BRIEF REPORT

A Variegated Squirrel Bornavirus Associated with Fatal Human Encephalitis

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SUMMARY

Between 2011 and 2013, three breeders of variegated squirrels (*Sciurus variegatoides*) had encephalitis with similar clinical signs and died 2 to 4 months after onset of the clinical symptoms. With the use of a metagenomic approach that incorporated next-generation sequencing and real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR), the presence of a previously unknown bornavirus was detected in a contact squirrel and in brain samples from the three patients. Phylogenetic analyses showed that this virus, tentatively named variegated squirrel 1 bornavirus (VSBV-1), forms a lineage separate from that of the known bornavirus species. (Funded by the Federal Ministry of Food and Agriculture [Germany] and others.)

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Beginning in late 2011, three men in succession (63, 62, and 72 years of age) from the state of Saxony-Anhalt, Germany, had a progressive encephalitis or meningoencephalitis that led to death within 2 to 4 months after the onset of clinical symptoms. The clinical course was characterized by fever, shivers, or both; progressive psychomotor slowing; confusion; unsteady gait; myoclonus, ocular paresis, or both; and finally, coma. All three patients had preexisting medical conditions (hypertension, diabetes, or obesity). In all three patients, the disease was also accompanied, at some point during the course of the illness, by bilateral crural-vein thrombosis, which led to pulmonary embolism in two patients. An analysis of the cerebrospinal fluid showed pleocytosis, and cranial imaging revealed edematous lesions in the cerebral cortical areas and basal ganglia or meninges that were increasing in size, a finding consistent with a viral infection (Fig. 1A and 1B). While the patients were alive, no infectious agent could be detected by means of microscopic, culture, molecular, or serologic investigations of cerebrospinal fluid samples, biopsy samples, or serum. All three patients were treated in intensive care units, had to undergo mechanical ventilation, and died despite receiving treatment with broad antiinfective chemotherapy. Biopsy and postmortem analysis of the affected brain areas showed tissue edema, necrosis, glial activation, and lymphocyte infiltration, often as perivascular cuffing, but no viral inclusions or any microorganisms. Details of the characteristics of the patients, the clinical symptoms, and the results of laboratory analyses are shown in Table 1, and in Tables S1 and S2 in the Supplementary Appendix, available with the full text of this article at NEJM.org.

All three patients were breeders of variegated squirrels (*S. variegatoides*). They were friends, had met privately on a regular basis, and had exchanged their squirrel breeding pairs on multiple occasions (a detailed description is provided in the Supplementary Appendix). At least two of the patients had been scratched by their squirrels in the past, and one had been bitten. Because the initial pathogen-specific screening of a squirrel from the breeding population of Patient 3 revealed no evidence of any of the tested pathogens (for details, see Table S3 in the Supplementary Appendix), a panel of samples from the squirrel was analyzed by means of metagenomic sequencing. All further analyses were based on the detection of several short sequence fragments with a strong similarity to *Mammalian 1 bornavirus*.

METHODS

SAMPLES OF TISSUE AND BODY FLUIDS

Organ, blood, oropharyngeal-swab, and chest-cavity fluid samples from a healthy variegated squirrel that had direct contact with Patient 3 were available for the analyses. In addition, archived formalin-fixed, paraffin-embedded (FFPE) brain tissue from Patients 1 and 2, as well as fresh-frozen brain samples, cerebrospinal fluid, and serum from Patient 3, were tested. FFPE brain tissue from 10 unrelated humans (tissue from patients with Alzheimer's disease, human immunodeficiency virus–induced encephalopathy, or herpes simplex virus–induced encephalitis, as well as normal brain tissue), materials from polymerase-chain-reaction (PCR)–negative variegated squirrels, and a Borna disease virus (BoDV)–infected horse brain were used as control material.

