LABORATORY INVESTIGATION OF VIRAL ETIOLOGIC AGENTS IN PEDIATRIC PATIENTS WITH ACUTE VIRAL ENCEPHALITIS

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Encephalitis is an acute inflammatory disease that effects the brain, and it almost always involves inflammation of the adjacent meninges. Thus, the term encephalitis or meningoencephalitis usually applies to the same disease. Encephalitis in hosts with intact immunity is most commonly caused by a variety of viral infections. Viral encephalitis is usually acute and occurs as a consequence of the destruction of the infected neuronal cells of the host immune system.

The common cause of viral encephalitis are herpes simplex virus (HSV), arboviruses (e.g., Japanese encephalitis virus(JEV), St.Louis encephalitis virus), enterovirus, rabies virus and mumps virus; and in a lesser extent: human herpesvirus 6 (HHV-6), cytomegalovirus (CMV) and Epstein-Barr virus (EBV).

The present study employed several laboratory methods to investigate the infection rate of viral etiologic agents in pediatric patients who were clinically diagnosed as acute viral encephalitis. The subjects were hospitalized at Siriraj Hospital during the period of February 1996 to October 1998, and included 36 cases of viral encephalitis, 23 cases of CNS infection, 61 cases of other manifestations and 43 cases of unknown diagnosis. The methods used in our study were 1) the detection of HSV and HHV-6 DNA genomes in CSF by PCR, and of JEV and enterovirus RNA genomes by RT/PCR; 2) determination for ratio of CSF: Serum HSV IgG as expressed in term of antibody specific index (ASI) by ELISA; 3) detection of IgG and IgM to JEV and Dengue Virus antigens in CSF and paired sera; 4) detection for a four-folded rise of hemagglutination inhibiting(HI) antibodies to JEV and dengue virus type 1 and 2 in paired sera; and 5) detection of specific antibodies in paired sera, i.e., HSV IgG and IgM, HHV-6 IgG and IgM, and enterovirus IgG, IgM and IgA Using various laboratory methods mentioned above. With limited amount of CSF samples and sera, we discovered the prevalence of 26.09%(6 of 23) of Japanese encephalitis,16.67% (6 of 36) of HSV encephalitis, 8.33%(3 of 36) of HHV-6 encephalitis and 3.13%(1 of 32) of enterovirus encephalitis. We also discovered four cases of JEV infection based on the criteria that the diagnosis of JEV infection should be given in cases of CSF not being available or cannot diagnosed but sera were positive by ELISA IgM or by a four-folded rise of HI antibody titer. Five cases(13.88%) of Dengue infection were diagnosed 36 patients who were at first presented with symptoms and signs suggestive of viral encephalitis but later progressed to dengue hemorrhagic fever. With information from routine laboratory two more cases of mumps, one case of varicella zoster encephalitis and one case of rabies encephalitis were also found in our subjects. We concluded that 25(69.44%) of 36 acute viral encephalitis cases were laboratory confirmed viral infections. Because of the laboratory methods, the diagnosis of viral encephalitis could not be achieved without investigation of the CSF samples, either by detection of viral genomes or of the specific antibodies. The present study suggested that PCR and RT/PCR are very useful tools in the diagnosis of CNS infections. And, since the above diseases are caused by many viral agents, multiplex PCR should be further developed in order to reduce materials and time consumed; and most of all when the specimens obtained are of very minute amount. However it should be kept in mind that PCRs are susceptible to contamination with extraneous DNA fragments that can be amplified and carried over through successive amplification rounds. Moreover, PCR may yield false positive result caused by nonspecific amplification of unrelated nucleic acid sequences. And the lack of gold standard against which to validate the PCR result makes the interpretation of positive PCR tests extremely difficult.

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วิโรจน์ ปองธนพิสิฐ : การสืบค้นทางห้องปฏิบัติการหาเชื้อต้นเหตุของ โรคไวรัสสมองอักเสบเฉียบพลันในผู้ป่วยเด็ก (LABORATORY INVESTIGATION OF VIRAL ETIOLOGIC AGENTS IN PEDIATRIC PATIENTS WITH ACUTE VIRAL ENCEPHALITIS). คณะกรรมการควบคุมวิทยานิพนธ์ : พิไลพันธ์ พุธวัฒนะ, Ph.D., พิสมัย โพธิผละ, M.Sc., อุไรวรรณ โฆษิตานนท์, M.Sc., สนทนา ศิริตันติกรณ์, Dr. rer. Nat., กุลกัลยา โชคไพบูลย์กิจ, M.D., บุญยศ เรืองสกุลราช, Ph.D. xxx หน้า. ISBN 974-663-436-4

โรคสมองอักเสบ เกิดจากอาการอักเสบเฉียบพลันของสมองและมักมีการอักเสบของเยื่อหุ้มสมองร่วมด้วย โรคสมองอักเสบเฉียบพลันมักมีสาเหตุจากเชื้อไวรัส ก่อให้เกิดการทำลายเซลล์ประสาทที่ติดเชื้อ และ เกิดการตอบสนองของระบบภูมิคุ้มกัน

เชื้อต้นเหตุ ของโรคไวรัสสมองอักเสบที่พบได้บ่อย เช่น เชื้อเริม หรือ Herpes simplex virus (HSV), Arboviruses (เช่น Japanese encephalitis virus, St. Louis encephalitis virus), Enteroviruses, โรคพิษสุนัขบ้า และ โรคคางทูม เชื้อที่พบได้น้อย เช่น Human herpesvirus 6(HHV-6), Cytomegalovirus (CMV), และ Epstein-barr virus (EBV).

