

## Original Article

# Encephalitis in Taiwan: A Prospective Hospital-Based Study

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**SUMMARY:** To investigate encephalitis in Taiwan, a multicenter study was conducted with patients who had acute severe neurological dysfunction and suspected encephalitis from May 2000 to December 2001. Demographic data such as age, sex, and seasons were analyzed. Polymerase chain reaction analyses were performed to determine the microbiologic diagnosis. The patients included 73 males and 54 females, with a peak age of 10-40 years old. Microbiologic diagnoses in 86 (69%) of 124 cases involved herpes simplex virus (HSV, 45 cases), varicella zoster (16 cases), *Mycobacterium tuberculosis* (10 cases), cytomegalovirus (8 cases), adenovirus (5 cases), influenza (1 case), and enterovirus (1 case). Pathogens were found in 69% of the cases. Encephalitis was most likely to occur in June and July. Based on the results, HSV is still the major viral cause of encephalitis in Taiwan.

## INTRODUCTION

Every year in Taiwan, many cases of encephalitis are reported. Previous research regarding adenovirus (ADE) (1), mycoplasma (2), measles (3), Japanese encephalitis (4), and an outbreak of enteroviral encephalitis in 1998 (5,6) have been reported.

The etiological pattern of encephalitis is poorly understood. The disease may lead to neural damage, and the sequela of encephalitis can induce socioeconomic problems in Taiwan (7,8). Specific treatments are available for herpes simplex virus (HSV) and other antigens; hence, a precise etiologic diagnosis is important.

To investigate encephalitis in Taiwan, a multicenter study was conducted from May 2000 to December 2001. This project was organized by 30 major hospitals and medical centers in Taiwan. The study targeted patients who had acute and severe neurological dysfunction and suspected encephalitis.

## MATERIALS AND METHODS

**Patients:** This multicenter study was conducted from May 2000 to December 2001 in Taiwan. During the period of investigation, data from the patients were recorded at 30 major hospitals and medical centers in different geographic regions in Taiwan (north, central, south, and east). At the end of the study, 30 hospitals were participating, including 35% of the major hospitals in Taiwan (Taiwan has 67 major hospitals and 17 medical centers).

The patients included those with acute severe neurological dysfunction and suspected encephalitis, in particular those with symptoms and signs of acute mental dysfunction, memory impairment, loss of consciousness, abnormal behavior, convulsions, and involuntary movement. All patients were required to report to the infectious diseases department of

their own hospital and were required to register at the Center for Disease Control, Taipei, Taiwan (CDC). Cerebrospinal fluid (CSF) and serum samples were placed in ice-boxes with dry ice, and the samples were sent to the CDC within 72 h of the patient's admission to the hospital.

**Clinical diagnosis:** Encephalitis was defined as follows: acute and severe neurological dysfunction in the context of suspected encephalitis, which included signs and symptoms of acute mental dysfunction, memory impairment, loss of consciousness, pareses, abnormal behavior, convulsions, and involuntary movements (9,10). The patients' electroencephalographic (EEG) and/or CT scans of the brain and CSF findings were also compatible with a diagnosis of encephalitis. Patients with high fever, headache, nausea, and vomiting were excluded by lumbar puncture, CT/magnetic resonance imaging (MRI), or EEG examination. Patients with other diseases, especially those with other systemic infection (sepsis), cerebral vascular accident, neoplasm of the brain, or psychiatric diseases that caused disturbed consciousness were also excluded by lumbar puncture, CT/MRI, or EEG examination.

**Microbiologic diagnosis:** Polymerase chain reaction (PCR) assays were performed using serum and/or CSF specimens. The microbiologic diagnosis was regarded as proven if the PCR examination detected the organism or its nucleic acid in the CSF; the diagnosis was suspected if the PCR examination detected the organism or its nucleic acid in the serum. Serological tests and viral isolation could not be performed at every hospital in Taiwan. This study aimed at the probability of PCR test in the screening of suspected encephalitis in Taiwan. Serological tests require both a considerable amount of time and repeat blood sampling. It is therefore more efficient to use PCR analysis rather than serological test or viral isolation (11).