METAGENOMICS, WHOLE-GENOME SEQUENCING, AND SEQUENCE ANALYSIS

A metagenomic analysis of squirrel samples was performed in accordance with a standard workflow, as described elsewhere.[1,2] Sequencing was performed with the use of a MiSeq instrument (Illumina). The obtained reads were analyzed with the use of RIEMS[3] for the detection of pathogens and with Genome Sequencer software, version 2.8 (Roche), for sequence assembly. The complete coding sequences, as well as the nucleoprotein (N) nucleotide and amino acid sequences, were used to analyze the evolutionary relationships among this newly discovered bornavirus, previously reported bornavirus species, and endogenous bornavirus-like sequences from

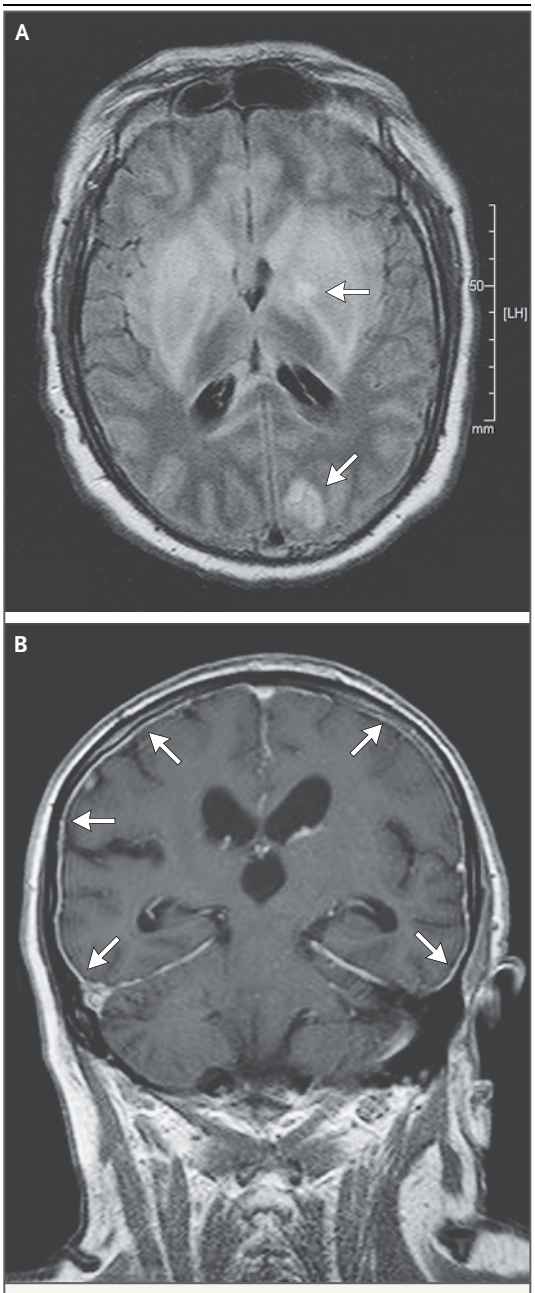

Figure 1. Magnetic Resonance Imaging.

In Panel A, T_2-weighted transversal imaging (with a fluid-attenuated inversion recovery [FLAIR] sequence) shows hyperintense lesions in the left basal ganglia and occipital lobe of Patient 2 (arrows). In Panel B, a T_1-weighted coronal image shows contrast enhancement of the meninges in Patient 3 (arrows).

Table 1. Characteristics and Symptoms of the Patients and Imaging Results.

Characteristic	Patient 1	Patient 2	Patient 3
Age (yr)	63	62	72
Clinical diagnosis	Encephalitis	Encephalitis	Meningoencephalitis
Month of disease onset	November 2011	June 2013*	November 2013
Estimated time from onset of clinical symptoms until death (mo)	3	2*	4
Preexisting medical conditions	Hypertension	Hypertension, type 2 diabetes, renal insufficiency	Hypertension, obesity
Initial symptoms	Severe fatigue, constipation, anorexia, nocturnal agitation, confusion, psychomotor slowing, shivers	Weakness, vertigo, unsteady gait, headache, abdominal pain, confusion, psychomotor slowing, shivers, fever	Unsteady gait, anogenital numbness, problems passing urine and stool, headache, confusion, psychomotor slowing, mood disturbances, fever
Later symptoms	Myoclonus, grimacing, divergent bulbi, tetraparesis, sopor, coma	Myoclonus, opsoclonus, sopor, coma	Ocular paresis, sopor, coma
Additional diagnoses during illness	Bilateral crural-vein thrombosis, hepatopathy, bronchopulmonary infection due to assumed aspiration	Bilateral crural-vein thrombosis leading to pulmonary embolism; bronchopulmonary infection due to assumed aspiration	Bilateral crural-vein thrombosis leading to pulmonary embolism; bronchopulmonary infection due to assumed aspiration
Magnetic resonance imaging timing and findings			
Initial	December 2011: normal	July 2013: normal	December 2013: dilated ventricles
Follow-up	3 weeks after initial scan: hyperintense (T_2-weighted imaging) lesions in temporal, parietal, and insular cortex and midbrain; spine not affected	10 days after initial scan: hyperintense (T_2-weighted imaging), partly symmetric lesions in temporal, parietal, and frontal cortex, basal ganglia, and brainstem; spine not affected	6 days after initial scan: meningeal contrast enhancement; spine not affected
Electroencephalography timing and findings	December 2011: delta activity with short alpha episodes	August 2013: low-voltage theta and delta activity	December 2013: delta and theta activity
Antiinfective treatment	Ceftriaxone, ciprofloxacin, carbapenems, doxycycline, glucocorticoids†	Ceftriaxone, ampicillin, clindamycin, carbapenems, erythromycin, acyclovir, glucocorticoids‡	Ceftriaxone, ampicillin, acyclovir, doxycycline, ribavirin, tuberculosis treatment§

