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A Cluster of Fatal Tick-borne Encephalitis Virus Infection in Organ Transplant Setting

Dariusz Lipowski,[1] Marta Popiel ,[2] Karol Perlejewski,[2] Shota Nakamura,[9] Iwona Bukowska-Ośko,[2] Ewa Rzadkiewicz,[1] Tomasz Dzieciątkowski,[3] Anna Milecka,[5] Wojciech Wenski,[7] Michał Ciszek,[4] Alicja Dębska-Ślizień,[6] Ewa Ignacak,[8] Kamila Caraballo Cortes,[2] Agnieszka Pawełczyk,[2] Andrzej Horban,[1] Marek Radkowski,[2] and Tomasz Laskus[2]

[1]Department of Infectious Diseases, [2]Department of Immunopathology of Infectious and Parasitic Diseases, [3]Department of Microbiology, and [4]Department of Immunology, Warsaw Medical University, [5]Regional Transplantation Coordination Center, and [6]Department of Nephrology, Transplantation and Internal Diseases, Gdańsk Medical University, [7]Intensive Care Unit, Regional Hospital, Elbląg, and [8]Department of Nephrology, Kraków Medical University Hospital, Poland; and [9]Department of Infection Metagenomics, Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Background. Tick-borne encephalitis virus (TBEV) infection has become a major health problem in Europe and is currently a common cause of viral brain infection in many countries. Encephalitis in transplant recipients, althrough rare, is becoming a recognized complication. Our study provides the first description of transmission of TBEV through transplantation of solid organs.

Methods. Three patients who received solid organ transplants from a single donor (2 received kidney, and 1 received liver) developed encephalitis 17–49 days after transplantation and subsequently died. Blood and autopsy tissue samples were tested by next-generation sequencing (NGS) and reverse transcription polymerase chain reaction (RT-PCR).

Results. All 3 recipients were first analyzed in autopsy brain tissue samples and/or cerebrospinal fluid by NGS, which yielded 24–52 million sequences per sample and 9–988 matched TBEV sequences in each patient. The presence of TBEV was confirmed by RT-PCR in all recipients and in the donor, and direct sequencing of amplification products corroborated the presence of the same viral strain.

Conclusions. We demonstrated transmission of TBEV by transplantation of solid organs. In such a setting, TBEV infection may be fatal, probably due to pharmacological immunosuppression. Organ donors should be screened for TBEV when coming from or visiting endemic areas.

Keywords. encephalitis; transplantation; TBEV.

Tick-borne encephalitis virus (TBEV) infection has become a major health problem in Europe and Asia and is currently a common cause of viral brain infection in many countries [1, 2]. Tick-borne encephalitis virus belongs to Flaviviridae and is transmitted by ticks to warm-blooded animals and occasionally to humans [3]. Over the last few decades, the number of reported encephalitis cases has increased due to a variety of factors, including global warming, which has extended the length of tick feeding season and its habitat range, and extensive reforestation efforts and an increase in outdoor activities [2, 3]. In Poland, the number of reported cases increased >2-fold, peaking at 351 in 2009 [4], and similar upward trends were observed in Germany, Czech Republic, Slovakia, and Switzerland [1]. However, there are major fluctuations in the number of cases from year to year, and the numbers have stabilized recently; in 2013 there were 227 cases of tick-borne encephalitis in Poland [5].

Tick-borne encephalitis virus infection can cause a wide spectrum of disease, ranging from asymptomatic to full-blown encephalitis and even death [2, 6]. Because the virus is at least transiently present in the blood, it could be hypothetically transmitted through blood transfusion or organ transplantation, more so because the majority of patients are either asymptomatic or have a mild febrile illness only [6, 7]. However, such an occurrence has not been demonstrated so far.

Here we report on the investigation of 3 patients who developed severe encephalitis after receiving solid organ transplants from a single donor. All 3 patients subsequently died and our investigation demonstrated TBEV transmission through transplanted organs.