**Specimen processing:** DNA and RNA were simultaneously extracted from each CSF or serum specimen. The DNA samples were purified from 200  $\mu$ l of the specimen by using the QIAamp blood kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions. Purified genomic DNA was then dissolved in 50  $\mu$ l of TE buffer. The RNA

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was purified from 150  $\mu$ l of the specimen with a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was dissolved in 50  $\mu$ l of RNase-free ddH<sub>2</sub>O. The RNA was then transcribed to cDNA by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions.

**PCR primers:** PCR primers were synthesized on a DNA synthesizer (Applied Biosystems 394; Foster City, Calif., USA). The 197 specimens were tested for a range of 17 pathogens, including the following: HSV, varicella-zoster virus (VZV), cytomegalovirus (CMV) (12), *Mycobacterium tuberculosis* (MTB) complex (13), enterovirus (ENT) (14), Japanese encephalitis virus (JEV), ADE, human herpesvirus 6 (HHV-6) (15), HHV-7 (16), Epstein-Barr virus (EBV) (17), *Mycoplasma pneumoniae* (18), *Campylobacter jejuni* (19), influenza A or B virus (INFA) (20), measles virus (21), mumps virus (22), and rubella virus (23) (Table 1).

**PCR assays:** The PCR reaction mixture (10  $\mu$ l, pH 8.4) contained 50 mM KCl; 10 mM Tris-HCl; 1.5 mM MgCl<sub>2</sub>; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1  $\mu$ l (100 ng/ $\mu$ l) of each primer; 2.5 units of Taq DNA polymerase; and 1  $\mu$ l of purified clinical or genomic DNA as the template. The PCR reaction was initiated by denaturation at 94°C for 1 min, followed by 60 cycles of denaturation at 94°C for 15 s,

annealing at 55°C for 15 s, and polymerization at 72°C for 30 s. Finally, the reaction was extended at 72°C for 2 min. The negative control contained all of the components, except for the target DNA. The positive controls contained all of the components and 1 ng of purified genomic DNA or cDNA. The PCR products were separated on a 2% agarose gel and were analyzed by means of ethidium bromide staining. When the first PCR results of the samples were negative, other PCR primers were used, as previously described.

**Statistics and variables analyzed:** To evaluate the demographic and clinical characteristics of the patients, descriptive statistical tests were performed. Age, sex, microbiologic diagnosis, month of onset, clinical symptoms, and MRI and EEG results were analyzed. To investigate the association between some of these variables, a two-way contingency table analysis was performed. The *t* test was used to compare the mean ages by different group by sex. One-way analysis of variance (ANOVA) was used to test the hypothesis that the mean ages of patients in the five microbial groups – HSV, VZV, MTB, CMV, and ADE + ENT + INFA – were equal. If the hypothesis was rejected at the 0.05 level of significance, then Duncan multiple range test for multiple comparisons was used to determine which pairs of microbial groups were different. The probability of clinical symptoms were analyzed by using a logistic regression model by age.