* This patient had peripheral facial nerve palsy that developed in April 2013; the palsy subsided after glucocorticoid treatment.
† This patient was highly positive for anti-Yo (Purkinje cell) autoantibody in the cerebrospinal fluid (CSF) but not in the serum and was therefore given a single high dose of methylprednisolone; however, a full-body computed tomographic scan did not reveal any neoplasia. Paraneoplastic autoimmune serologic testing of the serum and CSF in all three patients did not reveal any other autoantibodies, such as anti-Yo (except in Patient 1), anti-Hu, anti-Ri, anti-amphiphysin, anti–NMDA receptor, anti–glutamate receptor, and anti-CV2.
‡ Single-dose methylprednisolone was administered, because initially an autoimmune encephalitis was considered on the basis of the patient's clinical presentation.
§ This patient had positive results of a *Mycobacterium tuberculosis* interferon release assay and was treated with ethambutol, isoniazid, rifampin, and pyrazinamide.

humans and squirrels (Fig. S1 in the Supplementary Appendix).

REAL-TIME RT-QPCR DETECTION

On the basis of sequences obtained from a metagenomic analysis of the squirrel samples, two independent primer–probe RT-qPCR systems were established, corresponding to two different regions within the bornavirus genome (Table S4 in the Supplementary Appendix). Both systems used standard RT-qPCR reagents and cycling conditions and were combined with an internal control system, as described elsewhere.[4] To rule out endogenous DNA sequences as the source of virus sequence amplification, qPCR analysis was also performed without reverse transcription.

IMMUNOHISTOCHEMICAL ANALYSIS

A standard staining protocol was applied with the use of bornavirus-specific polyclonal and monoclonal antibodies that recognized the viral proteins N, X, and phosphoprotein (P), as described previously.[5,6] A BoDV-positive horse sample and the 10 above-mentioned unrelated human brain-tissue samples were used as controls (Fig. S2A, S2B, and S3 in the Supplementary Appendix).

INDIRECT IMMUNOFLUORESCENCE ASSAY

For the detection of bornavirus-specific IgG antibodies in the serum and in the cerebrospinal fluid from Patient 3, a persistently BoDV-infected cell line was used in a standard indirect immunofluorescence procedure (Fig. S4 in the Supplementary Appendix). The specificity of this serologic assay was confirmed through the investigation of 40 serum samples obtained from febrile patients; all of the samples tested negative. For confirmation, the serum was titrated in a validated routine immunofluorescence assay for the detection of bornavirus-specific antibodies.

RESULTS

METAGENOMIC ANALYSIS

The metagenomic analysis revealed five sequence fragments that had 70.3% to 81.2% identity with isolates of the species *Mammalian 1 bornavirus* in samples L00652 (liver, lung, and kidney) and L00651 (chest-cavity fluid) (Table S5 in the Supplementary Appendix). Targeted screening of the sequencing reads from the remaining pools additionally detected 23 reads related to *Mammalian 1 bornavirus* sequences (identities between 67.6% and 81.7%) (Table S5 in the Supplementary Appendix). The fragments were related to both mammalian and avian bornavirus sequences, and the virus was tentatively named variegated squirrel 1 bornavirus (VSBV-1). Gross pathological examination of the squirrel did not reveal any specific changes; histologically, however, the brain was found to have satellitosis and mild glial activation.

