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REVIEW ARTICLE

Epidemiology of *Borna disease virus*

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Introduction

Borna disease virus (BDV) is an enveloped virus with a negative-stranded non-segmented RNA genome of approximately 8·9 kb. It replicates and transcribes its genome in the nucleus and uses the cellular RNA splicing machinery to regulate gene expression. Mainly because of these features, BDV has been classified as the prototype virus of a newly established family, *Bornaviridae*, within the order *Mononegavirales*. Comprehensive recent reviews have summarized the basic molecular characteristics of BDV (Schwemmle *et al.*, 1999*b*; Gonzalez-Dunia *et al.*, 1997; de la Torre, 1994; Schneemann *et al.*, 1995).

BDV is the causative agent of Borna disease (BD), a mostly fatal meningoencephalitis originally detected among horses of Germany. Natural hosts of BDV are horses, sheep and other farm animals. Many other warm-blooded vertebrates ranging from rodents to non-human primates are susceptible to experimental infection with BDV. In these animals, BDV infection may either remain clinically inapparent, or it may lead to severe neurological abnormalities and eventually to death. Numerous studies with experimentally infected rats and mice have conclusively demonstrated that BD is caused by immunopathological mechanisms in which the antiviral T cell response results in neurological disorder. The immunological aspects of BD have, in part, already been reviewed (Bilzer & Stitz, 1996; Stitz *et al.*, 1995). Apart from providing an excellent model system for the analysis of virus-induced CNS immunopathology, BDV has recently been recognized as a valuable tool for studying virus-induced neurodevelopmental damage (Briese *et al.*, 1999; Gonzalez-Dunia *et al.*, 1997).

After a first report had suggested that BDV or a BDV-related virus might be associated with human psychiatric disorders (Rott *et al.*, 1985), questions regarding the epidemiology of BDV became the common interest of medical and veterinary virologists. Accumulating evidence indicated that BDV infections might occur worldwide in several vertebrate species, including humans. Most intriguingly, early follow-up studies seemed to confirm the link between BDV and certain human mental diseases. However, this initial enthusiasm has recently been dampened because several key experiments could not be reproduced by independent laboratories. Furthermore, it became increasingly clear that the currently available diagnostic tools are not well suited to studying the epidemiology of this unusual virus. The question of whether BDV infects humans and causes psychiatric diseases, as well as the issue of a possible worldwide distribution of BDV, have become highly controversial and the debate is still ongoing.

This review analyses our current knowledge regarding the epidemiology of BDV. It takes into account a wealth of early and some more recent publications in German that are otherwise not easily accessible. It deals with the genetic diversity of BDV isolates and discusses several hypotheses with regard to the natural reservoir of BDV. Finally, it critically evaluates the evidence of a worldwide presence of BDV in animals and the suggested links between BDV and human disease.

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Infection of animals

Borna disease in horses and sheep is restricted to central Europe

BDV infections can result in neurological disease that mainly affects horses and sheep in certain areas of Germany (Ludwig *et al.*, 1985; Grabner & Fischer, 1991; Bilzer *et al.*, 1996; Dürrwald, 1993). The endemic area also includes parts of the upper Rhine valley between Switzerland, Austria and the Principality of Liechtenstein (Weissenböck *et al.*, 1998*b*; Caplazi *et al.*, 1999). Between 1894 and 1896 a large epidemic of BDV-induced disease occurred among cavalry horses in the town of Borna in the state of Saxony (Germany). The disease and its inducing viral agent are named after the location of this first documented large outbreak (Zwick, 1939; Gellert, 1995; Rott & Becht, 1995; Dürrwald & Ludwig, 1997). In recent years, the number of animals diagnosed with classical BD was relatively low, usually affecting fewer than a total of 100 horses and 100 sheep each year (Herzog *et al.*, 1994; Dürrwald, 1993; Caplazi *et al.*, 1999). To our knowledge, until very recently, no confirmed cases of BD had been reported in horses or sheep outside the endemic areas described above. The exception is a BD case in a horse from eastern Austria

(Nowotny *et al.*, 2000). Unlike infected animals from the classical endemic areas, this horse was shown to be infected with a novel genotype of BDV. An unresolved issue of BDV epidemiology that will be discussed below is why the boundaries of the endemic regions remained essentially unchanged for decades in spite of unrestricted trade of horses and sheep.

Diagnosis of Borna disease

Reliable *intra vitam* diagnosis of BD is difficult. Horses and sheep with BD exhibit a variety of clinical symptoms, predominantly behavioural abnormalities, apathy and movement disorders, which are not specific for BD but may also be seen in animals infected with other microorganisms that invade the CNS. Cerebrospinal fluid (CSF) of animals with BD may display pathological alterations, such as increased protein content and mononuclear pleocytosis. However, these changes are not specific for BD but rather represent non-specific indicators of viral meningoencephalitis (Bilzer *et al.*, 1996; Grabner & Fischer, 1991; Hiepe, 1960). BDV-specific antibodies in serum and/or CSF are better indicators. Among the currently used methods of detecting these antibodies, indirect immunofluorescence assay (IFA) appears most reliable (Dürrwald, 1993; Nübling *et al.*, 1999). The percentage of horses and sheep with confirmed BD that scored positive in this serological assay varied considerably between different studies (Grabner & Fischer, 1991; Dürrwald, 1993; Herzog *et al.*, 1994; Bilzer *et al.*, 1996; Caplazi & Ehrensperger, 1998). Whereas all serum and CSF samples were found to contain BDV-specific antibodies in one study (Bilzer *et al.*, 1996), only 41% of the serum samples and 61% of the CSF samples were positive in another study (Grabner & Fischer, 1991). Herzog *et al.* (1994) detected BDV-specific antibodies in 100% of serum samples but only in 73% of CSF samples from horses with BD. More recently, BDV-specific antibodies were detected in only two out of three ponies with experimentally induced BD (Katz *et al.*, 1998). One reason for these non-uniform findings is that the titres of BDV-specific antibodies are usually very low in animals with BD (Metzler *et al.*, 1979; Dürrwald, 1993; Herzog *et al.*, 1994; Bilzer *et al.*, 1996; Katz *et al.*, 1998; Caplazi & Ehrensperger, 1998). Sensitivity differences in the cell systems used by the various laboratories for IFA might thus account partly for the discrepancies. For these reasons, *intra vitam* examination alone can usually not provide firm proof of BD. Post-mortem confirmation by histological analysis of brain tissue is required.

