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Aetiology of viral central nervous system infection, a Malaysian study

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Abstract

Over 100 viruses are known to cause acute viral encephalitis in human. In order to diagnose a viral central nervous system infection, various laboratory diagnosis methods have been used. In this study, we examined 220 cerebrospinal fluid samples that were received at the Diagnostic Virology Laboratory of University Malaya Medical Centre between year 2004 to 2006, by viral isolation, pathogen specific antibody ELISA, polymerase chain reaction (PCR) and Real-Time PCR. Majority of the samples were from patients <10 years old. Out of 220 samples, 3 were positive for viral isolation, 27 for PCR (inclusive for the 3 positive for viral isolation) and 39 for pathogen specific ELISA. The total positive detection rate of this study was 30%. Herpes virus was the most important aetiologic agent, responsible for 58% of infection, followed by paramyxovirus (especially measles virus) in 26% of infection, and 14% by enterovirus. Parvovirus and flavivirus were the other common viruses. Among the herpes viruses, herpes simplex and cytomegalovirus were the most common.

INTRODUCTION

Viral infections of the central nervous system (CNS) are often difficult to diagnose by conventional methods. Clinically, neurodiagnostic tests are often assisted by modern investigations such as electroencephalogram (EEG), computerized topography scans (CT) and magnetic resonance imaging (MRI). These tests, together with careful and continuous clinical assessment, can usually establish the presence of encephalitis but do not necessarily establish the aetiologic cause, which often remains unknown.[1,2]

Laboratory examination of cerebrospinal fluid (CSF) is an essential part of the diagnostic work-up in suspected CNS viral infection. Traditionally, this was done by viral culture and serology. Viral isolation followed by antigen detection and microscopy examination have low sensitivity. Enteroviruses such as echoviruses, polioviruses, and coxsackie B viruses are the viruses most frequently cultured from CSF. However, this may take up to 7 days.[3] Furthermore, the presence of specific antibody in CSF can interfere with viral isolation, yielding false negative result.[4,5]

Indirect diagnosis by detection of intrathecally produced antibodies generally has poor sensitivity during early stages of the infection. For most diseases, antibodies identified in the CSF are not useful diagnostically unless evaluated sequentially, requiring acute and convalescent CSF to demonstrate seroconversion. Such a delayed diagnosis however, is of little practical value in the decision whether to institute antiviral therapy, as well as being inconvenient. Nevertheless, this may retrospectively clarify the aetiology of infection and so have prognostic value.[5-7]

PCR, with its speed and high molecular sensitivity, has great potential for the diagnosis of CNS infections.[8] However, one of the difficulties in an assessment of the clinical usefulness of PCR is the lack of a satisfactory gold-standard test for comparison. Previous studies have established the usefulness of PCR in outbreaks of enteroviral meningitis[9,10] and Mollaret's meningitis[11], and have suggested that Epstein-Barr virus DNA can be used as a tumour marker in cases of AIDS-related primary lymphoma of the CNS.[12,13] PCR has an important role in herpes simplex encephalitis, because an early diagnosis has important implications for the management of patients. Lakeman *et al*[14] proposed that detection of herpes simplex virus DNA by PCR, rather than brain biopsy, should be the standard test for the diagnosis of herpes simplex encephalitis. However, the use of PCR for clinical diagnoses

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across a wide range of CNS viral infections has not been established.

We have, therefore, devised a protocol for the PCR amplification of CSF for four families of viruses associated with CNS disease. These include herpesvirus, flavivirus, enterovirus as well as paramyxovirus. The pan-herpesvirus PCR is for detecting herpes simplex virus type 1 and type 2, and varicella zoster virus. This study aims to assess the value of PCR, serology and viral culture in determining the etiologies of viral CNS infection in a Malaysian referral medical center.

METHODS

Sample Collection

This retrospective/prospective study was conducted based on all routine CSF samples sent during 2003 to 2005 to the laboratory of the Virology Unit of University Malaya Medical Centre (UMMC) with the clinical diagnosis of viral encephalitis. The laboratory serves as a reference centre for viral diagnosis and were receiving samples from all over the country including the Neurology and other wards of University Malaya Medical Centre (UMMC), other hospitals and private laboratories such as Subang Jaya Medical Centre, Pantai Medical Centre, Sunway Medical Centre, Assunta Hospital, Seremban General Hospital, Malacca Hospital, Tawakal Hospital, BP Lab and Path Lab. In total there were 220 CSF samples; 175 (79%) of which were furnished with adequate clinical details (including age, sex, race, CSF abnormalities), 45 (21%) samples were not.