REAL-TIME RT-QPCR ANALYSES

With the use of two independent RT-qPCR systems, VSBV-1 RNA was found in various sample materials from the squirrel and in the samples available from all three human patients (Table 2). High VSBV-1 RNA loads were observed in the squirrel brain, heart, lung, kidney, and oropharyngeal-swab samples. In contrast, in EDTA-treated blood and chest-cavity fluid, only low VSBV-1 RNA loads were detected. Intermediate viral genome loads could be ascertained in FFPE brain samples from Patients 1 and 2. An analysis of fresh-frozen material from Patient 3 revealed high VSBV-1 RNA loads in the brain (Table 2). In all the investigations, the VSBV-1 genome could be detected only when assays including reverse transcription were used; results remained negative in assays without reverse transcription. Moreover, all the control materials from patients with unrelated brain diseases and from healthy persons tested negative in both PCR systems.

WHOLE-GENOME SEQUENCING AND PHYLOGENETIC ANALYSIS

RNA from the brain samples obtained from the squirrel and from Patient 3 was deep-sequenced to determine the viral genomes for in-depth analyses. Sequencing yielded two nearly identical complete coding sequences (8798 nucleotides; accession numbers LN713680 and LN713681) with two synonymous exchanges at positions 1857 (amino acid 619) and 3702 (amino acid 1234) within the *L* gene. Annotation of these sequences revealed a canonical bornavirus genome structure (the genes encoding N, X, P, M, G, and L). Phylogenetic analyses of the complete coding sequence and *N* sequence showed that this novel bornavirus forms a distinct lineage within the bornavirus phylogeny in a sister relationship with the *Mammalian 1 bornavirus* lineage (Fig. 2, and Fig. S1 in the Supplementary Appendix). Further-

Table 2. Real-Time RT-qPCR Results for Samples Obtained from the Squirrel and the Three Patients.*

Origin and Sample	Quantification Cycle Value†	
	VSBV-1 Assay 6	VSBV-1 Assay 10
Squirrel		
EDTA-treated blood	35.7	33.0
Chest-cavity fluid	32.0	28.4
Brain	13.2	13.1
Heart	12.4	14.1
Lung	13.3	13.8
Liver	18.2	18.9
Spleen	17.2	17.4
Kidney	11.0	11.7
Colon	19.1	18.5
Oropharyngeal swab	14.7	15.5
Patient 1 FFPE brain	25.9	24.0
Patient 2 FFPE brain	20.8	19.0
Patient 3		
Serum	29.1	27.8
CSF	30.5	26.3
Brain	12.7	12.6

* For a description of the reverse-transcriptase quantitative polymerase-chain-reaction (RT-qPCR) assays, see the Supplementary Appendix. FFPE denotes formalin-fixed and paraffin-embedded, and VSBV-1 variegated squirrel 1 bornavirus.

† Quantification cycle values denote the cycle during qPCR in which a positive fluorescence signal can be differentiated from the background. Low values indicate higher initial genome copy numbers of the target, and higher values indicate smaller genome copy numbers. A difference by factor of approximately 3.3 in the value corresponds to a $\log_{10}$ difference in the number of genome copies.

more, previously described endogenous *N*-derived bornavirus-like sequences from squirrels[8] and humans[9] were distantly related to the previously unknown VSBV-1 sequences (Fig. S1 in the Supplementary Appendix). The relationships in the time-resolved phylogeny (Fig. S5 in the Supplementary Appendix), as well as the fact that the VSBV-1 is most closely related to BoDV of horse origin, imply that VSBV-1 emerged from a mammalian bornavirus rather than from an avian one. In accordance with the latest criteria proposed by the International Committee for Taxonomy of Viruses Bornaviridae Study Group,[7] on the basis of the phylogenetic analyses and the nucleotide sequence identities of less than 75% between this bornavirus and those of the most closely related classic *Mammalian 1 bornavirus* sequences, VSBV-1 can be classified as a new bornavirus species (Table S6 in the Supplementary Appendix).

IMMUNOHISTOCHEMICAL ANALYSES

Brain-tissue sections from the squirrel and from Patient 1 were analyzed and showed positive immunostaining in nuclei, cytoplasm, and neuronal and glial processes of brain cells, as well as in the neuropil, when polyclonal (monospecific) antibodies for the detection of the viral N, P, and X proteins were applied (Fig. 3), but not when the monoclonal anti-N antibody was used. Whereas the unrelated human tissue samples and the control squirrel samples were negative (Fig. S2A and S2B in the Supplementary Appendix), the BoDV-positive horse brain had reaction patterns in the brain cells that were similar to the reaction patterns in the squirrel and Patient 1 with all of the applied antibodies (Fig. S3 in the Supplementary Appendix).