Histologically, variable degrees of encephalitis are observed in brains of animals with BD (Gosztonyi & Ludwig, 1984; Bilzer *et al.*, 1995; Caplazi & Ehrensperger, 1998). Lymphocytic infiltrations are usually most prominent in the hippocampus, the brain stem and in parts of the cerebral cortex. Inflammation is usually absent or less prominent in the cerebellum. $CD4^+$ T cells are predominantly present at perivascular sites, whereas $CD8^+$ T cells are found both in the perivascular cuffs and in the brain parenchyma (Bilzer *et al.*, 1996; Caplazi & Ehrensperger, 1998). To clearly distinguish BD from encephalitis induced by other viruses, it is mandatory to prove that BDV infection of the CNS has occurred. However, isolation of infectious virus from brain tissue of diseased animals is not always successful, even if primary young rabbit brain cell cultures are used (Herzog *et al.*, 1994; Dürrwald, 1993). Traditionally, Joest-Degen inclusion bodies in nuclei of infected neurons have served as BDV-specific markers (Gosztonyi & Ludwig, 1995), but they cannot consistently be seen by routine histology in brains of diseased animals. Enhanced sensitivity of virus detection is achieved by visualizing BDV antigen in tissue sections using monoclonal antibodies. Since expression of viral markers (both RNA and antigen) may vary dramatically between brains of individual animals with BD (Bilzer *et al.*, 1995, 1996; Lebelt & Hagenau, 1996), confirmatory diagnostic work can be demanding. Immunohistological analyses of paraffin-embedded brain sections with monoclonal antibodies against the major BDV antigens p40 (nucleoprotein), p24 (phosphoprotein) or gp18 (putative matrix protein) consistently showed that virus-infected cells are non-uniformly distributed in brains of diseased animals and that antigen-positive neurons are found most frequently in the hippocampus (Bilzer *et al.*, 1995; Lebelt & Hagenau, 1996; Caplazi & Ehrensperger, 1998). Interestingly, in some animals with overt neurological disease only very few virus-infected cells can be visualized (Caplazi & Ehrensperger, 1998), indicating that sometimes BD cases might escape detection by this method.

To improve the sensitivity of BDV antigen detection, an antigen-capture ELISA was introduced which uses a cocktail of monoclonal antibodies and a monospecific polyclonal antiserum for antigen capture and detection, respectively (Dürrwald, 1993). It was found that a large fraction of brain samples which tested positive with this ELISA did not contain infectious virus (Dürrwald, 1993). Surprisingly, the antigen-capture ELISA also detected large amounts of viral antigen in PBMCs and peripheral organs like liver, kidney and spleen of some diseased horses (Dürrwald, 1993; Bode *et al.*, 1994*a*). Unfortunately, the specificity of this ELISA has never been compared systematically with more traditional BDV antigen detection systems. It thus remains unclear whether a positive result with this antigen-capture ELISA is firm proof of infection.

RT–PCR or RT-nested PCR analysis of native or formalin-fixed brain tissue is a sensitive alternative technique that may be used to confirm the clinical diagnosis of BD (Richt *et al.*, 1993; Binz *et al.*, 1994; Schneider *et al.*, 1994; Herzog *et al.*, 1994; Zimmermann *et al.*, 1994; Schüppel *et al.*, 1995; Sorg & Metzler, 1995; Lebelt & Hagenau, 1996; Bilzer *et al.*, 1996). However, since high-sensitivity PCR technology is prone to contamination artefacts, confirmatory laboratory diagnosis which relies exclusively on RT-nested PCR results should not be considered definitive. Another potential problem with RT-

nested PCR is that divergent viral genotypes may not be detected due to sequence differences in the target genes. In this context it is important to note that the recently identified new BDV genotype (see below) cannot readily be identified with standard PCR primers used for the classical European genotypes (Nowotny *et al*., 2000). A possibly more reliable, though less sensitive method to detect viral transcripts is the analysis of thin sections of paraffin-embedded brains by *in situ* hybridization using RNA probes complementary to the major transcripts of BDV (Carbone *et al*., 1991; Gosztonyi *et al*., 1991; Gosztonyi & Ludwig, 1995; Bilzer *et al*., 1995, 1996).

Borna disease in other animals

BD is not strictly limited to horses and sheep, although the frequency at which other animals get the disease appears to be very low. BDV was found in donkeys (Zimmermann *et al*., 1994; Dürrwald, 1993; Bilzer *et al*., 1995, 1996; Lebelt & Hagenau, 1996; Caplazi *et al*., 1999), goats (Caplazi *et al*., 1999) and cattle (Caplazi *et al*., 1994; Bode *et al*., 1994*b*) with neurological disease and strong lymphocytic infiltrations of the CNS. Some of the diseased bovines were from farms in regions of Germany in which BD is not endemic in horses and sheep (Bode *et al*., 1994*b*). BDV antigen and infectious virus was shown to be present in the CNS of two rabbits with neurological disease which originated from the endemic region in Switzerland (Metzler *et al*., 1978). An earlier report described the isolation of BDV from the brain of a rabbit with neurological disease (Otta & Jentzsch, 1960). BDV antigen and RNA were further found in brains of several zoo animals in Erfurt (Thuringia, Germany) that showed neurological disease (Schüppel *et al*., 1994, 1995). More recent work demonstrated BDV antigen and RNA in the brain of a dog with severe CNS inflammation and neurological disease that had lived in an endemic area in Austria (Weissenböck *et al*., 1998*a*).