การศึกษานี้ใช้วิธีการตรวจทางห้องปฏิบัติการหลายวิธี เพื่อสืบค้นอัตราการติดเชื้อไวรัสต่างๆซึ่งก่อโรคสมองอักเสบเฉียบพลันในผู้ป่วยเด็กซึ่งได้รับการรับตัวไว้รักษาเป็นผู้ป่วยในของโรงพยาบาลศิริราชในช่วงเวลาตั้งแต่เดือนกุมภาพันธ์ 2539 ถึงเดือนตุลาคม 2541 ผู้ป่วยเด็กที่นำมาศึกษาวิจัยถูกแบ่งออกเป็น 4 กลุ่มโดยกลุ่มที่ 1 เป็นผู้ป่วยโรคไวรัสสมองอักเสบจำนวน 36 ราย กลุ่มที่ 2 เป็นผู้ป่วยที่แสดงอาการติดเชื้อของระบบสมองส่วนกลางจำนวน 23 ราย กลุ่มที่ 3 เป็นผู้ป่วยโรคอื่นๆจำนวน 61 ราย (เช่นไข้และชัก 13 ราย, ลมบ้าหมู 7 รายและสมองอักเสบจากการติดเชื้อชนิดแพร่กระจาย 3 ราย,) กลุ่มที่ 4 เป็นผู้ป่วยที่ไม่สามารถติดตามประวัติการวินิจฉัยโรคได้จำนวน 43 ราย วิธีการศึกษาคือ (1)ตรวจหายีโนมของไวรัสในน้ำไขสันหลังได้แก่ ยีโนมของHSVและ HHV-6 โดยวิธีปฏิกิริยาลูกโซ่โพลี่เมอเรส (PCR) และของ JEV และ Enterovirus โดยวิธี Reverse Transcription/ Polymerase Chain Reaction (RT/PCR) (2) เปรียบเทียบค่าอัตราส่วนของ HSV IgG ที่อยู่ในน้ำไขสันหลัง และ ในซีรั่มโดยใช้ค่า ดัชนี Antibody Specific Index โดยวิธี ELISA (3) ตรวจหา IgG และ IgM ต่อเชื้อไข้สมองอักเสบเจอี และเด็งกี่ในน้ำไขสันหลังและซีรั่มคู่ (4) ตรวจหาระดับ แอนติบอดี้ที่เพิ่มขึ้นเป็น 4 เท่า โดยวิธี HI ต่อ JEV และDengue virus type 1 และ 2 ใน ซีรั่มคู่ (5) ตรวจหาระดับแอนติบอดี้ชนิด HSV IgG และ IgM, HHV-6 IgG และ IgM และ Enterovirus IgG, IgM และ IgA ผลการศึกษาพบอัตราการติดเชื้อ JEV 26.09%(6 ใน 23 ราย), เชื้อ HSV 16.67%(6 ใน 36 ราย), HHV-6 8.33%(3 ใน 36 ราย), เชื้อ Enterovirus 3.13%(1 ใน 32 ราย), เชื้อเด็งกี่ 13.88%(5 ใน 36 ราย), เชื้อคางทูม 5.56%(2 ใน 36 ราย), เชื้ออีสุกอีใส 2.78%(1 ใน 36 ราย), และเชื้อพิษสุนัขบ้า 2.78%(1 ใน 36 ราย) ในผู้ป่วยสมองอักเสบทั้งสิ้นจำนวน 36 รายนี้ ไม่สามารถศึกษาเชื้อต้นเหตุได้ครบทุกชนิดในผู้ป่วยทุกคน เนื่องจากปริมาณตัวอย่างตรวจมีจำกัด แต่ก็ได้พบเชื้อทั้งสิ้น 25 ชนิด คิดเป็นอัตราการติดเชื้อไวรัส 69.44% การวินิจฉัยทางห้องปฏิบัติการจะบ่งชี้สาเหตุของโรคไวรัสสมองอักเสบได้ต่อเมื่อใช้ตัวอย่างตรวจที่เป็นน้ำไขสันหลังเท่านั้น ส่วนการหาแอนติบอดี้จำเพาะอาจช่วยหรือไม่ช่วยสนับสนุนการวินิจฉัย

การศึกษาครั้งนี้เชื่อว่าการตรวจหา ยีโนมโดยวิธี PCR และ RT/PCR เป็นวิธีการที่มีประโยชน์มาก ในการตรวจวินิจฉัยโรคติดเชื้อไวรัสที่ระบบประสาทส่วนกลาง แต่เนื่องจากมีเชื้อก่อโรคหลายชนิด และปริมาณน้ำไขสันหลังมีน้อยจึงน่าจะได้มีการพัฒนาวิธี Multiplex PCR ขึ้นใช้เพื่อช่วยลดปริมาณการใช้น้ำไขสันหลังและประหยัดเวลาในการตรวจสอบ.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFRIMS	Armed Force Research Institute of Medical Sciences
ASI	Antibody specific index
BSA	Bovine serum albumin
bp	base pair
°C	degree Celcius
CF	Complement - fixation
CMV	Cytomegalovirus
CNS	Central nervous system
CPE	Cytopathic effect
CT	Computer tomogram
DEN	Dengue virus
DHF	Dengue hemorrhagic fever
DGV	Dextrose gelatin veronal
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EEG	Electroencephalogram
ELISA	Enzyme linked immunosorbent assay
FA	Fluorescent antibody
GRBC	Goose red blood cell

HA	Hemagglutination
HHV-6	Human herpesvirus 6
HRP	Horse raddish peroxidase
HI	Hemagglutination inhibition
HSV	Herpes simplex virus
IE	Immediate early gene
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
Kb	Kilobase
LLC-MK_2	A cell line derived from Rhesus monkey kidney
MAC-ELISA	μ chain antibody capture enzyme linked immunosorbent assay
MRI	Magnetic resonance image
MVE	Murray Valley encephalitis
μl	microliter
mg	milligram
ml	millilitre
min	minute
mRNA	Messenger ribonucleic acid
NHS	Normal human serum
ng	nanogram (10^{-9} grams)
NT	Neutralization test
NS	Nonstructral gene
O.D.	Optical density

ORF	Open reading frame
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline containing Tween 20
PCR	Polymerase chain reaction
PMK	Primary monkey kidney cell
RPHA	Reverse passive haemagglutination
RNA	Ribonucleic acid
RT/PCR	Reverse transcriptase/polymerase chain reaction
SDS	Sodium dodecyl sulfate
SLE	St. Louis encephalitis
VZV	Varicella zoster
WN	West Nile

CHAPTER I

INTRODUCTION

Viral, bacterial, fungal and parasitic agents can infect the central nervous system (CNS) and produce the devastating diseases with significant morbidity and mortality e.g., encephalitis, meningoencephalitis, aseptic meningitis and myelitis. Among the viral agents, the common ones reported are herpes simplex virus type 1 (HSV-1), enteroviruses, mumps virus, arboviruses, rabies virus and several uncommon viruses such as human herpesvirus 6 (HHV-6), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (1).

The diagnosis of viral infections in CNS is still the problems. The conventional laboratory methods such as viral culture and serology are cumbersome and the collection of clinical sample such as brain biopsy is invasive and unsatisfactory. Electroencephalogram (EEG), computer tomogram (CT) and magnetic resonance image (MRI) are useful for clinical diagnosis and define the severity and distribution of neurologic involvement. However, they are not specific for each viral etiologic agent (1-2).

Modern laboratories have simplified and improved the ability of polymerase chain reaction (PCR) to diagnose many viral CNS infections. PCR has been reported in the diagnosis of enteroviral, HSV, HHV-6, EBV and CMV infections in cerebrospinal fluid (CSF) (3-11). Primers also exist for the genome detection of

certain arboviral encephalitides e.g., California encephalitis, Japanese encephalitis. (JE) (3-4)

The successful detection of viral DNA or RNA in CSF is influenced by time at specimen collection, severity of the disease and the viral etiology. PCR assay in CSF is relatively rapid, and sensitive; and when method of specimen collection is concerned, collecting the CSF is less invasive than collecting the brain biopsy. Previous reports, as using PCR or RT/PCR to detect viral DNA or RNA in CSF samples from patients with CNS infections could show prevalence at a range of 3-26%, 2-8% and 6-15% for enterovirus, HSV and HHV-6 infections, respectively (11-19) .Meanwhile, the annual epidemiological surveillance of Ministry of Public Health, Thailand in 1996 reported the incidence of 519 cases of Japanese encephalitis, 18 cases of poliomyelitis and 77 cases of rabies. There were not reported for other viral etiologic agents that are due to limitation of laboratory investigation method.