Table 1. Pathogens assessed and PCR primers

Pathogen <sup>1)</sup>	Target gene	Primer <sup>2)</sup>	
		Forward (5'→3')	Reverse (5'→3')
HSV	Glycoprotein D	1 ATCACGGTAGCCCGCCGTGTGACA	1 CATACCGAACGCACCACACAA
		2 CCATACCGACCACACCGACGA	2 GGTAGTTGGTCGTTCCGCGCTGAA
VZV	ORF4	1 ACGGGTCTTGCCGGAGCTGGT	1 AATGCCGTGACCACCAAGTATAAT
		2 ACCTTAAAACTCACTACCAGT	2 CTAATCCAAGGCGGGTGCAT
CMV	IE	1 ACATCTTTCTCGGGTTCTCGTTGC	1 GTCCTCTGCCAAGAGAAAAGATGGAC
		2 TTGAGGGATTCTTCGGCCAACCTCTG	2 TCTCCTGTATGTGACCCATGTGCTT
MTB complex	IS6110	1 TGGCTAACCTGAACCGTGAG	1 TTCAGGTCGAGTACGCCTTCT
		2 GATGCACCGTCGAACGGCTGA	2 AGGTGGCCA TCGTGAAGCGA
ENT	5' Nontranslated region	1 CGGTACCTTTGTACGCCTGTT	1 GGACACCCAAAGTACGCCTGTT
		2 CAAGCACTTCTGTTTCCC	2 AGGCTCTTACACCATGT
JEV	C protein	1 AAGATTAGCCTGCGTCCGAT	1 GTCAAATTATCGGTTTGTCT
		2 AAGATTAGCCTGCGTCCGAT	2 GAGGGT TGATAGGTTAAGAGC
ADE	Heson	1 ATGACTTTTGGAGGTGGATCCCATGGA	1 GCCGAGAAGGGCGTGCAGGTA
HHV-6	Capsid protein	1 GTGTTTCCATTGTAAGTAAACCGGT	1 TAAACATCAATGCGTTGCATACAGT
		2 TTCTAGCGGATCGTTGACGTCTGTG	2 ACAGCGCAGCAACATGTTTCAGAGC
HHV-7	ORF U10	1 TATCCCAGCTGTTTTCATATAGTAAC	1 GCCTTGCGGTAGCACTAGATTTTTTG
		2 CAGAAATGATAGACAGATGTTGG	2 TAGATTTTTTGAAAAAGATTTAATAAC
EBV	Capsid antigen p23	1 CAGCTCCACGCAAAGTCAGATTG	1 ATCAGAAATTTGCACTTCTTTTGC
		2 TTGACATGAGCATGGAAGAC	2 CTCGTGGTCGTGTTCCCTCAC
<i>M. pneumoniae</i>	ATPase operon	1 AGGCTTGTAAATCGTC	1 TGGTTAATTGACTGG
<i>C. jejuni</i>	Hip O	1 GAAGAGGGTTTGGGTGGTG	1 AGCTAGCTTCGCATAATAAAGTCTG
Influenza A virus	Matrix protein	1 CCGTCAGGCCCTCAAAGC	1 TGCTGGGAGTCAGCAATCTG
		2 CAGAGACTTGAAGATGTCTT	2 CAGAGACTTGAAGATGTCTT
Influenza B virus	Matrix protein	1 TGTCGCTGTTTGGAGACACA	1 TGGCCTTCTGCTATTTCAAA
		2 GAAGGCAAAGCAGAAGTAGC	2 TGGCCTTCTGCTATTTCAAA
Measles virus	Nucleoprotein	1 CATTACATCAGGATCCGG	1 GTATTGGTCCGCCTCATC
Mumps virus	F	1 ATCAGTAATCATGAAG	1 ACCACTGCAGGCGTCAT
Rubella virus	E1 protein	1 TCAGAATTCCAATGGAAGCTATCGGA	1 AGTCACGAATTCGTGTAGTAGAGCAAGCAT

<sup>1)</sup>: HSV, herpes simplex virus; VZV, varicella-zoster virus; CMV, cytomegalovirus; MTB, *Mycobacterium tuberculosis*; ENT, enterovirus; JEV, Japanese encephalitis virus; ADE, adenovirus; HHV, human herpesvirus; EBV, Epstein-Barr virus.

<sup>2)</sup>: Primer 1 for each pathogen was used in the first PCR assay, and primer 2 was used in the second assay.

## RESULTS

**Patients:** A total of 127 patients with acute encephalitis were reported during the course of this study. The patients came from 20 of 22 administrative divisions in Taiwan, as recorded by the neurologist.

