No.	Sex	Age at encephalitis (Y)	Age at epilepsy onset (Y)	Age at time of study (Y)	Epilepsy syndrome	Seizure frequency (times/4 weeks)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)	Ratio of MMP-9/ TIMP-1	S-NT2	S-M3-4	S-CT1	C-NT2	C-M3-4	C-CT1	Albumin
26.	F	0.3	NA	28	LRE	NA	140.6	169.2	0.83	NA	NA	NA	NA	NA	NA	NA
27.	M	3	NA	14	LRE	NA	92.9	130.3	0.71	NA	NA	NA	NA	NA	NA	NA
28.	F	1	NA	20	SGE	NA	37.8	101.9	0.37	NA	NA	NA	NA	NA	NA	NA
29.	M	4.3	4.4	18	LRE	420	43	138.9	0.31	NA	NA	NA	NA	NA	NA	NA
		4.3	4.4	20	LRE	196	28.2	191.6	0.15	NA	NA	NA	NA	NA	NA	NA
30.	M	17.4	17.4	19	LRE	28	84.4	124.1	0.68	NA	NA	NA	NA	NA	NA	NA
		17.4	17.4	19	LRE	18	45.2	149.6	0.30	0.240	0.320	0.285	NA	NA	NA	NA
31.	F	21	23	23	LRE	NA	136.8	186.6	0.73	0.405	0.498	0.444	0.298	0.301	0.290	NA
32.	M	15	15	15	LRE	12	95.6	156.4	0.61	0.219	0.264	0.234	NA	NA	NA	19.0
33.	F	28	28	29	LRE	3	89.7	173.2	0.52	0.340	0.607	0.384	0.089	0.105	0.113	21.0
34.	F	0.4	2	13	West syndrome	NA	113.9	104.0	1.10	0.439	0.784	1.021	0.448	0.320	0.282	8.6
35.	M	3	NA	5	LRE	NA	107.4	236.3	0.45	0.633	0.939	0.850	1.104	1.149	1.000	22.4
36.	F	28	NA	29	LRE	0	40	134.0	0.30	0.428	0.519	0.482	0.466	0.431	0.438	NA
37.	F	26	26	31	LRE	3	56.1	173.6	0.32	0.616	0.634	0.776	NA	NA	NA	NA
		26	26	33	LRE	4	18.0	125.6	0.14	0.334	0.406	0.571	NA	NA	NA	NA
38.	M	6	6	7	LRE	NA	55.7	142.7	0.39	NA	NA	NA	NA	NA	NA	18.6
		6	6	7	LRE	140	52.4	135.5	0.39	NA	NA	NA	NA	NA	NA	NA
39.	F	11	11	23	LRE	6	36.3	110.2	0.33	NA	NA	NA	NA	NA	NA	NA
		11	11	23	LRE	2	29.5	117.4	0.25	NA	NA	NA	NA	NA	NA	NA
40.	F	1.1	1.5	5	LRE	120	47.0	114.6	0.41	NA	NA	NA	NA	NA	NA	NA
41.	M	6	6	11	LRE	31	24.9	105.8	0.24	1.226	NA	NA	0.345	0.299	0.302	23.2
		6	6	12	LRE	14	28.2	127.3	0.22	NA	NA	NA	NA	NA	NA	NA
42.	M	7	7	9	LRE	84	61.2	154.2	0.40	0.343	0.480	0.477	0.253	0.411	0.437	NA
		7	7	11	LRE	112	41.0	169.0	0.24	NA	NA	NA	NA	NA	NA	NA
43.	F	6	9	10	LRE	0	60.5	140.5	0.43	NA	NA	NA	0.156	0.163	0.298	12.0
44.	M	0.9	1.3	12	West syndrome	140	102.4	191.0	0.54	3.243	3.224	3.293	0.700	0.818	0.871	NA
45.	M	28	28	37	LRE	NA	208.1	113.1	1.84	NA	NA	NA	NA	NA	NA	NA
46.	F	8	8	11	LRE	1	69.5	106.9	0.65	NA	NA	NA	NA	NA	NA	NA

AEDs: antiepileptic drugs; C-CT1: antibodies against GluR epsilon 2-CT1 in the CSF; C-M3-4: antibodies against GluR epsilon 2-M3-4 in the CSF; C-NT2: antibodies against GluR epsilon 2-NT2 in the CSF; LRE: Localization-related epilepsy; NA: no data available; S-CT1: antibodies against GluR epsilon 2-CT1 in the serum; S-M3-4: antibodies against GluR epsilon 2-M3-4 in the serum; S-NT2: antibodies against GluR epsilon 2-NT2 in the serum; SGE: symptomatic generalized epilepsy; Y: years.

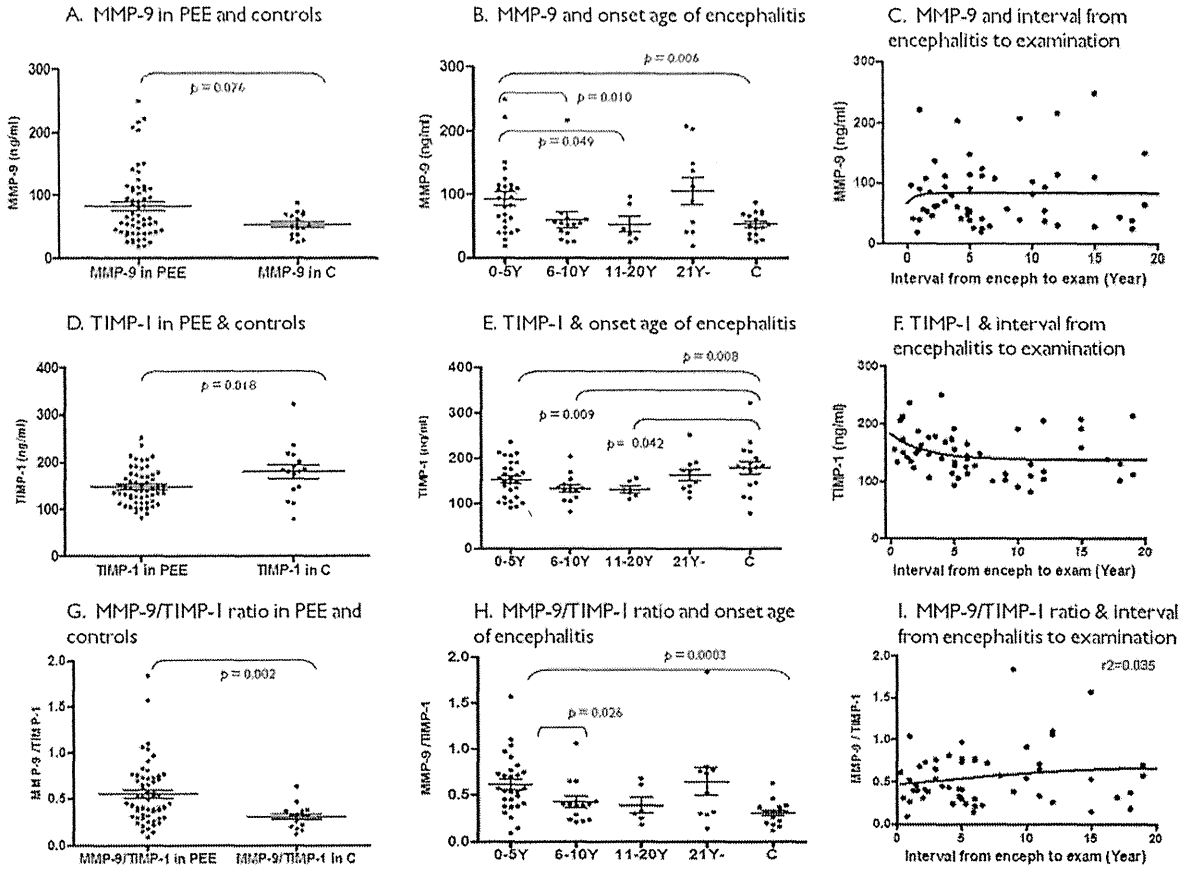

Figure 2. A – Serum MMP-9 levels in PEE patients and control subjects; **B** – Serum MMP-9 levels stratified by age of occurrence of encephalitis; **C** – Relationship between serum MMP-9 levels and time interval from encephalitis to study; **D** – Serum TIMP-1 levels in PEE patients and control subjects; **E** – Serum TIMP-1 levels stratified by age of occurrence of encephalitis; **F** – Relationship between serum TIMP-1 levels and time interval from encephalitis to study; **G** – Serum MMP-9/TIMP-1 ratios in PEE patients and control subjects; **H** –Serum MMP-9/TIMP-1 ratios stratified by age of occurrence of encephalitis; **I** – Relationship between serum MMP-9/TIMP-1 ratios and time interval from encephalitis to study. The longer horizontal bars represent mean levels, and the shorter horizontal bars represent mean ±standard error values.

C = control subjects; enceph. = encephalitis; PEE = postencephalitic epilepsy.

Table 2. Antibodies to GluR epsilon 2 in controls (9 patients)

Samples	Sera			CSF		
	NT2	M3-4	CT1	NT2	M3-4	CT1
Mean concentration (units?)	0.415	0.591	0.496	0.172	0.200	0.211
± SD	0.102	0.123	0.109	0.080	0.087	0.082
Number of samples	13	11	11	15	13	13

SD – standard deviation

croglia (Sredni-Kenigsbuch, 2002). These particular effects of IFN γ enhance the autoimmune cytotoxic process mediated by $CD8^+$ T cells in CNS. Elevation of these cytokines in CNS facilitated by excessive BBB permeability may result in increased seizure frequency.