CHAPTER II

OBJECTIVE

This study attempted to investigate the viral etiologic agents of CNS infection in pediatric patients by

1. developing PCR technique to detect HSV DNA and HHV-6 DNA in CSF.
2. developing reverse transcription / polymerase chain reaction (RT/PCR) technique to detect JEV RNA in CSF.
3. detecting enterovirus RNA in CSF by using commercial kit.
4. determining the rate of JEV, HSV, HHV-6, and enterovirus infection in pediatric patients aged one month to 12 years who were hospitalized to the Infectious Disease Unit, Department of Pediatrics, Siriraj Hospital according to the symptoms and signs of viral encephalitis during February 1996 to October 1998. Laboratory investigations were performed for detecting the viral genome in CSF together with the specific antibodies in sera or sera and CSF by various serological tests.

CHAPTER III

LITERATURE REVIEW

CNS infections can be caused by a spectrum of infectious agents including viruses, bacteria, fungi, parasites and prions. Major clinical manifestations of CNS infection are fever, headache, alteration of mental and focal neurological signs. However, these features may be of non-infectious origin. Clinical presentation of CNS infections can be encephalitis, meningitis, myelitis, etc., and may be either of acute subacute or chronic form. Diversity on etiologic agents and incidence of CNS infections vary on geography, cultural factors and frequency of exposure to vectors for viral transmission (1).

Pathological findings and pathogenesis of viral encephalitis.

Viral infections of CNS occur infrequently, and mostly result in a benign, self-limited disease. Nevertheless, death and neurological damage may be seen occasionally. A viral CNS disease is usually determined by tissue tropism and disease duration. Host physiology such as age, sex and genetics also influence viral infections and clinical course (20).

Encephalitis is an acute inflammatory disease that affects brain tissue and it almost always involves inflammation of the adjacent meninges. Encephalitis is most commonly caused by viral infection and manifests as either acute encephalitis or post

infectious encephalomyelitis. Acute viral encephalitis is caused by direct viral infection of neural cells and perivascular inflammation; and destruction of gray matter occur as a consequence of the host immune response. Meanwhile, post infectious viral encephalitis produces signs and symptoms of the disease following a viral systemic infection. The primary pathologic finding of post infectious viral encephalitis demonstrates demyelination of white matter and perivascular aggregation of immune cells suggesting an autoimmune etiology. Virus and viral antigen can not be demonstrated in this disease. The presence of immune cells distinguishes primary and post infectious encephalitis from an encephalopathy (1).

Viruses gain access to the CNS by two basic pathways : hematogenous and neuronal routes. Arboviruses, enteroviruses, measles virus and varicella virus encounter hematogenous pathway for entering the CNS, while HSV and rabies virus are the prototypes in neuronal pathway (20).

Hematogenous route

The initial steps involved in hematogenous spread to CNS begins with virus replication at the local site of entry and primary viremia which allows virus to seed distant locations in the body and frequently marks the onset of clinical illness. Infection of the distant organs permits secondary replication which is followed by extensive viremia for long period of time facilitating invasion to the CNS. Virus must localize in the blood vessel of the CNS before crossing the blood-brain or blood-CSF barrier, a network of tight endothelial junctions sheathed by glial cells that regulate molecular access to the CNS. Virus infects endothelial cells, leaks across damaged endothelium, passively transports through channels of endothelium or bridges the endothelium within migrating leukocytes. Cell-associated and cell-free viruses can

cross the endothelium and enter the brain parenchyma or CSF. In CSF, virus may remain only in the meninges or may enter the brain parenchyma by crossing either ependymal cells or the pial linings (20).

Neuronal route

Neuronal spread to the CNS can occur along peripheral or cranial nerves. The nerve shields the virus from immune regulation and allows access to the CNS. After primary replication at site of entry into the body, the virus such as rabies enters the peripheral nerve by crossing myoneural spindles or by entering the motor end plate, and then spreads along motor or sensory neural pathway to infect neurons in the brain stem. CNS infection through olfactory neurons of the cranial nerve system is unique since olfactory neurons are not protected by the blood brain barrier, thus, neuronal access to the brain can occur easily (20).

Epidemiology of viral encephalitis

Bases on epidemiological background, viral encephalitis is divided into two forms; 1) the epidemic encephalitis of which the incidence rate is dependent on seasonality and usually associated with dense population of the insect vector; and 2) the sporadic form of which the incidence rate does not change all year round. The examples of the epidemic form are St.Louis encephalitis which is the most common arboviral encephalitis of North America, and Japanese encephalitis which is common in East and Southeast Asia; and the examples of the sporadic form are HSV, rabies virus and enteroviruses.

Clinical diagnosis of viral encephalitis

Onset of viral encephalitis usually is acute. Clinical diagnosis of the disease relies on presence of sign and symptoms of high fever, headache, nausea, vomiting, neck pain and photophobia. Alteration of level of consciousness ranges from mild lethargy and confusion to coma. Generalized or focal neurological abnormalities may be stationary, progressive or fluctuating. Seizures also are common. Investigation by EEG, MRI and CT may facilitate diagnosis of encephalitis. Clinical presentation and patient's history, while frequently suggestive of a diagnosis, remain unreliable methods for determining the specific etiology (1-2, 20).

Laboratory investigation of viral encephalitis

CSF findings

Examination of CSF in a patient with viral encephalitis reveals a normal or evaluated opening pressure, a normal or elevated protein level and a normal glucose level. Other typical CSF finding include pleocytosis and a moderate elevation in white cell count of up to several hundred per cubic millimeter. Polymorphonuclear cells may predominate at early onset of the disease but are replaced by mononuclear cells within hours (1).

Virological findings

Current laboratory techniques for identifying viral etiology of CNS infection remain insensitive. Previous investigators failed to identify an agent in 33 to 75% of presumed CNS infection (21-22). Rates of virus isolation from CSF varied on the etiology. The disease can be diagnosed only presumptively by serological test or

isolation of virus from another anatomical site of the body. Detection of viral antigen and virus culture from brain biopsy was previously used as the gold standard for the disease diagnosis; and is now being replaced by PCR or other modern techniques for detection of viral genomes. The precised diagnosis well lead to the proper disease management and curation.