**Age and sex:** The study included 73 male patients (57%) and 54 female patients (43%). The male-to-female ratio was 1.4:1. The mean age of all 127 patients was 35.0 years old  $\pm$  2.0 (mean  $\pm$  SEM). The mean ages of the male (35.1 years old  $\pm$  2.7) and female (34.9 years old  $\pm$  2.8) patients were not significantly different ( $P = 0.95$ ,  $t$  test). When we divided the age variable into five categories, i.e., 1-15, 16-30, 31-45, 46-60, and >60 years old, the differences between the male-to-female ratios showed statistical significance ( $P = 0.001$ , Pearson's  $\chi^2$  test). The male-to-female ratio (3.5:1) was the highest in the >60 year-old group ( $n = 18$ ) and was the lowest (0.2:1) in the 46-60 year-old group ( $n = 21$ ). The number of female cases exceeded the number of male cases only in the 46-60 year-old group (Fig. 1).

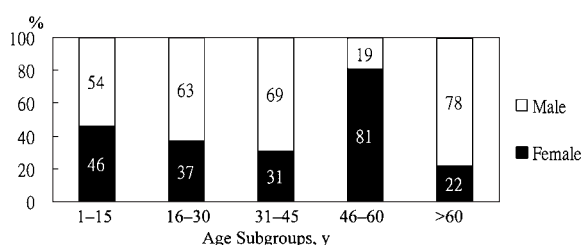


Fig. 1. Relative frequency of male and female patients by age groups.

**Microbial etiology:** The diagnosis of the CSF samples was established in 41 of the 127 patients, and serum sample diagnosis was established in 120 of the 127 patients. Thus, CSF and/or serum sample diagnosis was established in 124 of the 127 patients. The microbiologic diagnosis was proven in 21 (17%) of 124 patients and suspected in 65 patients (52%). That is, the microbial organism or nucleic acid was found in 86 (69%) patients. However, 38 cases (31%) remained without any known microbial etiology (Table 2, Fig. 2). HSV was the predominate pathogen in the patients' CSF (Table 3).

**1. Microbial etiology and sex:** The distributions of known microbial etiology by sex did not significantly differ ( $P = 0.363$ , Pearson's  $\chi^2$  test;  $P = 0.402$ , Fisher's exact test). The observed frequency distribution of microbes associated with sex showed that the number of male cases exceeded that of female cases for all microbial groups (Fig. 3).

**2. Microbial etiology and age:** The mean ages of the patients in the five main microbial groups revealed a significant difference ( $P = 0.005$ , ANOVA  $F$ -test). The Duncan multiple range test (significance level = 0.05) indicated that the mean ages of patients in the HSV (36.0 years old  $\pm$  3.3,  $n = 45$ ), VZV (34.1 years old  $\pm$  4.4,  $n = 16$ ), MTB (39.2 years old  $\pm$  6.3,  $n = 10$ ), and CMV (40.2 years old  $\pm$  6.4,  $n = 8$ ) groups were not significantly different. However, patients with ADE, ENT, or INFA (5.5 years old  $\pm$  2.9,  $n = 7$ ) were younger than those in the other four microbial groups (Fig. 4).

ADE, ENT, and INFA were found in patients who were younger than 30 years old. CMV was not found in children younger than 15 years old. In each age group, HSV was the major known virus related to encephalitis (Fig. 5).

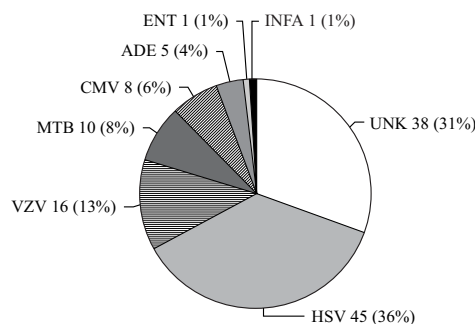


Fig. 2. Distribution of microbes for all 124 cases. UNK, unknown. Abbreviations are in Table 1.

Table 2. Distribution of diagnosis results from serum and CSF testing

Microbiologic detection	No. of cases $n = 124$ (%)	Diagnostic result for encephalitis
CSF positive result	21 (17)	Proven
Serum positive result	65 (52)	Suspected
Idiopathic	38 (31)	Unknown

CSF, cerebrospinal fluid.

