Epidemiology and host spectrum of Borna disease virus infections

Paula M. Kinnunen,1,2+ Airi Palva,1 Antti Vaheri2,3 and Olli Vapalahti1,2,3

1Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland
2Infection Biology Research Program Unit, Department of Virology, Haartman Institute, Faculty of Medicine, University of Helsinki, Finland
3HUSLAB, Helsinki University Central Hospital, Helsinki, Finland

Borna disease virus (BDV) has gained lot of interest because of its zoonotic potential, ability to introduce cDNA of its RNA transcripts into host genomes, and ability to cause severe neurobehavioural diseases. Classical Borna disease is a progressive meningoencephalomyelitis in horses and sheep, known in central Europe for centuries. According to current knowledge, BDV or a close relative also infects several other species, including humans at least occasionally, in central Europe and elsewhere, but the existence of potential ‘human Borna disease’ with its suspected neuropsychiatric symptoms is highly controversial. The recent detection of endogenized BDV-like genes in primate and various other vertebrate genomes confirms that at least ancient bornaviruses did infect our ancestors. The epidemiology of BDV is largely unknown, but accumulating evidence indicates vectors and reservoirs among small wild mammals. The aim of this review is to bring together the current knowledge on epidemiology of BDV infections. Specifically, geographical and host distribution are addressed and assessed in the critical light of the detection methods used. We also review some salient clinical aspects.

Introduction

Any infection or disease that is naturally transmissible between vertebrate animals and humans is, according to the definition of the World Health Organization, classified as a zoonosis (Acha, 1987). As many as 60–80 % of emerging human diseases are zoonoses (Cleaveland et al., 2001; Jones et al., 2008). Of the emerging zoonotic pathogens, 71.8 % originate from wildlife (Jones et al., 2008) or, more specifically, 58 % infect ungulates, 51 % carnivores, and 34 % rodents (Cleaveland et al., 2001), emphasizing the utmost importance of collaboration between veterinary, medical and wildlife sciences in the detection and battle against these threats.

This review addresses Borna disease virus (BDV). It is known as a veterinary pathogen that, in addition to ungulates and carnivores, also infects humans at least occasionally and has its suspected host among small wild mammals (reviewed by Staeheli et al., 2000; Kinnunen et al., 2007; Puorger et al., 2010). Animal Borna disease (BD) has classically been described as a chronic, progressive meningoencephalomyelitis, causing both neurological and behavioural symptoms in horses and sheep (reviewed by Dürwald & Ludwig, 1997). During recent decades, milder manifestations and a wider host range have also been seen (Lundgren et al., 1993; Berg et al., 1999; reviewed by Rott & Becht, 1995). In humans, numerous neuropsychiatric entities have been reported to be associated with potential markers of BDV infection, but the evidence remains inconclusive and the true prevalence and causative role are questionable (reviewed by Planz et al., 2002; Lipkin et al., 2011). This association has become even more intriguing since the recent observation of endogenization of ancient bornavirus genes in vertebrate genomes (Belyi et al., 2010; Horie et al., 2011).

The first descriptions of BD, then known as ‘heitige Kopfkrankeit der Pferde’ (‘heated head disease of horses’), stem from equine practitioners in Germany in the 18th century (von Sind, 1770). The disease was later named after an epidemic in cavalry horses in the city of Borna in Saxony at the end of the 19th century (reviewed by Dürwald & Ludwig, 1997), and its viral aetiology was established as early as 1924 (Zwick & Seifried, 1924). In addition to certain parts of Germany, Austria, Liechtenstein and Switzerland also constitute the classical endemic BD region where sheep, horses and rabbits have succumbed to the disease (reviewed by Dürwald & Ludwig, 1997). In the 1990s, however, researchers started to find BDV elsewhere in the world, as a result of mounting interest after the first reports of human BDV infections (Rott et al., 1985). Publications on BDV infection signs in multiple species, including cat, dog and...
cow, and especially in neuropsychiatric human patients, have now accumulated. Many of the early PCR findings have later been shown or suspected to be caused by contamination (reviewed by Sauder et al., 2002; Planz et al., 2003; Dürwald et al., 2007), and there have been specificity concerns with some of the serological methods (reviewed by Sauder et al., 2002; Weissenböck et al., 2002; Wolff et al., 2006). Nevertheless, carefully verified human BDV infections have also occurred rarely (de La Torre et al., 1996), but because transmission between man and vertebrate animals has not been demonstrated, BDV is a possible, not verified, zoonosis.

The aim of this review is to compile the current knowledge on BDV epidemiology. Specifically, geographical and host distribution, including possible reservoirs and vectors, are addressed and assessed in the critical light of the detection methods available. Furthermore, some historical and clinical aspects are described, and epidemiological significance of recently found endogenization of bornavirus genes is discussed. In addition to the more recent publications written in English, the most significant older German literature is also included.

**Properties of BDV**

BDV was for a long time the sole known member of the family Bornaviridae in the order Mononegavirales. Recently, however, its relatives, avian bornaviruses (ABVs), have been detected in psittacine birds with proventricular dilatation disease (Kistler et al., 2008; Honkavuori et al., 2008), and in Canada geese and trumpeter swans (Delnatte et al., 2011; Payne et al., 2011).

BDV is an enveloped, 80–100 nm, non-cytopathic and strictly cell-associated virus causing slowly progressing, chronic infections of neurons and occasionally glial cells in vivo, while infecting several cell lines in vitro (Danner et al., 1978; Danner & Mayr, 1979; reviewed by Pletnikov et al., 2002). BDV enters the target cell via receptor-mediated endocytosis (Clemente & de la Torre, 2009) and exits it by budding through the plasma membrane to the adjacent cell (most frequently) or intercellular compartment (Kohno et al. 1999; Ludwig et al., 1988). The 8.9 kb single-, negative-stranded RNA genome replicates, as an exception, in the nucleus (Briese et al., 1994; Cubitt et al., 1994). There it associates closely with chromosomes, which facilitates virus spread from cell to cell in mitosis (Matsumoto et al., 2012). By utilizing splicing and alternative, overlapping reading frames, BDV uses its small genome effectively (Briese et al., 1994; reviewed by Lipkin & Briese, 2007). The genome encodes six proteins: the nucleoprotein (p40, N) is the most abundant of these, although when the infection becomes chronic, its molecular ratio to the polymerase cofactor phosphoprotein (p24, P), with which it forms complexes, decreases (Watanabe et al., 2000). In addition to these most abundant proteins, the BDV genome encodes protein p10 (X), matrix protein p16 (M), glycoprotein p57 (G, gp94 when glycosylated) and RNA polymerase (L) (Walker et al., 2000; reviewed by Lipkin & Briese, 2007; Tomonaga et al., 2002). Polymerase activity is important in the adaptation of BDV to new hosts (Ackermann et al., 2007) and the X protein regulates viral polymerase activity and inhibits apoptosis, being essential for host survival (Poenisch et al., 2009). BDV uniquely limits its genome amplification by trimming the genome, which may favour non-cytopathic virus persistence and evasion of the antiviral host response (Habjan et al., 2008; Schneider et al., 2005). BDV infection produces an extremely low number of infectious virus particles per cell, although the cells express high BDV RNA and protein levels (Danner et al., 1978; reviewed by Tomonaga et al., 2002). Lipkin et al. (2011) recently reviewed BDV molecular biology in more detail.

