Human Pegivirus in Patients with Encephalitis of Unclear Etiology, Poland

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe evidence for human pegivirus (HPgV) infection in the central nervous system (CNS) among patients with encephalitis of unclear etiology, according to a case series
- Compare HPgV sequences in cerebrospinal fluid with those circulating in serum, according to a case series
- Determine clinical implications of evidence for HPgV infection in the CNS among patients with encephalitis of unclear etiology, according to a case series

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Human pegivirus (HPgV), previously called hepatitis G virus or GB virus C, is a lymphotropic virus with undefined pathology. Because many viruses from the family Flaviviridae, to which HPgV belongs, are neurotropic, we studied whether HPgV could infect the central nervous system. We tested serum and cerebrospinal fluid samples from 96 patients with a diagnosis of encephalitis for a variety of

pathogens by molecular methods and serology; we also tested for autoantibodies against neuronal antigens. We found HPgV in serum and cerebrospinal fluid from 3 patients who had encephalitis of unclear origin; that is, all the markers that had been tested were negative. Single-strand confirmation polymorphism and next-generation sequencing analysis revealed differences between the serum and cerebrospinal fluid–derived viral sequences, which is compatible with the presence of a separate HPgV compartment in the central nervous system. It is unclear whether HPgV was directly responsible for encephalitis in these patients.

Human pegivirus (HPgV) was originally described as a hepatitis virus by 2 independent groups of researchers and called GB virus C and hepatitis G virus (*1*,*2*). Whereas the infection was found to be common in patients with forms of chronic hepatitis, and particularly prevalent in patients with chronic hepatitis C infection, it is not associated with liver injury in the absence of concomitant infection with hepatitis C virus (HCV) or hepatitis B virus (HBV). Furthermore, the liver is not the primary replication site for this virus (*3*,*4*). This virus was recently renamed as pegivirus and assigned to a new genus (*Pegivirus*) within the family Flaviviridae (*5*).

Infection with HPgV is common worldwide; ≈5% of healthy blood donors in industrialized countries are viremic, whereas in some developing countries the prevalence of viremia among blood donors is ≈20% (*6*). There is evidence that HPgV is transmitted parenterally, sexually, and also vertically from mother to child (*7*). However, the high proportion of HPgV infection in apparently healthy blood donors and the general population suggests existence of nonparenteral routes. The reasons for the high prevalence of infection in developing countries are not entirely clear but could be related to overall poor hygienic conditions, as well as to the time of exposure. In sub-Saharan Africa, where HPgV is particularly common, this virus is transmitted mainly during childhood, which may facilitate the establishment of chronic infection (*7*). Because no association between HPgV and disease has been consistently identified, blood donors are not routinely screened for the virus.

Interest in the HPgV infection was revived when several studies identified its beneficial effect on the survival of HIV-infected persons (*8*,*9*); anti-HIV replication effects of HPgV were confirmed in vitro (*8*). Several in vivo and in vitro studies suggest that HPgV may directly interfere with HIV replication and affect host cell factors necessary for the HIV life cycle; specific mechanisms include modulation of cytokine and chemokine release and receptor expressions and lowering of T-cell activation and proliferation (*9*). However, infection with HPgV may not be totally benign; some studies found an association between infection and non-Hodgkin lymphoma (*10*,*11*), which could be the result of lowered immune activation.

Many viruses from the family Flaviviridae, most prominently arthropodborne viruses (arboviruses) such as West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), are neurotropic and a prominent cause of encephalitis in Europe and North America (*12*). These factors raise the question whether HPgV could be neurotropic and whether it could be an etiologic agent in neuroinfections. Of note, despite substantial progress in diagnostics, the etiology of encephalitis remains unclear in 40%–80% of patients (*13*,*14*). A plethora of pathogens may cause encephalitis; many of these pathogens are rare and thus testing is not performed to identify them, and others have not yet been identified.

Three recent case reports described HPgV RNA in the human central nervous system (CNS), demonstrating that the virus can be present in the brain under certain circumstances (*15*–*17*). In the first study, viral sequences were detected postmortem in brain tissue from a patient with multiple sclerosis, not encephalitis (*15*). In the second study, the presence of HPgV might have been related to a severely compromised blood–brain barrier; the patient was HIV-positive and had cerebral toxoplasmosis and fungal encephalitis (*16*). Although the full-length virus was recovered from the patient's brain tissue, it is unclear which cells harbored the virus and it was possible that the actual source was blood. Furthermore, the association of HPgV with multiple sclerosis could not be established because the study was limited to a single case. In the third study, HPgV was detected in serum and CSF of a patient with a severe form of encephalitis of unclear origin (*17*). Of these 3 studies, none included comparison of serum- and CNS-derived virus. We conducted a study of 96 consecutive patients with diagnosis of encephalitis (*18*) in Poland during 2012–2015 to determine whether HPgV could be found in the CNS.

Materials and Methods

Patients and Routine Diagnostics

We prospectively enrolled patients with encephalitis at the Warsaw Hospital for Infectious Diseases (Warsaw, Poland) from June 2012 through July 2015. The details of this study were published previously (*18*). We defined encephalitis as an acute-onset illness with altered mental status, decreased level of consciousness, seizures, or focal neurologic signs, together with ≥1 abnormality of the CSF (leukocyte count ≥4 cells/mm^2 or protein level ≥40 mg/dL). We obtained written informed consent from all patients or from close relatives of patients unable to give

consent due to their condition. The Internal Review Board of the Medical University of Warsaw approved the study.