Excessive BBB permeability results in increased levels of albumin in CNS. Direct brain exposure to serum albumin results in albumin uptake into astrocytes through transforming growth factor-beta receptors, induces NMDA-receptor–mediated neuronal hyperexcitability and subsequently epileptiform activity (Ivens et al.,

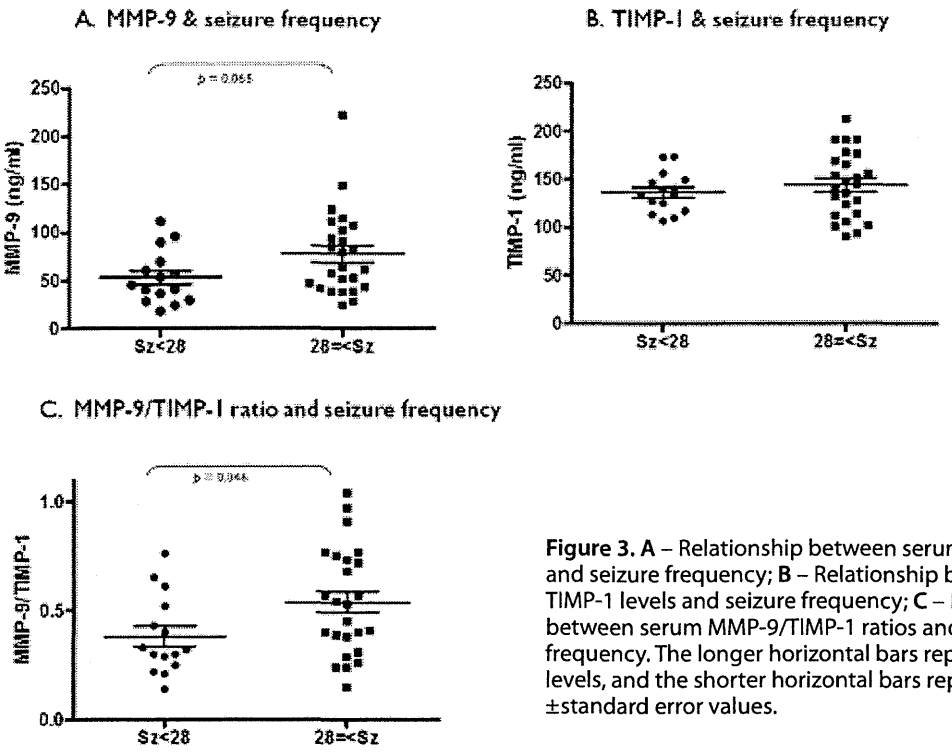

Figure 3. A – Relationship between serum MMP-9 levels and seizure frequency; **B** – Relationship between serum TIMP-1 levels and seizure frequency; **C** – Relationship between serum MMP-9/TIMP-1 ratios and seizure frequency. The longer horizontal bars represent mean levels, and the shorter horizontal bars represent mean ±standard error values.

2007). Therefore, elevated albumin levels in CNS may contribute to the pathological mechanisms of intractable epilepsy. However, in PEE, albumin levels in CSF were not correlated to seizure frequency (Figure 4I).

Our data reveals that antibodies against GluR epsilon 2-NT2 in CSF have a weak relationship with seizure frequency (figure 4G). Anti-double-stranded DNA antibodies in CSF from patients with systemic lupus erythematosus cross-react with the N-terminus of GluR epsilon 2 and cause neuronal apoptosis in the rat hippocampus (DeGiorgio et al., 2001). It is, therefore, possible that antibodies against GluR epsilon 2 may also cause apoptosis in the hippocampus. Furthermore, antibodies against the N-terminus of GluR epsilon 2 have been reported to cause hippocampal neuron damage with ensuing memory impairment (Kowal et al., 2006) and amygdala neuron damage with emotional behavior impairment (Huerta et al., 2006). In Rasmussen syndrome, antibodies against GluR epsilon 2 are detected in the acute stage with higher frequencies of epileptic seizures (Takahashi et al., 2009). Therefore, antibodies against GluR epsilon 2 may contribute to the intractability of epileptic seizures and neurological sequelae in PEE via induction of apoptosis.

A study in young patients with influenza-associated encephalopathy showed that a higher MMP-9/TIMP-1 ratio in the acute stage is related to a poorer clinical condition (Ichiyama et al., 2007). PEE patients with early onset encephalitis appeared to sustain BBB dysfunction late after encephalitis in the chronic epileptic stage. Sustained abnormality of MMP-9/TIMP-1 may contribute to a continuous influx of cytokines, antibodies, and other blood constituents into the CNS, and subsequently to higher frequencies of epileptic seizures and their neurological sequelae. Children are more prone to BBB dysfunction than adults, because BBB is not fully developed in children. BBB development and maintenance is tightly regulated by permanent interaction of endothelial cells with the tissue microenvironment (i.e. extracellular matrix) (Engelhardt, 2003). Acute encephalitis would disturb the maturation process, break down the BBB integrity and reduce the effectiveness of recovery.

Other than excessive permeability of BBB, MMP-9 contributes to the remodeling of dendritic spines and aberrant synaptogenesis, resulting in epileptogenesis in animal models of epilepsy (Wilczynski et al., 2008). These effects of MMP-9 in excitatory neuron network formation may be necessary for intractability of PEE.

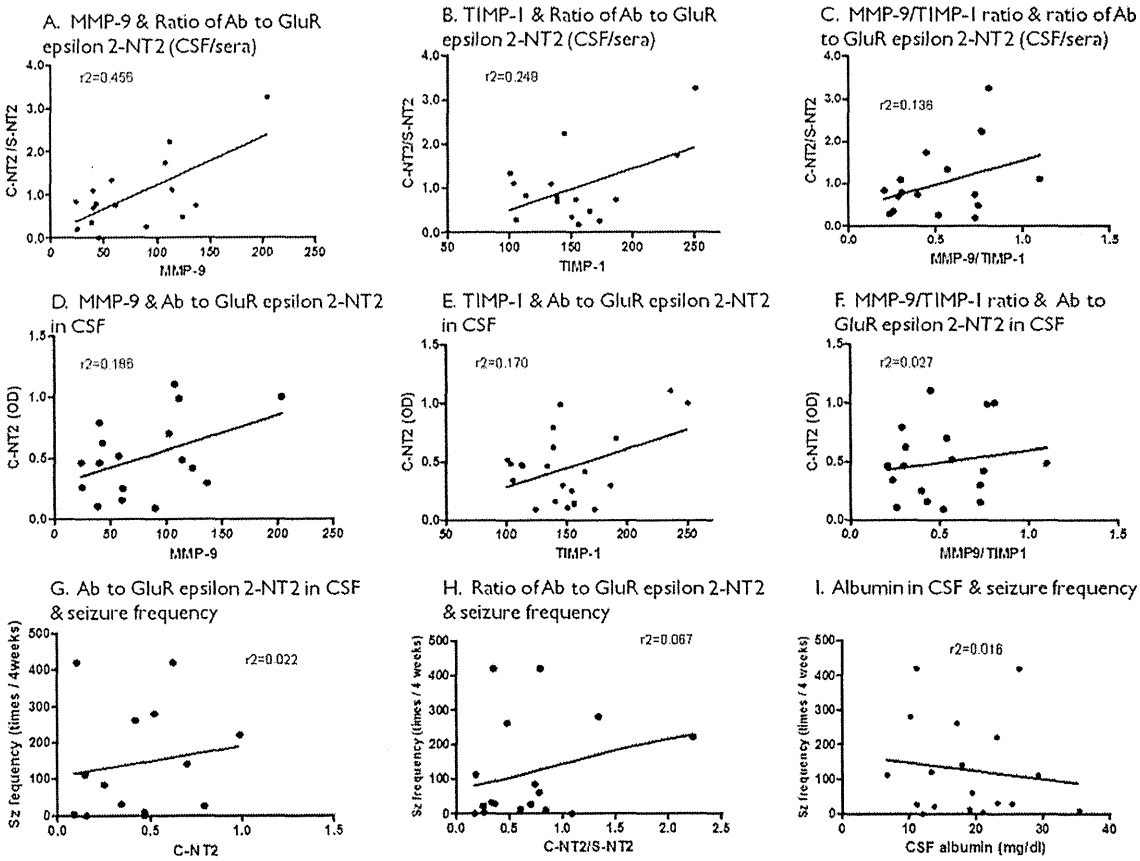

Figure 4. A – Relationship between serum MMP-9 levels and CSF/sera ratio of antibodies against GluR epsilon 2-NT2; **B** – Relationship between serum TIMP-1 levels and CSF/sera ratio of antibodies against GluR epsilon 2-NT2; **C** – Relationship between serum MMP-9/TIMP-1 ratios and CSF/sera ratios of antibodies against GluR epsilon 2-NT2; **D** – Relationship between serum MMP-9 levels and antibodies against GluR epsilon 2-NT2 in CSF; **E** – Relationship between serum TIMP-1 levels and antibodies against GluR epsilon 2-NT2 in CSF; **F** – Relationship between serum MMP-9/TIMP-1 ratios and antibodies against GluR epsilon 2-NT2 in CSF; **G** – Relationship between antibodies against GluR epsilon 2-NT2 in CSF and seizure frequency; **H** – Relationship between CSF/sera ratios of antibodies against GluR epsilon 2-NT2 and seizure frequency; **I** – Relationship between albumin levels in CSF and seizure frequency.