BDV remains stable for at least 1 year at −20 °C, for 3 months at +4 °C, for 1 week at +20 °C, and for 2 days at +37 °C (Danner & Mayr, 1979). BDV is sensitive to a pH <4 and to UV light, but rather resistant to desiccation.

**Ecology and epidemiology**

**Geographical distribution**

Signs of BDV infection, including antibodies, antigen, RNA and/or virus itself, have been reported from animals in many continents (Fig. 1). The highest clinical incidence in animals and the verified classical BD cases, however, are restricted to central Europe (reviewed by Dürwald et al., 2006a; Staeheli et al., 2000). As several PCR amplicons retrieved from animals and humans outside the classical endemic central European area resemble those of control strains, many PCR results have been suspected to be caused by contamination (Dürwald et al., 2006b; 2007). Furthermore, some epidemiological studies have been conducted based on a triple-ELISA method (Bode et al., 2001), the results of which have not been reproducible or confirmable by other methods (see ‘Immunity and detection of serological response’). However, these discrepancies cannot explain the detected BDV antigens or antibodies with other, reproducible methods, e.g. immunohistology and immunofluorescence assay (IFA). Thus, although it seems probable that not all BDV reports are reproducible, the virus or its close relative is distributed widely. Whether some of the findings are in fact caused by ABVs, endogenized bornavirus genes or as-yet-unknown bornaviruses remains to be shown.

**Species diversity**

BDV has long been known to infect and cause disease in horses (Zwick et al., 1927). Later, natural infections with BDV or a BDV-like agent were also verified in zoo animals [monkey, sloth, llama, alpaca, pygmy hippopotamus (Jacobsen et al., 2010; reviewed by Dürwald et al., 2006a; Ikuta et al., 2002); goat, deer (reviewed by Dürwald & Ludwig, 1997); sheep (Metzler et al., 1976); rabbit (Metzler et al., 1978); cat (Lundgren et al., 1993); cow (Caplazi et al., 1994); human (de La Torre et al., 1996); dog (Weissenböck et al., 2002)]. Recently, BDV has also been shown to cause disease in certain bird species, including psittacine birds (Delnatte et al., 2006; 2008; Honkavuori et al., 2008) and passerine birds (Delnatte et al., 2006; 2008; Honkavuori et al., 2008).
et al., 1998); lynx (Degiorgis et al., 2000); and, recently, the shrew (Hilbe et al., 2006). Evidence also exists of infections in ostrich (Malkinson et al., 1995), fox (Dauphin et al., 2001), mallard and jackdaw (Berg et al., 2001), wild voles (Kinnunen et al., 2007), macaque (Hagiwara et al., 2008) and raccoon (Hagiwara et al., 2009). In addition, this neurotropic virus experimentally infects tree shrew, rhesus monkey, chicken, rat, mouse, hamster, Mongolian gerbil, guinea pig (reviewed by Staeheli et al., 2000; Pletnikov et al., 2002) and bank vole (Kinnunen et al., 2011). Despite the wide possible host range, the incidence of BD in species other than horses and sheep appears low (reviewed by Dürrwald et al., 2006a; Weissenböck et al., 1998). As a sporadic and relatively poorly known disease, BD commonly seems to escape diagnosis.

**Phylogeny and molecular epidemiology**

The genomic sequence of BDV – then representing all known bornaviruses – was long thought to be extremely conserved, as the nucleotide sequences from isolates or PCR amplicons were >95% identical (reviewed by Dürrwald et al., 2006a; Schneider et al., 1994). Although BDV has an RNA genome and lacks the proofreading activity of its polymerase, it has also been demonstrated to be surprisingly stable in persistently infected cell cultures (Formella et al., 2000). A strict selection pressure, probably related to a complicated transmission chain including several species, such as that of rabies virus (Holmes et al., 2002), may provide constraints to genomic variation, as mutations may easily weaken the fitness of BDV in one or more hosts crucial for its survival (reviewed by Dürrwald et al., 2006a). More recently, however, one strain, No/98, from an Austrian region where BD had not previously been diagnosed, has been detected with only 85% identity to the previous variants (Nowotny et al., 2000) (Fig. 2). This finding indicates that additional BDV variants with distinct features may be present, but probably escape detection with primers and probes designed to amplify the known strains. Even the most similar central European BDV strains present with minor differences according to their geographical, but not host species, origin (Kolodziejek et al., 2005). Recently, a BDV-related novel virus species, ABV, was found by a pan-viral microarray screening method (Kistler et al., 2008) and, based on the established ABV-detection methods, another distinct bornavirus lineage was revealed in goose and swan (Delnatte et al., 2011; Payne et al., 2011). To date, seven ABV genotypes have been identified altogether (Nedorost et al., 2012). ABV isolates share a nucleotide identity of 91–100% within a genotype, 68–85% between genotypes, and 60–69% with known BDV strains (Fig. 2) (Kistler et al., 2008; Payne et al., 2011). Surprisingly, the recently observed bornavirus-like genes, estimated to have been integrated in vertebrate genomes 40 million years ago, have also retained their similarity to current BDV strains: the endogenous bornavirus-like (EBL) sequences are 40% identical to current viruses at the amino acid level (Horie et al., 2010).
Is BDV infection vector-borne?