Table 3. Distribution of CSF results by microbial group

Microbial group	No. of cases $n = 41$ (%)
HSV	14 (34)
MTB	3 ( 7)
VZV	2 ( 5)
ADE	1 ( 2)
CMV	1 ( 2)
Idiopathic	20 (49)

Abbreviations are in Table 1.

**Seasonal distribution:** Five medical centers recorded patient data during the entire study period from May 2000 to December 2001, and the other 25 hospitals started reporting cases beginning in March 2001. In the year 2000, the initial five medical centers reported the most cases (10 patients) in July. In the second year, they reported seven cases in January and seven cases in June. By observing the frequency distribution of onset by month, encephalitis was found to be most common from November to January and from June to July, with the lowest number of cases occurring between August and October, inclusively (Fig. 6).

**Clinical symptoms:** The most common clinical symptoms in all 127 patients were signs of meningeal irritation (78 patients, 61%), and the least common clinical symptom was pareses (17 patients, 13%, Table 4).

**1. Age and clinical symptoms:** The distributions of clinical symptoms by age groups (1-15, 16-30, 31-45, 46-60, and >60 years old) were significantly different for pareses and signs of meningeal irritation (for both,  $P < 0.001$ , Pearson's  $\chi^2$  test; for pareses,  $P = 0.010$ , Fisher's exact test; and for signs of meningeal irritation,  $P < 0.001$ , Fisher's exact test). These findings indicated that the probability of encephalitis patients with pareses or signs of meningeal irritation was not equal among the different age groups, according to a contingency-table analysis.

We assigned the scores by using the median for each age

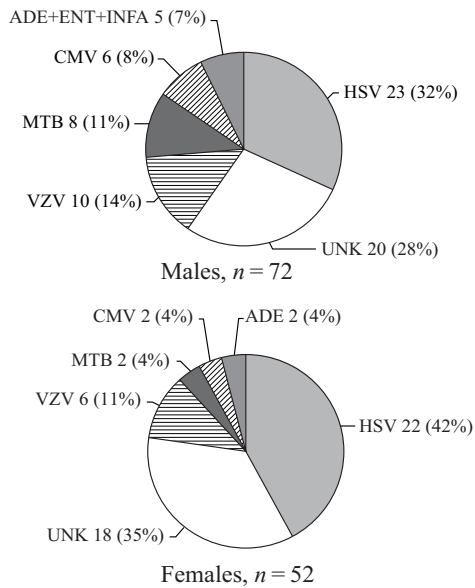


Fig. 3. Distribution of microbes by sex. Abbreviations are in Table 1.

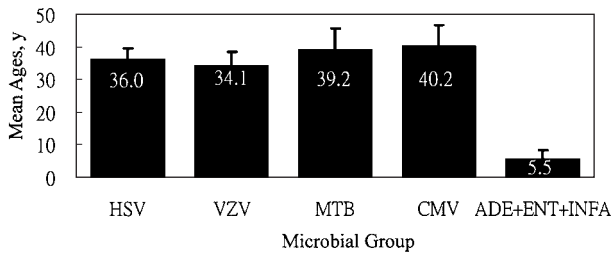


Fig. 4. Comparison of the mean ages of the patients by microbial group.