METHODS

Patients

The organ donor was a 44-year-old male who was hospitalized in September 2012 for multiple injuries related to a traffic accident. The patient was declared braindead after 5 days, and his organs were recovered on the same day. The donor lived in an area endemic for TBEV (North Eastern Poland), and it is unclear whether he had any symptoms of infection before

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Correspondence: T. Laskus, MD, PhD, Department of Immunopathology of Infectious and Parasitic Diseases, Warsaw Medical University, 3C Pawińskiego St, 02-106 Warsaw, Poland (tlaskus@yahoo.com).

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the accident. The results of routine obligatory screening tests for infection with cytomegalovirus (CMV), *Toxoplasma gondii*, human immunodeficiency virus (HIV), hepatitic C virus (HCV), and hepatitis B virus (HBV) were all negative. Autopsy revealed extensive traumatic injuries to the skull and brain, including brainstem.

Patient 1 was a 54-year-old male liver transplant recipient with alcohol-related end-stage liver cirrhosis. The patient did not travel to a TBEV-endemic area in recent months. The transplanted organ undertook function and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) normalized within 3 weeks after the procedure. Patient received standard immunosuppressive treatment consisting of corticosteroids and tacrolimus. Seventeen days after transplantation, the patient experienced fever up to 39ºC and headache and was readmitted to the hospital. At admission the patient presented with meningeal signs, but his cerebrospinal fluid (CSF) was normal (Table 1), and bacterial and fungi cultures of blood and CSF were negative. Polymerase chain reaction (PCR) and/or serological tests for the presence of CMV, *Mycobacterium tuberculosis*, herpes simplex virus 1 (HSV-1) and 2 (HSV-2), varicella zoster virus (VZV), and *T. gondii* were negative. The patient was treated with acyclovir and antibiotics, but he remained febrile, developed dysarthia, dysphagia, and eventually tetraplegia, and his mental status progressed to coma. He died 69 days after admission due to septic shock and multiorgan failure. Autopsy revealed multiple recent pulmonary emboli, endocarditis, and moderate widespread inflammatory changes in the brain parenchyma.

Patient 2 was a 27-year-old male kidney transplant recipient with end-stage renal disease due to membranoproliferative glomerulonephritis, which was diagnosed only 2 years earlier. After transplantation, the graft was functional, and the patient did not require dialysis. His immunosuppressive regimen consisted of tacrolimus, corticosteroids, and mycophenolate mofetil. Twenty-five days after transplantation, the patient was readmitted to the hospital with a 3-day history of fever up to 39.2°C and 1 day of headache, vertigo, and vomiting. There was no history of recent travel to a TBEV-endemic area or history of tick bite. At admission, the patient presented with meningeal signs: nystagmus, dysarthia and aphasia, paralysis of cranial nerves III and IV, and bilaterally positive Babinski sign. Analysis of the CSF showed increased cytosis of 160 but normal protein and glucose (Table 1); PCR tests for Epstein-Barr virus (EBV), CMV, human herpes virus 6 (HHV-6), HSV-1, HSV-2, and VZV were all negative. The patient progressed to coma within 2 days and eventually required mechanical ventilation. He died 36 days after admission. On autopsy, the brain showed diffused swelling and petechial bleedings in brainstem; microscopically there were widespread nodular and patchy mononuclear infiltrates with perivascular lymphocytic cuffing. There were also features of acute kidney allograft rejection.

Patient 3 was a 48-year-old male kidney transplant recipient with end-stage renal disease of unknown cause. After transplantation, the graft was fully functional. His immunosuppressive regimen consisted of tacrolimus, corticosteroids, and mycophenolate mofetil. Fifty-one days after transplantation, the patient was readmitted to the hospital with a history of 2 days of fever up to 39.5°C, complaints of headache, double vision, and weakness in lower extremities. At admission the patient presented with nystagmus and meningeal signs, and his consciousness was impaired. His CSF was normal (Table 1), and PCR testing for the presence of CMV and HSV-1 and HSV-2 in CSF were negative. Patient 3 was treated with acyclovir, but his mental status progressed to coma, and he eventually required assisted ventilation. The patient died 83 days after admission. Autopsy was not performed.