Although BDV infects numerous animal species, it is not very contagious within a domestic animal species, and no transmission from domestic animals to humans has been described. BD cases in even the most common victims, sheep and horses, are mainly sporadic (Danner, 1978; Priestnall et al., 2011; Vahlenkamp et al., 2002), and transmission within or between the domestic species occurs rarely, if ever (Kolodziejek et al., 2005; Metzler et al., 1979). Furthermore, the endemic areas have not essentially changed with human travelling and animal trading. These data point towards an infection source in nature. Whether from nature or from each other, the stablemates of BD animals frequently have or gain antibodies (Herzog et al., 1993; Vahlenkamp et al., 2002), and transmission within a domestic species is uncommon (Kolodziejek et al., 2005). Furthermore, BDV is transmitted in rodents: vertically in mice (Okamoto et al., 2003) and horizontally in rats via urine (Sauder & Staeheli, 2003). Direct evidence of BDV in wild small mammals also exists: BDV or a very closely related virus infects the bicoloured white-toothed shrew (Crocidura leucodon) (Hilbe et al., 2006), bank vole (Myodes glareolus) and root vole (Microtus oeconomus) (Kinnunen et al., 2007), and experimental infection of bank voles leads to chronic infection and excretion without major pathology or symptoms (Kinnunen et al., 2011). However, BDV infections are rare among wild rodents based on antibody screening. Several researchers have searched in vain for markers of BDV infection from small rodent numbers (Hagiwara et al., 2001; Hilbe et al., 2006; Puorger et al., 2010; Mizutani et al., 1999; Vahlenkamp et al., 2002), but a larger study has revealed confirmed seropositive wild rodents (Kinnunen et al., 2007; Kinnunen, 2011). This finding, combined with the epidemiological evidence, emphasizes the need for a future study using RT-PCR and immunohistology to identify wild, natural BDV carriers.

The incidence of BD in horses and sheep peaks in March to June and in cats between December and March, although cases in all of these species occur all year round (reviewed by Dürwald & Ludvig, 1997; Dürwald et al., 2006a; Lundgren et al., 1993). The temporal appearance of cases is strictly associated with the incubation period. The natural incubation period for BDV is long: based on practical experience, it is 2–3 months, but experimental data indicate that it may even be up to 143 days in horses (see ‘Infection and disease of production and companion animals’). If this holds true, then horses and sheep should become infected between November and April, at the time when the few living rodents seek shelter inside or start mating. However, as several factors affect the real incubation period, strict conclusions cannot be drawn.

The detection of BDV-positive shrews in Switzerland and BDV antibody-positive voles in Finland indicates that BDV are observed independently of the region and species at 2- to 5-year intervals (Zwick et al., 1927; reviewed by Dürwald & Ludvig, 1997; Dürwald et al., 2006a), which could be related to fluctuation in wild rodent numbers. Thirdly, BDV strains cluster geographically rather than according to the species or year of isolation, suggesting that transmission within a domestic species is uncommon (Kolodziejek et al., 2005). Furthermore, BDV is transmitted in rodents: vertically in mice (Okamoto et al., 2003) and horizontally in rats via urine (Sauder & Staeheli, 2003). Direct evidence of BDV in wild small mammals also exists: BDV or a very closely related virus infects the bicoloured white-toothed shrew (Crocidura leucodon) (Hilbe et al., 2006), bank vole (Myodes glareolus) and root vole (Microtus oeconomus) (Kinnunen et al., 2007), and experimental infection of bank voles leads to chronic infection and excretion without major pathology or symptoms (Kinnunen et al., 2011). However, BDV infections are rare among wild rodents based on antibody screening. Several researchers have searched in vain for markers of BDV infection from small rodent numbers (Hagiwara et al., 2001; Hilbe et al., 2006; Puorger et al., 2010; Mizutani et al., 1999; Vahlenkamp et al., 2002), but a larger study has revealed confirmed seropositive wild rodents (Kinnunen et al., 2007; Kinnunen, 2011). This finding, combined with the epidemiological evidence, emphasizes the need for a future study using RT-PCR and immunohistology to identify wild, natural BDV carriers.
or BDV-like viruses may have distinct hosts in different geographical areas. Apart from western and north-western Germany, the geographical distribution of bicoloured white-toothed shrews comprises all classical BDV-endemic regions in central Europe, as well as Israel and Turkey (Shenbrot et al., 2006), but does not comply well with the current BDV distribution data in northern Europe (Dauphin et al., 2001; reviewed by Staeheli et al., 2000; Wensman et al., 2008). In contrast, the distribution of the bank vole is wider and covers all European countries from which BDV infections have been reported (Amori et al., 2006). Thus, bank voles, together with bicoloured white-toothed shrews as reservoirs, could explain the geographical pattern of BDV infections, at least in Europe. Professional trapping and further screening of small mammals in known BDV-endemic areas, e.g. Germany, are the next steps toward uncovering the role of vector animals in BDV epidemiology.

Despite the frequency of BDV antibodies (Table 1), BD is sporadic. Its incidence, even in the best-known hosts, horse and sheep in the classical endemic region, has

### Table 1. Manifestations of BD or BDV infection reported, and examples of seroprevalence in natural host animal species