We collected CSF and serum samples from patients at admission (5–7 days after symptom onset) and kept them frozen at -80°C until analysis. We tested the samples from all 96 patients for the presence of 5′ untranslated region (UTR) HPgV RNA. We performed real-time quantitative PCR (qPCR) or real-time quantitative reverse transcription PCR (qRT-PCR) to detect human herpesvirus (HHV) 1 and 2, varicella zoster virus (VZV), cytomegalovirus (CMV), HHV-6, enteroviruses (coxsackievirus A9, A16, B2, B3, B4, B5; echovirus 5, 6, 9, 11, 18, 30; and enterovirus 71), TBEV, WNV, and human adenovirus (HAdV) in CSF samples. We used commercial serologic tests to test CSF and paired serum samples for HHV-1, HHV-2, VZV, TBEV, and WNV as described (*18*). We detected autoantibodies against neuronal surface antigens using the Autoimmune Encephalitis Mosaic 6 assay (Euroimmun AG, Luebeck, Germany).

HPgV 5′ UTR and E2 Amplification

We extracted total RNA with TRIzol LS (ThermoFisher Scientific, Waltham, MA, USA) from 400 µL of CSF or serum and suspended RNA in 20 µL of water, 5 µL of which was subsequently used for each amplification reaction. We amplified the HPgV 5′ UTR by nested reverse transcription PCR (RT-PCR) as described previously (*19*), resulting in 421 bp-length product; we amplified the E2 region following the RT-PCR protocol published by Smith et al. (*20*). The final product was 422 bp in length.

Single-Strand Conformation Polymorphism

We subjected the amplified PCR products to single-strand conformational polymorphism analysis, as described previously (*21*). In brief, we purified HPgV 5′ UTR and HPgV-E2 PCR products by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). We then subjected the products to thermal denaturation, ran them on nondenaturing 1% polyacrylamide gels at 400V in 25°C, fixed them with acetic acid, and stained them with silver stain.

Next-Generation Sequencing

We reamplified RT-PCR products with primers specifically designed for the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Each primer contained the following: sequences complementary to the adapters on a flow cell; an 8-nt index sequence; sequences corresponding to the Illumina sequencing primers; and sequence-specific nested primers for the 5′ UTR and E2 region. Amplification of the 5′ UTR region included initial denaturation at 94°C for 5 min, 20 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and final elongation at 72°C for 10 min. HPgV E2 amplification included denaturation at 94°C for 5 min followed by 20 cycles of 94°C for 18 s, 55°C for 20 s, 72°C for 90 s, and 1 cycle at 72°C for 10 min. We trimmed the libraries by using the LabChip XT apparatus (PerkinElmer, Waltham, Massachusetts, USA) with the DNA 300 Assay Kit (PerkinElmer); the range of fraction collection was 370–430 bp for 5′ UTR and 460–530 bp for E2.

We assessed the quality and average length of next-generation sequencing libraries by using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We equimolarily pooled the indexed samples and sequenced them on Illumina MiSeq with 301 bp-end reads according to the manufacturer's protocol.

Data Analysis

We trimmed raw reads using cutadapt version 1.2.1 (https://github.com/marcelm/cutadapt/); (*22*), then used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) for additional processing. We removed all Phred quality score reads <20 using FASTQ/A Artifacts Filter and preprocessed the remaining reads (grouping, counting, and frequency arrangement) using R scripts (*23*). To diminish the contribution of false positive variants to genetic diversity, we applied the experimentally established sequencing error cutoff of 1.22%. Finally, we aligned remaining sequences and generated phylogenic trees with ClustalX version 2.0 (http://www.clustal.org/clustal2/) (*24*). We assessed nucleotide diversity per site and the number of substitutions with respect to the dominant serum sequence in each patient using DnaSP version 6.11.06 (*25*). We predicted the RNA secondary structures of the 5′ UTR using Mfold version 3.2 (http://unafold.rna.albany.edu/?q=mfold) (*26*), and searched for putative B cell epitopes within the E2 region using BepiPred-2.0 (http://www.cbs.dtu.dk/services/BepiPred/) (*27*).

Results

Four patients were positive for 5′ UTR HPgV RNA in serum, and 3 of these patients were also positive in CSF. We analyzed the samples from those 3 patients; their samples were collected at admission, which was 5–7 days after symptom onset. We diagnosed encephalitis of unclear origin for all 3 patients because they were negative for all the pathogens tested (Table 1). The small number of HPgV-infected patients did not allow for statistical analysis, but these patients were not strikingly different from other encephalitis patients. We initially suspected 1 patient, who had a severe illness with prolonged hospitalization, of having HHV infection, but tests did not confirm HHV. All 3 patients recovered without any neurologic sequelae.

We compared serum- and CSF-derived 5′ UTR and E2 amplicons from the 3 patients by single-strand conformational polymorphism analysis (Figure 1). Because this

Table 1. Comparison of clinical characteristics of 3 encephalitis patients infected with human pegivirus compared with patients with other forms of encephalitis, Poland, 2012–2015*