Cerebrospinal fluid from patients 1 and 3 were retrospectively tested with PCR/reverse transcription-PCR (RT-PCR) for HSV-1, HSV-2, HSV-6, CMV, VZV, West Nile virus (WNV), and adenovirus and found negative. Neither serum nor CSF samples were available from patient 2 or donor, and their analysis was confined to autopsy tissue samples.

Table 1. Clinical and Tick-borne Encephalitis Virus Detection Results in 3 Transplant Recipients With Encephalitis and Their Donor

Patient	Age, y/ Sex	Organ transplanted	Time from transplantation to onset of symptoms, d	Time from transplantation to death, d	Detection of TBEV by RT-PCR and NGS[a]				Immediate cause of death	Cerebrospinal fluid		
					Brain tissue	CSF	Serum	Other tissues[b]		Protein, mg/dL	Cell count, per mm^3	Glucose, mg/dL
Donor	44 / M	NA	NA	NA	Pos	NA	NA	NA	Mechanical brain injury	NA	NA	NA
Patient 1	54 / M	liver	17	86	Pos	Neg	Neg	Neg	Sepsis	37	1	73
Patient 2	27 /M	kidney	22	61	Pos	NA	NA	Neg	Encephalitis	300	160	40
Patient 3	48 / M	kidney	49	134	NA	Pos	Neg	NA	Encephalitis	30	1	44

Abbreviatons: NA, not available; CSF, cerebrospinal fluid; M, male; NA, not available; NGS, next-generation sequencing; RT-PCR, ; TBEV, tick-borne encephalitis virus.

[a]Tick-borne encephalitis virus RNA sequences were detected independently by RT-PCR and next-generation sequencing (NGS) with the exception of the donor, for whom only paraffin blocks were available and only RT-PCR was successful.

[b]Lung, kidney, lymph node. liver, and spleen were analyzed only by RT-PCR.

Multiple autopsy samples were available from patients 1 and 2 and consisted of kidney, liver, lung, spleen, lymph nodes, and cortex from the brain frontal region. No autopsy was performed in patient 3, and the availability of his samples was confined to CSF and serum. Only paraffin block of brain tissue (prefrontal cortex) was available for analysis from the donor.

Next-Generation Sequencing and Data Analysis

Total RNA was extracted from 500 mL of CSF (2 x 250-mL extractions were combined) using TRIzol LS Reagent suspended in 5 µL of water and amplified using Single Primer Isothermal Amplification (SPIA). Brain tissue samples were extracted using TRIzol Reagent, and 1 µg of total RNA was subjected to cytoplasmic and mitochondrial rRNA removal using Ribo-Zero Gold rRNA Removal Kit, after which 50 ng of RNA was amplified with SPIA. Samples for next-generation sequencing (NGS) were prepared using Nextera XT Kit as described in detail previously [8]. Finally, samples were pooled equimolarily and sequenced on Illumina HiSeq 1500 (100 nt, paired-end reads).

Raw reads were trimmed by the following procedures: (1) adaptor removal using cutadapt-1.2.1 [9] ; (2) artifact sequences removal using fastx_artifacts_filter; and (3) trimming bases with quality below Q20 (phred quality score) from 3' end of each read and removing reads shorter than 50 basepairs by fastq_quality_trimmer [10]. Next, trimmed sequences were mapped to the human reference sequence (hg19) using Stampy [11]. The unmapped sequences were compared using blastn program against unfiltered National Center for Biotechnology Information nucleotide database (NCBI-nt database) with e-value cutoff of 1e-5. The taxonomic information of each sequence was assigned, and the abundance of identified microorganisms was presented by text mining of blastn output files using BioRuby scripts [12].