<table>
<thead>
<tr>
<th>Species</th>
<th>Clinical manifestation</th>
<th>Pathological manifestation</th>
<th>References</th>
<th>Examples of seroprevalences by IFA*</th>
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<tr>
<td>Horse</td>
<td>Begins with excitability or depression; continues with swallowing difficulties, ataxia, imbalance, decreased sensory, proprioceptive and reflex functions; ends with severe excitability, aggressiveness or lethargy, and circling, paresis, paralysis, somnolence, stupor and coma</td>
<td>Meningoencephalitis (myelitis)</td>
<td>Bilzer et al. (1996); Grabner &amp; Fischer (1991); reviewed by Dürwald &amp; Ludwig (1997); Ikuta et al. (2002); Richt et al. (2000)</td>
<td>USA, 2.7%; Germany, 29–100%; Finland, 0–14%; Kao et al. (1993); Kinnunen (2011); Richt et al. (1993); Vahlenkamp et al. (2000)</td>
</tr>
<tr>
<td>Horse</td>
<td>Ataxia, narcolepsy, behavioural changes, apathy, colic, abnormal movements</td>
<td>Not analysed</td>
<td>Berg et al. (1999); Bode et al. (1994)</td>
<td></td>
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<tr>
<td>Sheep</td>
<td>Altered behaviour, progressing ataxia, swallowing difficulties, wall-pressing, somnolence, dyskinesia (seizures)</td>
<td>Encephalitis (meningitis, myelitis)</td>
<td>Metzler et al. (1976); reviewed by Ikuta et al. (2002); Ludwig &amp; Bode (2000)</td>
<td>Germany, 16%; Finland, &lt;4.6%; Kinnunen et al. (2007); Vahlenkamp et al. (2000)</td>
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<tr>
<td>Cow</td>
<td>Anorexia, anxiety, ataxia, paresis, circling, paralysis</td>
<td>Encephalitis</td>
<td>Caplazi et al. (1994); Okamoto et al. (2002a)</td>
<td>Finland, &lt;1.9%; Kinnunen et al. (2007); Kinnunen (2011)</td>
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<tr>
<td>Alpaca</td>
<td>Lack of sexual desire, convulsions, prostration, death</td>
<td>Meningoencephalitis</td>
<td>Jacobsen et al. (2010)</td>
<td></td>
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<tr>
<td>Rabbit</td>
<td>Anorexia, apathy, somnolence, gait disorders, paralysis (seizures)</td>
<td>Meningoencephalitis, myelitis</td>
<td>Metzler et al. (1978)</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>Salivation, mydriasis, circling, coma</td>
<td>Meningoencephalitis</td>
<td>Okamoto et al. (2002b); Weissenböck et al. (1998)</td>
<td>Finland, 0–33%; Kinnunen et al. (2007)</td>
</tr>
<tr>
<td>Cat</td>
<td>Mental and behavioural changes, hindleg ataxia, paresis, anorexia, hyperaesthesia (seizures)</td>
<td>Meningoencephalomyelitis</td>
<td>Johansson et al. (2002); Lundgren et al. (1993)</td>
<td>Germany, 7–34%; Sweden: symptomatic, 17–44%; Finland, 0–3.3%; Huebner et al. (2001); Kinnunen (2011); Lundgren et al. (1993)</td>
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*IFA, Immunofluorescence assay. IFA results (commonly combined with verification analyses) were chosen because this method has been used most widely, and the results are reproducible and repeatable (Rott et al., 1985; Staeheli et al., 2000; Sauder et al., 2002). In addition to IFA, BDV antibodies have also been detected using other serological methods, but as the results are quite incomparable with each other (Galabru et al., 2000; Johansson et al., 2002; Sauder et al.; 2002; Kambich et al., 2008), comparison of prevalences makes no sense.
declined from the large outbreaks, which affected thousands of animal patients in the 19th century, to 21–69 cases year⁻¹ (reviewed by Staeheli et al., 2000; Friedrich Loeffler Institut, 2012). Whether this results from improved hygiene (including rodent control) of stables, decline of a particular vector species and/or other factors remains to be elucidated.

Rodents, including wild rodents and some pets, carry and transmit several rodent-borne viral zoonoses (roboviruses) in direct or indirect contact with humans: hantaviruses, arenaviruses, encephalomyocarditis picornavirus, sandfly fever phlebovirus, tanapox virus, as well as orthopoxviruses (Meerburg et al., 2009). In addition, rodents are important maintenance hosts in several vector-borne viral zoonoses, such as tick-borne encephalitis (Tonteri et al., 2011). Recent studies (Kinnunen et al., 2007; Puorger et al., 2010) have nominated BDV as a candidate for the newly described (EDENext, 2011), wider epidemiological group of rodent- and insectivore-borne ‘rainboviruses’.

Arthropods and birds have also been suggested as remotely possible BDV reservoirs (reviewed by Rott & Becht, 1995; Dürrwald et al., 2006a). Arthropods are probably not to blame, as BDV does not cause the high viraemia needed for the transmission of arboviruses, and 93 Ixodes ricinus ticks fed experimentally with a high number \((2–6 \times 10^5)\) of BDV-infected Madin–Darby canine kidney cells maintain BDV RNA for only 1–24 days (Schindler, 2004). As for birds, BDV RNA positivity of mallards and jackdaws has been reported (Berg et al., 2001). It is possible that birds host and transmit BDV in addition to the phylogenetically closely related ABVs, but this remains to be shown. It is also possible that BDV has complex transmission chains involving several reservoir or vector species or both, or that different (possibly novel) BDV-like viruses hide in different reservoirs.

**Diseases caused by BDV**

**Infection route and dissemination**

BDV infects animals experimentally via various routes: most efficiently intranasally and intracerebrally, and also intramuscularly, subcutaneously and intradermally, but not intragastrically (Carbone et al., 1987). Interestingly, the intravenous route is rather inefficient, and a nerve ending is probably needed for BDV to be infectious through this route. Natural transmission routes are unproven. Nonetheless, successful experimental intranasal infection of rats (Carbone et al., 1987; Morales et al., 1988; Stitz et al., 2002), mice, sheep and horses (reviewed by Staeheli et al., 2000) and the presence of BDV antigen or RNA in the olfactory epithelium, nerves and bulb of naturally infected horses point strongly towards an olfactory route, although trigeminal and pharyngeal nerve routes cannot be excluded (Bilzer et al., 1996). Infection most probably occurs via excreta: experimentally chronically infected rats excrete BDV abundantly in urine (and less in tears and saliva; Table 2), and can infect other rats in close contact (Morales et al., 1988; Stitz et al., 1998; Sauder & Staeheli, 2003). In addition to urine, experimentally chronically infected wild bank voles seem to excrete BDV even more commonly in faeces (Kinnunen et al., 2011). In a few naturally infected horses and sheep, BDV RNA is found in salival, nasal and conjunctival fluids, but infectious virus is non-existent in asymptomatic animals (Herzog et al., 1994; Lebelt & Hagenau, 1996; Richt et al., 1993; Vahlenkamp et al., 2002) and rarely exists in symptomatic horses (Herzog et al., 1994), further indicating possible transmission from a reservoir. In addition to horizontal transmission, vertical transmission of BDV has been observed in experimentally infected mice (Okamoto et al., 2003) and may have occurred from a pregnant mare to her fetus (Hagiwara et al., 2000).