Characteristic	Encephalitis patients infected with pegivirus			Other encephalitis patients	
	Patient 1	Patient 2	Patient 3	Infectious cause identified, n = 41†	Unknown cause or autoimmune illness, n = 52‡
Male sex	No	No	Yes	30 (73)	23 (44)
Median age, y (range)	55	28	20	38 (19–85)	38 (20–82)
Pharmacological immunosuppression present	0	0	0	2 (5)	3 (6)
HIV positive	0	0	0	0	2 (4)
Cancer	0	0	0	6 (15)	1 (2)
Median length of hospital stay, d (range)	41	30	8	12 (5–97)	11.5 (6–79)
Fever ≥38°C	Yes	Yes	No	26 (63)	18 (35)
Headache	Yes	Yes	No	22 (54)	25 (48)
Altered mental status	Yes	Yes	No	36 (88)	47 (85)
Focal neurologic signs	No	No	Yes	9 (22)	10 (20)
Seizures	No	No	Yes	12 (29)	16 (31)
Stiff neck	No	No	No	7 (17)	12 (23)
CSF analysis					
Median leukocyte count, cells/mm^2 (range)	3	4	3	41 (1–1225)	18 (1–362)
Median protein level, g/L (range)	0.68	0.26	1.63	0.57 (0.16–3.21)	0.56 (0.11–3.33)
Death	0	0	0	1 (2.4)	0

*Values are no. (%) unless otherwise indicated. CSF, cerebrospinal fluid
†Identified infections were human herpesvirus 1 (n = 22), enterovirus (n = 6), varicella zoster virus (n = 5), tick-borne encephalitis virus (n = 6), and cytomegalovirus (n = 2).
‡All markers of viral infection were negative, but 5 patients had antibodies against N-methyl-D-aspartate receptor and a diagnosis of autoimmune encephalitis.

analysis suggested the presence of differences between the serum- and CSF-derived viral sequences in individual patients, we subjected all amplicons to next-generation sequencing. After filtering, the mean number of reads per sample was 70,759 (range 2,706–183,046) (Table 2).

When we compared 5′ UTR and E2 sequences phylogenetically, we found that serum- and CSF-derived sequences clustered together in individual patients; no sequence was found in multiple patients (Figure 2). Of note, we found several variants to be unique in the CSF compartment. These sequences comprised 2.28%–29.32% of all variants for 5′ UTR and 0%–41.78% of all variants for E2 (Table 2; Figure 3). Unique CSF-derived sequences were also present when we analyzed the E2 region on the amino acid level (Figure 4). The changes were serine to phenylalanine at aa position 508 in patient 2 and proline to leucine at position 572 in patient 3. Both changes were within the predicted B cell epitopes (aa 506–522 and 559–572).

We analyzed all 5′ UTR sequence variants to determine the predicted stability of their secondary RNA structure. We deemed the effect of variations minor because most were localized in the nonbasepaired parts (data not shown), and free energies of the hypothetical secondary structures were only occasionally and mildly affected (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/10/18-0161-Techapp1.pdf) (*28*).

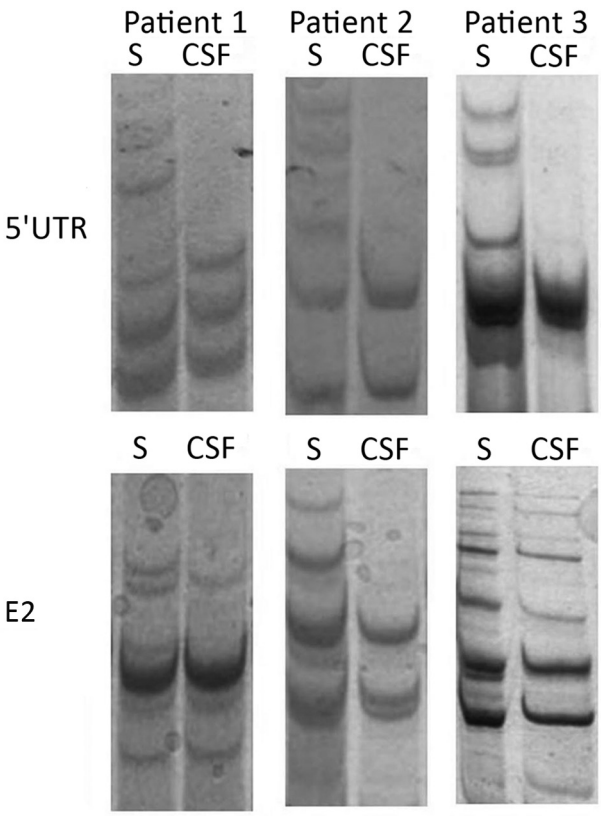

Figure 1. Single-strand conformation polymorphism analysis of 5′ UTR and E2 region human pegivirus amplicons from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. CSF, cerebrospinal fluid; S, serum; UTR, untranslated region.

Discussion

We detected HPgV sequences in CSF from 3/96 patients with encephalitis. We classified all 3 cases as encephalitis

Table 2. 5 Human pegivirus variants in serum and cerebrospinal fluid in 3 patients with encephalitis of unknown origin, Poland, 2012–2015*

RNA region	Patient 1		Patient 2		Patient 3	
	Serum	CSF	Serum	CSF	Serum	CSF
5′ untranslated region						
No. reads before error cutoff	124,987	110,331	108,002	183,046	101,240	145,411
No. reads after error cutoff	61,783	57,668	49,075	109,061	55,328	75,998
No. nucleotide variants†	3	7	3	3	2	3
No. unique nucleotide variants in CSF†	–	5 (29.32)	–	1 (2.28)	–	1 (2.49)
No. nucleotide substitutions	2	7	2	2	1	2
Nucleotide diversity per site	0.004	0.007	0.004	0.004	0.003	0.004
E2 region						
No. reads before error cutoff	70,460	38,025	77,656	76,918	2,706	82,738
No. reads after error cutoff	26,720	20,619	26,558	42,609	453	34,887
No. nucleotide variants†	8	4	8	9	4	7
No. unique nucleotide variants in CSF†	–	0	–	5 (41.78)	–	3 (27.28)
No. nucleotide substitutions	4	2	5	5	2	3
Nucleotide diversity per site	0.007	0.004	0.006	0.007	0.004	0.005
No. amino acid variants†	2	2	2	5	2	4
No. unique amino acid variants in CSF†	–	0	–	3 (27.28)	–	2 (27.28)