In addition, 30 serum samples from other hospitals sent for Japanese encephalitis (JE) diagnosis were also obtained from the UMMC Virology Unit.

All archived samples were examined prospectively by PCR for a range of viruses. These include pan-herpesviruses (herpes simplex 1 and 2, vericella zoster virus, Epstein-Barr virus, cytomegalovirus, human herpes Virus 6), flaviviruses, enteroviruses (Coxackie A 16, coxackie B 5, enterovirus 71), paramyxovirus (mumps, measles, Nipah viruses). Later the assay was then adapted to real-time PCR version by using SYBR-Green I for higher sensitivity. Each CSF samples was also subjected to viral isolation and antigen specific ELISA.

Primer for PCR and RT-PCR

For pan-herpesvirus PCR, primers were designed to target the consensus region of DNA polymerase gene of the human herpesvirus. First, the sequence of DNA polymerase gene of 6 major types of human herpesvirus: herpes simplex virus 1, herpes simplex virus 2, Epstein-Barr virus, cytomegalovirus, varicella zoster virus and human herpes virus 6 were obtained from National Centre for Biotechnology Information (NCBI). After performing the homology analysis (Clustal X 1.83), two set of primers were designed in which the first set of primer Hp1/Hp2 were targeted at herpes simplex virus 1, herpes simplex virus 2, Epstein-Barr virus and cytomegalovirus. Second set of primer Vp1/Vp2 were targeted at varicella zoster virus and human herpes virus 6.

For pan-flavivirus, pan-enterovirus, pan-paramyxovirus RT-PCR (FEP RT-PCR), 3 sets of primers were also designed targeting at the consensus region. All primers were designed using the same parameters so that they would have similar melting temperatures (Tm), and GC content (GC %), so that all of them can work synchronously under identical conditions. All the primers were then blasted against the NCBI database to ensure it specificity. The primer sequences are shown in Table 1.

Amplification of virus genome

The viral nucleic acid (both DNA and RNA) was separately isolated by using QIAamp DNA and QIAamp RNA Blood Mini Kit (QIAGEN, Cat. No.52904). The extracted samples were then subjected to a pan-herpesvirus PCR and pan-RNA virus RT-PCR by using AccuPower PCR Premix (Bioneer, cat. No. K-2054) and RT-PCR PreMix (Bioneer, cat. No. K-2055). This premix contains optimal concentration of all the components necessary for cDNA synthesis as well as amplification in a single 0.2ml tube. Each premix tube also contains a stabilizer and is preserved in a lyophilized form. Thus the premix can be used easily by simply distributing the 15µl of diluted primer mix into each tube followed by adding the 5µl of RNA template (approximately 1.0 µg).

The RT step was performed at 50°C for 30 min. The rest of the thermal cycling profile are the same with both PCR and RT-PCR assay in which 15 min of *Taq* polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C denaturation for 30s, 60°C of annealing for 30s and 72°C extension for 1 min. The PCR results

Table 1: **Primer sequences used to detect four families of common CNS virus.**

Virus	Genome region	Primer name	Sequence
HSV-1	gp46	Hp1/Hp2	
HSV-2	gp33	Hp1/Hp2	Hp1: 5'--GTGGTGGACTTTGCCAGCCTGTACCC
CMV	gp60	Hp1/Hp2	
EBV	gp30	Hp1/Hp2	Hp2: 5'--TAAACATGGAGTCCGTGTCGCCGTAGATGA
VZV	gp133	Vp1/Vp2	Vp1: 5'-- GTCGTGTTGGATTTTCCAAGTTTGTATCCA
HHV-6	gp51	Vp1/Vp2	Vp2: 5'-TAAACACACAATCCGTATCACCATAAATAACCT
Flavivirus	5' UTR	FL-5F	ATGGCCATGACTGACACCACNCCTTT
		FL-6R	GTGTCCCATCCAGCGGTGTCATCAGC
Enterovirus	5' UTR	EV-8F	CAA GCA CTT CTG TTT CCC CGG
		EV-5R	ATGGCCAATCCAATAGCTATATGGTAACAA
Paramyxovirus	L gene	pmxF	TAC TGC CTN AAT TGG AGA TAT GA
		pmxR	CCT TCT ATA CCC CCT CTA GGA TA