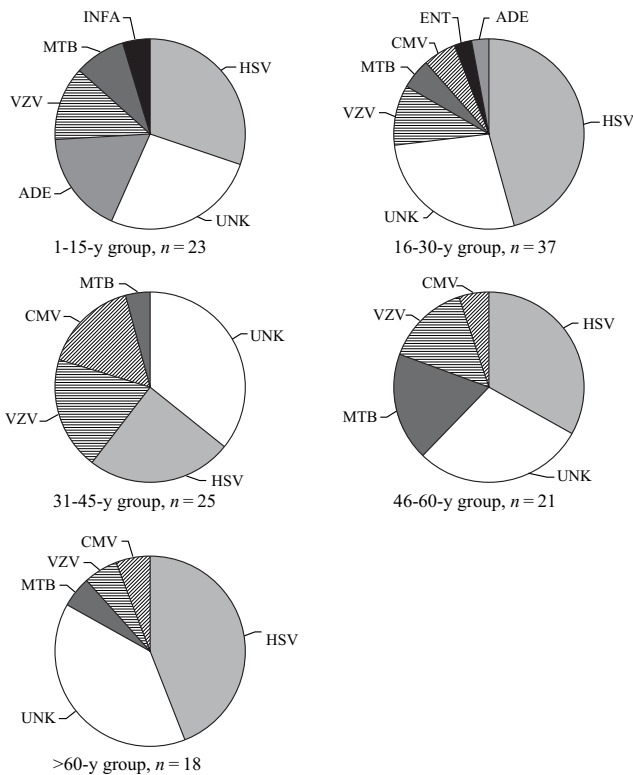


Fig. 5. Distribution of microbes by age group. Abbreviations are in Table 1.

group, i.e., 3.3, 24.6, 37.6, 51.0, and 73.4. The logistic regression model was used to fit the observed probability of encephalitis in patients with pareses or signs of meningeal irritation. Patients who had pareses were primarily in the >60 or 1-15 year-old groups, with a relatively low percentage in the 16-45 year-old group (Fig. 7). The patients who had signs of meningeal irritation were for the most part 16-45 years old, with a relatively low percentage in the 1-15 or >60 year-old groups (Fig. 8).

**2. Sex and clinical symptoms:** The distributions of each clinical symptom by sex did not show a significant difference (for all groups,  $P > 0.05$ , Pearson's  $\chi^2$  test).

**3. Microbial etiology and clinical symptoms:** The distributions of each clinical symptom by microbial etiology did not show a significant difference (for all groups,  $P > 0.05$ , Pearson's  $\chi^2$  test).

**MRI and EEG findings:** A total of 46 patients were examined by means of MRI, and 35 of them (76%) had

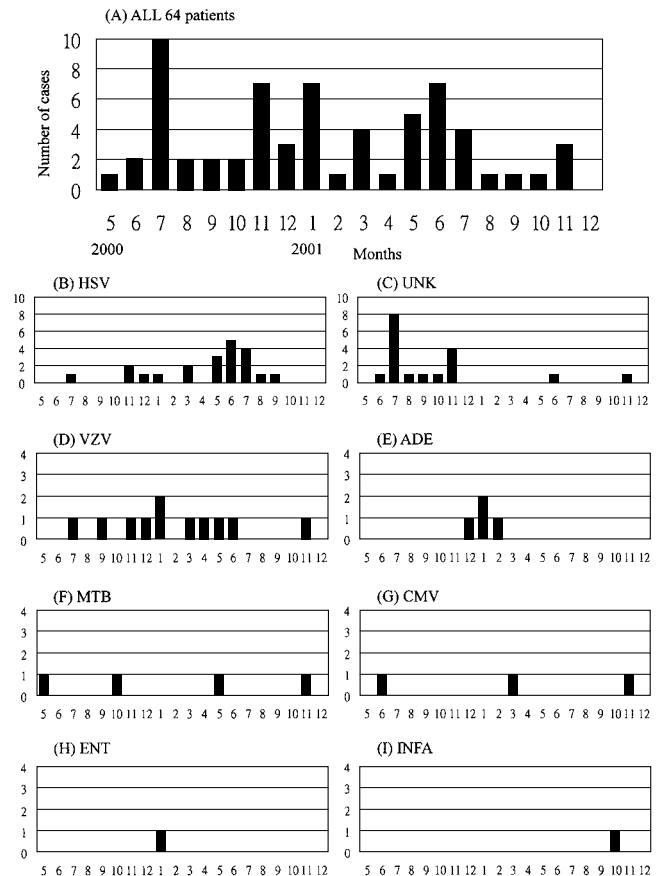
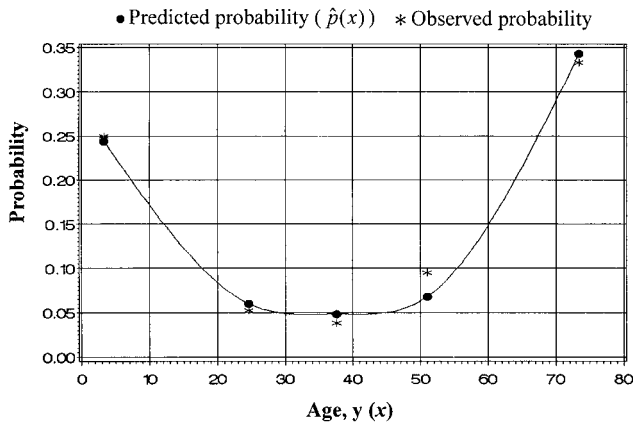