Detection of Tick-borne Encephalitis Virus RNA by reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol LS or TRIzol from CSF or autopsy brain tissue as described previously [13]. One microgram of tissue RNA was routinely used for RT-PCR, whereas the amount of CSF extracted RNA loaded into the reaction mixture corresponded to 250 µL. Extracted RNA was incubated for 30 minutes at 37°C in 15 µL of reaction mixture containing 25 pM of random hexamers, 1× PCR buffer, 5 mM magnesium chloride, 5 mM dithiothreitol, 1 mM dNTP, and 10 U of Moloney murine leukemia virus (MMLV) reverse transcriptase. The enzyme was deactivated by heating to 99°C for 10 minutes. Two microliters of the reverse transcription product were directly added into 18 µL of real-time PCR mix containing 25 pM each of specific primers (5'-AGATTTTCTTGCACGTGCAT-3' nt 1 to 20 and 5'-CTCTTTCGACACTCGTCGAGG-3' nt 195 – 175; NC_001672.1). Amplification was run in LightCycler as follows: initial denaturation and activation of enzyme for 5 minutes at 95°C, followed by 35 cycles of 95°C for 30 secones, 54°C for 5 seconds, and 72°C for 30 seconds. The above assay was capable of detecting approximately 100 viral copies as established on control samples (Langat virus strain provided by Prof Heinrich Neubauer, Friedrich-Loeffler-Institut, Jena, Germany). Each amplification was followed by melting curve analysis to ensure that a single-size product was amplified and no significant primers-dimers were present. In addition, amplification products were run on agarose gel to confirm the correct product size. Negative controls included tissue samples from uninfected subjects and normal sera.

Only paraffin block of brain tissue from the frontal cortex was available from the donor, and RNA was extracted using RecoverAll Total Nucleic Acid Isolation kit.

PCR amplification products were sequenced directly by the Sanger method. Phylogenetic trees were constructed according to the Maximum Likelihood method based on the Tamura-Nei model [14] usingMEGA 5.0 [15].

The study was approved by the local institutional review board.

RESULTS

After sequencing and quality trimming, the number of NGS reads ranged from 24.0 mln to 51.7 million per analyzed sample (Table 2). Human sequences were the most abundant in all samples (51.6%–99.8% of all reads), followed by bacterial, fungal, protozoan, plant, and other, as well as sequences not matching any sequences deposited in the GenBank. The number of viral sequences was very small, ranging 29–5461 per sample. Athough a number of different viruses were identified, only TBEV was present in at least 1 sample in all 3 recipients (Table 3).

Because the NGS analysis suggested TBEV as the common causative agent of encephalitis in all 3 recipients, all available patients' samples were tested for the presence of TBEV RNA by specific RT-PCR (Table 1). Tick-borne encephalitis virus RNA was detected in brain tissue in both transplant recipients for whom such samples were available (patients 1 and 2) and in CSF from the third transplant recipient (patient 3), for whom autopsy was not performed. All available serum samples as well as other autopsy samples available for patients 1 and 2 (lung, kidney, lymph node, liver, spleen) were negative. Importantly, the paraffin block brain tissue sample from the donor was also TBEV RNA positive.

The PCR products from the donor and all 3 recipients were sequenced directly. As seen in Figure 1, these represented the same TBEV sequence, which clustered differently from a number of other European TBEV strains.

DISCUSSION

We demonstrated transmission of TBEV through transplanted organs from a single donor to 3 recipients. The donor was retrospectively found to be infected with TBEV, and recipients

Table 2. Results of Next-Generation Sequencing in Autopsy Brain Tissue, Serum, and Cerebrospinal Fluid From 3 Transplant Recipients Who Developed Encephalitis