Ab – antibody; S – serum.

Our study suggested serum MMP-9/TIMP-1 and CSF/sera ratio of GluR epsilon 2-NT2 antibodies as probable markers of BBB dysfunction. S100B protein has reported as the serum marker of BBB dysfunction from data in patients with osmotic disruption of BBB (Marchi et al., 2007, 2012). However, data in traumatic brain injury or subarachnoid hemorrhage could not show any contribution of a compromised BBB or blood-CSF barrier to S100B serum levels (Kleindienst, et al., 2010). Therefore there is a need to identify more reliable serum markers of BBB disruption.

The results of this study may bring new insight into the pathogenesis and intractability of PEE, and may have important implication on the choice of intervention for PEE. Agents that alter BBB permeability are candidates for PEE prevention and the control of epilepsy progression (Dormán et al., 2007; Yamaguchi et al., 2007). Recently, we have reported that pranlukast may reduce epileptic seizures by normalizing serum levels of MMP-9 (Takahashi et al., 2012). Further studies of BBB dysfunction and intractability of epileptic seizures using levels of MMP-9 in CSF are needed.

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Statement of compliance with the Journal's guidelines for ethical standards in publishing

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

DISCLOSURE OF CONFLICTS OF INTEREST

None of the authors has any conflicts of interest to disclose.

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Characteristics of epilepsy and immunological markers in epileptic patients after influenza-associated encephalopathy

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Abstract

Objective: This study aimed to elucidate the electro-clinical characteristics of epilepsy and immunological markers in patients with epilepsy after influenza-associated encephalopathy/encephalitis (IAE). *Methods:* Eighteen patients with epilepsy after IAE (8 males, 10 females; mean age of onset 6.4±6.4 years) were studied. Antibodies to glutamate receptor (GluR) ε2 (NR2B) were examined by immunoblot and ELISA. Cytokines were measured by BioPlex. *Results:* Mean interval between IAE and epilepsy onset was 63.2 ± 95.0 days (mean ± SD). In 16 of 18 patients, complex partial seizures were observed. Most complex partial seizures were of short durations and showed few lateralizing signs. Interictal discharges were seen in the frontal area in 7 of 14 patients. Ictal EEG showed rapid propagation to bilateral hemispheres. Patients with higher cerebrospinal fluid levels of anti-GluRε2 antibodies, higher cerebrospinal fluid levels of IL-1β, soluble tumor necrosis factor receptor 1 and IFN-γ during chronic stage, had higher frequency of epileptic seizures.
Conclusion: This study indicates that the frontal lobes are susceptible to rapid epileptogenesis after IAE, and that epileptic partial seizures after IAE had characteristics resembling generalized seizures. Presence of anti-GluRε2 antibodies and elevated IL-1β, TNFα, and IFN-γ in cerebrospinal fluid may be associated with intractability of epileptic seizures.

INTRODUCTION

Sequelae affecting the central nervous system are common after influenza-associated encephalopathy / encephalitis (IAE). The death rate of acute disease is approximately 30%. Even among the surviving patients, approximately 25% have neurological impairment[1], and epilepsy occurs at a high frequency.[2] Rather than a direct effect of viral infection, TNFα and other inflammatory cytokines are reported to play an important role in the mechanism of IAE, and rapid progressive apoptosis of neuron is responsible for IAE.[3] Therefore, the mechanism of epilepsy after IAE may differ from epilepsy after encephalitis with direct viral involvement of the cerebral parenchyma, as seen in herpes simplex virus (HSV)-1 encephalitis. The semiological characteristics, pathophysiology and seizure outcome of epilepsy after IAE remain unclear.

Glutamate receptor (GluR) is a receptor for glutamic acid, a neurotransmitter, and is classified into ion channel types and metabotropic types.[4,5] Ion channel type GluRs are classified pharmacologically into NMDA and non-NMDA types. NMDA type GluRs (NR) have a tetrameric structure of heterogeneous subunits, composed of the essential GluRζ1 (NR1) subunits and GluRε1-4 (NR2A-2D) or GluRχ1-2 (NR3A-3B) subunits. Antibodies to GluRε2(NR2B) have been detected in patients with various conditions such as Rasmussen syndrome and non-herpetic acute limbic encephalitis. Antibodies to NMDA type GluRs have been reported to cause internalization of NMDA type GluR complexes, resulting in dysfunction of various neurological pathways.[6]

Recent immunological research in epilepsy has revealed the contributions of cytokines to epileptogenesis and ictogenesis.[7] Interleukin (IL)-1 and tumor necrosis factor (TNFα) are known to be proconvulsant factors. Here we studied the clinical features, ictal and interictal EEGs, MRI findings and outcome of epileptic seizures after IAE, and examined immunological markers

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including anti-GluRε2 antibodies and cytokines in cerebrospinal fluid (CSF). Since TNFα is unstable, soluble tumor necrosis factor receptor 1 (sTNFR1), which is the receptor of TNFα and indicates the levels of TNFα was also measured in our study.

METHODS

Patient background

Between January 2000 and July 2009, 15 patients were treated in our hospital for epilepsy after IAE, and 8 patients had samples and clinical data sent to our center for the purpose of autoantibody testing. Of these 23 patients, those with only convulsions induced by fever and those with a follow-up period less than 3 months were excluded. Eventually, 18 patients (8 males, 10 females) were studied. The age (mean ± SD) at the last clinical follow-up for epilepsy was 11 ± 9 years, and the interval (mean ± SD) from IAE to last follow-up was 5.4 ± 6.3 years. The type of IAE was widespread encephalitis in 4 patients, localized encephalitis in one patient, acute necrotizing encephalopathy in one patient, Reye syndrome in one patient, mild type encephalopathy in 2 patients, unknown in 8 patients.

Influenza virus infection was diagnosed by a positive rapid test for influenza antigen in 17 of 18 patients, and by a two-fold increase of serum antibodies to influenza virus in the remaining patient. The causative influenza virus was type A in 8 patients, type B in 2 patients, and unknown in the remaining 8 patients because the information was not provided by the referring doctors. Three patients received influenza vaccination for that season, 6 patients did not receive vaccination, and no information was available from the remaining 9 patients.

Soon after the onset of influenza, all patients subsequently developed encephalopathy/ encephalitis. The age (mean ± SD) at onset of IAE was 6.4 ± 6.4 years, and 11 patients (61%) were aged 1-5 years at onset (Figure 1A). The first neurological symptom of IAE was febrile convulsive seizures in 50% of patients with onset age at 1-5 years, and impaired consciousness in 25% of patients with onset age at 6-15 years (Figure 1B). Behavioral abnormality was frequently the first neurological symptom in

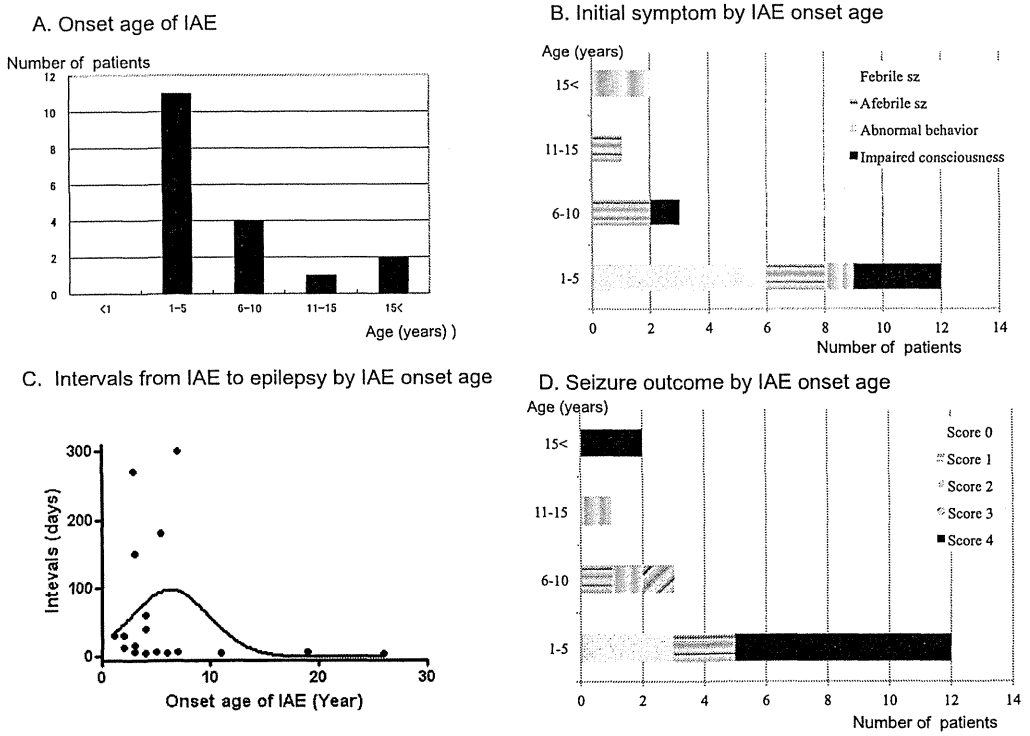


Figure 1. Clinical data of patients with epilepsy after influenza-associated encephalopathy (IAE). **A**: Onset age of IAE. **B**: Initial symptom of IAE according to IAE onset age. **C**: Intervals from IAE to epilepsy according to IAE onset age. **D**: Seizure outcome according to IAE onset age. Score 0 (daily seizures), 1 (weekly seizures), 2 (monthly seizures), 3 (yearly seizures), 4 (no seizures).

patients with onset age older than 11, which mimics the initial symptom of limbic encephalitis. Of 11 patients whose initial neurological symptom was convulsive seizure, 4 had status epileptics. Motor development outcome demonstrated a bipolar trend, with three of 16 patients having severe quadriplegia, and 13 patients free from motor function impairment. The degree of cognitive outcome varied.