*Numbers in parentheses are percentages. CSF, cerebrospinal fluid
†After applying the 1.22% sequencing error cutoff.

of unknown origin because they were negative for serologic and molecular markers of common CNS pathogens. Furthermore, we demonstrated that these viral sequences differed from those circulating in serum. The presence of viral RNA in CSF could be due to a compromised blood–brain barrier, which was possible in these patients with encephalitis. However, the presence of differences in circulating and CSF-derived sequences is more compatible with the existence of separate viral compartments and thus independent replication. Similar compartmentalization in which distinct blood and CNS viral populations indicate separately evolving populations has been described for other viruses, most prominently for HIV (*29*) but also for HCV (*30*,*31*) and human BK polyomavirus (*32*).

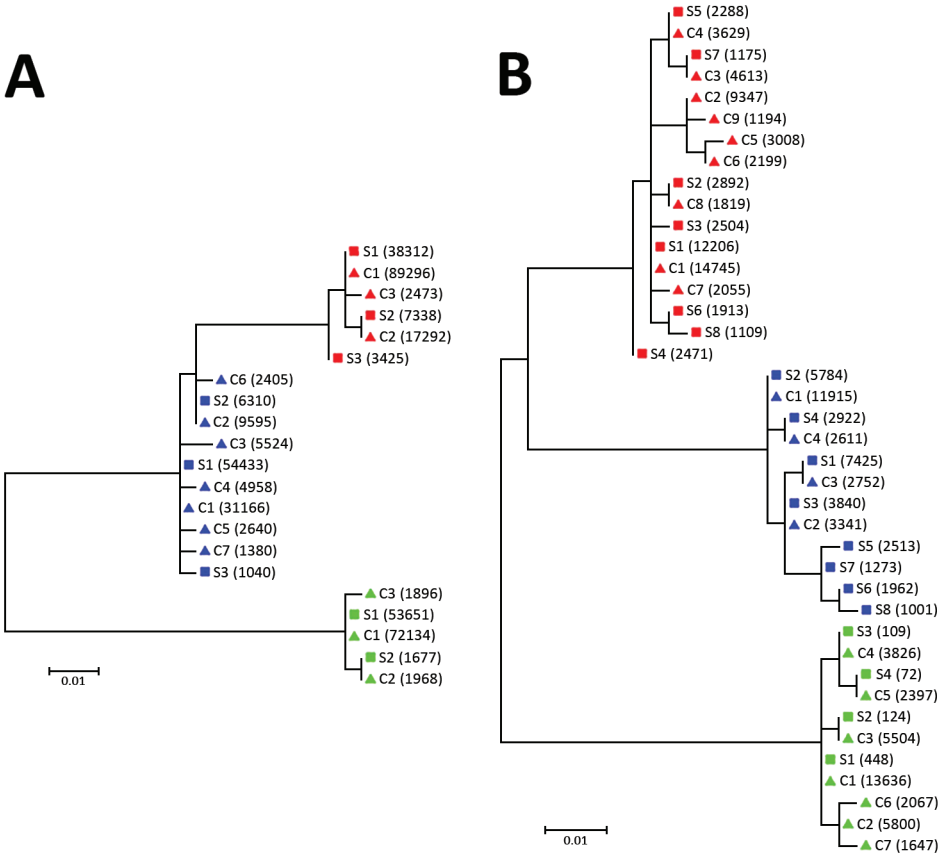

Figure 2. Phylogenetic analysis of A) 5′ UTR and B) E2 region sequences of human pegivirus from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Phylogenic trees were generated using ClustalX version 2.0 (http://www.clustal.org/clustal2/). Viral variant frequencies follow haplotype number. Red indicates patient 1; blue, patient 2; green, patient 3. Scale bars indicate number of nucleotide substitutions per site. C, cerebrospinal fluid; S, serum; UTR, untranslated region.

```
Patient 1 1839
                 |
S1(7425)    TCCTACACCA TGACCAAAAT CCGGGACTCC TTACACTTGG TGAAATGTCC CACACCAGCC ATTGAGCCTC CCACCGGGAC GTTTGGGTTC TTCCCCGGAG TGCCGCCCCT TAACAACTGC
S2(5784)    ......G... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(3840)    ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(2922)    .......... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S5(2513)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S6(1962)    ......G... ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S7(1273)    ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S8(1001)    .......... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(11915)   ......G... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(3341)    ......G... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(2752)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(2611)    .......... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

S1(7425)    ATGCTGTTGG GCACGGAAGT GTCTGAGGCA TTGGGTGGAG CTGGCCTCAC GGGGGGGTTC TATGAACCCC TGGTGCGCAG GTGTTCGGAG CTGATGGGGC GCCGGAATCC GGTCTGCCCG
S2(5784)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(3840)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(2922)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S5(2513)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S6(1962)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S7(1273)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S8(1001)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(11915)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(3341)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(2752)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(2611)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                                                                                                                                  2178
S1(7425)    GGGTATGCAT GGCTCTCCTC GGGACGGCCT GATGGGTTTA TACATGTTCA GGGACACTTG CAGGAGGTCG ATGCTGGCAA CTTCATCCCT CCCCCACGCT
S2(5784)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(3840)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(2922)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S5(2513)    .....C.... .......... .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
S6(1962)    .....C.... .......... .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
S7(1273)    .....C.... .......... .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
S8(1001)    .....C.... .......... .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
C1(11915)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(3341)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(2752)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(2611)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Patient 2 1839
                 |
S1(12206)   TCCTACGCCA TGACCAAGAT CCGAGACTCC TTACATCTGG TGAAATGTCC CACACCAGCC ATTGAGCCTC CCACCGGGAC GTTTGGGTTC TTCCCTGGGA CGCCGCCCCT CAACAACTGT
S2(2892)    ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(2504)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(2471)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S5(2288)    .......... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S6(1913)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S7(1175)    ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S8(1109)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(14745)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(9347)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........C
C3(4613)    ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3629)    .......... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(3008)    ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... .........C
C6(2199)    .......... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... .........C
C7(2055)    .......... .......... .......... .......... .......... .......... .......... .......... .C........ .......... .......... ..........
C8(1819)    ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C9(1194)    ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........C

S1(12206)   ATGCTGCTGG GCACGGAAGT GTCTGAGGCA TTGGGTGGGG CTGGCCTCAC GGGGGGGTTC TATGAACCCC TGGTGCGCAG GTGTTCGGAG CTGATGGGGC GCCGGAATCC AGTCTGCCCG
S2(2892)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(2504)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(2471)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S5(2288)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S6(1913)    ........A. .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S7(1175)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S8(1109)    ........A. .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(14745)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(9347)    .......... .......... ..T....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(4613)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3629)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(3008)    .......... .......... ..T....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C6(2199)    .......... .......... ..T....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C7(2055)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C8(1819)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C9(1194)    .......... .......... ..T....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                                                                                                                                  2178
S1(12206)   GGGTATGCAT GGCTTTCCTC GGGCCGGCCT GACGGGTTTA TACATGTCCA GGGCCACTTG CAGGAGGTCG ATGCTGGCAA CTTCATCCCT CCGCCACGTT
S2(2892)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(2504)    .......... .......... .......... .......... .......... .......... .........T. .......... .......... .......... ..........
S4(2471)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......C.. ..........
S5(2288)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S6(1913)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S7(1175)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S8(1109)    .......... .......... .......... .......... .......... .......... .......... .......... .......... ........C. ..........
C1(14745)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(9347)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(4613)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3629)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(3008)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C6(2199)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C7(2055)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C8(1819)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C9(1194)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

Patient 3 1839
                 |
S1(448)     TCCTACGCCA TGACCAAGAT CCGAGACACC CTACATCTGG TGAAATGTCC CACACCAGCC ATTGAGCCTC CCACCGGGAC GTTTGGGTTC TTCCCCGGGAC ACCGCCCCT TAACAACTGT