Japanese encephalitis virus

JE is a serious acute disease accompanied by high fatality and grave sequalae with neuropsychiatric disorders among half of the survivors. Its causative agent, JE virus, is a member of the family *Flaviviridae*, genus *Flavivirus* (23-24). All flaviviruses contain a serologically cross-reactive, group specific epitope located on the virion envelope protein. Cross-neutralization with polyclonal sera has been utilized to classify the flaviviruses into eight antigenic complexes (25-26). JE virus is the prototype of an antigenic complex that includes St.Louis encephalitis (SLE), Murray Valley encephalitis (MVE), West Nile (WN) viruses and several other flaviviruses of lesser medical importance. Among flaviviruses that cause human encephalitis, JE virus is the most common. The first JE virus isolate was recovered from the brain of an encephalitis patient in Tokyo in 1935 and was virologically and serologically established as the prototype Nakayama strain. During the early 1930s, it was suspected that the virus was transmitted by mosquito vector, *Culex tritaeniorhychus* which breeded in watered rice fields. The disease is endemo-

epidemic in many countries in East, Southeast and South Asia where plenty of rice fields provide ample breeding sites for vector mosquitoes (23, 26). Frequent epidemics with 10,000-20,000 human cases occurs in Asia annually. Since 1960 when vaccine programs were instituted in some regions in Asia and the orient, the number of human infections has dropped from thousands to only a few dozen each year (27). Mortality rates of 25-50% are observed during epidemics and neurological morbility is high.

In an ecological study of JE in Thailand during 1992 to 1993, two JE virus isolates were obtained from each pool of *C tritaeniorhynchus* and *C vishnui* captured in Chiang Mai Area, Northern Thailand (23, 26). RT-PCR results and nucleotide sequence analysis of their PrM protein genes confirmed that these two isolates belonged to JE virus genotype but conventional serological tests could not identify them into either of the two previously known JE isolates, Nakayama or JaGAr 01 immunotype (25).

At least two immunotypes of JE virus have been repeatedly distinguished; Nakayama and JaGAr 01 isolated from *Culex* mosquitoes in Japan in 1959. (Viruses isolated in 1969 were immunologically placed between these two types). To further complicate the matters, antigenic differences have been shown between substrains of Nakayama virus. The recognition of antigenic strain variation has led to altered strategies for vaccine preparation.

1. Virion structure

JE virion is spherical in shape with diameter about 50 nm. It has a spherical nucleocapsid surrounded by lipid bilayer envelope with small surface projection of 5 to 10 nm. in length, representing E glycoprotein dimers anchored to the virus

membrane. The E protein is thought to be involved in virion assembly, receptor binding, membrane fusion, and neutralization by specific antibodies. The second envelope protein, the membrane (M) protein, is also found on the surface of the virions. Intracellular virions contain a precursor of M, designated Pr M, whereas virions being released into the culture fluid contain the mature M protein. The icosahedral nucleocapsid about 30 nm. in diameter is composed of multiple copies of the capsid protein (10.5 - 11.7 kDa) and a copy of the genomic RNA (120 to 140S, buoyant density 1.30 to 1.31 g/ml) which can be isolated after solubilization of the envelope with nonionic detergents (26). The released extracellular virus particles are morphologically indistinguishable from cell-associated particles found within intracellular vesicles. The immature particles contain exclusively unprocessing Pr M and are less infectious than the released virions.

2. Genome structure

The genome of JE virus is a single-stranded, positive-sense, infectious RNA, about 11 kb long. Genome-length RNAs appear to be the only virus-specific messenger RNA (mRNA) molecules in JE-infected cells. The major portion of the genome RNA consists of a long open reading frame (ORF) of more than 10,000 bases (28). Flanking this ORF are 5′ and 3′ nontranslated regions (NTR) containing conserved RNA elements which may play an undefined role in RNA replication. The 5′ terminus presents the cap structure while the 3′ terminus presents the stable stem-loop structure. These terminal structures are thought to function as a signal for initiation of RNA synthesis. The nonstructural proteins (NS) of JE virus is devided into seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (26).

The NS1 glycoprotein exists in cytoplasma and at cell surface, and it is also secreted slowly as extracellular nonvirion forms from the manmalian cells, but not from the mosquito cells. The secreted form is antigenic and contains both virus-specific and cross-reactive epitopes. Antibodies to NS1 do not react with the virion and exhibit no neutralizating activity. However, protective immunity is presumably mediated by antibody and complement mediated lysis of cells bearing NS1 targets (26, 29).

The NS3 is highly conserved and is believed to be an enzymatic component of the RNA replication unit probably due to its function as protease, helicase, and RNA triphosphatase activities. The NS3 protein is present on cell membranes and serves several functions, i.e., stimulation of virus-specific T-cell responses and acting as a target for an attack by cytotoxic T cells. NS3 also contains multiple dominant epitopes for CD4+ and CD8+ T lymphocytes (30).

The NS5 is the largest and most highly conserved flavivirus protein. The conserved domain characteristics of an RNA polymerase is found within the C-terminal portion of NS5 such that it functions both as the RNA dependent RNA polymerase in virus replication and as a methyltransferase in 5′ cap methylation.

The other four nonstructural proteins, NS2A, NS2B, NS4A and NS4B, are each relatively small and hydrophobic. These proteins are postulated to be the components of the viral replication complexes that function as the membrane anchors. NS2A has been implicated in the processing of NS1, while the presence of NS2B is required for NS3 protease activity (28, 31).

3. Pathogenesis

Most JE mosquito vectors are zoophilic, preferring animals to human. The JE virus replicates in salivary gland of mosquitoes such as *C. tritaeniorhynchus*, *C. gelidus* and *C. fuscocephala*. JE virus is transmitted mainly to domestic and wild animals including birds. Pigs and birds are the principle amplified hosts, and mosquitoes, especially *C. tritaeniorhynchus,* are responsible for transmission between these vertebrates and from vertebrates to human. After man is biten by the infected mosquito, the virus replicates locally and then spreads by low-level primary viremia to lymphoreticular organs where additional replication occurs: and is followed by secondary viremia with viral dissemination to target organs such as brain, kidney, and liver. The method of invasion of CNS is unknown, but pathologic studies showed the major areas of involvement to be the cerebral cortex, cerebellum, and spinal cord. However, functional and structural changes due to hypertension, cerebrovascular diseases, and head trauma have also been suggested as factors contributing to neuroinvasion. In addition, viral isolates recovered from brain tissue of fatal human cases in Thailand differed in an RNA fingerprint analysis from the contemporare isolates from pigs and mosquitoes, suggesting that the human isolates represented distinct neuroinvasive prototypes (33-34).

4. Clinical manifestation

After the incubation period of 4-14 days, JE virus produces a rapid onset of a prodromal symptom of high fever, headache, abdominal pain, nausea, diarrhea and vomiting lasting several days precedeing the signs of CNS infections such as unconsciousness, confusion, ranging from the mild mental clouding to drowsiness and stupor, or agitation and delirium. Generalized seizures occur in 20% of the pediatric

cases but are much less frequent in adults. Some patients develop a prominent parkinsonian like, extrapyramidal syndrome with tremor, masked faces, and rigidity. Facial weakness, extraocular movement abnormalities, ataxia and dysphasia have also been recorded. In severe cases, coma develops within a few days of onset. The majority of deaths occur by the fifth to tenth days or during a more protracted course with cardiopulmonary complications. Mortality rates range from less than 10% in healthy young adults to as high as 50% in young children and patients older than 65 years of age. In nonfatal cases, the course of acute illness lasts for up to 2 weeks followed by a lengthly convalescent phase. Neuropsychiatric sequalae occured in 45% to 70% of the survivors and are particularly severe in children. Sequalae include parkinsonism, convulsive disorders, paralysis, mental retardation, and psychiatric disorders. The social prognosis of survivors is generally poor. Sequalae are more frequent in patients whose acute disease is severe, prolonged, and associated with coma and localizing neurological signs (24, 26).