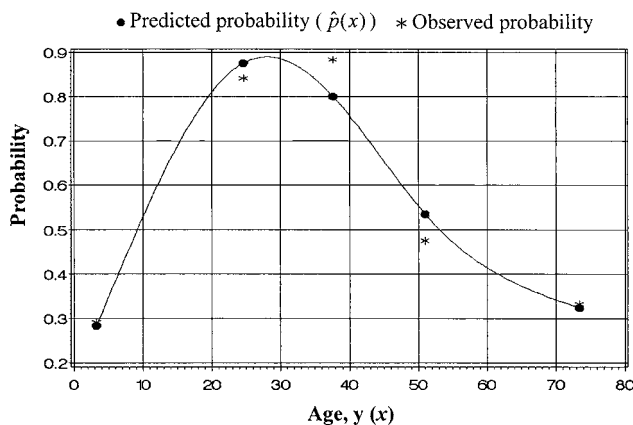
Fig. 6. Seasonal distribution of 64 patients reported by the first five medical centers.

Clinical symptom	No. of cases n = 127 (%)
Acute mental dysfunction	53 (42)
Paresis	17 (13)
Convulsion	35 (28)
Meningeal irritation sign	78 (61)
Involuntary movement	20 (16)
Other	21 (17)



1. Model:  $\log[\hat{p}(x)/1-\hat{p}(x)] = -0.7429 - 0.1233x + 0.0017x^2$
2. Likelihood-ratio test statistic for the goodness-of-fit was 0.331 (df=2,  $P=0.847$ )

Fig. 7. The probability of paresis symptoms by age.



1. Model:  $\log[\hat{p}(x)/1-\hat{p}(x)] = -2.0100 + 0.3600x - 0.0098x^2 + 0.0001x^3$
2. Likelihood-ratio test statistic for the goodness-of-fit was 1.988 (df=1,  $P=0.370$ )

Fig. 8. The probability of signs of meningeal irritation by age.

Table 5. Distribution of MRI results by microbial group

Microbial group	Abnormal (%)	Normal	Total
Idiopathic	13 ( 87)	2	15
HSV	10 ( 71)	4	14
VZV	5 ( 83)	1	6
MTB	2 ( 50)	2	4
CMV	3 (100)	0	3
ADE + ENT + INFA	2 ( 50)	2	4
Total	35 ( 76)	11	46

Abbreviations are in Table 1.

Table 6. Distribution of EEG results by microbial group

Microbial group	Abnormal (%)	Normal	Total
Idiopathic	10 ( 83)	2	12
HSV	11 ( 92)	1	12
VZV	2 ( 67)	1	3
MTB	1 ( 33)	2	3
CMV	0 ( 0)	1	1
Total	24 ( 77)	7	31

EEG, electroencephalographic.  
Abbreviations are in Table 1.

abnormal results, including gyral swelling, high signal intensity on T2-weighted images in the lobular region, and hydrocephalus, among other findings (24). A total of 31 patients were examined by EEG, and 24 (77%) had abnormal results, including diffuse background or local slow waves. The MRI results associated with known microbial etiology were not significant ( $P = 0.593$ , Fisher's exact test, Table 5). However, the EEG results associated with known microbial etiology showed a significant difference ( $P = 0.049$ , Fisher's exact test, Table 6). The proportion of abnormal EEG findings among patients with MTB was slightly lower than that of patients with HSV or VZV.