S2(124)     .......... ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(109)     ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(72)      ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(13636)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(5800)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(5504)    .......... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3826)    ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(2397)    ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C6(2067)    ......A... .......A.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C7(1647)    ......A... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

S1(448)     ATGCTCTTGG GCACGGAAGT GTCCGAAGCA CTTGGCGGGG CTGGTCTCAC GGGGGGGTTC TATGAACCCC TGGTGCGCAG GTGTTCGGAG CTGATGGGAC GCCGCAATCC AGTCTGTCCG
S2(124)     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(109)     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(72)      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(13636)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(5800)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C3(5504)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3826)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(2397)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C6(2067)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C7(1647)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                                                                                                                                  2178
S1(448)     GGGTTTGCAT GGCTCTCCTC GGGCAGACCT GACGGGTTTA TACATGTTCA GGGCCACTTG CAGGAGGTAG ATGCTGGCAA CTTCATCCCT CCTCCACGCT
S2(124)     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S3(109)     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
S4(72)      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C1(13636)   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C2(5800)    .......... .......... .......... .......... .......... .......... .......... .......... .......... ....T..... ..........
C3(5504)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C4(3826)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C5(2397)    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
C6(2067)    .......... .......... .......... .......... .......... .......... .......... .......... .......... ....T..... ..........
C7(1647)    .......... .......... .......... .......... .......... .......... .......... .......... .......... ....T..... ..........
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Figure 3. Comparison of E2 region human pegivirus sequences amplified from serum and cerebrospinal fluid from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Numbers in parentheses represent the number of reads representing a given sequence. Shading indicates sequences unique to cerebrospinal fluid. Nucleotide numbering follows the reference strain published by Linnen et al (*2*) (GenBank accession no. NC_001710.1). C, cerebrospinal fluid; S, serum.

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Patient 1
                461                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (7425)       SYTMTKIRDSLHLVKCPTPAIEPPTGTFGFFPGVPPLNNCMLLGTEVSEALGGAGLTGGFYEPLVRRCSELMGRRNPVCPGYAWLSSGRPDGFIHVQGHLQEVDAGNFIPPPR
S2 (5784)       ..A.....................................................................................................................
S3 (3840)       ..A.....................................................................................................................
S4 (2922)       ........................................................................................................................
S5 (2513)       ........................................................................................................................
S6 (1962)       ..A.....................................................................................................................
S7 (1273)       ..A.....................................................................................................................
S8 (1001)       ........................................................................................................................
C1 (11915)      ..A.....................................................................................................................
C2 (3341)       ..A.....................................................................................................................
C3 (2752)       ........................................................................................................................
C4 (2611)       ........................................................................................................................
Patient 2
                461                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (12206)      SYAMTKIRDSLHLVKCPTPAIEPPTGTFGFFPGTPPLNNCMLLGTEVSEALGGAGLTGGFYEPLVRRCSELMGRRNPVCPGYAWLSSGRPDGFIHVQGHLQEVDAGNFIPPPR
S2 (2892)       ..T.....................................................................................................................
S3 (2504)       ........................................................................................................................
S3 (2471)       ........................................................................................................................
S5 (2288)       ........................................................................................................................
S6 (1913)       ........................................................................................................................
S6 (1175)       ..T.....................................................................................................................
S8 (1109)       ........................................................................................................................
C1 (14745)      ........................................................................................................................
C2 (9347)       ..........................................F.............................................................................
C3 (4613)       ..T.....................................................................................................................
C4 (3629)       ........................................................................................................................
C5 (3008)       ..T.......................................F.............................................................................
C6 (2199)       ..........................................F.............................................................................
C7 (2055)       ............................S...........................................................................................
C8 (1819)       ..T.....................................................................................................................
C9 (1194)       ..T.......................................F.............................................................................
Patient 3
                461                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (448)        SYAMTKIRDTLHLVKCPTPAIEPPTGTFGFFPGTPPLNNCMLLGTEVSEALGGAGLTGGFYEPLVRRCSELMGRRNPVCPGFAWLSSGRPDGFIHVQGHLQEVDAGNFIPPPR
S2 (124)        ........................................................................................................................
S3 (109)        ..T.....................................................................................................................
S4 (72)         ..T.....................................................................................................................
C1 (13636)      ........................................................................................................................
C2 (5800)       ........................................................................................................................L.
C3 (5504)       ........................................................................................................................
C4 (3826)       ..T.....................................................................................................................
C5 (2397)       ..T.....................................................................................................................
C6 (2067)       ........................................................................................................................L.
C7 (1647)       ..T.....................................................................................................................L.
```