HSV-1: Herpes simplex virus type 1; HSV-2: Herpes simplex virus type 2; CMV: cytomegalovirus; EBV: Epstein-Barr virus; VZV: Vericella-zoster virus; HHV-6: Human herpes virus 6
Hp1/Hp2 together with Vp1/Vp2 was used in pan-herpesvirus PCR. Primer FL-5F/FL-6R, EV-8F/EV-5R and pmxF/pmxR are consensus primer that used to detect 3 families of RNA viruses; the flavivirus, enterovirus and paramyxovirus.

were then analyzed by gel electrophoresis. 5µl of the 20µl PCR product were loaded into a 1.5% (W/V) SeaKem LE Agarose gel in 0.5 X TBE buffer with a 100-bp ladder as molecular weight marker.

The same sets of primers were then adapted to real-time SYBR Green I PCR/RT-PCR assay by using one-step QuantiTect SYBR Green kit (Qiagen). This assay was performed in iCycler (BioRad). After optimization, all the pre-collected were assayed in a 25µl reaction containing 5µl of the sample RNA, optimal concentration of primer (75nM each), and 3mM of $MgCl_2$ as final concentration. The thermal cycling profile is the same as mention above.

The diagnosis was regarded as probable if specific intrathecal antibody or virus-specific IgM was detected in CSF. Regarding the serology study of the CSF samples, normally a diagnosis can be regarded as confirmed if the intrathecal antibody of a specific virus is detected in CSF. However, the presence of intrathecal antibody may reflect contamination either by blood from traumatic tap or by serum when blood brain barrier breaking down. Therefore, intrathecal synthesis of the antibodies is assumed when the ratio of a virus specific antibody in serum/CSF is less than 20 by end point titration. Since there was no concurrent sample of CSF and serum was taken in this study, detection of virus-specific antibody in CSF was interpreted as probable CNS infection.

RESULTS

Two hundred and twenty CSF samples with clinical suspicion of viral CNS infections were evaluated with the newly developed assays. Out of these, 175 CSF samples had adequate clinical details, 46 samples from patients referred from other hospitals or laboratories did not.

Figure 1a shows the age distribution of the patients. As shown, most patients were in paediatric age group of less than 10 years old. Figure 1b shows the ethnic and gender distributions of the patients. As shown, the highest incidence was Malays, followed by Chinese, Indians and other ethnic groups. This corresponded to the ethnic composition of the general population. There were slightly more males than females.

Of the 220 CSF samples, 30% (n=66) were found to be consistent with CNS infection; 59% (n=39/66) had detectable anti-viral antibodies, 41% (n=27/66) were PCR positive, and 3 were culture positive (one each for herpes simplex 1, herpes simplex 2 and coxsackie B) (Table 2).