Electro-clinical evaluation of epileptic seizures

Ictal semiology of epileptic seizures was evaluated by reviewing medical records and video-EEG recordings (seven patients). Ictal EEGs were evaluated by reviewing video-EEG recordings in seven patients.

Outcome evaluation

Seizure outcome was classified into five categories, score 0 (daily seizures), 1 (weekly seizures), 2 (monthly seizures), 3 (yearly seizures) and 4 (no seizures).

Examination of antibodies to GluRε2 (NR2B)

Antibodies against the GluRε2 molecule were assayed as described previously, by immunoblot using whole molecule of GluRε2 (NR2B) protein as antigen (anti-GluRε2 antibody)[8] and by ELISA using synthetic peptides of the N-terminal and C-terminal of GluRε2 as antigens (anti-GluRε2-NT2 antibody and anti-GluRε2-CT antibody, respectively).[9] Control levels of anti-GluRε2 antibodies were obtained by measuring the antibody levels in the CSF of control patients without epilepsy or IAE (n = 9).

Measurements of cytokines, chemokines and growth factors

The following immunological markers were measured by the BioPlex system (BioRad): IL-1β, IL-1r , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, granulocyte colony-stimulating factor (CSF), granulocyte macrophage CSF, interferon (IFN)-γ, interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)β, regulated upon activation, normal T cell expressed and secreted (RANTES), TNFα, and vascular endothelial growth factor (VEGF). Soluble TNFR1 was determined using an ELISA kit (Cosmo Bio BMS03). Control levels of these markers were obtained by measuring the respective levels in the CSF of control patients without epilepsy or IAE (n = 9)

Statistical analyses

Statistical analyses were performed by Mann-Whitney test. Data are expressed as mean ± SD.

RESULTS

Clinical features of epilepsy

The mean interval from IAE to onset of epilepsy was 63.2 ± 95.0 days (Table 1). Epilepsy occurred within one month after IAE in two-thirds of all patients, but some patients with onset of IAE around 5 years of age had longer intervals of more than 100 days (Figure 1C).

The epileptic seizure type at the last follow-up could be studied in 17 of 18 patients, while the data of one patient was not available. The seizure type was complex partial seizure (CPS) alone in 9 patients, CPS plus secondary generalized tonic-clonic seizure (sGTC) in 5, CPS plus simple partial seizure (SPS) in 2, and sGTC alone in one.

Epilepsy was classified as symptomatic localization-related epilepsy in all 17 patients with confirmed seizure types.

Interictal EEG

Interictal discharges could be studied in 14 patients. Discharges in bilateral frontal lobes were observed in 3 patients, discharges in unilateral frontal lobe in 2 patients, and bilateral independent frontal spikes in 2 patients (Table 1). In 2 patients who had no interictal discharge, seizures were controlled for several years after IAE.

Semiology of epileptic seizures

Of 2 patients with SPS, one manifested nausea, and the other inability to sustain thoughts. No patients had "postural seizures"; i.e., supplementary motor seizures, focal motor seizures or other typical seizures of frontal lobe origin, although interictal discharges were frequent in frontal regions. In seizures documented by video-EEG recordings, lateralizing signs were not observed in the very initial stage, but 4 of 7 patients became to show lateralizing signs after initial stage in the recorded seizures (Table 1).

Table 1: Clinical characteristics of the study patients

Pt	Sex	Type of IAE	Onset of epilepsy		Classification		Interictal EEG	Video- EEG recordings		Antibodies to GluRε2 (CSF)				Seizure outcome	
			Age (yr)	Latency from IAE (days)	Seizure	Epilepsy		Ictal EEG	Ictal semiology	IgG (IB)	IgM (IB)	NT2 (ELISA)	CT (ELISA)	Age	Score
1	M	ANE	2.9	270	sGTC	unknown	bilateral frontal sharp, bilateral diffuse spike	ND	ND	ND	ND	ND	ND	9	4
2	F	unknown	2	30	CPS	SPE	multifocal spikes & multifocal sharp waves	Fig. 3A: small spikes in bilateral frontal→diffuse attenuation→ low voltage rhythmic waves in F3F4Fz	slight extension of shoulders and body trunk→anteflexion of head and trunk→elevation of upper limb forward	ND	ND	ND	ND	4	0
3	F	unknown	3	7	unknown	unknown	unknown	ND	ND	ND	ND	ND	ND	4	4
4	F	unknown	7	7	CPS	SPE	unknown	ND	ND	-	-	ND	ND	7	2
5	F	WE	11	6	CPS, sGTC	SPE	bilateral frontal spike with slow	Fig. 3B: sharp wave in F3→ diffuse attenuation→low voltage rhythmic waves in bil frontal regions	open eyes→extension of right upper extremities, twitching right mouth corner→right version of eyes and right limb hypermovement	ND	ND	ND	ND	25	2
6	F	WE	4	6	SPS, CPS	SPE	multifocal spikes & multifocal sharp waves	ND	ND	-	-	0.34	0.378	4	4
7	M	unknown	4	60	CPS	SPE	bilateral frontal dominant spikes	Fig. 3C: slow waves predominantly in bilateral frontal regions→diffuse attenuation	vibration of bilateral upper limbs in sleep	+	-	0.624	0.619	15	0
8	F	unknown	5.4	180	CPS	SPE	multifocal spikes with slow waves	Fig. 3D: sharp waves in Fp1Fp2 →diffuse attenuation	head pulled back→dropdown	+	-	0.136	0.168	7	0
9	F	unknown	6	5	CPS	SPE	left frontal spikes	ND	ND	-	-	0.156	0.298	10	3
10	M	unknown	1.1	30	CPS, sGTC	SPE	left temporal spikes (T3T5 spike)	slow waves in the F3FP1→slow waves in left hemisphere	motionless→right rotation of head, tonic convulsion of right upper limb	-	-	0.28	0.372	8	4
11	M	Reye syd.	3	150	CPS	SPE	right frontal sharp waves	ND	ND	ND	ND	ND	ND	28	4
12	M	mild	2.1	14	CPS, sGTC	SPE	unknown	ND	ND	+	+	0.237	0.237	2.5	1
13	M	LE	26	4	CPS , SPS	SPE	no interictal discharge	ND	ND	ND	ND	ND	ND	32	4
14	M	mild	3	16	CPS sGTC	SPE	no interictal discharge	ND	ND	ND	ND	ND	ND	6	4
15	M	WE	5	7	CPS	SPE	bilateral independent frontal spikes	Sp2 rhythmic waves at onset	loss of consciousness→tonic convulsion of right upper limb	+	+	0.345	0.302	13	1
16	F	unknown	7	300	CPS, sGTC	SPE	bilateral independent frontal spike with slow waves	Continuous Sp-W or slow waves in left frontal region	loss of consciousness→right version of eyes	+	-	0.554	0.563	7	1
17	F	WE	19	7	CPS	SPE	unknown	ND	ND	ND	ND	ND	ND	19	4
18	F	mild	4	40	CPS	SPE	T5	ND	ND	ND	ND	ND	ND	10	4

ANE; acute necrotizing encephalopathy; WE, widespread encephalitis; LE, localized encephalitis; SPS, simple partial seizure; CPS, complex partial seizure; sGTC, secondarily generalized tonic-clonic seizure; ND, not done; CSF, cerebrospinal fluid; Sp-W, spike with slow; Age, years old; Latency, latency from IAE to epilepsy onset (days); score, score of epileptic seizure in Table1; IB, immunoblot assay; SP, sphenoidal electrode.

Ictal EEG

The time lag (mean ± SD) from the start of ictal discharge to appearance of clinical seizure symptoms was 1.90 ± 1.36 seconds. The mean duration of ictal event was 80.1 ± 73 seconds. In 4 (Patient. 2, 5, 7 and 8) of 7 patients evaluated for ictal EEG, initial epileptic discharges appeared simultaneously in 4 or more leads of the frontal regions (Table 1). The frontal dominant ictal discharges were followed by diffuse or focal attenuation of electrical activity bilaterally, with or without fast activities for usually several seconds (Figures 2A-D). In 2 other patients (Pts. 10, 16), initial ictal discharges appeared in the left frontal region. In the remaining patient (Patient 15), initial ictal discharges were recorded as rhythmic waves in right sphenoidal electrode. Multifocal epileptic foci were not found on ictal EEG in any of the patients, although multifocal or bilateral and diffuse interictal discharges were observed in almost all patients.

MRI

In the chronic stage of epilepsy, atrophic MRI lesions were observed in 5 of 18 patients, and high intensity lesions on FLAIR MRI were found in 2 of 18 patients. The lesions were frontal lobe dominant in 3 of the 7 patients (atrophy in one, and high intensity lesion in 2).

There was no correlation between characteristics of MRI and clinical manifestation of IAE.