DETECTION OF BORNAVIRUS-SPECIFIC ANTIBODIES

Bornavirus-specific IgG antibodies were detected in serum and cerebrospinal fluid from Patient 3 with the use of an indirect immunofluorescence assay (Fig. S4 in the Supplementary Appendix). In both the cerebrospinal fluid and the serum, bornavirus-specific IgG antibody titers (1:2560 and 1:5120, respectively) were identified in a routine immunofluorescence assay that was validated for the detection of antibodies against BoDV.

DISCUSSION

We describe the detection of a variegated squirrel–derived bornavirus associated with the death of three people. The three patients had similar central nervous system (CNS) symptoms and died of progressive meningoencephalitis or encephalitis; bilateral crural-vein thrombosis also developed in all three during the clinical course of their illness. The reason for the thrombosis remains unclear. The spinal cord was not affected in any of the patients; all the lesions were found in the cortical areas, basal ganglia, or brainstem. All three patients were squirrel breeders and members of the same private squirrel-breeding association. Although these findings do not meet Koch's postulates, the fact that the complete coding sequences generated from the squirrel and the human sample material were almost

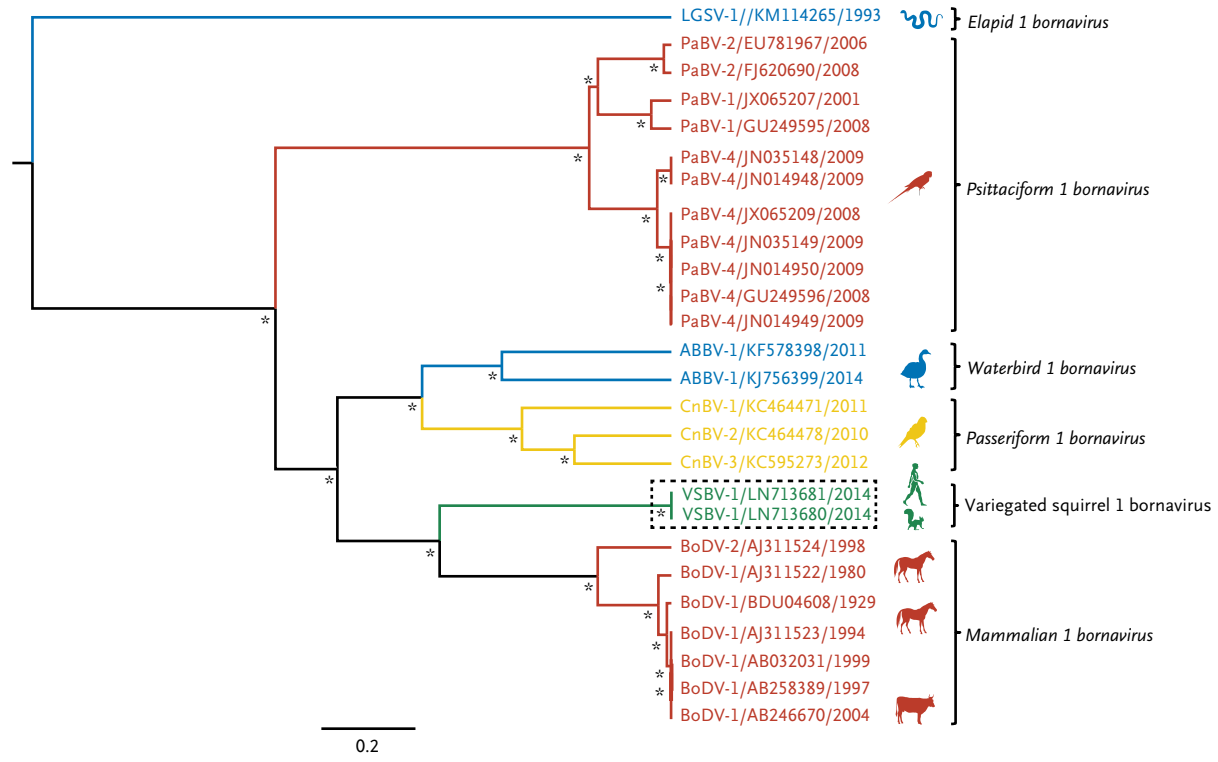

Figure 2. Phylogenetic Analysis of the Members of the Bornavirus Genus, Including the Putative Variegated Squirrel 1 Bornavirus (VSBV-1) Species.