	Patient 1		Patient 2		Patient 3	
Sample	Brain tissue[a]	Serum	CSF	Brain tissue	Serum	CSF
Total reads	41 950 640	36 648 876	26 289 366	41 017 044	64 300 644	62 606 188
Reads after trimming	39 064 947	33 530 062	23 957 100	33 133 366	51 690 985	49 732 554
Human	38 871 183 (99.50%)	32 502 188 (96.93%)	12 605 295 (52.61%)	33 078 990 (99.84%)	51 580 174 (99.79%)	49 572 372 (99.68%)
Viral	29 (0.00007%)	357 (0.001%)	1551 (0.006%)	1595 (0.005%)	5461 (0.01%)	3021 (0.006%)
Bacterial	7268 (0.02%)	547 799 (1.63%)	7 027 639 (29.33%)	1525 (0.005%)	12 936 (0.03%)	24 092 (0.05%)
Fungal	119 (0.0003%)	29 971 (0.09%)	400 372 (1.67%)	20 (0.00006%)	94 (0.0002%)	35 (0.00007%)
Protozoan	14 (0.00004%)	4457 (0.01%)	31 357 (0.13%)	1 (0.000003%)	57 (0.0001%)	5 (0.00001%)
Other[b]	4435 (0.01%)	236 653 (0.71%)	2 972 623 (12.41%)	14 903 (0.04%)	7857 (0.02%)	3579 (0.007%)
No match	181 899 (0.47%)	208 637 (0.62%)	918 263 (3.83%)	36 332 (0.11%)	84 406 (0.16%)	129 450 (0.26%)

Sequences were compared with the National Center for Biotechnology Information nucleotide database.

Abbreviation: CSF, cerebrospinal fluid.

[a]Prefrontal cortex.

[b]Sequences related to plants, plant viruses, synthetic constructs.

became ill 17–49 days after transplantation, developed severe encephalitis, and subsequently died. The donor came from North East Poland, which is an endemic area for TBEV, and because his organs were procured in early October, he was most likely to have acquired the infection a month earlier, at a time of high tick activity.