Epileptic seizure outcomes

At the last follow-up, epileptic seizures persisted in 8 of 18 patients, and patients with younger onset of IAE tended to have frequent seizures (Figure 1D). Patients with onset age up to 5 years had bipolarized seizure outcome: almost half (7 of 12) of these patients were seizure-free, while the other half had high seizure frequencies at weekly or daily levels.

Nine patients received prophylactic antiepileptic drugs after IAE, but their epileptic seizure outcome was not different from that of patients not treated prophylactically. Antiepileptic drugs succeeded to control seizures in 9 patients: carbamazepine was effective in 4 patients, and valproic acid in 5 patients.

Seizure outcome and immune markers in CSF

In the chronic stage after IAE, CSF study was performed in 9 patients at 46 ± 53 months (mean ±SD) after onset of IAE. Of 9 patients, 5 were positive for anti-GluRε2 IgG antibodies (immunoblot), and 2 were positive for anti-GluRε2 IgM antibodies (immunoblot). Seizure frequency (score of epileptic seizures) was not related to the presence of anti-GluRε2 IgM antibodies in CSF ($p = 0.13$), but was significantly higher in patients with anti-GluRε2 IgG antibodies in CSF ($p = 0.02$) (Figure 3A, B). Seizure frequency was not related to the presence of IgM or IgG antibodies to GluRε2 in serum (Figure 3C, D). Measurements of CSF levels of anti-GluRε2-NT2 and -CT antibodies in CSF showed that patients with higher levels of antibodies to GluRε2 appeared to have poorer seizure outcome (Figure4A, B). Exceptionally, Patient 8 had antibody levels of control subjects, but this patient had daily seizures (score 0) (Table 1).

Study of cytokines in CSF showed that seizure outcome appeared to be poorer in patients with higher levels of IL-1β, IFNγ and sTNFR1 in CSF (Figure 4C-E). Other cytokines, chemokines or growth factors examined by BioPlex were not related to seizure outcome (data not shown). Although Patient 8 who had daily seizures had low anti-GluRε2 antibodies at control levels, she had extremely high levels of IL-1β (2.22 pg/ml), IFNγ (81.52 pg/ml), and sTNFR1 (1.16 ng/ml). Patient 7 who also had daily seizures had high levels of anti-GluRε2 antibodies and low sTNFR1 at control level. Patient 16 with weekly seizure had medium levels of antibodies to GluRε2, and low IL-1β, IFNγ and sTNFR1 at control levels. Patient 15 with weekly seizure had higher levels of antibodies to GluRε2-NT2, and low cytokines and sTNFR1 at control levels. Patient 12 with weekly seizure had low anti-GluRε2 antibodies and cytokines at control levels.

DISCUSSION

Epileptic seizures in patients with epilepsy after encephalitis/ encephalopathy (EAE) are often intractable. Among 383 pediatric patients admitted between 1993 and 1994 to our epilepsy center for the treatment of intractable epilepsy, the most frequent causal disease was EAE (10.4%).[10] In 67 consecutive patients with EAE in our center, the major causal microbe was influenza virus (21%), followed by HSV (10%).[10] Therefore, understanding of the clinical characteristics and pathophysiology of epilepsy after IAE is important to improve outcome of the disease.

The mean Interval from IAE to epilepsy onset was 63.2 days, and all patients had onset of epilepsy within one year after IAE. On the other

Figure 2A

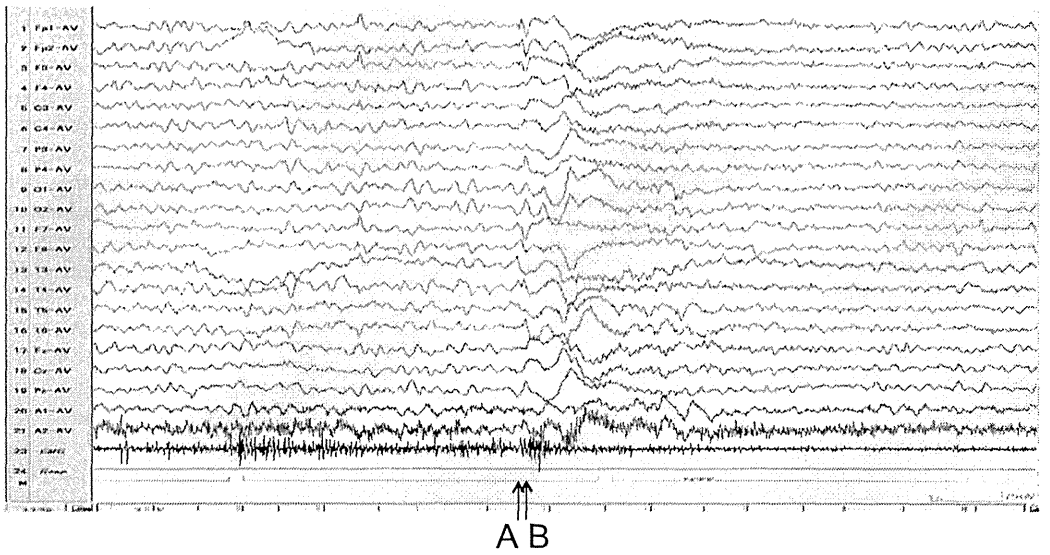
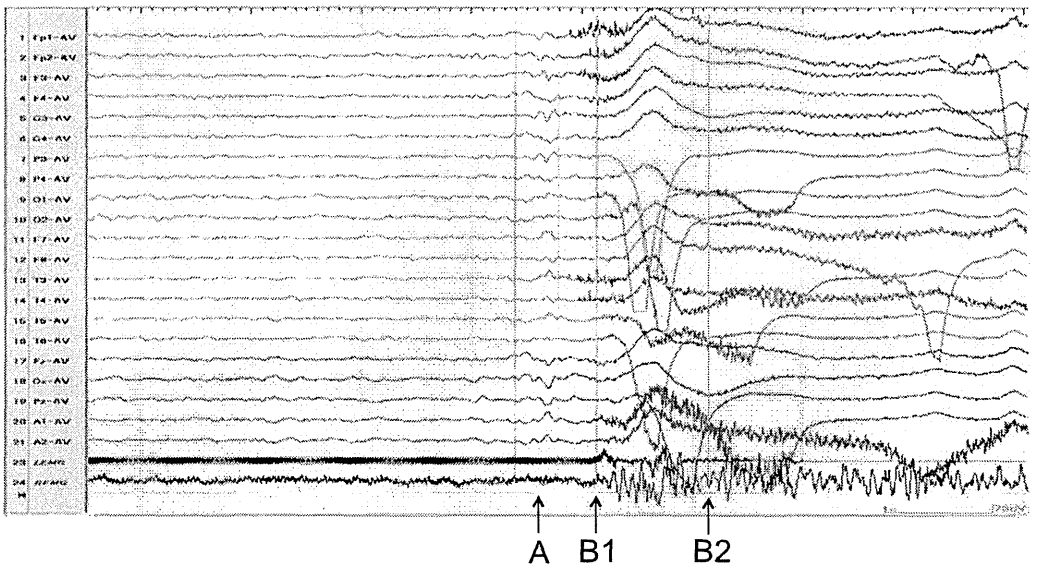


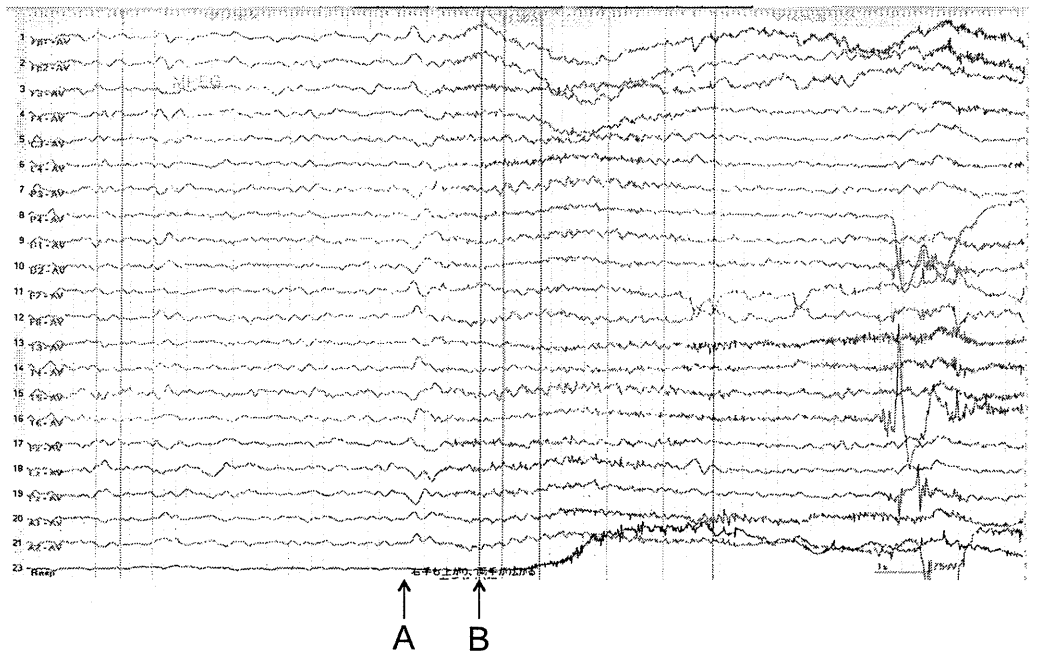
Figure 2. Ictal EEG recordings of patients with epilepsy after influenza-associated encephalopathy (IAE).
A: Ictal-EEG of Patient 2 (female) recorded at 2 years of age. This patient developed IAE on the first day of Influenza infection and epilepsy 30 days later. Almost immediately after ictal EEG onset (A), seizure appeared as slight extension of shoulders and body trunk (B) followed by anterior flexion of head and body trunk, and tonic contraction of upper limbs forward.