The phylogenetic tree was inferred on the basis of complete coding sequences with the use of the Bayesian Markov chain Monte Carlo method and, in parallel, the neighbor-joining and maximum-likelihood methods. Statistical support of grouping from Bayesian posterior probabilities (clade credibilities ≥90%) and 1000 neighbor-joining and maximum-likelihood bootstrap replicates (≥70%) is indicated with an asterisk. The taxon information includes the virus abbreviation, GenBank accession number, and year of detection. Branches are colored according to lineage (within the bornavirus species classification and nomenclature proposed by the International Committee for Taxonomy of Viruses Bornaviridae Study Group,[7] with the exception of VSBV-1 [tentative, unclassified bornaviruses]). The VSBV-1 sequences generated during this study are highlighted. The scale bar represents nucleotide substitutions per site.

identical, the detection of viral RNA in the brain tissue of all three patients, the results of bornavirus antigen immunostaining, the similarity of the clinical picture among the three patients, the anti-bornavirus IgG titers in the serum as well as in the cerebrospinal fluid of Patient 3, and the epidemiologic link among all three cases support VSBV-1 as the likely causative agent.

Borna disease is described mainly in association with natural infections of horses and sheep, in which BoDV has a main tropism for the CNS, infecting neurons, astrocytes, oligodendrocytes, and ependymal cells.[10-13] The infection is noncytolytic and persistent, and in the natural hosts, mood, sensorium, sensibility, motility, and the autonomous nervous system are simultaneously or successively affected, with a fatal outcome in up to 90% of infected animals.[14] BoDV-like viruses, the so-called *Psittaciform 1 bornavirus* and *Passeriform 1 bornavirus*, have been described in association with fatal proventricular dilatation disease, mainly in psittacine birds.[6,15-17] Moreover, endogenous BoDV-like sequence fragments have already been detected in humans as well as in other species, including squirrels.[8,9] However, on the basis of the PCR data (suggesting high RNA loads) combined with the whole-genome information from the RNA fraction and the antibody response, it is unlikely that the virus sequences detected in this study are endogenous BoDV-like sequences.

In the 1990s, controversy arose regarding whether BoDV was a zoonotic agent responsible for human psychiatric disorders.[14,18] However,