The question of whether BDV induces 'staggering disease' in cats is more complicated. First reports showed that a high percentage (44%) of Swedish cats suffering from this encephalitic disease had serum antibodies to BDV (Lundgren *et al*., 1993; Lundgren & Ludwig, 1993). As judged by immunohistochemistry, viral markers were expressed at very low levels in the brains of only three out of 24 diseased cats (Lundgren *et al*., 1995*a*, *b*). In contrast, cats with staggering disease from Austria were reported to lack detectable levels of BDV markers in the CNS (Nowotny & Weissenböck, 1995). A report from Japan (Nakamura *et al*., 1999) showed that low levels of BDV-specific antigen and RNA were present in the brain of a cat with CNS inflammation and neurological symptoms distinct from those of staggering disease. In another study from Switzerland, only one of 180 brains of cats with histologically confirmed CNS disease showed evidence of BDV infection by immunohistochemistry (Bornand *et al*., 1998). The other 179 cat brains were negative for BDV markers by immunohistochemistry and RT–PCR (Melzer, 1999). More recently, BDV-specific nucleic acid was demonstrated by *in situ* hybridization in neurons of the cerebral cortex of a cat with paralytic disease but without CNS inflammation (Berg & Berg, 1998). The viral nucleic acid which was amplified by RT–PCR from the cat brain tissue showed the classical European genotype, closely related in sequence to prototype strain He/80. Finally, BDV-specific RNA was found by RT-nested PCR in the brains of some diseased cats in the United Kingdom (Reeves *et al*., 1998), but BDV infection of these animals was not confirmed by other methods. A conservative interpretation of these various results is that although natural BDV infections of cats may occur occasionally, this virus is probably not the aetiological agent of staggering disease.

A recent case report from Sweden (Berg *et al*., 2000) described a free-ranging lynx with non-suppurative meningoencephalitis similar to equine BD. Astrocytes from the brain of the lynx stained positive for BDV antigen by immunohistochemistry. The sequence of a BDV p24 fragment amplified by RT-nested PCR from the brain of this animal differed by more than 2% from known BDV strains. This observation emphasizes the notion that the host range of BDV is wider than previously thought.

A neurological disease affecting a large number of ostriches in Israel is also believed to have resulted from infection with BDV or a BDV-like virus (Malkinson *et al*., 1993; Ashash *et al*., 1996). The diagnosis was mainly based on the fact that sera of diseased animals contained antibodies which readily recognized BDV-encoded proteins. Since no histological and immunohistochemical analyses were performed, these results are rather preliminary and have to be considered with caution.

Non-symptomatic BDV infections: how frequent are they?

Since BD is endemic in some parts of central Europe and since it is well known from experimental infections of horses that an average of 6 weeks may pass between BDV infection and the first signs of neurological disease (Heinig, 1964; Katz *et al*., 1998), it is clear that infected animals lacking neurological symptoms must exist, at least temporarily, on farms with sporadic BD cases. Metzler *et al*. (1979) performed an 18-month-long follow-up study with eight healthy sheep from a flock in which several animals had died from classical BD. Five of the eight animals were seropositive at the beginning of the study. Two of these animals developed classical BD within 2 months. The other three sheep seroconverted within 8 months, but they remained healthy. Interestingly, neither the seven lambs from seropositive sheep nor three control sheep which were housed together with the infected animals contained BDV-specific antibodies at the end of the 18 months observation period, indicating that sheep-to-sheep transmission of BDV had not occurred and that the seroconverted animals had acquired the BDV infection in the field before the study was initiated. Unfortunately, sensitive techniques to analyse the brains for BDV antigen or nucleic acid at the end of the experiment were not available in 1979. Similar

experiments performed by Matthias (1958) also failed to provide evidence for transmission of BDV between horses, sheep and cattle by cohabitation.

Herzog *et al*. (1994) performed a prospective study with more than 100 healthy seropositive horses by requesting health reports from the owners 1 year after the initial examination. According to these reports, about 20% of the animals started to exhibit neurological symptoms within 1 year, and about a third of these animals had to be euthanized because of BD. Caplazi and coworkers (P. Caplazi, V. Bracher, K. Melzer, R. Goetzmann, U. Braun & F. Ehrensperger, unpublished results) examined the brains of six healthy animals (three sheep, one horse, one donkey and one mule) that had remained in a stable after a local BD epidemic had killed several other animals on the farm. Mild encephalitis and serum antibodies to BDV antigens were found in all six animals. BDV antigen was detected by immunohistochemical analysis in the brains of four animals, and BDV-specific RNA was detected by RT-nested PCR in five brains. These studies clearly established that non-symptomatic BDV carriers do exist, at least temporarily, on farms with sporadic BD cases.

In two other studies, brains of 283 (Rohner-Cotti, 1992) and 109 (Goetzmann, 2000) randomly selected sheep from endemic regions in Switzerland and the Principality of Liechtenstein were analysed for BDV infection by histology and, selectively, by immunohistochemistry and RT-nested PCR. Both tissue collections included brains of animals from flocks with a history of sporadic BD. Interestingly, no BDV-infected animals were identified in either study. It thus seems that the frequency at which non-symptomatic virus carriers can be found is highly variable, depending on the geographical location and, most likely, additional unknown factors.

