A CLUSTER OF FATAL TICK-BORNE ENCEPHALITIS VIRUS INFECTION IN ORGAN TRANSPLANT SETTING

Running head: TBEV in transplantation

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ABSTRACT

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, while 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 (two received kidney and one 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 RT-PCR.

Results. All three recipients were first analyzed in autopsy brain tissue samples and/or cerebrospinal fluid (CSF) by NGS, which yielded 24-52 mln sequences per sample, and 9 to 988 matched Tick-Borne Encephalitis Virus (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 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.

Key words: encephalitis, transplantation, TBEV

INTRODUCTION

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]. TBEV 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 extended the length of tick feeding season and its habitat range, as well as to extensive reforestation efforts and the increase in outdoor activities [2, 3]. In Poland, the number of reported cases increased over two-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 TBE in Poland [5].

TBEV infection can cause a wide spectrum of disease ranging from asymptomatic to full-blown encephalitis and even death [2, 6]. As the virus is at least transiently present in the blood it could be hypothetically transmitted through blood transfusion or organ transplantation, the more so, as 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 three patients who developed severe encephalitis after receiving solid organ transplants from a single donor. All three patients subsequently died and our investigation demonstrated TBEV transmission through transplanted organs.

PATIENTS

The organ donor was a 44 years-old male who was hospitalized in September 2012 for multiple injuries related to a traffic accident. The patient was declared brain-dead 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 prior to 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 TBEV endemic areas in recent months. The transplanted organ undertook function and ALT and 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 39 ^{o}C and headache and was readmitted to the hospital. At admission the patient presented with menigeal signs, but his CSF was normal (Table 1) and bacterial and fungi cultures of blood and CSF were negative. 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 Toxoplasma 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 hospital with a three-day history of fever up to 39·2 C, and one day of headache, vertigo, and vomiting. There was no history of recent travel to 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 Babinski sign was bilaterally positive. 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 two 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, and his immunosuppressive regimen consisted of tacrolimus, corticosteroids and mycophenolate mofetil. Fifty-one days after

transplantation the patient was readmitted to hospital with a history of 2 days of fever up to 39·5 C, complains of headache, double vision, 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.

CSF from patients 1 and 3 were retrospectively tested with 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.

Multiple autopsy samples were available in 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.

METHODS

Next-Generation Sequencing (NGS) and data analysis. Total RNA was extracted from 500 ml of CSF (2 x 250 ml extractions were combined) using TRIzol LS Reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA), suspended in 5 µl of water and amplified using SPIA (Ovation RNA-Seq V2; NuGEN, San Carlos, USA). Brain tissue samples were extracted using TRIzol Reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA) and one µg of

total RNA was subjected to cytoplasmic and mitochondrial rRNA removal using Ribo-Zero Gold rRNA Removal Kit (Illumina, San Diego, USA) after which 50 ng of RNA was amplified with SPIA (Ovation RNA-Seq V2; NuGEN, San Carlos, USA). Samples for NGS were prepared using Nextera XT Kit (Illumina, San Diego, USA) 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, 3) trimming bases with quality below Q20 (phred quality score) from 3' end of each read and removing reads shorter than 50 bp 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 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 TBEV RNA by RT-PCR. Total RNA was extracted with TRIzol LS or TRIzol (ThermoFisher Scientific, Waltham, Massachusetts, USA) from CSF or autopsy brain tissue as described previously [13]. One µg of tissue RNA was routinely used for RT-PCR, while the amount of CSF extracted RNA loaded into the reaction mixture corresponded to 250 µl. Extracted RNA was incubated for 30 min at 37 ^{0}C in 15 µl of reaction mixture containing 25 pM of random hexamers (Invitrogen, Carlsbad, USA), 1x PCR buffer II (Perkin Elmer), 5 mM $MgCl_2$, 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 ^{0}C for 10 min. Two µl of the reverse transcription product were directly added into 18 µl of real-time PCR mix

(LightcCycler FastStart DNA Master SYBR Green I; Roche Diagnostics, Switzerland) 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 (Roche) as follows: initial denaturation and activation of enzyme for 5 min at 95 ^{0}C, followed by 35 cycles of 95 ^{0}C for 30 sec, 54 ^{0}C for 5 sec and 72 ^{0}C for 30 sec. 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 (Ambion Ltd., Cambridgeshire, UK).

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 mln 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 as it ranged from 29 to 5461 per sample. While a number of different viruses were identified, only TBEV was present in at least one sample in all three recipients (Table 3).

Since the NGS analysis suggested TBEV as the common causative agent of encephalitis in all three recipients, all available patients' samples were tested for the presence of TBEV RNA by specific RT-PCR (Table 1). TBEV RNA was detected in brain tissue in both transplant recipients in whom such samples were available (patients 1 and 2) and in CSF from the third transplant recipient (Patient 3) in whom autopsy was not performed. All available serum samples as well as other autopsy samples available in 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 three 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 three recipients. The donor was retrospectively found to be infected with TBEV and recipients became ill 17-49 days after transplantation, developed severe encephalitis and subsequently died. The donor came from a North East part of Poland, which is an endemic area for TBEV, and as his organs were procured in early October, he was most likely to have acquired the infection a month earlier, at the time of high tick activity.