Figure 4. Comparison of amino acid composition of E2 region human pegivirus sequence variants amplified from serum and cerebrospinal fluid from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Numbers in parentheses represent the number of reads representing a given sequence. Shading indicates sequences unique to cerebrospinal fluid. C, cerebrospinal fluid; S, serum.

It is unclear how HPgV could access the CNS. Although initially HPgV was thought to be a hepatotropic virus, the viral negative strand, which is the putative replicative intermediate of the virus, was not detected in liver tissue (*3*); however, it was found in bone marrow and spleen (*33*,*34*). The virus is now considered lymphotropic because it can be detected at a low level in multiple lineages of peripheral leukocytes (*35*,*36*), and it has been speculated that the primary target could be a progenitor hematopoietic stem cell. The route for CNS access could be through infected leukocytes; all basic groups (T cells, B cells, macrophage/monocytes, and NK cells) have the ability to enter the brain under certain conditions (*37*). Certain monocyte family members are constantly replaced as part of normal physiology (*38*,*39*), while the entry of T cells and B cells depends largely on their activation state (*40*,*41*). Such a phenomenon related to trafficking of infected leukocytes through the blood–brain barrier has been long postulated for HIV-1 neuroinfection (*42*).

Whether HPgV was the causative factor of encephalitis in the patients we describe is not clear. Viral pathogens are typically present only transiently in CSF and this window could be easily missed, particularly when the spinal tap is done too late in the course of illness (*43*). If HPgV encephalitis exists, it could be a rare phenomenon. In a previous study of 17 encephalitis and aseptic meningitis cases of unknown cause, no CSF sample was positive (*44*). However, even infections with well-known neurotropic agents from the Flaviviridae family, such as WNV or TBEV, are usually subclinical or asymptomatic; clinical signs and symptoms develop only in 5%–30% of cases (*45*,*46*). Of note, in 2 of the cases we describe, the encephalitis was mild, and all 3 patients recovered without any neurologic sequelae. Obviously, the mere presence of a pathogen in the CNS in patients with encephalitis does not prove causality; for example, HCV sequences are commonly detected in brain and CSF of infected patients without any accompanying evidence of encephalitis (*29*). In our study, we detected HPgV sequences in CSF only in patients without an obvious cause of encephalitis and in none of the patients in whom a known pathogen was identified.

The identified 5′ UTR and E2 region sequence differences between CSF and serum compartments could have biological meaning. The 5′ UTR contains an internal ribosomal entry site that allows cap-independent viral translation (*28*). Such structures were identified within the 5′ UTR of the picornaviruses and were shown to interact with cellular proteins, thus affecting the host range of individual viruses (*47*). Research has also shown that, for HCV, translation efficiencies of brain-derived internal ribosomal entry site variants are generally lower than those found in serum, which could be a viral strategy favoring latency in the CNS (*31*). Taking this into consideration, we speculated that at least some of the 5′ UTR changes in the patients we report represent tissue-specific adjustment. Viral adaptive changes could be relatively small and yet make a huge difference; for example, it has been demonstrated for lymphocytic choriomeningitis virus in mice that variants differing by a single amino acid substitution are competitively selected either by the liver and spleen or by neurons (*48*).

On the amino acid level, we saw 2 unique E2 region changes in CSF variants compared with serum: in patient 2, serine was changed to phenylalanine at aa position 508, and in patient 3, proline was changed to leucine at position 572. Both were within regions predicted to contain B-cell epitopes, thus suggesting that they were the effect of immune pressure. Furthermore, the change in patient 3 was located in the region of E2 that was experimentally shown to contain a strong antigenic site and likely to be involved in cell binding or fusion (*49*).