Figure 2 shows the positive serology and PCR

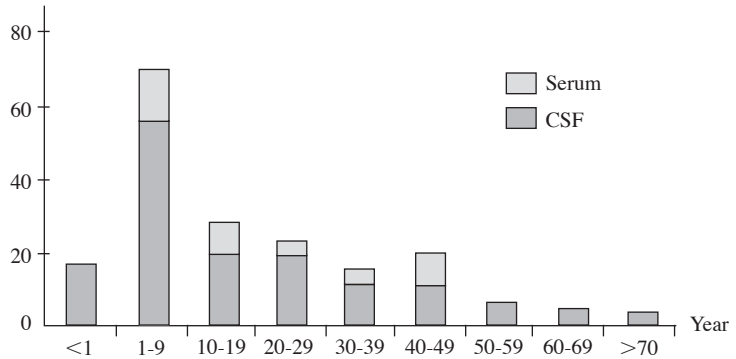

Fig. 1a Age distribution of patients

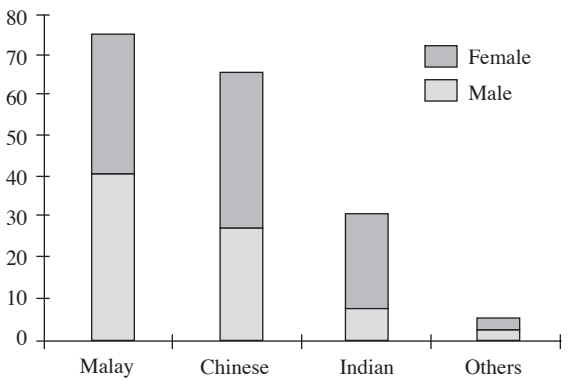

Fig. 1b Race and gender distribution of patients

according to the groups of viruses. As shown, 39 samples were positive by ELISA and 27 by PCR. Thus, of the 220 CSF samples, the aetiology was confirmed to be viral in 27, and another 39 were probable viral CSN infection. Thus, of the 66 positive serology and PCR, 39 (58%) were pan-herpesvirus (31% herpesvirus, 23% cytomegalovirus, 5% vericella virus), 17 (26%) were pan-paramyxovirus (23% measles virus and 2% rubella virus), 9 (14%) were pan-enterovirus, and 1 (2%) parvovirus. In addition, pan-flavivirus RNA was found in 10/30 serum samples by FEP RT-PCR.

Excluding samples without adequate clinical data, the overall results remained the same; there were 59 (instead of 66) positive samples, 33 (55.9%) were positive for pan-herpesvirus, 17 (28.8%) for pan-paramyxovirus, 8 (13.6%) for pan-enterovirus, and 1 (1.7%) for parvovirus.

DISCUSSION

In many patients with presumptive viral CNS infection, the underlying causes are often not found. Furthermore, the possible viral aetiology agents are many and screening for large numbers of viruses is not only prohibitive in cost, but also impractical.[15] This study used consensual segment PCR. By using consensual segment PCR, we could screen a large number of clinical specimens for a broad range of viruses quickly and accurately, thus probably increased the positive rate of the investigations.

Our results showed that there was broad agreement between PCR and ELISA, in that viruses commonly detected by ELISA were also detected by PCR. This suggests that the PCR results were genuine. PCR and ELISA were more sensitive than viral culture in diagnosing CNS infection. The rate of positive rate of our PCR examination, 27/220 CSF samples (12%), corresponds with published data. The specific viral agents found to be responsible were also broadly similar to those found elsewhere.[16,17]

ELISA appeared to be more sensitive than PCR except for enterovirus. For example, in measles, the detection by ELISA was 12 compared to RT-PCR which was 2. This was probably because

Table 2: Positive detection of CSF and serum samples by viral isolation, PCR and ELISA

		CSF Samples (220)			Serum Samples (30)		
		Confirmed		Probable	Confirmed		Probable
Virus Family	Virus	Isolation	PCR	ELISA	Isolation	PCR	ELISA
Pan-herpesvirus	CMV	0	6	9	0	0	0
	Herpes	2[a]	10	11	0	0	0
	VZV	0	0	3	0	0	0
Pan-flavivirus	Flavi	0	0	0	0	10[d]	0
Pan-enterovirus	EV	1[b]	7	2	0	0	0
Pan-paramyxovirus	Measles	0	4[c]	12	0	0	0
	Rubella	0	0	1	0	0	0
Others	Parvovirus	0	0	1	0	0	0
	Total	**3**	**27**	**39**	**0**	**10**	**0**

CMV: cytomegalovirus; VZV: Vericella-zoster virus; EV: enterovirus

[a] One for herpes simplex type 1 and herpes simplex type 2 was isolated in CSF samples. Viral isolation was done by Virology Laboratory UMMC.

[b] Coxackie B was isolated in of the CSF samples.

[c] Pan-paramyxovirus was detected by FEP RT-PCR in 4 of the CSF samples. DNA sequencing of the PCR product confirmed the specific virus infection.

[d] Pan-flavivirus was detected by FEP-RT-PCR, the DNA sequencing results confirmed the specific virus infection to be Japanese encephalitis virus.

the samples were taken late. With the rise of neutralizing antibodies, viral particles might have been cleared by the immune system and hence were not detected by PCR. On the other hand, in enteroviral infections, there were 7 patients confirmed by PCR versus 2 by serology. The specimens could have been taken earlier accounting for the higher sensitivity of PCR as compared to ELISA.