Detection of clinically inapparent BDV infections by serological methods

To determine the frequency of clinically inapparent BDV infections of horses in Germany by more simple means, large-scale serological studies using sensitive IFA were performed (Lange *et al*., 1987; Herzog *et al*., 1994). These studies showed that approximately 12% of the horses from both endemic and non-endemic regions had serum antibodies which recognized BDV antigen. As observed with sera from diseased horses, the titres of BDV-specific serum antibodies in healthy animals were also low (1:5 to 1:320). Using IFA, Western blot analysis or ELISA, variable proportions (ranging from 3% to 58%) of horses from the United States of America, Japan, Iran and Sweden were found to be seropositive (Herzog *et al*., 1994; Kao *et al*., 1993; Nakamura *et al*., 1995; Yamaguchi *et al*., 1999; Bahmani *et al*., 1996; Berg *et al*., 1999). By contrast, a survey by IFA of healthy horses from an endemic region in Switzerland suggested that only about 1% were seropositive (Rohner-Cotti, 1992).

BDV-specific antibodies were detected in less than 4% of healthy sheep from the same endemic region in Switzerland (Rohner-Cotti, 1992; Goetzmann, 2000). Seropositive sheep (Hagiwara *et al*., 1997) and dairy cattle (Hagiwara *et al*., 1996) were also found in Japan. The prevalence of serum antibodies to BDV antigens in cats from Switzerland was 29%, with no significant difference between healthy and diseased cats nor between cats originating from endemic or non-endemic regions (Melzer, 1999). Large numbers of seropositive cats were also reported from Japan (Nishino *et al*., 1999; Nakamura *et al*., 1999).

Since most of the presently used serological assays have been optimized for highest sensitivity, it remains unknown whether they might occasionally detect cross-reactive antibodies which do not originate from encounters with BDV. In fact, a recent nationwide comparison in Germany showed that serological assays used by bornavirus laboratories varied considerably with respect to sensitivity and specificity (Nübling *et al*., 1999).

Intra vitam diagnosis of BDV infection by RT–PCR

Direct identification of viral nucleic acids in accessible body fluids would seem to represent a straightforward way to determine the frequency of non-symptomatic persistent infections with BDV. Nasal secretions, saliva and conjunctival fluid of some healthy seropositive horses in Germany contained BDV-specific RNA as assessed by RT–PCR analysis (Richt *et al*., 1993; Herzog *et al*., 1994). A similar approach using BDV p24 gene-specific RT–PCR for analysis of the same body fluids of horses with confirmed BD yielded positive results in only two out of seven animals (Lebelt & Hagenau, 1996). Unfortunately, no follow-up studies with larger numbers of animals were reported. Thus, the diagnostic value of these tests remains unclear.

Since viral RNA is consistently found in the blood of immunologically tolerant, persistently infected rats (Sierra-Honigmann *et al*., 1993; Sauder & de la Torre, 1998), many laboratories set out to screen blood samples by RT-nested PCR. The results of such studies in animals and humans (see below) are highly controversial. In Japan, BDV-specific RNA was detected in blood samples from 29·8% of healthy horses (Nakamura *et al*., 1996), 10·8% of healthy dairy cows (Hagiwara *et al*., 1996), between 16·7% and 31% of healthy sheep (Hagiwara *et al*., 1997), 8·3% of healthy cats (Nakamura *et al*., 1996) and 53·3% of cats with neurological disease (Nakamura *et al*., 1999). Similarly, 23·6% of healthy horses in Iran (Bahmani *et al*., 1996) and 28·6% of horses with various neurological diseases in Sweden (Berg *et al*., 1999) were reported to contain BDV-specific RNA in the blood. A recent study (Reeves *et al*., 1998) indicated that four out of five cats with neurological disease, but none of five healthy cats in the United Kingdom had BDV-specific RNA in the blood. In marked contrast to these reports, we failed to find BDV-specific nucleic acid in blood samples of several horses with classical BD (P. Caplazi, W. Hallensleben, F. Ehrensperger & P. Staeheli, unpublished results). Since our RT-nested PCR assay could detect as few as

200 BDV p40 RNA molecules in 5 μg of total RNA, it is unlikely that insufficient sensitivity can explain our negative results. Similarly, the analysis of blood from a large number of healthy and diseased horses in Germany consistently yielded negative results in another laboratory (S. Herzog, personal communication). We further used RT-nested PCR to monitor viraemia in experimentally infected mice. We found no evidence for BDV in blood at any time-point in disease-susceptible or disease-resistant strains of mice (Lieb *et al.*, 1997). Since the reasons for the striking discrepancies between the different laboratories are unclear at present, epidemiological studies based on RT-nested PCR analysis of blood should be considered with great caution. We believe that accidental sample contamination might be a simple explanation for at least some of the positive results.

Rodents as a virus reservoir?