BDV spreads intraaxonally from the inoculation site into the central nervous system (CNS) (Carbone et al., 1987). When in the rodent CNS, the virus spreads further and can be demonstrated in all cortical and brainstem areas 2 weeks post-infection (p.i.) (Stitz et al., 2002). Naturally infected horses and sheep brains are also widely BDV-positive (Bilzer et al., 1996; Caplazi & Ehrensperger, 1998), which may be followed by intra-axonal centrifugal spread to peripheral nerves and, occasionally, to parenchymal cells (Lebelt & Hagenau, 1996; reviewed by Ikuta et al., 2002). The pathogenesis was reviewed recently in more detail by Lipkin et al. (2011).

**Infection and disease of production and companion animals**

In cats infected experimentally with a high dose \((2–6 \times 10^5\) f.f.u.) of BDV by intracerebral (i.c.) inoculation, the first symptoms may arise 2 weeks p.i., but it may take up to 2.5 months or a cat may remain asymptomatic for at least 6 months, regardless of the strain used (V or a feline isolate; Lundgren et al., 1997). Ponies infected experimentally with \(1 \times 10^3–2 \times 10^4\) TCID₅₀ (median tissue culture infective dose) of BDV strain CR3 by i.c. inoculation had an incubation time of 15–26 days (Katz et al., 1998). Older studies proposed experimental incubation periods of 24–143 days in horses, 32–85 days in sheep, 18 days to months in rabbits, 20–390 days in guinea pigs, 20–90 days in adult rats and 36 days in hens (Mayr & Danner, 1974). However, experienced veterinary clinicians estimate a natural incubation time of 2–3 months in horses (reviewed by Dürrwald et al., 2006a), and recent data indicate a minimum of 2 months in alpaca (Jacobsen et al., 2010). Persistent subclinical infections, resulting either from a very long incubation period or the low-pathogenic character of the BDV infection, or both, are common. Indeed, during a follow-up of 1–2 years in naturally infected animals, only a proportion of the antibody-positive individuals (20% of horses, ≤40% of sheep) developed symptoms (Herzog et al., 1994; Vahlenkamp et al., 2002). It is therefore worth noting that the incubation period may vary considerably according to several factors such as virus strain, titre,
### Table 2. Comparison of BDV infection of small mammal species

CNS, Central nervous system; i.c., intracerebral; i.n., intranasal; NA, data not available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Proven infection route</th>
<th>BDV antibodies</th>
<th>Distribution in CNS</th>
<th>Peripheral distribution</th>
<th>Symptoms</th>
<th>Excretion</th>
<th>References</th>
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<tr>
<td><strong>Experimental BDV infection of rodents</strong></td>
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<tr>
<td>Laboratory mouse</td>
<td>i.c.</td>
<td>Almost always</td>
<td>Wide</td>
<td>Rare, slow, restricted, only neuronal</td>
<td>Vary from symptomless or mild (commonly in strains C57BL/6, CBA, BALB/c, C3H) to fatal meningoencephalitis (commonly strain MRL)</td>
<td>NA</td>
<td>Rubin <em>et al.</em> (1993); Hallensleben <em>et al.</em> (1997); Enbergs <em>et al.</em> (2001); Ackermann <em>et al.</em> (2010); and others</td>
</tr>
<tr>
<td>Laboratory rat</td>
<td>i.c., i.n.</td>
<td>Almost always</td>
<td>Wide</td>
<td>Wide, common, mainly neuronal</td>
<td>Neonatally infected: mild (locomotor hyperactivity, learning deficits and abnormal social behaviour) Adult, immunocompetent: fatal encephalitis (strains Lewis and Wistar) or asymptomatic (strain black hooded)</td>
<td>Urine (tears, saliva)</td>
<td>Narayan <em>et al.</em> (1983); Carbone <em>et al.</em> (1987); Morales <em>et al.</em> (1988); Herzog <em>et al.</em> (1991); Stitz <em>et al.</em> (1998); Hornig <em>et al.</em> (1999); Weissenböck <em>et al.</em> (2000); Furrer <em>et al.</em> (2001a); Sauder &amp; Staeheli (2003); and others</td>
</tr>
<tr>
<td>Laboratory gerbil</td>
<td>i.c.</td>
<td>Sometimes (0–100 % depending on dose, duration and antigen)</td>
<td>Wide</td>
<td>NA</td>
<td>Neonatally infected: from asymptomatic (very common with low dose) to fatal encephalitis (common with higher doses) Young: 25 % die</td>
<td>NA</td>
<td>Watanabe <em>et al.</em> (2001); Lee <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>Wild bank vole</td>
<td>i.c.</td>
<td>Sometimes (41 %)</td>
<td>Wide</td>
<td>Wide, common, mainly neuronal</td>
<td>Neonatally infected: mild or symptomless; severe disease rare</td>
<td>Urine, faeces</td>
<td>Kinnunen <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><strong>Natural BDV infection of insectivores and rodents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild bicoloured white-toothed shrew</td>
<td>NA</td>
<td>NA</td>
<td>Wide</td>
<td>Wide, mainly neuronal</td>
<td></td>
<td>NA</td>
<td>No</td>
</tr>
<tr>
<td>Wild voles</td>
<td>NA</td>
<td>Verified in a few bank/root voles</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td>Kinnunen <em>et al.</em> (2007)</td>
</tr>
</tbody>
</table>
infection route and the immunological status of infected animals.

In naturally infected, diseased animals, BDV infection manifests as a peracute, acute or subacute disease with non-purulent meningoencephalitis, although milder manifestations are also seen (Table 1). Different combinations of simultaneous or successive neurobehavioural alterations are noticed (Metzler et al., 1976; Grabner & Fischer, 1991; Bilzer et al., 1996; reviewed by Richt et al., 2000; Ikuta et al., 2002). Paralysis is common and, in the classical BD form, death occurs in 60–80 % of animals by 5 weeks after the onset of symptoms. Spontaneous recovery is possible, but is often accompanied by altered behaviour for the rest of the animal’s life, and occasionally leads to the recurrence of severe encephalitis (Grabner & Fischer, 1991; reviewed by Ikuta et al., 2002).

Interestingly, BDV pathology and clinical outcome are partially related to the T-cell-mediated immune response and a delayed-type hypersensitivity reaction, although sole production of certain pro-inflammatory cytokines may also contribute (Stitz et al., 2002; Wensman et al., 2011). Based on ex vivo studies, BDV pathology also involves alteration and impairment of nerve-cell functions, affecting stimulus-induced synaptic plasticity (Prat et al., 2009). The pathogenesis was reviewed recently in more detail by Lipkin et al. (2011).