Figure 2B



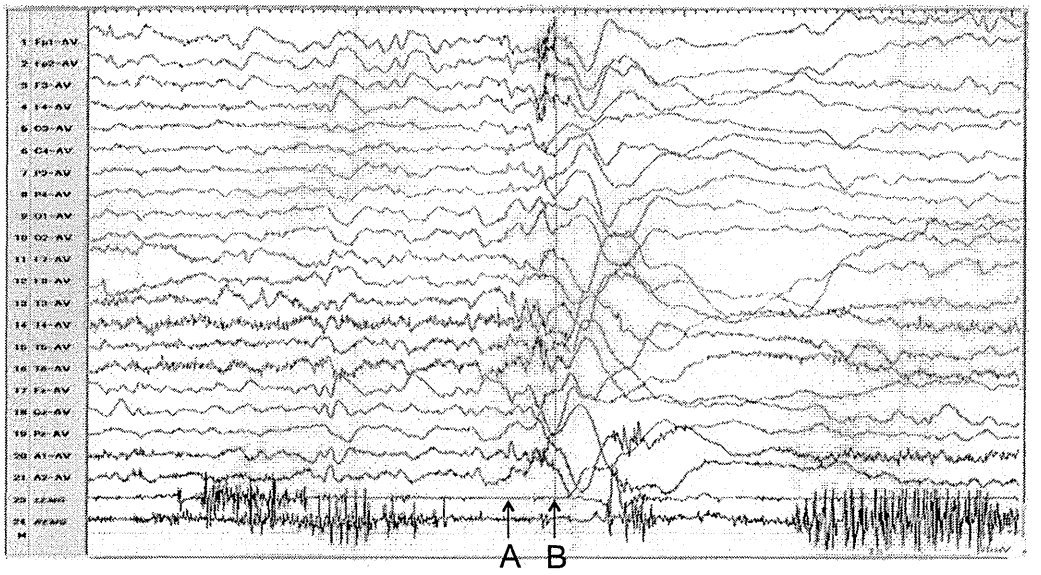
B: Ictal-EEG of Patient 5 (female) recorded at 11 years of age. This patient developed IAE on the 5th day of influenza A infection, and afebrile seizure on the 6th day. Soon after ictal EEG onset (A), seizure appeared as eye-opening (B1) followed by extension of right upper extremity (B2), twitching of right mouth corner, and abduction and hyperactive motor movement of right upper limb.

Figure 2C



C: Ictal-EEG of Patient 7 (male) recorded at 4 years of age. The patient developed IAE two days after influenza A infection, followed by afebrile seizure 60 days after the influenza infection. Soon after ictal EEG onset (A), seizure appeared as synchronous vibratory movement of bilateral upper limbs in sleep (B).

Figure 2D



D: Ictal-EEG of Patient 8 (female) recorded at 5 years of age. The patient developed IAE on the first day of influenza A infection, and subsequently developed epilepsy at 180 days after IAE. Soon after ictal EEG onset (A), seizure appeared as tilting of the head backward (B), followed by falling forward.

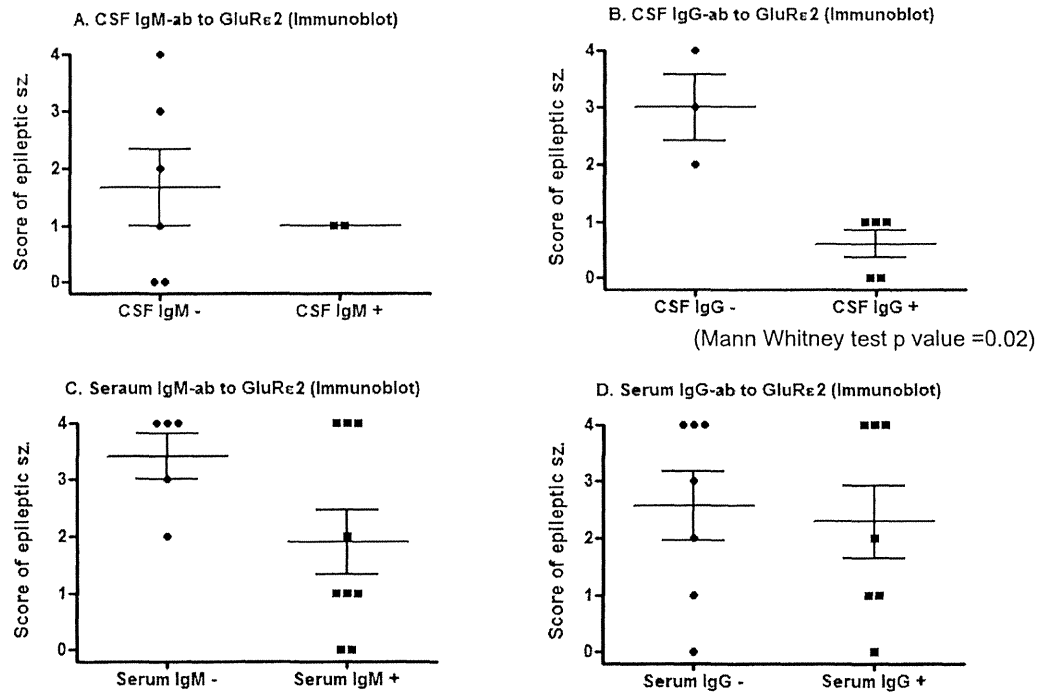


Figure 3. Seizure outcome and antibodies to GluRε2 detected by immunoblot
CSF IgM, IgM antibodies to GluRε2 in CSF; CSF IgG, IgG antibodies to GluRε2 in CSF; Serum IgM, IgM antibodies to GluRε2 in serum; serum IgG, IgG antibodies to GluRε2 in serum.

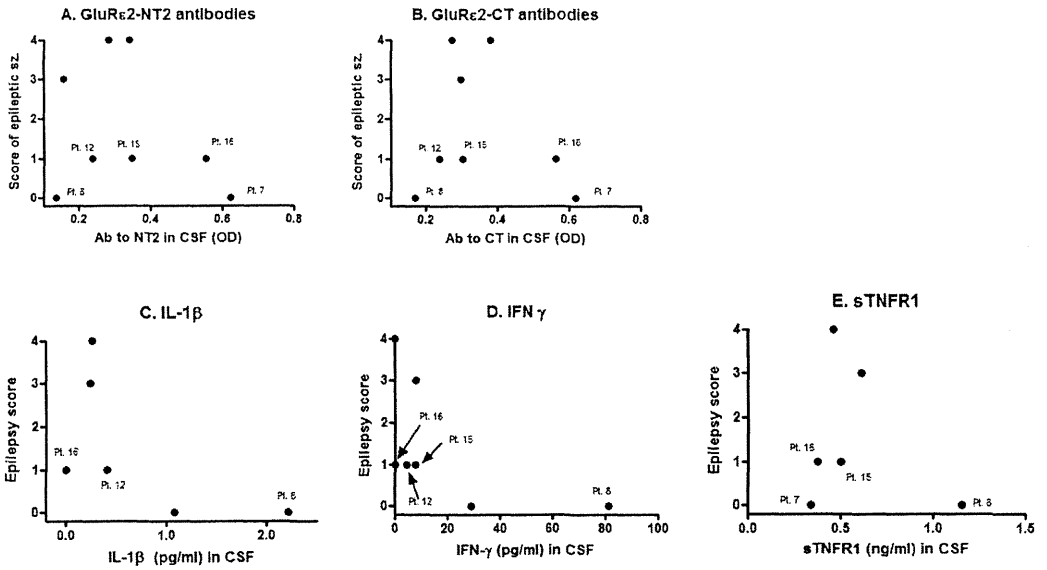


Figure 4. Relationship between seizure outcome and levels of anti-GluRε2 antibodies (ELISA) and cytokines in CSF. **A**: Antibodies to GluRε2-NT2 in CSF. Antibody level is expressed as optical density (OD) of ELISA. The OD (mean ± SD) in CSF of control patients (n = 9) was 0.162 ± 0.055. **B**: Antibodies to GluRε2-CT in CSF. Mean OD in CSF of control patients (n = 9) was 0.189 ± 0.061. **C**: IL1β in CSF. Mean level in control patients (n = 9) was 0.6 ± 0.7 pg/ml. **D**: IFNγ in CSF. Mean level in control patients (n = 9) was 24.0 ± 53.4 pg/ml. **E**: sTNFR1 in CSF. Mean level in control patients (n = 9) was 0.488 ± 0.095 ng/ml.

hand, the mean interval from EAE to epilepsy onset in 67 patients was 6.4 months (0 month to 7 years and 3 months).[11] This finding suggests that epileptogenesis might take a shorter time after IAE comparing with EAE by microbes other than influenza.[12] Acute encephalitis with refractory, repetitive partial seizures (AERRPS) is known to show continuous evolution from encephalitis to residual epilepsy without a latent period.[13,14] Epilepsy after IAE and AERRPS may share common factors such as immunological factors that contribute to early establishment of epileptogenesis.