Because experimental infection of rats, mice, sheep and horses is readily achieved by intranasal application of virus (Matthias, 1958; Heinig, 1969; Carbone *et al.*, 1987, and own unpublished results), it is reasonable to assume that natural infection with BDV might occur by this route. The natural source of infectious virus, however, has still not been determined. An obvious possibility is that farm animals with BD may shed infectious virus. However, since diseased animals do usually not live very long and since BD occurs mostly sporadically, it is unlikely that virus transmission solely by diseased animals could maintain the infection chain. BDV could further be transmitted by horses, sheep and other farm animals in which, for unclear reasons, the infection might have taken a non-symptomatic but highly productive persistent course. An argument in favour of this possibility is that BDV RNA was detected by RT–PCR in nasal secretions, saliva and conjunctival fluid of a small fraction of infected horses (Richt *et al.*, 1993; Herzog *et al.*, 1994). It should be noted that, in contrast to earlier reports (Heinig, 1969), all attempts to demonstrate infectivity in secretions of horses have failed (Richt *et al.*, 1993; Herzog *et al.*, 1994; Lebelt & Hagenau, 1996). Furthermore, the above discussed follow-up study with sheep from a flock with BD which were housed together with uninfected animals (Metzler *et al.*, 1979) yielded no evidence for sheep-to-sheep transmission of BDV. Epidemiological data further question the transmission of BDV from horse to horse. For example, it is difficult to understand why BD has remained restricted rather tightly to a few endemic areas in central Europe for many decades in spite of largely unrestricted animal trade and frequent contacts of riding horses with animals from other parts of the world at national and international sport events. It is also difficult to understand why BD is more frequently seen in horses from traditional farms which are housed together with other animals than in horses kept in modern facilities with higher hygiene and management standards (Heinig, 1969; Dürrwald, 1993; F. Ehrensperger, unpublished observations). Furthermore, the proposed mode of virus transmission cannot readily explain why most BD cases in horses and sheep are recorded in early summer (Heinig, 1969; Dürrwald, 1993; Caplazi *et al.*, 1999; Rott & Becht, 1995). Finally, the absence of species-specific mutations in BDV strains from horses and sheep or other farm animals (see below) seems to argue in favour of a common source of virus in an as yet unknown animal reservoir. The territorial factor, the enhanced frequency of BD in stables with poor hygiene and the seasonal periodicity would all be compatible with the existence of a rodent reservoir.

A recent serosurvey of 106 wild rats from Hokkaido (Japan) yielded no evidence of BDV infection of these animals (Tsujimura *et al.*, 1999). To our knowledge, systematic searches for BDV in rats and other small rodents have to date not been performed in the endemic regions of central Europe using sensitive detection techniques. In this context it is of importance to note that newborn infected rats that do not develop overt neurological disease contain infectious virus in urine and possibly other body excretions (Morales *et al.*, 1988). Such experimentally infected animals can transmit BDV to non-infected littermates and to their mothers (Morales, 1988; Dürrwald, 1993). Thus, animal feed contaminated with urine of persistently infected rats or other rodents could represent a source of infectious BDV.

Genetic diversity of BDV

High genetic similarity between isolates from central Europe

Several BDV strains were isolated from infected horses, sheep and other animals, and their sequences have been determined either partly or completely. Furthermore, sequences of BDV genome fragments amplified by RT–PCR from the brains of horses, sheep, donkeys, dogs and cats have been published (Binz *et al.*, 1994) or deposited in the EMBL/GenBank database. Part of this information was used to construct the phylogenetic tree shown in Fig. 1, which is based on nucleotide sequences of a 333 nucleotide fragment of the BDV p24 gene. This sequence comparison revealed that most strains are highly related to each other. Their genomes differ by less than 5%. Intriguingly, nucleotide exchanges in the genome of classical European BDV strains are not distributed randomly. Rather, they cluster to well defined nucleotide positions in the p24 gene (Fig. 2) as well as other parts of the viral genome. Since these sites frequently map to third-codon (wobble) positions of the open reading frames, the exchanges do not usually affect the amino acid sequence of the viral proteins. It thus seems likely that some unrecognized functional constraints including secondary or tertiary RNA structures restrict sequence alterations to certain hot spots. Alternatively, the non-random distribution of base exchanges in the genomes of central European BDV strains might simply indicate that all known field isolates originate from just a handful of genetically stable progenitor strains.

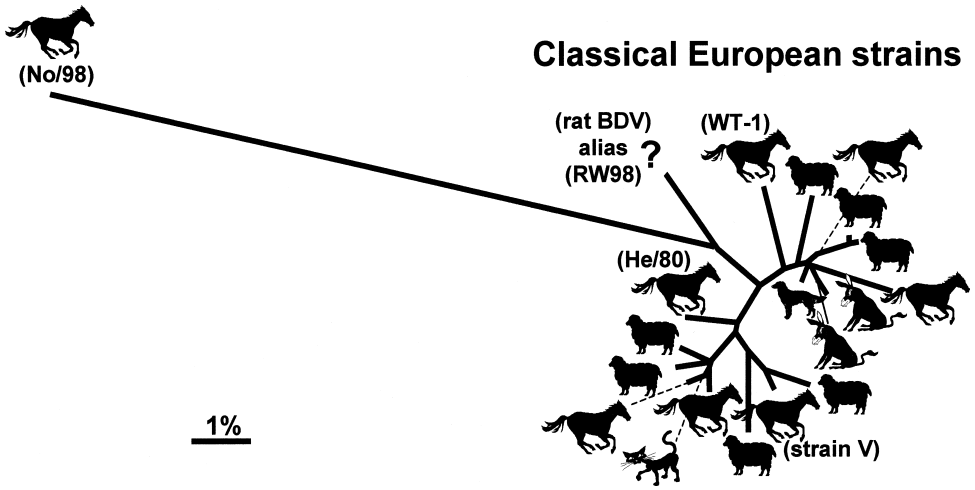

Fig. 1. Phylogenetic tree of BDV field strains. The tree is based on nucleotide sequences encoding amino acids 71–181 of the P protein of BDV. Note that all classical European strains are closely related, although they originate from different species. Also note the remote position of the eastern Austrian horse isolate No/98 in the phylogenetic tree. Sequence information was from Binz *et al.* (1994) and from EMBL/GenBank accession numbers U94868, L27077, U04608, U94884, U94885, U94872, U94864, U94880, AJ250178/AF158630, AJ277119, AJ277120, AF136236, S67507, U94883 and U94876.

ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGA GAC ATC TCG GCT CGT	Strain V (U04608)
ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGG GAC ATC TCG GCT CGC	He/80 (L27077)
ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGA GAC ATC TCG GCT CGC	Horse 4*
ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGA GAC ATC TCG GCT CGC	Cat (U94864)
ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGA GAC ATC TCG GCT CGT	Sheep (AJ277119)
ATC GAG GCT GAG GAG GTG CGG GGC ACT CTT GGA GAC ATC TCG GCT CGC	Sheep (AJ277120)
ATA GAG GCT GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	WT-1 (S67507)
ATA GAG GCT GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCG GCT CGC	Sheep 1*
ATA GAG GCT GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	Horse 2-2*
ATA GAG GCT GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	Donkey 1*
ATA GAG GCC GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	Horse 1-1*
ATA GAG GCT GAG GAG GTA CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	Dog (U94880)
ATA GAG GCT GAG GAG GTG CGG GGC ACC CTT GGG GAC ATC TCA GCT CGC	Donkey (U94872)
ATT GAG GCC GAG GAG GTG CGG GGT ACC CTT GGA GAC ATC TCG GCT CGC	RW98 (AF158631)

Fig. 2. Non-random distribution of nucleotide exchanges in classical European BDV strains. A short stretch of viral sequence (nucleotides 1533–1580) from the p24 gene is shown. Third-codon (wobble) positions at which nucleotide exchanges were observed most frequently are marked by arrows and red colour. Rare exchanges are highlighted with green colour. For unknown reasons, mutations at these positions are highly preferred over mutations at certain other third-codon positions which would also not result in amino acid substitutions of the viral protein. Sequence variations of this type might be expected if functional constraints on secondary or tertiary RNA structures existed. Similar patterns of non-random nucleotide exchanges were also observed in other parts of the viral genome. Individual sequences are identified by their accession numbers, except those reported by Binz *et al.* (1994), which are marked by asterisks.

Sequence comparison further showed that BDV strains from various host species seemed to lack species-specific signatures. Viruses from horses did not show a higher degree of similarity to each other than to viruses from sheep, donkeys or other hosts (Fig. 1). If BDV mainly spread from horse to horse and from sheep to sheep, species-specific patterns of nucleotide exchanges would be expected. Since this was not observed, the data rather seem to point towards a single source from which the various farm animals acquired the virus. In the first part of this review we speculated that persistently infected rodents or other wild animals might serve as a virus reservoir, and that farm animals might contract the BDV infection by feed which is contaminated with rodent urine.

A novel BDV genotype from eastern Austria

The only BDV strain known to date whose sequence differs markedly from viruses of the classical European group is strain No/98 (Fig. 1). This strain originates from a diseased horse in eastern Austria where no cases of BD had previously been recognized. No/98 differs from all other strains by about 15 %

at the nucleotide level (Nowotny *et al.*, 2000). Interestingly, conservation at the amino acid level is very high (93–98%) for all viral proteins except for p10 (also designated protein X), which is only about 81% identical to its counterparts in other BDV strains.

From an epidemiological point of view, the discovery of No/98 is of great importance. It shows that some natural variants of BDV may easily escape detection by RT–PCR when standard primer sets derived from sequences of classical European strains are used. It is possible, therefore, that infections with BDV are more frequent than previously believed. If, as in the case of No/98, the major antigens of other, yet unidentified, BDV strains are also conserved, those viruses could be identified by immunohistochemical methods using a cocktail of monoclonal antibodies rather than by RT–PCR technology.

The work with No/98 further suggests that additional BDV variants with distinct structural and possibly biological features might be present outside central Europe. Unexpectedly, however, recent reports have indicated that BDV strains in horses, sheep, cats and humans of Japan, Taiwan, Iran, the United Kingdom and the United States of America are almost identical to laboratory strains derived from central European isolates (Iwata *et al.*, 1998; Kishi *et al.*, 1995*b*, 1996; Czygan *et al.*, 1999; Reeves *et al.*, 1998; Berg *et al.*, 1999; Bahmani *et al.*, 1996, Hagiwara *et al.*, 1997). These data allow for at least two different interpretations. One possibility is that classical European BDV strains are predominantly present in horse breeds that are traded most extensively worldwide. In this context it is of interest to note that a variant of a central European BDV isolate adapted to grow in rabbits had been in use as an attenuated live vaccine (Zwick & Witte, 1931; Zwick, 1939) in some parts of Germany. Because its efficacy was questionable (Dürrwald, 1993), the use of this vaccine was discontinued around 1980 in West Germany and a few years later in East Germany. Thus, the remote possibility exists that the non-pathogenic vaccine strain (or escape virus of it) gave rise to present-day field isolates in the above-mentioned countries. However, since there is no clear evidence that transmission of BDV from horse to horse does occur, this scenario seems quite unlikely. Alternatively, it remains possible that the reports on the detection of BDV in animals and people from non-European countries represent artefacts resulting from accidental contamination of samples with laboratory virus strains. It is also conceivable that complex mixtures of strains do indeed exist worldwide, but because everyone uses the same techniques to detect the virus, only a few strains become visible.