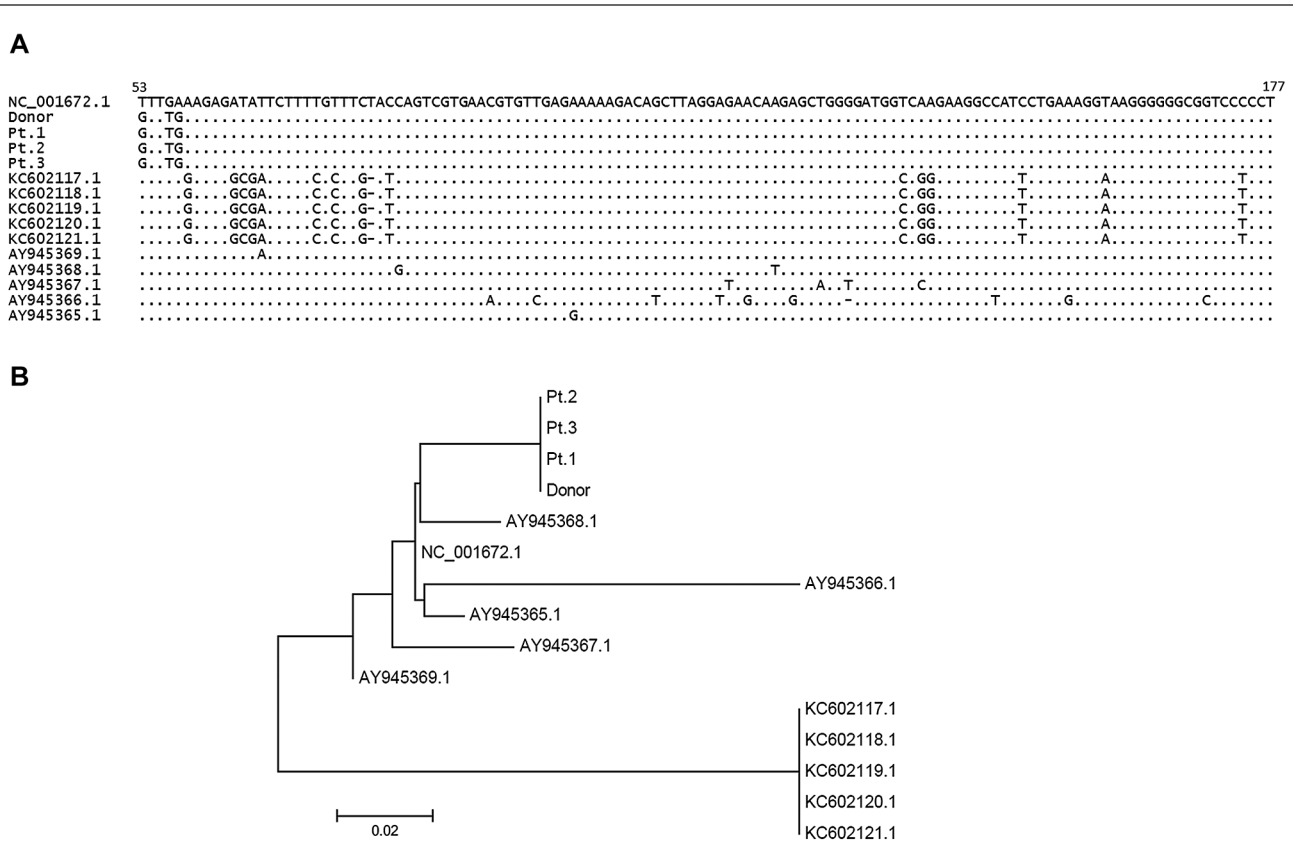

Figure 1. Nucleotide sequence comparison of the tick-borne encephalitis virus (TBEV) fragments recovered from donor and 3 organ recipients (Pt.1, Pt. 2, Pt. 3). *A,* The sequences are aligned to the sequence published by Wallner et al [27] and shown on the top line and with some other European TBEV strains deposited in the GenBank. Dots indicate identity and dashes indicate gaps introduced to maximize the alignment. *B,* Phylogenetic analysis of the above sequences. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model [14]. Evolutionary analysis was conducted using MEGA 5.0 [15].

Table 3. Most Frequently Identified Viral and Bacterial Sequences in Autopsy Brain Tissue, Serum, and Cerebrospinal Fluid From 3 Transplant Recipients Who Developed Encephalitis

	Viruses	Bacteria
Patient 1		
Brain tissue	TBEV (9)	Staphylococcaceae (2413)
	Vaccinia virus (9)	Bacillaceae (1417)
	Human herpesvirus 5 (2)	Enterobacteriaceae (1185)
		Alteromonadaceae (269)
		Moraxellaceae (248)
Serum	Torque teno virus (139)	Moraxellaceae (79 083)
	Bell pepper endornavirus (45)	Microbacteriaceae (11 799)
	Brome mosaic virus (33)	Enterococcaceae (69 539)
	Pepino mosaic virus (22)	Micrococcaceae (60 913)
	Moumouvirus (14)	Flavobacteriaceae (35 163)
CSF	Bell pepper endornavirus (1162)	Streptococcaceae (2 008 652)
	Human herpesvirus 4 (280)	Moraxellaceae (847)
	Trichoderma hypovirus (5)	Micrococcaceae (695 253)
	Human picobirnavirus (5)	Pseudomonadaceae (388 851)
		Staphylococcaceae (310 417)
Patient 2		
Brain tissue	TBEV (988)	Alteromonadaceae (539)
	Betapapillomavirus 1 (5)	Bacillaceae (217)
	SEN virus (1)	Rhodobacteraceae (134)
		Propionibacteriaceae (89)
		Enterobacteriaceae (82)
Patient 3		
Serum	Torque teno virus (4626)	Alteromonadaceae (10 404)
	SEN virus (388)	Xanthomonadaceae (511)
	Torque teno midi virus (223)	Propionibacteriaceae (209)
	TTV-like mini virus (132)	Pseudomonadaceae (150)
	Micro Torque teno virus (12)	Burkholderiaceae (144)
CSF	Torque teno virus (2648)	Alteromonadaceae (22 594)
	SEN virus (227)	Micrococcaceae (267)
	Torque teno midi virus (47)	Propionibacteriaceae (251)
	TTV-like mini virus (13)	Burkholderiaceae (146)
	TBEV (9)	Pseudomonadaceae (85)

Sequences were compared with the National Center for Biotechnology Information nucleotide database. Numbers in parenthesis represent the number of reads.
Abbreviations: CSF, cerebrospinal fluid; TBEV, tick-borne encephalitis virus; TTV, torque teno virus.