RNA viruses in particular are characterized by a high degree of genetic heterogeneity; probably because the lack of proofreading 3′ 5′ exonuclease activity in viral RNA polymerases causes low fidelity. As a result, viruses circulate in the infected host as a population of closely related but nonidentical genomes, referred to as quasispecies (*50*). It is unclear whether the observed high HPgV variability developed in the patients we describe de novo after infection or if most or all variants were transmitted from the infecting host; both mechanisms could occur together. However, because viral transmission is typically accompanied by narrowing of the quasispecies spectrum (known as the bottleneck phenomenon), some extent of postinfection evolution is highly likely.

In summary, we detected HPgV sequences in the CSF of 3 patients with encephalitis of unclear origin, and these sequences from CSF differed from those circulating in serum. These findings are compatible with the presence of a separate viral compartment in the CNS. Determining if the pegivirus was responsible for encephalitis or if it was present along with another cause of encephalitis will require further research, including histopathological analysis.

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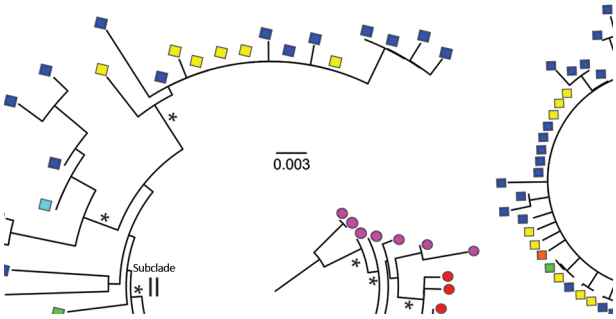
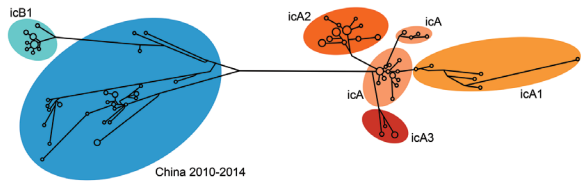
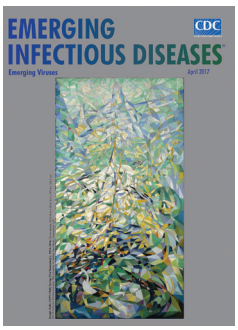
- Biologic Evidence Required for Zika Disease Enhancements by Dengue Antibodies
- Neurologic Complications of Influenza B Virus Infection in Adults, Romania
- Implementation and Initial Analysis of a Laboratory-Based Weekly Biosurveillance System, Provence-Alpes-Côte d'Azur, France
- Transmission of Hepatitis A Virus through Combined Liver–Small Intestine–Pancreas Transplantation
- Influence of Referral Pathway on Ebola Virus Disease Case-Fatality Rate and Effect of Survival Selection Bias
- *Plasmodium malariae* Prevalence and *csp* Gene Diversity, Kenya, 2014 and 2015



- Presence and Persistence of Zika Virus RNA in Semen, United Kingdom, 2016
- Three Divergent Subpopulations of the Malaria Parasite *Plasmodium knowlesi*
- Variation in *Aedes aegypti* Mosquito Competence for Zika Virus Transmission
- Outbreaks among Wild Birds and Domestic Poultry Caused by Reassorted Influenza A(H5N8) Clade 2.3.4.4 Viruses, Germany, 2016
- Highly Pathogenic Avian Influenza A(H5N8) Virus in Wild Migratory Birds, Qinghai Lake, China



- Design Strategies for Efficient Arbovirus Surveillance
- Typhus Group Rickettsiosis, Texas, 2003–2013
- Detection and Molecular Characterization of Zoonotic Poxviruses Circulating in the Amazon Region of Colombia, 2014
- Reassortment of Influenza A Viruses in Wild Birds in Alaska before H5 Clade 2.3.4.4 Outbreaks
- Incidence and Characteristics of Scarlet Fever, South Korea, 2008–2015
- Markers of Disease Severity in Patients with Spanish Influenza in the Japanese Armed Forces, 1919–1920
- Molecular Identification of *Spirometra erinaceieuropaei* in Cases of Human Sparganosis, Hong Kong
- Zika Virus Seroprevalence, French Polynesia, 2014–2015
- Persistent Arthralgia Associated with Chikungunya Virus Outbreak, US Virgin Islands, December 2014–February 2016
- Assessing Sensitivity and Specificity of Surveillance Case Definitions for Zika Virus Disease
- Detection of Zika Virus in Desiccated Mosquitoes by Real-Time Reverse Transcription PCR and Plaque Assay
- Surveillance and Testing for Middle East Respiratory Syndrome Coronavirus, Saudi Arabia, April 2015–February 2016



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Article DOI: https://doi.org/10.3201/eid2410.180161

Human Pegivirus in Patients with Encephalitis of Unclear Etiology, Poland

Technical Appendix

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Patient 1 155
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (54433) CACCTTGGTA GCCACTATAG GTGGGTCTTA AGAGAAGGTT AAGATTCCTC TTGTGCCTGC GGCGAGACCG CGCACGGTCC ACAGGTGTTG GCCCTACCGG TGTGAATAAG GGCCCGACGT
S2 (6310)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
S3 (1040)  .......... .......... .......... .......... .......... .......... .......... .........T .......... .......... .......... .......... 
C1 (31166) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (9595)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C3 (5524)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......G.. .......... 
C4 (4958)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C5 (2640)  .......... .......... .........C.... .......... .......... .......... .......... .......... .......... .......... .......... 
C6 (2405)  .......... .......... .......... .......... .......... .......... .......... .......... G......... .......... .......... .......... 
C7 (1380)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 