Since the etiology of encephalitis was initially unclear, and there are over 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 being 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 have 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 sub-clinical or asymptomatic and clinical symptoms develop only in 5%-30% of cases [6, 7]. The mortality in clinically overt cases is usually around 0·5%-2% [2] and in one large series of over 600 Polish patients was as low as 0·6% [20], which is in striking contrast to our report, in which all three 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 to 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 an almost universal feature of TBEV encephalitis [7, 22, 23], were present in only one out of three 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].

TBEV 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 one week after which a second phase of symptoms directly related to the CNS develops [6, 22, 23]. Notably, our patients had monophasic disease, which was previously associated with a more severe form of encephalitis [22]. TBEV viremia may be transient and confined to the early stage of infection, as viral RNA was detected in brain and CSF, while 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, while rare, is becoming a recognized complication. In a recent survey six clusters of WNV encephalitis, two clusters of rabies encephalitis, three clusters of lymphocytic choriomeningitis virus, and three clusters of Balamuthia granulomatous amebic encephalitis were indentified among solid organ transplant recipients in the USA 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.

CNS infection was identified in 12 donors, six of whom transmitted infection to 10 of 15 exposed recipients and five recipients subsequently died. Responsible pathogens included Balamuthia mandrillaris, Cryptococcus neoformans, lymphocytic choriomeningitis virus, and WNV [26].

In summary, it seems that organ donors should be screened for TBEV who live or have recently visited endemic areas, particularly during the high tick activity season, the more so as 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.

Figure 1a. Nucleotide sequence alignment of the TBEV fragments recovered from donor and three organ recipients (Pt.1 -3). The sequences are compared with 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. Figure 1b shows 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].

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Table 1. Some clinical and TBEV detection results in three transplant recipients with encephalitis and their donor

Pt	Age (yrs)/ Gender	Organ transplanted	Time from transplantation to onset of symptoms (days)	Time from transplantation to death (days)	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
Pt. 1	54 / M	liver	17	86	Pos	Neg	Neg	Neg	Sepsis	37	1	73
Pt. 2	27 /M	kidney	22	61	Pos	NA	NA	Neg	Encephalitis	300	160	40
Pt. 3	48 / M	kidney	49	134	NA	Pos	Neg	NA	Encephalitis	30	1	44

NA, not available; CSF, cerebrospinal fluid.
[a] TBEV RNA sequences were detected independetly by RT-PCR and next-generation sequencing (NGS) with the exception of donor, in whom only paraffin blocks were available and only RT-PCR was successful. [b] Lung, kidney, lymph node. liver, spleen were analyzed only by RT-PCR

	Brain tissue[b]	Serum	CSF	Brain tissue	Serum	CSF
Total reads	41950640	36648876	26289366	41017044	64300644	62606188
Reads after trimming	39064947	33530062	23957100	33133366	51690985	49732554
Human	38871183	32502188	12605295	33078990	51580174	49572372
	(99.503995%)	(96.934470%)	(52.616114%)	(99.835887%)	(99.785628%)	(99.677913%)
Viral	29	357	1551	1595	5461	3021
	(0.000074%	(0.001065%	(0.006474%	(0.004814%	(0.010565%	(0.006074%
Bacterial	7268	547799	7027639	1525	12936	24092
	(0.018605%)	(1.633755%)	(29.334264%)	(0.004603%)	(0.025026%)	(0.048443%)
Fungal	119	29971	400372	20	94	35
	(0.000305%)	(0.089385%)	(1.671204%)	(0.000060%)	(0.000182%)	(0.000070%)
Protozoan	14	4457	31357	1	57	5
	(0.000036%)	(0.013293%)	(0.130888%)	(0.000003%)	(0.000110%)	(0.000010%)
Other[c]	4435	236653	2972623	14903	7857	3579
	(0.011353%)	(0.705794%)	(12.408109%)	(0.044979%)	(0.015200%)	(0.007196%)
No match	181899	208637	918263	36332	84406	129450
	(0.465632%)	(0.622239%)	(3.832947%)	(0.109654%)	(0.163290%)	(0.260292%)

Table 2. Results of next-generation sequencing (NGS) in autopsy brain tissue, serum and cerebrospinal fluid CSF) from three transplant recipients who developed encephalitis[a]

[a]Sequences were compared to the NCBI-nt database; [b] Prefrontal cortex; [c]Sequences related to plants. plant viruses. synthetic constructs

Table 3. The most frequently identified viral and bacterial sequences in autopsy brain tissue, serum and cerebrospinal fluid from three transplant recipients who developed encephalitis[a]

	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 (79083)
	Bell pepper endornavirus (45)	Microbacteriaceae (11799)
	Brome mosaic virus (33)	Enterococcaceae (69539)
	Pepino mosaic virus (22)	Micrococcaceae (60913)
	Moumouvirus (14)	Flavobacteriaceae (35163)
CSF	Bell pepper endornavirus (1162)	Streptococcaceae (2008652)
	Human herpesvirus 4 (280)	Moraxellaceae (1232847)
	Trichoderma hypovirus (5)	Micrococcaceae (695253)
	Human picobirnavirus (5)	Pseudomonadaceae (388851)
		Staphylococcaceae (310417)
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 (10404)
	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 (22594)
	SEN virus (227)	Micrococcaceae (267)
	Torque teno midi virus (47)	Propionibacteriaceae (251)
	TTV-like mini virus (13)	Burkholderiaceae (146)
	TBEV (9)	Pseudomonadaceae (85)

[a]Sequences were compared to the NCBI-nt database.