Infections of rodents and shrews

In infected laboratory rats, BDV has several pharmacological effects in the brain, and studies have revealed additional mechanisms by which BDV interacts with host cells, impairs neuronal functions and affects innate immunity (Habjan et al., 2008; Prat et al., 2009; Qian et al., 2010; Solbrig, 2010; Weissenböck et al., 2000). Adult immunocompetent rats succumb to severe meningoencephalitis, whereas neonatally infected or immunocompromised rats and bank voles cope with BDV despite widespread virus, high antigen amount, subtle symptoms and occasional mild inflammatory reactions (Table 2; reviewed by Pletnikov et al., 2002). The fact that BDV mainly causes no or only subtle clinical signs in young, chronically infected animals is consistent with these species being a possible reservoir; specifically, the reservoir species is unlikely to be severely affected, in order to be able to spread a pathogen. The epidemiology and immunological mechanisms in rodents may resemble those of the lymphocytic choriomeningitis arenavirus, which infects wild mice prenatally, leading to asymptomatic, chronic infection, immunological tolerance and lifelong virus shedding, resulting occasionally in zoonotic transmission (Bonthius & Perlman, 2007). Studies are needed to elucidate whether BDV is transmitted vertically and subsequently shed in wild rodents.

Interestingly, although BDV-infected laboratory mice and rats almost invariably mount an antibody response, only a minority of experimentally infected bank voles are seropositive (Table 2). Such differences may relate to the fact that the bank vole represents a wild animal population, probably with fully functioning immune systems. This is not always the case in laboratory-bred rodent colonies: inbred rat strains differ in virus susceptibility, as shown with Rift Valley virus in laboratory rats (Ritter et al., 2000) and West Nile virus in laboratory mice (Mashimo et al., 2002), as well as with BDV (Table 2).

Following experimental i.c. or intranasal infection of rats and bank voles, BDV soon spreads centrifugally to the peripheral nerves (Table 2). For example, axons in nerves innervating the alimentary tract, salivary gland, smooth muscle and urinary bladder are commonly BDV-positive. Naturally infected shrews also demonstrate wide BDV distribution (Table 2). In laboratory mice, however, the peripheral distribution is rare, slow and restricted; this has not been studied in mice with natural immune systems.

In addition to the spread in peripheral nerves, laboratory rats and experimentally infected wild bank voles also excrete BDV (Table 2; also see ‘Infection route and dissemination’). Like Puumala hantavirus, which is commonly excreted by bank voles (Hardestam et al., 2008), BDV is peripherally spread and consecutively excreted in bank voles, but transmission studies are needed to elucidate whether they could function as a reservoir.

‘Human BD’?

As BDV infects a wide variety of animal species with a predilection for the limbic system, and occasionally induces persistent emotional, cognitive and behavioural alterations in experimental animals, including primates and their ancestors tree shrews (Tupaia glis) (Sprankel et al., 1978; Stitz et al., 1980), the question has arisen of whether the virus could also be a human pathogen and cause neuropsychiatric disorders. The first evidence of human infections was, indeed, reported in 1985: 0.43–12.5 % of patients with diverse psychiatric disorders had BDV antibodies in serum, whereas the seroprevalence in the control group was <0.5 % (Rott et al., 1985). The seropositive patients were diagnosed to have a primary major depressive disorder. The majority of later studies have also reported higher BDV marker prevalence in some patient groups than in controls, i.e. in unspecified psychiatric inpatients, neurotic, personality-adjustment and mood-disorder patients, schizophrenics and patients with chronic fatigue syndrome (summarized or meta-analysed by Planz et al., 2002; Chalmers et al., 2005; Lipkin et al., 2011; Arias et al., 2012). However, signs of infection are not present in all patient groups, and control or comparison groups also sometimes harbour signs of BDV infection. Furthermore, despite the association of detected BDV markers with some psychiatric diseases in numerous (but not all) studies, conclusions on the causal relationships are impossible to draw. The situation is even more complicated by the facts that no consensus exists on the method that should be used for BDV diagnosis (Wolff et al., 2006; reviewed by Chalmers et al., 2005; Sauder et al., 2002;
Bode, 2008) and that, based on sequence identity, many PCR results and even virus isolation from human peripheral blood cells have been suspected to have arisen from contamination by a control strain (Lieb et al., 1997; Schwemmle et al., 1999; Planz et al., 2003; reviewed by Sauder et al., 2002; Dürrwald et al., 2006b). A recent study using extremely tight criteria for BDV positivity found neither BDV RNA nor verifiable BDV antibodies in the blood samples of Californian psychiatric patients (Hornig et al., 2012).

Despite the controversies, there are researchers who consider that the existence of potential ‘human BD’ has been demonstrated and is common (Patti et al., 2008; reviewed by Ludwig & Bode, 2000; Bode & Ludwig, 2003). Especially controversial, however, are their findings based on the triple-ELISA method (see ‘Immunity and detection of serological response’). Additionally, other serological BDV methods also have been suspected to produce unspecific results (Weissenböck et al., 2002; reviewed by Sauder et al., 2002); this suspicion seems to be more common for BDV than for other viral infections. Nonetheless, a very few human BDV infections have been carefully documented with a combination of reproducible and controlled methods (de La Torre et al., 1996; Kinnunen et al., 2007). This suggests that BDV can indeed infect humans, but the frequency of human infections, the role of endogenized bornaviral genes and, most importantly, the existence of potential ‘human BD’ are still questionable.

**Infection kinetics and diagnosis of BDV infections**

**Immunity and detection of serological response**

BDV antigen has been reported to be present in the peripheral blood and to bind frequently to BDV-specific antibodies, so forming circulating immunocomplexes (CICs) (Bode et al., 2001). CICs, antigen and antibodies have been detected using a reverse-type sandwich triple ELISA by several associated research groups reporting high BDV prevalences among humans, horses and cats (reviewed by Bode, 2008). Unfortunately, this method was published without negative controls, and other researchers and methods have been unable to reproduce its results (De Bosschere et al., 2004; Wolff et al., 2006). Lower prevalences are found using more conventional methods, of which the immunofluorescence assay (IFA) is the most commonly used (Rott et al., 1985; reviewed by Sauder et al., 2002). Given the progress in the BDV molecular field, efforts should be made to improve this approach. It is now possible to use cells expressing isolated BDV proteins only, and to include negative controls pre-adsorbed with the same proteins. In addition to IFA, immunoblotting, immune precipitation and the electrochemiluminescence immunoassay, as well as different versions of the ELISA method, are used for BDV antibody detection from serum and cerebrospinal fluid (reviewed by Sauder et al., 2002).