5. Laboratory diagnosis

5.1 Virus isolation

JE virus is rarely isolated from blood at early illness, usually not later than 6 to 7 days after onset. It can be cultured from fresh, acutely infected brain tissue obtained by biopsy or at autopsy. However, JE virus may be isolated from CSF in up to one-third of the patients by inoculating the CSF specimens collected at acute phase onto C6/36, a cell line from *Aedes albopictus* (32, 35). The isolation rate is correlated with a poor prognosis. The JE virus can replicate in a wide variety of primary and continuous cell cultures devired from hamster, porcine, chicken, monkey, and mosquito in origins. Vero and LLC-MK_2 cells are useful for plaque assays (36).

Infant mice are highly susceptible to lethal infection by all routes of inoculation. Weanling mice succumb to intracerebral virus inoculation, but there is variation in pathogenicity by virus strain as using intraperitoneal route.

5.2 Virus antigen detection

The rapid diagnosis for JE virus infection may be achieved by immunofluorescence staining of viral antigen in mononuclear cells recovered from CSF (37). A monoclonal antibody-based reverse passive haemagglutination test (RPHA) described by Ravi *et. al.,* was used for detection of soluble JEV antigen in CSF (4). Based on immunofluorescence test, when JE antigen was localized only to neurons of brain tissue, the recovery rate of JE virus from CSF was 10% or lower.

5.3 Virus antibody detection in serum and CSF

Serologic diagnosis in JE infection depends on the demonstration of a four-folded or greater rise in specific antibody titer by HI, NT and CF, and also on the demonstration of specific IgM by ELISA (38-41). These tests may provide a clear diagnosis in patients with their first flavivirus infection, but uninterpretable results may be found in patients with multiple previous flavivirus infections acccording to the cross reactivity of the antibody. Immunizations with JE and yellow fever vaccines may also interfere with the interpretation of serologic results.

The standard method to diagnose JE is IgM capture ELISA, which has a sensitivity approaching 100% when both CSF and serum samples are tested together, and samples from 1 to 2 weeks after onset are used (39). The detection of locally synthesized IgM antibody in CSF is especially well suited for the definite diagnosis of virus infection of the CNS. CSF specific IgM is routinely used to differentiate between JEV encephalitis and extraneural JEV infection. A four-folded rise in serum

antibody titer or the presence of serum specific IgM can be observed in both categories, but CSF specific IgM will be present in CNS infected cases only. Thus, assay for JEV IgM in CSF is of diagnostic value especially when considered that almost of JEV infection is asymptomatic. IgM antibody synthesis may persist in CSF for weeks after recovery, indicating persist antigenic stimulation (4). Seventy-two patients suffering from meningo-encephalitis were studied, clinically, biochemistry, microbiologically and virologically. Evidence of rising titer in serum and CSF of antibodies to JEV virus were present in 50% of the cases.

5.4 Virus genome detection

In current year, the rapid RT-PCR was carried out to diagnose JEV encephalitis. The CSF specimens were first screened for flaviviral genomes by using flaviviral cross-reacting primer pairs, then the PCR product was further identified by using primer pairs specific to NS3 region of JEV genome (31, 42).

Herpes simplex virus

HSV belongs to the family of large DNA-containing viruses, *Herpesviridae*, which comprises more than 90 members known to infect a wide range of hosts varying from fungi to animals and humans. There are nine herpesviruses that infect humans; eight are human herpesviruses but the ninth one, B virus is zoonotic. The eight human herpesviruses are herpes simplex virus type 1, herpes simplex virus type 2, varicella-zoster virus, cytomegalovirus, Epstein Barr virus, human herpesvirus-6,

human herpesvirus-7 and human herpesvirus-8 or kaposi's sarcoma associated herpesvirus (KSHV) (43-45).

Herpes means "to creep" in Greek. Initially this term was utilized as a description for a variety to cutaneous, spreading lesions including the classic form of fever blisters recorded by Hippocrates and Herodotus, 25 centuries ago. In 1912, Gruter *et.al.*, demonstrated successfully the cultivation of HSV on rabbit cornea, and revealed that the infection could be transported within nervous tissue and ultimately caused encephalitis. During the 1940s and 1950s, HSV were found to be associated with many diseases including gingivostomatitis, herpetic whitlow, keratoconjuntivitis, neonatal herpes, visceral herpes and herpetic encephalitis. In 1968, Nahmias and Dowdle could distinguish HSV-1 from HSV-2 by basing on their antigenic and biological differences (44).

1. Virion structure

Virion of HSV is spherical in shape. The core of mature virion contains the viral genome which is tightly pack as a "ball wool". The envelope varies from 150 to 250 nm in size and is derived from the nuclear membrane. The envelope glycoprotein appears as protrusions or spikes of approximately 8 nm in length. At least 10 different glycoproteins designated gB, gC, gD, gG, gH, gL, gJ,gK, gL and gM are known. gB and gC functions in attachment of the cell receptor, gD functions in virus entry into the cells whereas gE and gI form a potent Fc receptor. The part between the under surface of the envelope and the surface of the capsid is designated as the tegument which appears as an amorphous in the thin section. An icosahedral nucleocapsid of 100 nm in diameter is composed of 162 hollow capsomers containing most of the virion protein. The HSV DNA genome is composed of approximately 152,000 base pairs, estimated to

contain about 75 genes which extraordinary high in the guanine cytosine content (68% for HSV-1 and 69% for HSV-2) (42-44,46).

2. Genome structure

The genome of HSV forms the two covalently linked components designated as L (Long) and S (Short) segments, constituting 82% and 18% of the genome, respectively. Each segment inverts upon itself leading to the formation of four isomers. The transcription of viral DNA takes place in the nucleus and is accomplished by host cell RNA polymerase II in conjunction with viral gene products. The regulation of HSV gene expression is coordinately regulated in sequential order. Three classes of mRNA molecules are produced : α, β and γ mRNAs. The α or immediately early genes are the first to express. Five proteins, ICP0, ICP4, ICP22, ICP27 and ICP47, regulate the expression of the rest of the viral gene, whereas α 47 blocks the presentation of antigenic peptides on the infected cell surfaces (44-47). The β or early genes are divided into β1 and β2. The β1 proteins, exemplified by polypeptides ICP6 (the large component of the viral ribonucleotide reductase) and ICP8 (the major of component DNA binding protein) appear very early after infection in the past have been mistaken for α protein. The β2 polypeptides include the viral thymidine kinase (TK) and DNA polymerase. Most of the β proteins function in viral nucleic acid metabolism, and the appearance of β proteins signal the onset of viral DNA synthesis The γ or late proteins can be divided into two groups, γ1 and γ2. The γ1 genes specifying gB and gD and γ 34.5 express early in the replication cycle, and occur in the absence of viral DNA synthesis. The γ2 genes (e.g.gC) expressed late in the replication cycle, and its expression is inhibited in

the presence of the inhibitors of viral DNA synthesis. In another word, replication of viral DNA commences after synthesis of β class of proteins (43-44).