Because the etiology of encephalitis was initially unclear and there are >100 known potential viral pathogens causing encephalitis, [16] we resorted to NGS, which was previously successfully used by others to identify novel and rare pathogens in various clinical settings, including central nervous system infections and transplantation [17]. Although CSF is considered sterile, we detected reads that mapped to different categories (bacterial, viral, fungal, protozoan). These findings are consistent with observations from other metagenomic studies reporting the common presence of DNA/RNA contamination, which originates from commercial reagents or has an environmental source [18, 19]. Thus, it seems necessary to verify any NGS detection of a specific pathogen by an independent method. In our patients, the initial identification of TBEV RNA by NGS was independently confirmed by specific RT-PCR.

Most TBEV infections are subclinical or asymptomatic, and clinical symptoms develop in only 5%–30% of cases [6, 7]. The mortality in clinically overt cases is usually around 0.5%–2% [2], and in 1 large series of >600 Polish patients was as low as 0.6% [20], which is in striking contrast to our report, in which all 3 infected patients died. It is highly likely that organ recipients who take immunosuppressive drugs are at a higher risk for developing severe forms of TBEV-related encephalitis, as has been previously described for another Flavivirus, WNV [21]. Other unusual features, which were most likely related to pharmacological immunosuppression, were extended incubation period, which was 17–49 days in our patients, and was reported to be typically only 7–10 days on average [7], and the paucity of CSF changes. In our patients, CSF pleocytosis and increased protein concentration, which are almost universal features of TBEV encephalitis [7, 22, 23], were present in only 1 of 3 patients. Thus, TBEV infection

in transplant recipients seems to be characterized by increased severity, extension of incubation period, and low CSF pleocytosis. These characteristics are similar to those of other encephalitis infections in the immunosuppressive setting [21, 24].

Tick-borne encephalitis virus infection caused by European strains is typically biphasic in the vast majority of patients, with the first viremic phase consisting of cold-like illness lasting 2–4 days followed by a symptom-free interval of about 1 week after which a second phase of symptoms directly related to the central nervous system develops [6, 22, 23]. Notably, our patients had monophasic disease, which was previously associated with a more severe form of encephalitis [22]. Tick-borne encephalitis virus viremia may be transient and confined to the early stage of infection because viral RNA was detected in brain and CSF, whereas tissues such as liver, kidney, lung, lymph node, and spleen were negative in both recipients in whom multiple autopsy samples were available for testing.

Encephalitis in transplant recipients, although rare, is becoming a recognized complication. In a recent survey, 6 clusters of WNV encephalitis, 2 clusters of rabies encephalitis, 3 clusters of lymphocytic choriomeningitis virus, and 3 clusters of Balamuthia granulomatous amebic encephalitis were indentified among solid organ transplant recipients in the United States between 2002 and 2013 [25]. A review of all potential donor-derived disease transmission events in the Organ Procurement and Transplantation network in the years 2008–2010 provided similar findings. Central nervous system infection was identified in 12 donors, 6 of whom transmitted infection to 10 of 15 exposed recipients, and 5 recipients subsequently died. Responsible pathogens included *Balamuthia mandrillaris*, *Cryptococcus neoformans*, lymphocytic choriomeningitis virus, and WNV [26].

In summary, it seems that organ donors who live or have recently visited endemic areas, particularly during the high tick activity season, should be screened for TBEV, the more so because the clinical course of disease among the organ recipients may be fatal. Whether this should be extended to testing blood donors is currently unclear, but further increase in TBEV infection incidence might justify such measures.

Notes

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