In contrast to many other viruses, antibodies do not seem to play a protective role in persistent BDV infections: if infected horses, sheep and cats mount an antibody response, the titre is low and neutralizing antibodies are detected late, if ever (Grabner & Fischer, 1991; Katz et al., 1998; Johansson et al., 2002; Vahlenkamp et al., 2002; reviewed by Sauder et al., 2002). Although the relevance of neutralizing antibodies is difficult to determine in natural infections, neutralizing antibodies against the major glycoprotein prevent infection and encephalitis in laboratory rats if administered prophylactically (Furrer et al., 2001b), but not if administered simultaneously with the virus (Stitz et al., 1998). Immunosuppressed rats mount a humoral, but not a cellular immune response (Stitz et al., 1998).

The most probable explanation for poor induction of antibody responses is the special biology of BDV: the tight association with cells, neurotropism, persistence and non-cytopathogenicity (Stitz et al., 1998). In addition, the endogenization phenomenon could have led to molecular mimicry of the infecting agent with host antigens, leading to immunotolerance.

Horses and cats infected experimentally with BDV through i.c. inoculation seroconvert 1 month p.i. (Johansson et al., 2002; Katz et al., 1998). Overall, the serological response is stronger in experimentally infected than in naturally infected animals, although it is not constantly detected in either animal group (Morales et al., 1988; Herzog et al., 1991; Rubin et al., 1993; Johansson et al., 2002). As BDV antibodies can only be found in the sera of 30–40% of verified, infected animals, seroprevalences are clearly an underestimate of the existing BDV infections.

Measurement of the avidity of antibodies has long been used in the timing of primary infections: a high proportion of strongly bound, urea-resistant antibodies indicates long-term (weeks to years old) adaptive immunity, whereas in acute infection, the proportion is low (Hedman et al., 1993).

High avidity has also been utilized as an indicator of the specificity of the antibody response (Allmang et al., 2001; Billich et al., 2002). Although most BDV antibody findings in humans have been of low avidity (Allmang et al., 2001), high-avidity serological responses also exist (Billich et al., 2002; Kinnunen et al., 2007). Measurement of avidity would most probably promote the understanding of BDV epidemiology, as rough timing of the infection would become possible. To validate the measurement for timing of BDV infections in each species, serial serum samples from experimentally infected animals (Gavrilovskaya et al., 1993) or from a well-known outbreak (Basso et al., 2010) should be studied.

Special care is necessary concerning specificity of serological BDV methods, even at the cost of sensitivity. For instance, although IFA is considered the most reliable method for BDV antibody detection (reviewed by Staeheli et al., 2000), it may be less sensitive, but, even more importantly, more specific than ELISA (Johansson et al., 2002). Unfortunately, no serum panel, verified using agreed methods, exists for exact validation of any assay. Hence, to classify a sample as verified BDV-positive, additional independent methods or repeated sampling, or both, need to
be employed (reviewed by Ludwig & Bode, 2000; Sauder et al., 2002).

**Viral kinetics and detection**

Reliable diagnosis of BD is currently not possible *intra vitam*. RT-nested PCR methods have amplified the viral genome from peripheral blood, but the results are of low diagnostic value, as numerous asymptomatic animals and humans are also reported to harbour BDV RNA in blood (reviewed by Ikuta et al., 2002), and BDV viraemia overall is controversial (reviewed by Staeheli et al., 2000). RNA in blood might, however, be indicative of BD if pursued carefully, avoiding contamination, and when combined with the clinical picture and antibody detection. In addition, the detection of BDV antigen in the peripheral blood has been reported, but remains to be authenticated (see 'Immunity and detection of serological response').

Definitive BD diagnosis is possible only post-mortem by demonstrating the presence of virus or viral markers in the brain. Virus isolation is a reliable confirmatory method, although slow and complicated by neurotropism of the virus and low virus titre (Danner et al., 1978; Lebelt & Hagenau, 1996). The diagnosis can be made earlier if based on the detection of viral antigen by immunohistology (the most popular confirmation technology) and/or viral RNA with *in situ* hybridization or RT-PCR (Lebelt & Hagenau, 1996; reviewed by Sauder et al., 2002). Real-time RT-PCR methods are available, with better control of contamination (Schindler et al., 2007; Wensman et al., 2007). As usual with non-cytopathic, cell-associated, persistent virus infections such as BDV, the results from different methods are not always congruent, reflecting the low amount of virus (Lebelt & Hagenau, 1996). BDV is most often present in the olfactory bulb, caudate nuclei, hippocampus, lateral ventral cerebral cortex and medulla oblongata (Lebelt & Hagenau, 1996), so these represent the most important sampling sites for BDV detection.

**Endogenization of BDV sequences**

In 2010, EBL sequences (EBLs) were detected in the genomes of humans and several other vertebrates (Belyi et al., 2010; Horie et al., 2010). The most commonly found EBL resembled the N gene (EBLN), and was the only EBL found in primates as well as in guinea pig, squirrel, lamprey and a few exotic animal species. In the genomes of lemur, mouse, rat, microbat, wallaby and a few fish species, however, either the L or M gene was detected in addition to or instead of the N gene (Belyi et al., 2010). These results suggest that endogenization was an ancient event in vertebrate evolution. Recently, further light was shed on this phenomenon from an experimental BDV infection of bank voles: in as many as 66% of voles, BDV N RNA had been reverse-transcribed into BDV N DNA in brain tissue and, in one vole, BDV P DNA was also detectable (Kinnunen et al., 2011). This demonstrates that reverse transcription of BDV RNA, which is the first, necessary step for an RNA virus gene insertion or endogenization, is common *in vivo*.

A recent study revealed that BDV ribonucleoproteins interact directly with host chromosomes throughout the cell cycle (Matsumoto et al., 2012). In addition to promoting the maintenance of chronic, nuclear BDV infection, this stable and close association may promote the endogenization of cDNA transcripts of BDV RNA into host chromosomes. This interesting process needs further clarification.