We found that epileptic seizure outcome is poorer in patients positive for anti-GluRε2 IgG antibodies (immunoblot) in CSF, and that CSF levels of anti-GluRε2-NT2 and anti-GluRε2-CT antibodies are negatively associated with seizure outcome. We also found that seizure outcome was apparently poorer in patients with higher CSF levels of the inflammatory cytokines IL-1β, sTNFR1, and IFNγ. In 2 patients with daily epileptic seizures, one (Patient 8) had increased levels of cytokines only, and another (Patient 7) had increased levels of both cytokines and anti-GluRε2 antibodies. In 3 patients with weekly epileptic seizures, 2 (Patients 15 and 16) had increased anti-GluRε2 antibodies levels only, and one (Patient 12) had normal levels of antibodies and cytokines in CSF. These data suggest that antibodies to GluRε2, IL-1β, IFNγ, and TNFα in CSF may be associated with the intractable seizures, in an additive manner. Because patients with daily seizure frequently had increased levels of proinflammatory cytokines, these cytokines (IL-1β, IFN-γ, TNFα) in CSF may have stronger impact on seizure frequency than anti-GluRε2 antibodies. Cytokines in brain flow into subarachnoid space, and are diluted by CSF produced in choroid plexus to significant degree. Although the increased levels of cytokines were slight in epileptic patients after IAE, the actual increased cytokine levels in brain was assumed to be dozens of times of those in CSF.

IL-1β secretion is prolific in perivascular astrocytes and is thought to destroy tight junctions, induce production of NO or matrix metalloproteinases (MMPs) in vascular endothelial cells and increase permeability of the blood-brain barrier, resulting in elevated albumin concentration in the central nervous system.[15] Increased albumin level in the central nervous system leads consequently to neuronal excitability.[16] IL-1β activates the NR2A/NR2B subunits in N-methyl-D-aspartate (NMDA) type GluR complex, thereby contributing to glutamic acid-induced neurodegeneration.[17] IL-1β is known to inhibit glutamic acid uptake by glia and enhance glutamic acid release from glia mediated by TNFα production, leading to elevated glutamic acid concentration in the synaptic gaps and ultimately to neuronal excitation.[15,18] These findings suggest that IL-1β may contribute to neuronal excitation by multiple mechanisms, probably also in epilepsy after IAE.

This study revealed that sTNFR1 in CSF may be associated with seizure frequency in epileptic patients after IAE. SolubleTNFR1 level in CSF has been reported to affect pediatric neurological prognosis in acute encephalitis / encephalopathy.[19] TNFα is the ligand of sTNFR1, and dose-dependently regulates the seizure threshold. A certain concentration range of TNFα is thought to enhance susceptibility to acute seizure.[20-22] High concentrations of TNFα have been shown to increase excitotoxic death of neurons by increasing synaptic AMPA receptors and decreasing GABA receptors[23], and cause spasms in TNFα transgenic mice.[24] Based on these findings, it is possible that TNFα gradually increases neuronal excitability and contributes to epileptogenesis in patients after IAE.

This study suggested that INFγ in CSF might impact seizure frequency in epileptic patients after IAE. INF-γ predicts poor prognosis in HSV encephalitis[25], and appears in early stages of Rasmussen syndrome.[26] INF-γ may support production of TNFα in microglia or act on tight junctions. These findings suggest that INFγ may contribute to the production of TNFα, resulting in neuronal excitabilities.

Several functions of antibodies to NMDA-type GluR have been reported. Antibodies to NMDA-type GluR complex cause internalization of NMDA-type GluR complex resulting in dysfunction of various neurological pathways.[6] Antibodies to ds-DNA, which cross-react with GluRε2, induce apoptosis of neurons, resulting in memory dysfunction and behavioral changes.[27-29] The diverse functions of anti-GluR antibodies may contribute to cognitive impairment and behavioral changes in epileptic patients after IAE. Probable association of seizure outcome and the levels of antibodies to GluR and inflammatory cytokines may suggest that immunological injury by antibodies and inflammatory cytokines is causally related with pathogenesis of epilepsy after IAE. There was a study that active neuro-inflammation and marked cellular injury occur in pediatric epilepsy and may play a common pathogenic role

or consequences in childhood epilepsy of diverse etiologies.[30]

In conclusion, we report the clinical characteristics and prognostic factors of epilepsy after IAE in 18 patients. Anti-GluRε2 antibodies and proinflammatory cytokines including IL-1β, TNFα, and INFγ in CSF may impact seizure outcome in an additive manner.

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DISCLOSURE

Conflicts of interest: None

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Human Bocavirus in Patients with Encephalitis, Sri Lanka, 2009–2010

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We identified human bocavirus (HBoV) DNA by PCR in cerebrospinal fluid from adults and children with encephalitis in Sri Lanka. HBoV types 1, 2, and 3 were identified among these cases. Phylogenetic analysis of HBoV1 strain sequences found no subclustering with strains previously identified among encephalitis cases in Bangladesh.

Encephalitis is a serious infection causing high rates of illness and, in industrialized countries, has a case-fatality rate of 6.5%–12% (*1*,*2*). However, the situation in developing countries is largely unknown. Globally, the causes remain unrecognized in 60%–85% of encephalitis cases (*1*,*2*). Recently, human bocavirus (HBoV) has been implicated in causing life-threatening encephalitis in Bangladeshi children (*3*). In Sri Lanka, information about the causative agents of encephalitis is scarce. The aim of this study was to determine the occurrence of HBoV and other possible pathogens in children and adults with encephalitis admitted to a tertiary care hospital in Sri Lanka.

The Study

The study was conducted at Colombo North Teaching Hospital, Ragama, Sri Lanka, during July 2009–November 2010. A total of 233 patients (110 adolescents/adults ≥12 years of age and 123 children) were enrolled. Adolescents and adults were admitted to adult wards. Cerebrospinal fluid (CSF) samples were available from 191 patients. Criteria for enrolment were as follows: any combination of the triad of fever, headache, and vomiting, along with altered level of consciousness, seizures, focal neurologic deficits, altered behavior, and signs of meningeal irritation. Clinical and laboratory information was available for 164 patients. The male:female ratio for adolescents/adults was 1.3:1; ages ranged from 12 to 90 years (mean 42 years); For children, the male:female ratio was 0.7:1; ages ranged from 2 to 144 months (mean 48 months). The ethics committees of the University of Kelaniya and Oita University approved this study.

CSF samples were subjected to macroscopic examination, total and differential leukocyte counts, bacterial culture, Gram staining, and measurement of protein and glucose. Blood was cultured for bacteria and examined for total and differential leukocyte counts, erythrocyte sedimentation rates, and hemoglobin and C-reactive protein levels.

Classical encephalitis-causing pathogens (Table) and diarrheagenic viruses, such as HBoV, rotavirus, astrovirus, norovirus, parechovirus, and human adenovirus (HAdV), were determined in CSF by PCR (online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/11/12-1548-Techapp1.pdf) (*3*–*5*). Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis was diagnosed by on-cell Western analysis (*6*). For HBoV PCR-positive patients, HBoV types 1–4-specific IgG and IgM responses in CSF samples were measured by enzyme immunoassays (*7*).

Nucleotide sequences of all amplicons were determined to confirm the PCR products, to distinguish genotypes, and to perform phylogenetic analysis (*3*). BLAST analysis (www.ncbi.nlm.nih.gov/blast) was used to identify the viruses and genotypes. Multiple sequence alignment was conducted by using ClustalW2 (www.ebi.ac.uk/clustalw). The phylogenetic analysis was done with a neighbor-joining tree by using MEGA5 (www.megasoftware.net). A bootstrap analysis of 1,000 replicates was performed to test the reliability of the branching pattern.

The causes of encephalitis were type 2 dengue virus in 1 (0.5%) patient, human echovirus (HEcoV) type 9 or 25 in 2 (1%), HBoV (Table) in 5 (3%), and HAdV 41 in 7 (4%): all were sole detections. None of the other viruses and no bacteria were detected. Samples positive for HBoV by primers designed from viral protein 1/2 also were positive by primers designed from nonstructural protein (NP) 1 gene. HEcoV was detected in 2- and 9-year-old children. HAdV 41 was not confined to children; ages of infected patients ranged from 13 months to 55 years. Of 81 CSF samples, anti-NMDAR encephalitis was detected in 2 (2%) adults (42 and 72 years of age). All patients in this study recovered and were discharged, except for one 13-month-old boy with HAdV 41 encephalitis who left the hospital against medical advice.

