
Fox brain 213	0	6 (12/196)	4 (11/273)	4 (11/273)	3·3 (9/273)	3·3 (9/273)	1·8 (5/273)
Fox brain 189		0	5 (10/198)	5 (11/198)	5 (11/198)	5 (11/198)	6·5 (13/198)
Blood 4			0	0·3 (1/294)	3·4 (10/294)	3·4 (10/294)	4·4 (13/294)
Blood 6				0	3·7 (11/294)	3·7 (11/294)	4·8 (14/294)
Blood 7					0	0	3 (9/294)
He/80						0	—
Strain V							0

(*b*) p40 alignment (200 nucleotide long)

	Horse brain	Bovine brain	Blood 25	Blood 16, 18/ brain 198, 204	He/80	Strain V
Horse brain 63	0	2	2·5	4	2	4
Bovine brain		0	0·5	3·5	0	3
Blood 25			0	4	0·5	3·5
Blood 16, 18/brain 198, 204				0	3·5	1·5
He/80					0	—
Strain V						0

and from horse blood samples was sequenced. The PCR products were directly sequenced. The determined sequences were aligned and compared to the He/80 strain, to the No/98 strain and also to strain V. Considering that only short sequences were successfully aligned (200 and 294 nucleotides for the p40 and the p24 sequences, respectively), significant variations at the nucleotide level between the detected sequences and the reference strain sequences were observed (Table 3). No amino acid substitutions between any of the compared p40 sequences were observed (data not shown). The sequence divergencies determined from comparing our BDV sequences and the reference strains ranged between 0 and 6·5 % (fox brain 189). This degree of genetic variability (based on short nucleotide sequences of 294 and 200 nucleotide fragments of the BDV p40 and p24 genes, respectively) is in accordance with the general finding of sequence divergency among BDV isolates (Binz *et al.*, 1994; Iwata *et al.*, 1998; Schneider *et al.*, 1994; Vahlenkamp *et al.*, 2000; Staeheli *et al.*, 2000).

The amino acid sequences of the BDV PCR products (obtained from fox brain samples 189 and 213, and horse blood samples 4, 6 and 7) were identical except for fox brain sample 189, which showed four substitutions when compared with reference strain He/80 or strain V, and fox brain 213, which showed one substitution when compared with He/80. This variation constitutes a strong argument in favour of a true positive detection and is in total accordance with the genetic variability of all BDV strains already described and characterized in the literature (Iwata *et al.*, 1998; Staeheli *et al.*, 2000).

In order to determine the degree of variation of the He/80 strain cultivated in our laboratory, we sequenced directly the PCR products of three RT–nested-PCR assays carried out on this He/80 strain. Sequences obtained from the He/80 PCR products were 100 % identical to the corresponding sequence published by Cubitt & de la Torre (1994) (data not shown). This identity confirmed the high degree of sequence conservation between different sources of the same BDV strain and the low number of mutations induced by our RT–nested-PCR protocol.

Three aliquots of horse blood 4 were both p40 and p24 positive by PCR. This horse was also BDV seropositive. This seropositivity (established 6 months before PCR detection) was previously determined by three different laboratories and by three methods: Western blot, indirect immunofluorescence assay and ELISA (Galabru *et al.*, 2000). Positive blood samples 6, 7 and 26 to 30 originated from the same stud in southwest France. Ponies 6 and 7 presented with nervous disorders while the other ones in contact with them were in good health. Vahlenkamp *et al.* (2000) also detected BDV p40 RNA in healthy horses living with BDV-infected horses. BDV trans-

mission (through salival, nasal or conjunctival secretions) is possible through close contact between animals (Richt *et al*., 1997). The fact that, among a majority of 196 randomly collected samples, BDV RNA was detected in animals living on the same farm could confirm a focused transmission of the virus. Moreover, the fact that a mare (no. 16) and her foal (nos 8 and 15) are both PCR positive may indicate vertical transmission. This route of transmission has recently been described (Hagiwara *et al*., 2000).

This study also reported BDV p40 RNA detection from one cow (no. 175) which showed neurological disorders (Table 1). The sequence of the p40 PCR product obtained from cattle brain was found to be identical to the He/80 sequence. This sequence identity between the cattle BDV sequence and the horse BDV isolates has already been described by Hagiwara *et al*. (1996).

In conclusion, this study reports for the first time the presence of the BDV genome in France. It also presents the first detection of the BDV genome in the brain of red foxes (*Vulpes vulpes*) (Table 2). Unfortunately, we had no information about the clinical status of these animals before death. This wild animal species has never been described as naturally infected with BDV. These data add to the epidemiological knowledge about BDV, confirming that this virus has a wide distribution and host range, including domestic and wild animals (Dürrwald & Ludwig, 1997; Richt *et al*., 1992; Sauder *et al*., 1996; Nakamura *et al*., 2000). Further studies are necessary in order to improve knowledge about this possible zoonotic pathogen, and in particular about the role of wild animals in BDV epidemiology (Herzog *et al*., 1997; Dürrwald & Ludwig, 1997).

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