Evolution of BDV in experimentally infected animals

Experimental work with BDV originating from brains of diseased horses indicated that it is possible to generate laboratory strains with different biological properties. Strains were described which induce either a standard biphasic disease

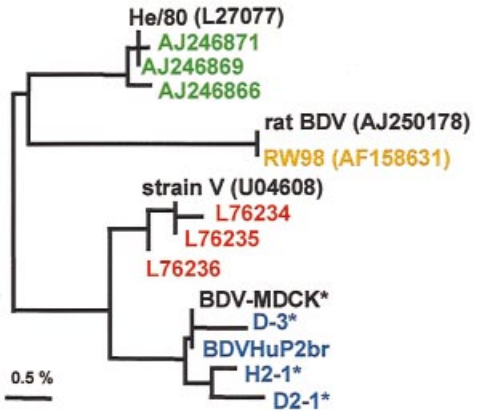

Fig. 3. Relationship between BDV laboratory strains and selected viruses reported to originate from human blood or brain tissue. A rooted phylogenetic consensus tree was generated by the neighbour-joining method using nucleotide sequences derived from regions encoding BDV protein p24 (nucleotides 1482–1814). The exception was isolate BDVHuP2br, for which sequence information was restricted to a fragment comprising nucleotides 1573–1772 (Nakamura *et al.*, 2000). The tree was constructed using the software package Clustal-X. Bootstrap analysis was applied using 100 values. Viral sequences from human tissues reported by laboratories frequently working with laboratory strains V, He/80, rat BDV or MDCK-BDV are marked with red, green, orange and blue colour codes, respectively. Individual sequences are identified by their accession numbers. Sequences reported by Iwata *et al.* (1998) are marked by asterisks.

pattern or obesity syndrome in rats (Herden *et al.*, 2000). It will be of interest to determine the genetic alterations responsible for these alternative phenotypes.

Recent work in our laboratory further showed that BDV strains might undergo surprising changes during passage in animals. We observed that several stocks of BDV which were supposed to consist of laboratory strain He/80 did not contain this virus but rather a new variant (designated rat BDV in Fig. 1) whose similarity to He/80 at the nucleotide level is only about 97% (Schwemmle *et al.*, 1999*a*; Formella *et al.*, 2000). The phylogenetic tree shown in Fig. 1 suggests that rat BDV has not evolved directly from strain He/80 by mutation. More likely, it was already present at a low concentration in the horse brain from which He/80 was isolated. It was presumably selected during passage in rat brains due to its superior growth characteristics in this animal host. These observations suggest that the CNS of diseased animals may be infected with more than one BDV variant and that they can be selected with different efficacy in different experimental hosts. Circumstantial evidence for simultaneous infections of horses with two different strains of BDV has previously been presented (Binz *et al.*, 1994).

Extraordinary stability of the BDV genome in persistently infected cell cultures

When BDV is maintained in persistently infected cell cultures, its genome appears invariant for hundreds of cell generations. Since polymerases of RNA viruses have no proof-

reading activity (Domingo & Holland, 1997), such behaviour indicates either that all virus variants which may be generated in the culture have lower fitness than the resident virus or that some unknown mechanisms exist which prevent the replacement of resident virus by novel variants. Recent evidence from our laboratory suggests that the latter possibility may be true.

To test whether mutant viruses were generated but failed to show up in persistently infected cell cultures, we designed bottleneck experiments in which single infected cells were cultured together with a large excess of uninfected cells, thus allowing virus populations of individual cells to spread unhindered. Of 30 virus-infected cultures resulting from such bottleneck experiments, one was found to contain a BDV variant with two point mutations in the G gene, demonstrating that virus variants can readily appear under appropriate conditions (Formella *et al.*, 2000).

These results are of importance for the correct interpretation of epidemiological data. Minor sequence variations of BDV strains isolated from human specimens had previously been regarded as strong evidence that contamination with laboratory strains had not occurred. However, since contamination of samples presumably occurs at extremely low virus doses, such laboratory accidents may closely mimic the conditions of our bottleneck experiments, which obviously allow the outgrowth of variant viruses.

Infections of humans

How conclusive is the serological evidence for BDV infection of humans?

An early serological survey by IFA showed that sera of some psychiatric patients contained IgG specific for BDV antigens (Rott *et al.*, 1985). Because such antibodies were found much less frequently in sera of healthy controls, this pioneering study suggested that BDV infections might be associated with human psychiatric disorders. Using this or other serological techniques, several researchers subsequently came to similar conclusions (Bode *et al.*, 1988, 1992; Bechter *et al.*, 1992; Fu *et al.*, 1993; Waltrip *et al.*, 1995; Auwanit *et al.*, 1996; Takahashi *et al.*, 1997; Kishi *et al.*, 1995*b*; Sauder *et al.*, 1996; Iwahashi *et al.*, 1997; Chen *et al.*, 1999*b*; Gonzalez-Dunia *et al.*, 1997). Although the percentage of reactive sera in patient and control groups differed widely between the various laboratories, a consensus emerged which indicated that BDV-specific antibodies are present at enhanced rates in psychiatric patients. The discrepant numbers of positive individuals in the different studies were explained by the facts that non-standardized in-house assay systems were employed by the various laboratories and that the titres of reactive antibodies were usually very low. Western blot analyses with recombinant BDV proteins indicated that, unlike sera from animals with confirmed BD, reactive human sera usually recognized only one, rather than both, of the major BDV antigens (Sauder *et al.*, 1996; Iwahashi *et al.*, 1997; Chen *et al.*, 1999*b*; Fu *et al.*, 1993; and own unpublished findings). Since multicentre studies revealed discrepancies between findings of different laboratories who analysed the same set of human sera (Nübling *et al.*, 1999), and since such discrepancies were encountered much less frequently with sera of animals with confirmed BDV infections, the diagnostic relevance of human serological data has remained controversial. Nonetheless, the presence of serum antibodies with the above-mentioned properties is taken as strong evidence for infection with BDV by some researchers (Bode *et al.*, 1992; Bechter *et al.*, 1992; Nakamura *et al.*, 2000). However, it should be noted that it is unclear whether the presence of reactive antibodies indicates a previous infection with BDV. The alternative possibility remains that these antibodies were induced by infection with an antigenically related microorganism of unknown identity or exposure to some other related immunogen. In fact, recent data from our laboratory indicate that reactive human sera exhibit surprisingly low avidity for BDV antigens (Allmang *et al.*, 2000), favouring the second possibility.