Serum sample showed that PCR was more sensitive than ELISA and viral culture in the diagnosis of JE (Table 2). These serum samples were collected by other centres and were send to UMMC for JE diagnosis. Ten out of 30 serum samples were positively detected by RT-PCR for JE virus. This was not false positive as subsequent viral genome sequencing confirmed the presence of JE virus.

JE virus was only detected in serum sample but not in CSF. This was due to the nature of arboviruses which spread haematogenously. Thus, in our study, JE virus genome was detected in serum during the primary viraemic phase.

There have been few studies published on the aetiology causes of CNS infections from this region. Our study yielded interesting epidemiology results. The total positive diagnosis rate (viral isolation, serology and molecular diagnostic) for CSF samples was 30% (66 out of 220), a figure similar to or slightly lower compared with those of other studies.[17-20]

In our study, 70% of patients diagnosed clinically with CNS viral infections had no microbiological diagnosis despite intensive investigations with consensual segment PCR, serology and viral culture. This could be due to the fact that our consensual segment PCR could not detect these viruses. The other possibility was that our consensual segment PCR test could be falsely negative because the specimens were obtained late and the rising antibody titre had effectively cleared the circulating viral particles in the cerebrospinal fluid or serum. Since we were not able to perform ELISA for all the viruses screened by PCR, some of the viral agents could have been missed.

In our CSF study, the commonest causes of viral CNS infection were the herpesviruses (herpes simplex virus, varicella zoster virus and cytomegalovirus), measles virus, enteroviruses,

Fig. 2a Positive detection of CSF and serum samples by ELISA and PCR

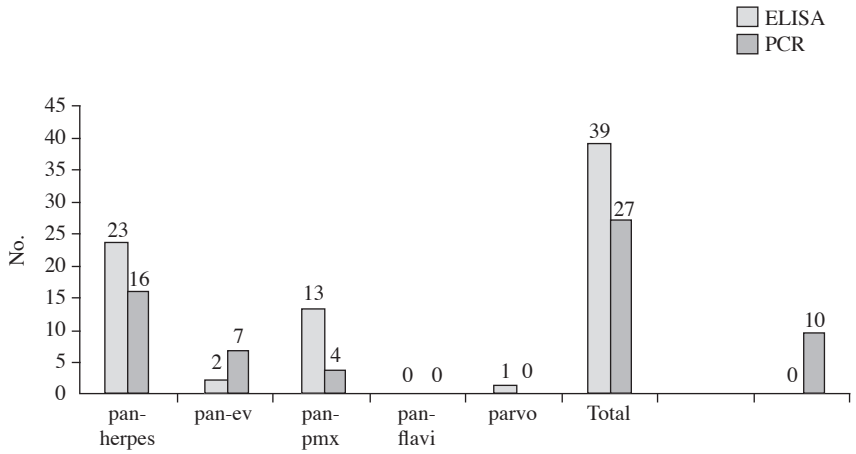

Figure shows that 39 samples were detected to be positive by ELISA and 27 by PCR.
Pan-ev: Pan-enterovirus; Pan-pmx: Pan-paramyxovirus

rubella and parvoviruses. This was consistent with in other studies. In a study of 40 patients with viral encephalitis in Thailand, viral agents were identifiable in a total of 26 (65%), with dengue (8), JE (6), herpes simplex (4), human herpes 6 (3), mumps (2), enterovirus, varicella zoster and rabies (1 each) being the agents found. In a large, prospective, epidemiological study in Finland, 10.3% of viral encephalitis had confirmed virology diagnosis. Another 52.6% had suggested virology diagnosis; with varicella zoster virus (22%) being the commonest, followed by respiratory (20%), enterovirus (19%), and herpes simplex, adenovirus, Epstein-Barr and rotavirus (5% each). In another radiological and serological study in Switzerland, the overall positive rate was 61/104 (60%), with varicella zoster, rubella, Epstein-Barr, herpes simplex, adenovirus, flavivirus, measles, respiratory syncytial and cytomegalovirus being the culprits.[17-20]

Our study showed that the herpes simplex virus was the commonest cause of CNS viral infection where the aetiology agent could be identified; though it caused only 10% of all CNS infection. JE virus, the commonest cause of viral encephalitis in Asia, was less common among our patients, probably because most of the patients were from urban areas, and were not exposed to animals which were the amplifying hosts of the virus.

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