3. Pathogenesis

Infection with HSV is extremely common and widespread, occuring in a world wide distribution with equal frequency in both sexes and in both developed and developing countries (46). It is important to recognize that HSV can infect any mucosal or cutaneous surface in normal human hosts. During the initial phases of HSV infection, the virus enters parabasal and intermediate epithelial cells where it multiplies, eventually producing cell lysis. A local inflammatory response is seen, and regional lymph nodes may become involved. The pathogenesis of HSV encephalitis remains unclear. Both primary and recurrent HSV infections can cause diseases of the CNS. In animal models, HSV gains access to CNS via olfactory nerve and result in a localization of the infection in the orbitofrontal region of brain (48-49). The ability of HSV to establish latency in neurons has also been recognized. Following primary infection, the virus is transported to the dorsal root ganglia. After viral attachment and uptake to the sensory nerve ending and removal of envelope, the HSV nucleocapsids spread to the cell stromas by retrograde axonal flow probably utilizing the cellular skeleton of microtubules. The virus has been isolated from trigeminal and sacral ganglia, and occasionally from cervical and vagus ganglia, when the CNS shows signs of active infection. Demonstration of HSV DNA by hybridization techniques has been reported only rarely in the CNS of asymptomatic individuals nor has it been found in human peripheral tissue. During latency, in neuronal ganglia, viral DNA persists in a circular concatameric structure composed of a unit-length molecule of DNA linked together in a head-to-tail configuration around

a nucleosome. Thus, the individual remains infected lifelong in the ganglia, where the viruses seem to be well tolerated to the attacks of an immune system and antiviral therapy; and HSV remains in the silent state during latency. Reactivation from latency state can be accomplished by a number of stimuli such as hormonal change, stress, fever, and sunburn and lead to the initiation of viral replication. The reactivated viruses may or may not elicit any disease symptom (50).

4. Clinical manifestation

HSV is a ubiquitous agent responsible for a wide variety of human infections such as gingivostomatitis, pharyngitis, genital herpes, herpetic whitlow, conjunctivitis, keratitis, and CNS diseases. HSV is the most common cause of sporadic encephalitis and it accounts for 2 to 19% of human encephalitis (48, 51-52).

There are a variety of the clinical presentations for HSV infections of the CNS. HSV-1 mainly causes focal encephalitis. It has been shown to exist in the mild forms and is likely that a number of such cases escape the diagnosis. HSV-1 can be isolated occasionally from CSF from cases of meningitis. The spectrum of HSV-2 induced CNS disease is also wide. Among newborns, the neurovirulent potential of HSV-2 is welll known. Brain involvement in HSV-2 infections has been also described in older-immunocompomised patients.

Clinical signs and symptoms of HSV encephalitis reflect localized temporal lobe disease. Focal encephalitis is usually associated with altered consciousness, disorientation, headache, personality changes, aphasia, generalized seizures, focal seizures, hemiparess, cranial nerve deficits, memory loss, motor disturbances, visual field cut, and papilledema. Fifteen percent of the cases present without evidence of focal abnormalities, and therefore all patients with fever and altered consciousness

compatible with viral cause should be considered as suspect for HSV infection. Other unusual but important clinical presentations include increased intracranial pressure with an imagine appearance of a temporal lobe tumor, prominent brain stem or mid-brain involvement. Encephalitis in patients without fever or focal signs is rare. The course of the disease is highly variable. Some patients progress rapidly to coma within 1-3 days, and others stabilize with encephalopathic symptoms and do not reach a full comatose state. Several of these cases were well documented by antigen detection and virus isolation in brain biopsy, and also by CSF serology (44,52).

5. Laboratory diagnosis

5.1 Virus isolation

Virus isolation is the standard diagnostic method for HSV infection. HSV is successfully isolated from clinical specimens such as lesion swabs or vesicular fluid, but it is rarely recovered from CSF from adult cases with HSV encephalitis. The isolation rate in CSF is on 5-10% of the samples study, but it may be higher in pediatric cases (51). Children with encephalitis without disseminated infection have skin vesicles in approximately 60% of the cases at anytime during the disease course and virus can be cultured from CSF in 25-40% of all cases. The cell types most commonly used for isolation of HSV include rabbit kidney, human embryonic kidney, human fibroblasts, and Vero cells. CPE tends to develop within 24 to 72 hour. after inoculation with specimens containing infectious viruses. The defection of viral antigens in cell culture before the appearance of CPE has been employed as a rapid method of virus identification and confirmation. The brain biopsy specimens may yield culturable virus, but to collect the clinical specimens by surgical procedure is so

invasive. Thus, the clinical diagnosis of herpes encephalitis viruses is wrong in half of the suspected cases(44).

5.2 Cytopathological study

Papanicolaou stain and Tzanck smear are used to demonstrate the cytopathological changes such as occurrence of multinucleated cells and ground glass appearance of the intranuclear inclusion body in the cell scrape samples. However, cytological changes cannot differentiate between HSV-1, HSV-2 and VZV infections.

5.3 Virus antigen detection.

Immunofluorescence study of the infected tissue represents the most sensitive histologic diagnostic technique. The use of monoclonal antibodies will also allow HSV typing. However, the method of antigen detection is not applicable to diagnose HSV encephalitis (55).

5.4 Virus antibody detection

A variety of assays for detection of antibodies to HSV are available. However, the tests are only helpful in the diagnosis of primary infection, because all of individuals with established recurrent diseases have persistent circulating HSV antibodies. The common tests used for measurement of HSV antibodies are CF, passive hemagglutination, neutralization, immunofluorescence, immune adherence hemagglutination, solid phase radioimmunoassay and ELISA (56-57) .The more recent development of type specific antibody assays by ELISA and Western blot are likely to replace many of the older systems. Serologic diagnosis of HSV infection is not of great clinical value because 85% of normal adults have HSV-specific antibodies in their serum. The intrathecal synthesis of HSV antibody occurs in HSV encephalitis and can be assessed by testing for specific antibody titers in CSF and

serum samples collected at sometime during illness. The result is presented as antibody specific index (ASI) which can be calculated from the ratio of a serum : CSF anti-HSV antibody. It was reported that 90% of the patients with biopsy-proven HSV encephalitis had serum : CSF anti-HSV ratios of ≤20:1 (52, 59). However, an increase in CSF antibody in order to reach a ratio of ≤20:1 does not occur soon enough to be useful in early disease diagnosis.