Significance of viral nucleic acid and infectious BDV in human samples

Bode *et al.* (1995) presented the first direct evidence that BDV might infect humans. Using highly sensitive RT-nested PCR, they detected BDV-specific nucleic acid in the peripheral blood of several psychiatric patients. This report was followed by a series of conflicting results from other groups. Some researchers detected BDV-specific RNA in blood samples (2–50%) from psychiatric and chronic fatigue syndrome patients, but also in some healthy blood donors (0–4·6%) (Kishi *et al.*, 1995*a*, *b*, 1996; Sauder *et al.*, 1996; Igata-Yi *et al.*, 1996; Nakaya *et al.*, 1996, 1999; Kitani *et al.*, 1996; Iwata *et al.*, 1998; Planz *et al.*, 1998; Iwahashi *et al.*, 1998; Chen *et al.*, 1999*a*; Nowotny & Kolodziejek, 2000; Kubo *et al.*, 1997). Other groups failed to find BDV in human blood (Lieb *et al.*, 1997; Richt *et al.*, 1997; Kim *et al.*, 1999; Bachmann *et al.*, 1999). Bode *et al.* (1996) reported the isolation of replication-competent BDV strains from the blood of three psychiatric patients. Successful isolation of BDV strain RW98 from the blood of a psychiatric patient was also reported by Planz *et al.* (1999). We questioned the human origin of RW98 (which we designated rat BDV) and most other BDV isolates by showing (Fig. 3) that their genomes were most strongly related to BDV strains frequently used for experiments in the various reporting laboratories (Schwemmle *et al.*, 1999*a*).

BDV antigen and/or RNA has also been detected in human autopsy brain samples from individuals with a history of mental disorder (de la Torre *et al.*, 1996; Haga *et al.*, 1997*a*; Salvatore *et al.*, 1997) and apparently normal controls (Haga *et al.*, 1997*b*). These data contrasted with a report from our group in which we failed to find BDV-specific nucleic acid in autopsy samples from various brain regions of 86 patients with various psychiatric disorders and 52 healthy controls (Czygan *et al.*, 1999). However, using non-nested RT–PCR, we were able to

confirm the presence of both BDV p40 and p24 transcripts in the brains of three psychiatric patients. In these brains, BDV had previously been shown to be present by means of RT-nested PCR, *in situ* hybridization and immunohistochemistry (de la Torre *et al*., 1996). To our surprise, we found that the genomes of the viruses present in the brains of these patients (Fig. 3) were almost identical to that of laboratory strain He/80 (Czygan *et al*., 1999). Since all three patients lived in the United States of America, it remains an unsolved puzzle why they were infected with virtually the same strain of BDV that killed a horse in Germany more than 20 years ago.

A recent report described BDV in the brain of a schizophrenic patient from Japan (Nakamura *et al*., 2000). Viral RNA was detected in scattered neurons of three brain regions by *in situ* hybridization using a BDV p24-specific RNA probe. Furthermore, several neurons in the hippocampus were stained using a polyclonal mouse antiserum. Moreover, infectious BDV was recovered by intracerebral injection of extracts of this human brain into newborn gerbils. The genome of this virus isolate (designated BDVHuP2br) was found to differ by about 2% from several standard BDV laboratory strains. However, the published p24 sequence of BDVHuP2br (Fig. 3) is identical to the corresponding region of laboratory strain BDV-MDCK (Iwata *et al*., 1998).

In summary, a critical evaluation indicates that no laboratory has to date been able to present solid evidence that BDV is infecting humans. Contamination problems may have clouded reality far more seriously than previously acknowledged.

Open questions and perspectives

Although many laboratories have engaged in BDV diagnostic work during the last 5 years, several fundamental questions of BDV epidemiology still wait to be answered. It is still not clear, for example, whether BDV infection of farm animals is indeed mainly restricted to central Europe. If it occurs worldwide, we will have to explain why infection of farm animals is symptomless in most parts of the world. Another fascinating issue in BDV epidemiology relates to the central question of which wild animal serves as the virus reservoir.

Due to intrinsic difficulties of BDV diagnostics, epidemiological studies are not straightforward. Since the humoral immune response to natural infection with BDV is usually weak, it is necessary to push the limits of presently available serological assays with the drawback of reduced specificity. Therefore, diagnostic laboratories should initiate a concerted action to carefully evaluate the various serological tests for specificity and sensitivity. Until such standardization has been achieved, the interpretation of serological studies will remain difficult.

Since BDV is strongly associated with the CNS, its *intra vitam* detection by virological methods is not readily possible. The question of whether viral nucleic acid and infectivity is present in blood has tremendous importance for laboratory diagnosis of BDV infections and for the safety of human blood products. To achieve an agreement between the various laboratories on this point, multicentre studies should be organized in which pre-PCR handling of samples is performed by a neutral laboratory with no history of experimental or diagnostic work on BDV, and in which multiple samples of human specimens are stored for independent testing.

In spite of discrepant views between laboratories on the interpretation of RT–PCR results, a wealth of serological data continues to indicate that BDV or an antigenically related agent might be associated with human psychiatric disorders. Challenging tasks for future research include confirming the role of BDV in human disease or else identifying the related immunogen.

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