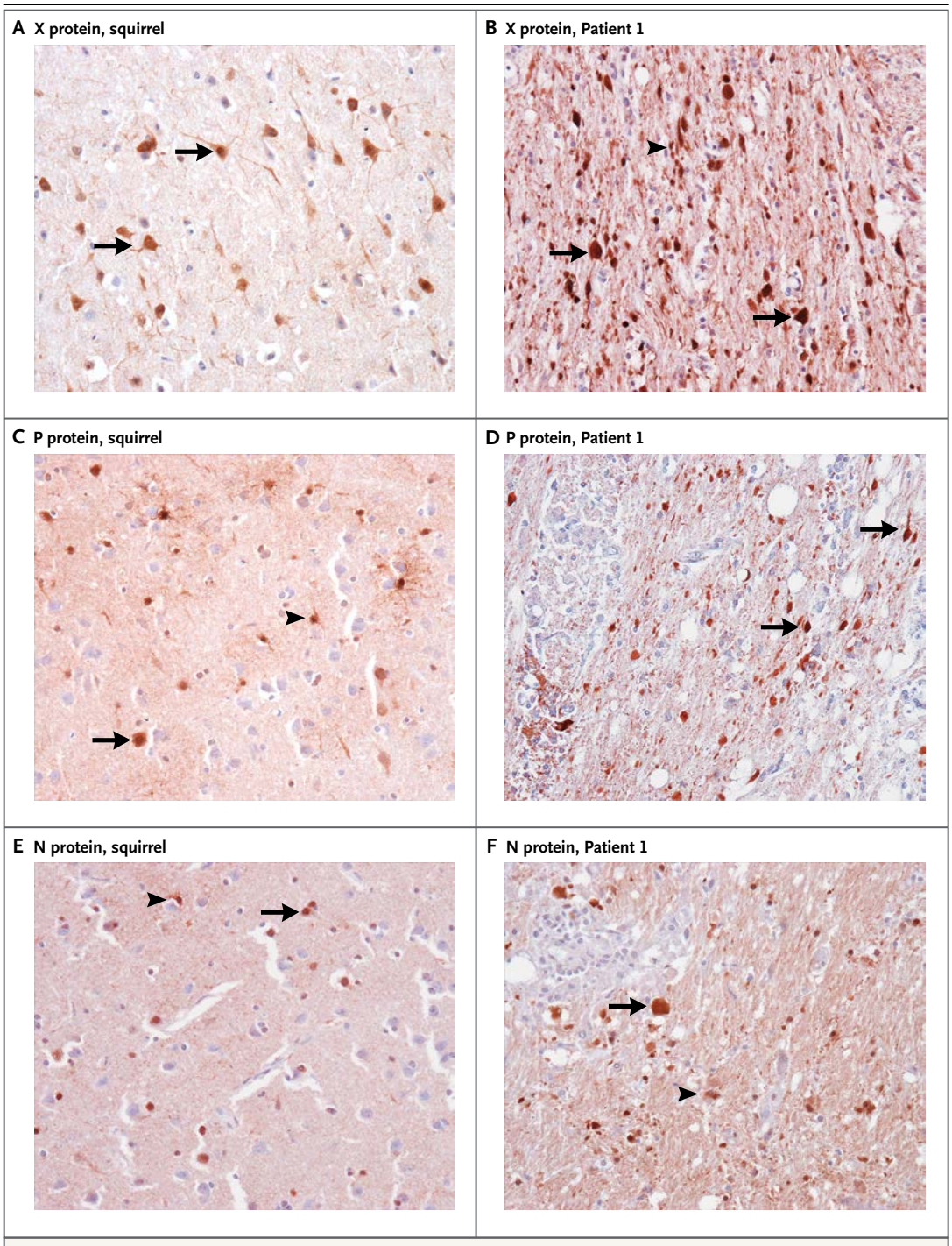

Figure 3. Viral Antigen Detected by Means of Immunohistochemical Analysis.

The presence of viral antigen in the squirrel (Panels A, C, and E) and Patient 1 (Panels B, D, and F) was shown in an immunohistochemical analysis with the use of monospecific polyclonal antibodies against the bornavirus proteins X (Panels A and B), P (Panels C and D), and N (Panels E and F). Viral antigen can be seen in neurons (black arrows), glial cells (black arrowheads), and neuropil. Viral antigen is present in nuclei and cytoplasm.

several studies have questioned whether BoDV-induced clinical disease actually occurs in humans,[18-21] and the general consensus has been that it does not.[14,22] In contrast, VSBV-1 is different from the classic BoDV, and the RNA loads and antigen loads detected in all three human case patients were high, which allowed for whole-genome sequencing and immunohistochemical detection. Therefore, the human infections described in our study are quite different from those studied in previous investigations or referred to in previous discussions, and VSBV-1 is likely to be a previously unknown zoonotic pathogen transmitted by the variegated squirrel. Rodents, particularly exotic ones, are not uncommon as pets, as is reflected in the high number of such animals imported to Europe and other parts of the world.[23] In our study, all three patients were private squirrel breeders who had close contact with these animals. For two of the patients, skin injuries due to squirrel bites and scratches were reported by family members. However, the route of zoonotic transmission from the squirrels to the patients remains uncertain. The high RNA load in the oropharyngeal-swab sample from the squirrel might support the hypothesis of transmission through scratches or bites. The epidemiologic aspects of BoDV disease are not well understood either,[24] and in natural cases, infection through the olfactory route is suspected.

Of note, all three patients were older than 60 years of age and had preexisting medical conditions, which may have conferred a predisposition to clinical infection with this unusual agent. BoDV has been detected in shrews, which might be also able to transmit the virus to other hosts.[25-27] In addition, it remains to be elucidated whether VSBV-1 was carried by the squirrels when they were imported from Latin America or whether it originated from other small mammals that had contact with the breeding facility.

In conclusion, VSBV-1, a zoonotic bornavirus from a variegated squirrel, was associated with three fatal CNS infections in humans. Further studies, including seroepidemiologic and molecular studies in putative animal reservoirs and human patients, in particular those with unexplained encephalitis or meningoencephalitis, are needed.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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