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Table. Characteristics of patients with HBoV encephalitis, Sri Lanka, 2009–2010*

Characteristic	Sample no. 93018	56684	84770	64502	285
Virus in CSF					
Virus detected†	HBoV1	HBoV1	HBoV1	HBoV2	HBoV3
HBoV IgM and IgG	Neg	Neg	Neg	Neg	Neg
Patient demographic					
Sex	F	F	M	M	F
Age	66 y	46 y	5 mo	17 y	8 mo
Place of residence	Kaleliya	Wattala	Mirigama	Makola	Heiyanthiduwa
Hospitalization					
Time between illness onset and hospitalization	NA	48 h	24 h	48 h	48 h
Duration of hospitalization	7 d	4 d	12 d	4 d	3 d
CSF test result‡					
Color	Clear	Clear	Clear	Clear	Clear
Leukocyte count, cells/μL	1	0	380	0	0
PMNs	0	0	130	0	0
Lymphocytes	1	0	250	0	0
Protein, mg/dL	NA	113	170	38	25
Glucose, mg/dL	65	160	48	63	83
Results of Gram stain	Neg	Neg	ND	Neg	Neg
Bacterial culture	ND	ND	Neg	ND	ND
Blood tests§					
Leukocyte count, cells/μL	10,000	15,200	36,500	15,900	13,200
PMNs, %	63.2	70	62	ND	52
Lymphocytes, %	21.6	21	35	ND	47
Hemoglobin, g/dL	12.2	12	7.7	13.2	13.2
ESR, mm/h	27	68	ND	ND	ND
CRP, mg/dL	ND	ND	>12	ND	<6
Glasgow coma score <15	No	Yes, 12	No	No	No
Outcome	Discharged	Discharged	Discharged	Discharged	Discharged

*HBoV, human bocavirus; CSF, cerebrospinal fluid; Neg, negative; NA, not available; PMN, polymorphonuclear neutrophil: ND, not done; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
†The following viruses were tested for herpes simplex virus (HSV) type 1, HSV-2, varicella-zoster virus (HSV-3), Epsetin-Barr virus (human herpesvirus [HHV] type 4), cytomegalovirus (HHV-5), (HHV-6), HHV-7, HHV-8, dengue virus, Japanese encephalitis virus, rubella virus, West Nile virus, yellow fever virus, tick-borne encephalitis virus, Nipah virus, measles virus, mumps virus, parainfluenza virus, respiratory syncytial virus, metapneumovirus, Chikungunya virus, Sindbis virus, Semliki Forest virus, eastern equine encephalitis virus, western equine encephalitis virus, poliovirus, Coxsackie virus, echovirus, enterovirus, lyssaviruses, and Chandipura virus. Bacteria were tested by PCR amplification of 16S rRNA, followed by sequencing.
‡Reference values: leukocyte count <5 cells/mm^3 and all lymphocytes; PMNs, none; protein, 20–45 mg/dL; glucose, 50–80 mg/dL or >50% of blood glucose level.
§Reference values: leukocyte count, 4,000–11,000 cells/mm^3; PMNs, 40–60% of leukocyte count; lymphocytes, 20%–40% of leukocyte count; hemoglobin, men: 14–18 g/dL, women, (12–15 g/dL, children: 11–16 g/dL; ESR, <20 mm in1st hr., CRP, <12 mg/dL.

The severity of symptoms in the HBoV-positive patients did not differ from those of patients with other infections. None of the patients who had positive PCR results for HBoV1–3 had corresponding HBoV1–4 IgM or IgG in their CSF. Phylogenetic analysis (Figure) of the viral protien 1/2 gene showed that the Sri Lanka HBoV1 strains did not subcluster with encephalitis-associated Bangladesh strain, although they had 97%–98% nt identities. The Sri Lanka HBoV1 strains had 98%–99% nt identities among themselves and with other HBoV1 strains. The Sri Lanka HBoV2 strain was closely related to the Tunisia strain (96% nt identity). The Sri Lanka HBoV2 had 90%–91% nt identities with the Bangladeshi encephalitis-causing strains and 90%–96% nt identities with other HBoV2 strains. The Sri Lanka HBoV3 strain was closely associated with the cluster formed by viruses from the United Kingdom, Australia, Tunisia, and China and had 96%–97% nt identities with those strains. The sequence of NP1 gene is conserved and had 98%–100% nt identities among the Sri Lanka strains.

Conclusions

The study in Bangladesh suggested that HBoV-associated encephalitis might be restricted to malnourished children (*3*). However, our study demonstrates that HBoV also can be detected in well-nourished children and adults with encephalitis. How HBoV might trigger encephalitis is unclear. HBoV viremia has been documented, and the virus might therefore have the potential to cross the blood–brain barrier. The NP1 of HBoV inhibits interferon-β production by the host, suggesting evasion of the innate immune response during infection (*8*).

Unlike the Bangladesh study, where 2 of 4 encephalitis patients in whom HBoV was detected died (*3*), all patients in our study recovered. In addition to HBoV1 and HBoV2, we detected HBoV3 in a child with encephalitis, which to our knowledge, has not been reported as a cause of the disease. Although HBoV infections occur mainly in children, among the 5 Sri Lanka patients with HBoV encephalitis, 3 were adults or adolescents. None of the patients with HBoV encephalitis had HBoV IgM or IgG in their CSF, indicating

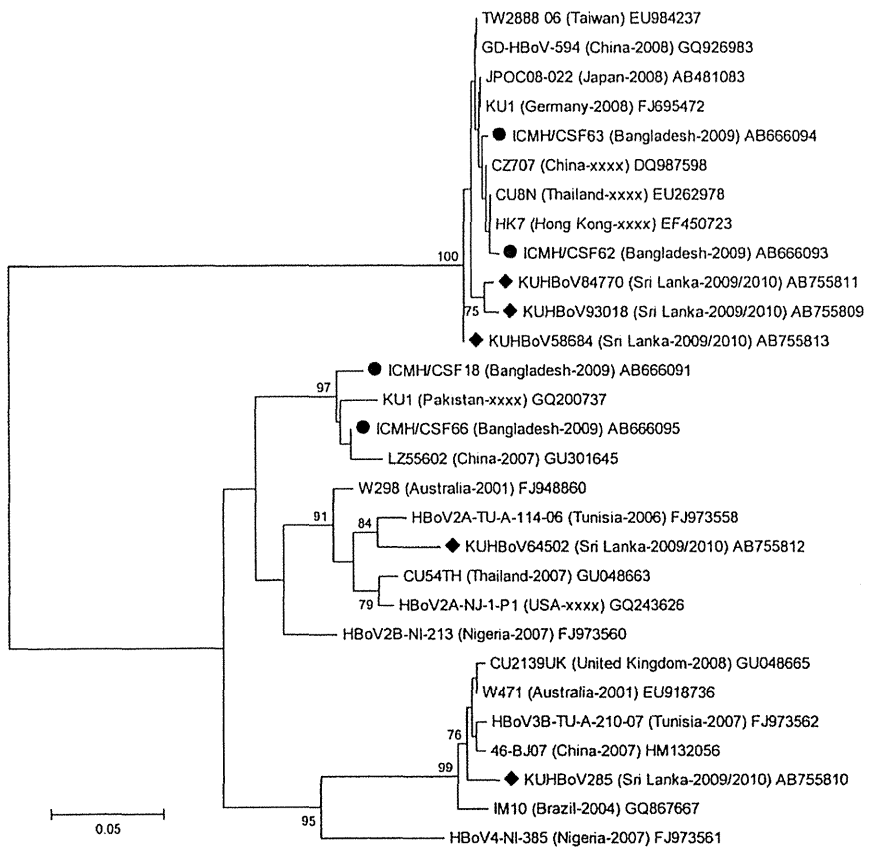

Figure. Phylogenetic tree of the partial VP 1/2 gene (nucleotide positions 3233–3808, amplicon size 575 bp) of HBoVs constructed by using nucleotide sequence by neighbor-joining method. The strain name is followed by country of origin and year of sample collection in parentheses, followed by the DDBJ/EMBL/GenBank accession no.; xxx indicates that the year of sample collection is undocumented. Sri Lanka and Bangladesh encephalitis-causing bocaviruses are indicated by circles and diamonds, respectively. The number adjacent to the node represents the bootstrap value, and values <70% are not shown. Scale bar indicates genetic distance expressed as nucleotide substitutions per site. (The DDBJ/EMBL/GenBank accession numbers of nonstructural protein 1 gene of strains KUHBoV93018, KUHBoV285, KUHBoV84770, KUHBoV64502 and KUHBoV58684 were AB822999, AB823000, AB823001, AB823002 and AB823003, respectively).

how rapidly disease onset occurred and how little time the immune system had to respond. Generally, the specific seroprevalence rate of HBoV1 antibodies in infected persons is 59%, followed by HBoV2, 3, and 4 (34%, 15%, and 2%, respectively) (*7*).

Our detection rate of viruses as a cause of encephalitis was 7.5%, and adding anti-NMDAR encephalitis, the detection rate increased to 10%, which is similar to that of another study (*9*). Anti-NMDAR encephalitis is becoming a dominant cause of encephalitis in certain population (*10*); however, in Sri Lanka, it is 1%–4%, similar to other studies (*11*).

Dengue virus is the leading endemic cause of encephalitis in Brazil (*12*). This infection is also endemic to Sri Lanka and, before our study, dengue encephalitis was suspected but unconfirmed in the population. Enteroviruses frequently cause CNS infection, and the HEcoV 9 and 25 found here are known to cause encephalitis (*13*).

Among the HAdVs, serotype F is mainly responsible for gastroenteritis, whereas encephalitis is caused mainly by serotypes B, C, and D (*14,15*). The large number of HAdV 41 encephalitis cases indicates a unique epidemiology in Sri Lanka.

Herpes simplex and varicella-zoster viruses are implicated as the major causes of encephalitis. However, these viruses were not responsible for encephalitis in our study or in the studies in Bangladesh. HBoV is dominant in both Bangladesh and Sri Lanka. The limitation of our study is that causation could not be proven by the presence of HBoV antibody during infection or the absence of HBoV DNA in the CSF when recovered. The HBoV DNA detected in our study may represent persistent DNA from past infection; however, history of recent respiratory or diarrheal infection was absent. Future studies using quantitative PCR and serology are warranted to better establish the etiologic role of HBoV infection and encephalitis.

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