In addition, the ratio of serum : CSF anti-HSV antibody of ≤20:1 may be suggestive but not conclusive for HSV encephalitis, since this ratio will fall if the serum antibody transudates into the CSF through a leakage of the blood-brain barrier.

5.5 Virus genome detection

Detection of HSV DNA in CSF of patients with HSV encephalitis by PCR was first reported by Rowley *et al.* (59). In some study, HSV DNA could be detected as early as at day 4 after onset of illness. PCR primers derived from DNA polymerase gene, glycoprotein gene or TK gene were employed in several reports, and primers common to both types of HSV could be used (5,59-61). Gutfond and colleagues (62) found that the detection of HSV-DNA in CSF by PCR was more sensitive than the antigen or antibody assays in the diagnosis of HSV encephalitis. However, the sensitivity of particular PCR assay varied according to the DNA preparation technique, primers, buffers, and PCR cycling condition and whether a single or nested PCR technique is used. In nested PCR, the product of first amplification are reamplified with a second set of primers nested between the first a set of primer. This procedure increases the sensitivity and the specificity of detection (63).

Human Herpesvirus 6

HHV-6 was first discovered by Salahuddin and colleagues in 1986 after identifying herpes-like particles in peripheral blood of patients with AIDS and lymphoproliferative diseases (64). Then, HHV-6 was classified as a member in the *Herpesviridae* family with genetic characterization similar to human cytomegalovirus (HCMV). Although lymphocyte tropism of HHV-6 places it into the gamma-herpesviruses, genomic analysis supports its classification as a new beta-herpesvirus. Later study showed that all HHV-6 isolates belong to one of the two variant groups, A or B (HHV-6A or HHV-6B). Variant A and B sequences diverge by about 4% enabling the variants to be distinguished on the basis of restriction fragment length polymorphism. Differential reactivities to identy HHV-6 variant have been reported with in *vitro* growth characteristics, monoclonal antibodies and PCR (65 - 70).

1. Virion structure

HHV-6 is an enveloped virion with icosahedral capsid containing 162 capsomers which enclose a core of approximately 60 nm. in diameter. The core is uniform in whickness but varies in shape from perfectly round to ellipsoidal and consists of punctate and / or filamentous structures. In nucleus of infected cells, most of capsids have many cores of low density with diameter of about 90 - 110 nm, while in the cytoplasm, the capsids are surrounded by a distinct tegument of uniform thickness with 20-40 nm. in diameter. The tegument is the most striking morphologic feature of HHV-6 virions as compared with those of other herpesviruses such that it is a component of free, unenveloped particles in the cytoplasm, and also of the budding particles present at

vacuole membranes. The latter particles undergo envelopment in cytoplasmic vacuoles yeilding a number of mature virions which are then released as the completed particles, into the extracellular space. An extracellular HHV-6 virions are 160-200 nm. in diameter as observed by both thin and scanning electromicroscopy (65, 71).

2. Genome structure

The HHV-6 genome is a linear, double-stranded DNA molecule with a size of 167 kbp in length; it consists of a 143 kb unique region flanked by a pair of a 13-kb directly repeated sequences (DR). The density of genome DNA is approximately 1,702 g/cm^3 and the mean G+C content of 43% (65,71). By nucleotide sequence analysis of the HHV-6A and HHV-6B, the organization of the conserved genes are found to be genetically colinear. The DNA sequences of open reading frames (ORF) in the immediate early (IE) gene of HHV-6A (U1102 strain) and HHV-6B (HST strain) are highly conserved except for some occasional base changes. Deletion of 108 and 228 base pairs was found in the ORF of IE gene of HHV-6A (U1102 strain). Furthermore, the ORF of IE gene of HHV-6B (HST strain) was 65 codons shorter than the ORF of HHV-6A (U1102 strain) at the 3′ end. In overall, the ORFs of IE gene of HHV-6B (HST strain) and HHV-6A (U1102 strain) shared approximately 85% DNA homology and 70% amino acid homology. HHV-6 possesses homologue of genes that encode for structural and regulatory proteins which are common to beta-herpesviruses. These genes include the major DNA-binding protein, major capsid protein, large tegument protein, DNA-polymerase and IE genes (70, 72-74).

3. Pathogenesis

HHV-6 is the causative agent of roseola infantum also known as roseola or examthem subitum (ES) or rose rash of infant or sixth disease. The disease with an average incubation period of 10 days is characterized by high fever for a few days and followed by the appearance of rash coinciding with subsidence of fever . The mode of HHV-6 transmission was found to be horizontal person to person contact. Oral secretions appear to be the most probable source, as the virus has been detected in the saliva in a significant proportion of healthy adult (75). Sexual transmission is important, vertical transmission and reactivation of virus replication during pregnancy have been documented serologically, but no syndrome of congenital infection has yet been described (76). The virus has been detected in donated organs and in a variety of cell types present in the clinical specimens including macrophages, lymphocytes, endothelial cells, and epithelial cells (69, 73, 77). Cultures of infected peripheral blood mononuclear cells and T-lymphocytes showed specific cytopathic effect with intracytoplasmic and intranuclear inclusion bodies containing viral particles that could be observed by electron microscopy. The infected cells also expressed viral antigens that could be detected by indirect immunofluorescence assay. Immunologic and molecular analysis revealed that, although the CD4 molecule was not the cell receptor for HHV-6 infection, CD4+T lymphocytes were the predominant target cells by direct and indirect infections.

HHV-6 infection may modulate the immune system in such a way that CD4+ T cells and NK cells were up-regulated, while CD3+ T cells were down regulated and the infection also induced the release of IFNα and IL-1β from the infected cells (67-

69,77). The alteration of polyclonal cell stimulation and cytokine activities might contribute to the development of lymphoproliferative disorder including lymphoma and leukemia. HHV-6 viral particles may invade CNS and infect glial cells which may lead to the development of encephalitis (10, 78). Nevertheless, recurrent of febrile convulsion and febrile seizure may be associated with reactivation of HHV-6 (71).

4. Clinical manifestation

HHV-6 causes a number of childhood diseases as it has been reported to be a major cause of febrile seizures, neurologic diseases and CNS complications. In adults, primary HHV-6 infection is less common. The virus implicates in cases of infectious mononucleosis, hepatitis, chronic fatigue syndrome, and is probably associated with certain human malignancies and progression to AIDS in human immunodeficiency virus infected cases.

HHV-6A plays a minor role in disease association, while HHV-6B is the major cause of roseola infantum and related illness (79).

Roseola infantum is characterized by high fever (>39°C), and macular or maculopapular rash. The other clinical features include erythematous papules called Nagayama's spot on the mucosa of soft palate, bulging fontanelle, seizures, diarrhoea, cough, oedematous eyelids and cervical lymphadenopathy (71,80). The most common complication of roseola infantum is convulsive seizures, but encephalitis and other complications of CNS are rare (10).