S1 (54433) CAGGCTCGTC GTTAAACCGA GCCCGTCACC CACCTGGGCT AACGACGCCC ACGTACGGTC CACGTCGCCC TTCAATGTCT CTCTTGACCA ATAGGTTTAT CCGGCGAGTT GACAAGGACC
S2 (6310)  .......... .......... ......T... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
S3 (1040)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C1 (31166) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (9595)  .......... .......... ......T... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C3 (5524)  .......... .......G.. .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C4 (4958)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .G........ 
C5 (2640)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C6 (2405)  .......... .......... ......T... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C7 (1380)  .......... .......... .........T .......... .......... .......... .......... .......... .......... .......... .......... .......... 
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (54433) AGTGGGGGCC GGGGGTCATG GGGATGGACC CCAGACCCTG CCCTTCCCGG TGGGCCGGGA AÅ 456 ΔG (kcal/mol)
S2 (6310)  .......... .......... .......... .......... .......... .......... .. -76.0
S3 (1040)  .......... .......... .......... .......... .......... .......... .. -76.0
C1 (31166) .......... .......... .......... .......... .......... .......... .. -76.0
C2 (9595)  .......... .......... .......... .......... .......... .......... .. -76.0
C3 (5524)  .......... .......... .......... .......... .......... .......... .. -76.1
C4 (4958)  .......... .......... .......... .......... .......... .......... .. -76.0
C5 (2640)  .......... .......... .......... .......... .......... .......... .. -76.0
C6 (2405)  .......... .......... .......... .......... .......... .......... .. -76.0
C7 (1380)  .......... .......... .......... .......... .......... .......... .. -73.0

Patient 2 155
S1 (38312) CACCTTGGTA GCCACTATAG GTGGGTCTTA AGAGAAGGTT AAGATTCCTC TTGTGCCTGC GGCGAGACCG CGCACGGTCC ACAGGTGTTG GCCCTACCGG TGAGAATAAG GGCCCGACAT
S2 (7338)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
S3 (3425)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C1 (89296) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (17292) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....A.... 
C3 (2473)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .....A.... 

S1 (38312) CAGGCATGTC GTTAAACCGA GCCCGTTACC CGCCTGGGCT AACGACGCCC ACGTACGGTC CACGTCGCCC TTCAATGTCT CTCTTGACCA ATAGGTTTAT CCGGCGAGTT GACAAGGACC
S2 (7338)  .......... .......... .......... .......... .......... .......... .......... .......... .......C.. .......... .......... .......... 
S3 (3425)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C1 (89296) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (17292) .......... .......... .......... .......... .......... .......... .......... .......... .......C.. .......... .......... .......... 
C3 (2473)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (38312) AGTGGGGGCC GGGGGTTATG GGGAAGGACC CCAGACCCTG CCCTTCCCGG CGGACCGGGA AÅ 456 ΔG (kcal/mol)
S2 (7338)  .......... .......... .......... .......... .......... .......... .. -71.0
S3 (3425)  .......... ......C... .......... .......... .......... .......... .. -73.6
C1 (89296) .......... .......... .......... .......... .......... .......... .. -71.0
C2 (17292) .......... .......... .......... .......... .......... .......... .. -71.0
C3 (2473)  .......... .......... .......... .......... .......... .......... .. -66.1

Patient 3 155
S1 (53651) CATCTTGGTA GCCACTATAG GTGGGTCTTA AGAGAAGGTT AAGATTCCTC TTGCGCCTGC GGCGAGACCG CGCACGGTCC GCAGGTGTTG GCCCTACCGG TGTGAATAAG GGCCCGACGT
S2 (1677)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C1 (72134) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (1968)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C3 (1896)  .......... .......... .......... .......... .......... .......... .......... .........T. .......... .......... .......... .......... 

S1 (53651) CAGGCTCGTC GTTAAACCGA GCCCGTCACC CACCTGGGCA AACGACGCCC ACGTACGGTC CACGTCGCCC TTCAATGTCT CTCTTGACCA ATAGGCTTAG CCGGCGAGTT GACAAGGACC
S2 (1677)  ....T..... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C1 (72134) .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C2 (1968)  ....T..... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
C3 (1896)  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
                                                                                                                                                                                                                                                                                                                                                                                                                                                
S1 (53651) AGTGGGGGCC GGGGGCTTGG AGAAGGACTC CAAGTCCCGC CCTTCCCGGT GGGCCGGGAA AT 456 ΔG (kcal/mol)
S2 (1677)  .......... .......... .......... .......... .......... .......... .. -79.0
C1 (72134) .......... .......... .......... .......... .......... .......... .. -79.0
C2 (1968)  .......... .......... .......... .......... .......... .......... .. -79.0
C3 (1896)  .......... .......... .......... .......... .......... .......... .. -79.0
```

Technical Appendix Figure. Comparison of 5′ untranslated region human pegivirus sequences amplified from serum and cerebrospinal fluid from 3 patients with encephalitis of unclear origin, Poland, 2012–2015. Numbers in parentheses represent the number of reads representing a given sequence. Shading indicates sequences unique to cerebrospinal fluid. Nucleotide numbering follows the reference strain published by Linnen et al (*2*) (GenBank accession no. NC_001710.1). Free energies of the predicted secondary RNA structures were calculated by using Mfold version 3.2 (*26*), and the structures themselves followed the model proposed by Simons et al. (*28*). C, cerebrospinal fluid; S, serum.