## DISCUSSION

The etiologic pattern of encephalitis in Taiwan is poorly understood, except for a few reports regarding ADE, mycoplasma, measles, JEV, and a recent outbreak of enterovirus encephalitis in 1998. The diagnosis of encephalitis can be supported by documenting the clinical signs and symptoms, slow-wave background activity on EEGs, and pleocytosis in the CSF. Because a large number of organisms can cause encephalitis and because specific treatment is available for infections with HSV and other antigens, a precise etiologic diagnosis is important (25).

According to the results of the demographic analysis, encephalitis was proven or suspected in patients aged 6 months to 80 years old in Taiwan. The total number of cases was quite consistent among the different age groups. The number of female patients exceeded the number of male patients only in the 46-60 year-old group.

The patients' mean ages also revealed that those with ADE, ENT, or INFA (5.5 years old  $\pm$  2.9) were significantly younger than those with the other four microbes (HSV, VZV, MTB, and CMV). Of note, three of five children with ADE were younger than 1 year old. The eight patients with CMV infection were all older than 25 years old. Other patients with HSV, VZV, and MTB included those from the youngest group (1-15 years old) to those from the oldest group (>60 years old).

The clinical symptoms indicated that older people and children were more likely to have paresis than middle-aged individuals. Conversely, middle-aged patients were more likely to have signs of meningeal irritation than older people and children.

The percentage of patients in whom the etiology remained unknown varied from 26% (1-15 year-old group) to 39% (>60 year-old group). ADE, ENT, and INFA were seen in patients younger than 30 years old. CMV was not seen in children younger than 15 years old. In each age group, HSV was the major known virus related to the encephalitis.

The search for an etiology can be challenging, but the causative viral agent can usually be identified by means of serological testing and viral culture. Serum titers for antibody against any neurotrophic agent are usually diagnostic, though the absence of a high antibody titer in the central nervous system (CNS) does not eliminate a potential viral pathogen. PCR assay could be a useful, rapid, and noninvasive test to confirm a diagnosis of encephalitis (26).

PCR assays are used to detect HSV DNA in the mouth, skin, serum, and CSF, and HSV may be principally spread via viremia. Kimura et al. (27) performed PCR in patients with HSV. Serum assays revealed positive results for HSV DNA in five of seven neonates, and all CSF results were positive for HSV (28). It remains a matter of debate whether

HSV DNA detected in the CSF is derived from intact virus or from viral DNA not associated with mature virions. However, the detection of HSV DNA is considered to be associated with viral replication and HSV infection of the CNS.

Failure of PCR in these patients was most likely a reflection of when the CSF samples were obtained during the course of the disease. The high specificity of the assay was demonstrated by the lack of false-positive results in a study of 708 cases, in which other causes for the neurological symptoms had been identified during follow-up (29,30).

In the past decade, the management of acute HSV encephalitis has dramatically improved with the advent of PCR, a method which has become the criterion standard for the diagnosis of HSV encephalitis.

An outbreak of neurological complications associated with an enterovirus 71 epidemic occurred in Taiwan in 1998. At three major hospitals, 41 children with culture-confirmed enterovirus 71 infection and acute neurological manifestations were identified. The spinal cord and brainstem were the main targets of enterovirus 71 in the fatal cases in this outbreak; however, the heart and pancreas can also be involved. Because the amino acid sequences in the P1 region are conserved (97% identity) among the three enterovirus 71 strains, as compared with other enteroviruses and polioviruses (31), these enterovirus 71 neurovirulent strains might share the same mechanisms of neurovirulence, and the mechanisms might be different from those in polioviruses. However, only one case was reported to have enterovirus 71 encephalitis in our studies.

In conclusion, PCR is an effective and quick method of detecting the organism responsible for idiopathic encephalitis. HSV encephalitis is still the major cause of idiopathic encephalitis in Taiwan.

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