EBLs, especially the most commonly found EBLN, might play a role in the epidemiology of BDV, being possibly advantageous and probably enabling a species to function as a reservoir. Most EBLs contain numerous stop codons, thus remaining untranslated, but at least one human EBLN interacts with cellular proteins, indicating a natural function (Horie et al., 2010). The possible biological function of EBLs remains to be elucidated, but hypotheses have arisen: species containing EBL sequences are more likely to be resistant to a lethal infection than those that do not (Belyi et al., 2010). This might result from protection mediated by the expression of endogenous BDV N or other components. Indeed, the nucleocapsid components of BDV, i.e. N, P and X proteins, convey resistance to BDV infection in cell lines (Geib et al., 2003). Furthermore, specific N epitopes are crucial for sufficient activation of the CD8+ T-lymphocytes mediating early control of BDV in rats and mice (Furrer et al., 2001a; Schamel et al. 2001). The delicate balance between pathology and protection depends on these particular CD8+ T-cells (Furrer et al., 2001a; Baur et al., 2008), thus warranting further studies both in the light of recent EBLN (and other EBL) findings and also by sequence determination of possible reservoir/vector species, including the bank vole and bicoloured white-toothed shrew. Their whole genomes are not yet available to determine whether they contain EBLs, as in some related species, other rodents and another shrew (Belyi et al., 2010; Horie et al., 2010).

**Treatment and prevention of BD**

Supportive and symptomatic treatments so far remain the only therapy for BD, although several approaches have been attempted to control it. As severe losses were suffered, vaccination against BDV was applied. When inactivated vaccines were shown to be ineffective, virulent virus was applied subcutaneously (reviewed by Dürrwald & Ludwig, 1997; Ludwig & Bode, 2000). Rigorous vaccination was compulsory in the former German Democratic Republic in 1962–1992, but the campaign proved ineffective. Nowadays, infections caused by these unsuccessful vaccinations possibly contribute to the fact that the incidence of clinical BD is still highest in horses in Germany (reviewed by Dürrwald et al., 2006a; Ludwig & Bode., 2000); the same is implied by BDV molecular epidemiology (Kolodziejek et al., 2005). No vaccination against this persistent infection has since been recommended, although new recombinant vaccine
candidates expressing BDV N mediate protection from the disease (but not from infection) in mice (Hausmann et al., 2005).

Amantadine sulphate is an antiviral affecting influenza viruses, and it is also used in the therapy of Parkinson's disease. Its use in the treatment of BD has been debated (Bode et al., 1997; Cubitt & de la Torre, 1997; Hallensleben et al., 1997; Dietrich et al., 2000). As amantadine sulphate is rather safe and readily available, it is used at the dose of 2–4 mg kg⁻¹ for a minimum of 12 weeks in the treatment of veterinary patients showing aggravating symptoms (Dieckhöfer, 2008). This is despite the lack of controlled and blinded studies on its effects, and it being ineffective in BDV-infected cells and mice (Cubitt & de la Torre, 1997; Hallensleben et al., 1997).

Intracerebrally administered ribavirin interferes with BDV replication and results in clinical improvement, although not in viral clearance in rodents (Jordan et al., 1999; Solbrig et al., 2002; Lee et al., 2008). However, its use is compromised by side effects. Some other antivirals have also been developed against BDV, but these trials have so far remained in the initial phase (Planz et al., 2001; Bajramovic et al., 2004; Volmer et al., 2005; Qian et al., 2010).

Because of the lack of effective antivirals and vaccines, the prevention of contact with possible virus sources remains the only way to reduce the incidence of BDV. Historically, improvements in general hygiene in animal premises and the separation of horses and sheep have provided some protection, but have not led to the elimination of BDV (reviewed by Ludwig & Bode, 2000).

Concluding remarks and prospects

The geographical distribution of BDV seems wide, but definite verifications based on virus isolation are rare and are concentrated in Europe. BDV infects several mammal species, including wild and domestic animals, and evidently humans, although verified human infections are very few. Although BD cases are rare and the classical (fatal) form again is concentrated in Europe, it is likely that there are two to three times more BDV-infected animals than can be deduced from the antibody prevalences. As the reliable diagnosis of BD is not possible intra vitam, proper post-mortem samples are needed to verify the true clinical relevance of BDV. Because of the chronic and persistent nature of the infection, and considering that unspecific results have been reported, the diagnosis of BD or BDV infection must be based not only on a single method, but on a combination of relevant techniques. The most reliable techniques involve detection of antigen or viable virus in nervous tissue.

Wild small mammals, such as the bank vole and bicoloured white-toothed shrew, are probably involved in the circulation of BDV and have been anecdotally associated with clinical cases (Hilbe et al., 2006; Kinnunen et al., 2007). Further infection and transmission experiments should be conducted in wild rodents and insectivores and, later, between small and large mammals to find out whether interspecies transmission indeed occurs.

What else could be the BDV reservoir or vector other than voles and shrews? Bats, in particular, host several related zoonoses caused by viruses of the order Mononegavirales (de Jong et al., 2011). One bat species (Myotis lucifugus) hosts EBLN and EBLL in its genome (Belyi et al., 2010), which is hypothesized to be an advantage for a possible reservoir species. Bats therefore warrant screening for BDV infections. Moreover, the role of EBLs in BDV epidemiology needs further clarification.

In addition to searching for BDV in species that have not previously been implicated, the timing of the peaks of primary infections, instead of the peaks of clinical cases, and their comparison with and plotting against probable host or vector numbers and activities would be crucial for elucidating the obscure transmission cycle of BDV. Longitudinal sampling of susceptible sentinel animals with the detection of seroconversion or avidity measurement could be useful in the timing, as long as the incubation period is not properly known. Whatever the reservoir(s) and vector(s), it seems clear that the BDV transmission cycle needs it/them, as the virus is not easily transmitted horizontally.

Crucial parts of BDV epidemiology remain a mystery. However, during the last few years, several cornerstones have been found: (i) ABVs, which may explain some earlier obscure findings in birds (Malkinson et al., 1995); (ii) rodent and shrew infections, elucidating the role of wild animals; and (iii) the phenomenon of endogenization, resulting in fascinating new questions about the relationship of EBLs, BDV and BD, including in humans. These findings are to be further specified by e.g. studies concerning the mechanisms of transmission, reverse transcription and endogenization and, moreover, uninvestigated wildlife species, e.g. bats, are to be screened for thorough elucidation of the epidemiology. A critical attitude, however, is still needed when interpreting results originating from single BDV-detection methods, the specificity of which has not been carefully verified.

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