HHV-6 can invade CNS and causes neurological diseases. Now several cases of encephalitis associated with HHV-6 infection have been reported (10-11, 81-82). CNS infections with HHV-6 have also been described in bone marrow transplant patients, in

adult and children with AIDS and in an elderly woman with no known underlying immunodeficiency. HHV-6 DNA can be detected in CSF during or after primary infection by PCR (11, 83). The nested PCR has become an accepted method for diagnosing HHV-6 infection, especially from CSF of patients with clinical and laboratory evidence of focal encephalitis.

5. Laboratory diagnosis

HHV-6 infection can be diagnosed by several means as follows.

5.1 Virus isolation

HHV-6 can be isolated from peripheral blood mononuclear cell (PBMC) population and in some occasions from saliva of patients with roseola on the first and second days of the disease. Blood samples are collected in tubes containing anticoagulant are processed by Ficoll-hypaque gradient. Then, the PBMC collected are cocultured with cord blood mononuclear cells. The CPE with its characteristic feature of balloon-like syncytia usually appears 2 to 4 days postinfection. HHV-6 antigens can be identified in the cultured cells by immunofluorescence test using HHV-6 specific antibody (71).

5.2 Virus antigen detection.

HHV-6 infected cells can be demonstrated in lung tissue from patients by immunohistochemical staining and HHV-6 soluble antigen in serum could be detected by using the antigen capture ELISA (84).

5.3 Virus antibody detection.

Measurement of specific antibody is employed by indirect and anti-complement immunofluorescence, neutralization test, competitive radioimmunoassay, and ELISA (85-86). These methods usually required acute and convalescent serum samples for

determination of a four folded or greater rise in the virus specific IgG antibody titer. Detection of IgM specific HHV-6 in a single serum sample would also indicate an acute infection in a young child. The titer of specific IgG antibody also rises during the reactivation of the infection in adults, but specific IgM antibody may not appears regularly at the same time.

Antibody to HHV-6 was found in 52 to 100% of adults , and in 89 to 100% of infants as a consequence of actively transported, transplacentally acquired maternal antibody . Subsequently, the majority of infants apparently were infected shortly after maternal antibody wanes, with anti HHV-6 seroprevalence rate of 63 to 92% by 9 to 12 months of age . Thereafter, anti HHV-6 antibody levels declined with age, and may fall below the range detectable by current methodology (71).

5.4 Virus genome detection.

DNA hybridization and PCR were used to demonstrate the presence of HHV-6 DNA in tissue or CSF samples of patients with encephalitis (87-88). PCR become an acceptable method for diagnosing HHV-6 infection, especially in patients with roseola infantum

HHV-6 could be classified into two groups by PCR as using primers which cover the area deleted in HHV-6A (U1102 strain), then the HHV-6 isolates were easily identified as variant A or B on the basis of the sizes of the amplified products (79). In addition, to the PCR methods using other set of primers, restriction analysis or sequence specific oligonucleotide probe hybridization after amplification are used to differentiate the variants (69, 79, 88).

Enterovirus

The genus Enterovirus of the *Picornaviridae* family comprises 68 serotypes of viruses that are pathogenic for man. These include poliovirus serotypes 1-3, coxsackie A virus serotypes 1-22 and 24, and coxsackie B virus serotypes 1-6, echovirus serotype 1- 9, 11-27 and 29-31 and enterovirus serotypes 68-71. (coxsackievirus A23, echovirus 10 and 28, and enterovirus 72 have been reclassified.) These viruses produce various disease manifestations ranging from respiratory tract infection, heart diseases to neurological diseases. So far all members of the genus are able to cause a disease in the CNS. In the mid 1700S, a syndrome retrospectively identificable as paralytic poliomyelitis was reported in medical literature and in the mid to late 1800S, polioviruses were widely distributed throughout most of the world population. Large epidemic spreaded across the United States and Europe in the first half of the twentieth century. In the summer of 1916, more than 27,000 persons in the United States were reported to be paralysed, with 6,000 deaths. Coxsackieviruses and echoviruses have had a shorter history. Epidemic pluerodynia was described clinically in 1735 by Hannaeus more than 200 years before the coxsackie viral etiology of this disease was discovered. In 1948, Dalldorf and Sickles first reported the isolation of coxsackievirus, using suckling mouse inoculation (89). At present, 30 different isolates from coxsackieviruses are classified into two groups (coxsackiesvirus types A and B) on the basis of differences in pathogenesis. In 1948, enteric viruses that produced cytopathic changes when inoculated on cultures such as simian-derived tissue culture cells but that failed to produce illness in laboratory animals were discovered. They were called orphan viruses. Later, several agents

were grouped together and called enteric cytopathogenic human orphan viruses, or echoviruses (89).

1. Virion structure

Enteroviruses are nonenveloped viruses with single strand RNA genome of positive polarity. The viruses are small in size about 27-30 nm. in diameter and have 1.34 g/ml bouyant density. Four different virus-encoded capsid proteins, VP1, VP2, VP3 and VP4 are packaged into an icosahedral protien shell of the virus. VP1 to VP3 are responsible for the antigenic diversity among the enteroviruses. Most of neutralization sites are densely clustered on VP1. VP4 is not present on the viral surface, rather, it is in close association with the RNA core, functioning as an anchor to the capsid (90).

2. Genome structure

The genomic RNA of the enteroviruses constitutes approximately 30% of the virion mass and is about 7.4 kb in length. Genomic RNA serves as a template for both viral protein translation and RNA replication. The genome contains 5′ noncoding region that is followed by a single long open-reading frame, a short 3′ noncoding region, and a poly (A) tail of 60 or more bases in length (90). The 5′ untranslated region (5′-UTR) is highly conserved among human enteroviruses, allowing the detection of almost all of the serotypes with a single pair of oligonucleotide primers that can effectively amplify the majority of known enteroviruses in the PCR test. Such PCR assays have been applied, on a limited scale, to the diagnosis of enteroviral CNS diseases (91-92).

3. Pathogenesis

The pathogenesis of enterovirus infections has been studied at molecular, cellular and organ system levels; while much has been learned, much more remain unexplained. After initial acquistion of virus by the oral or respiratory routes, implantation occurs in pharynx and lower alimentary tract and virus may be shed for up to several weeks or months. The incubation period is usually between 7 and 14 days with a range of 2 to 35 days. Within 1 day, the infection extends to the regional lymph nodes. Viremia may occur on the third day and lead to further virus proliferation in the cells of reticuloendothelial system and finally to involvement of the target organs including spinal cord, brain, meninges, myocardium and skin. Multiplication of virus in secondary infection sites coincides with the onset of clinical symptoms. Illness can vary from minor infections to fatal ones. Major viremic occurs during the period of multiplication of virus in the secondary infection sites; this period usually lasts from the third to the seventh day of infection. In many echovirus and coxsackievirus infections, CNS involvement apparently occurs at the same time as other secondary organ involvement does. Occasionally the CNS symptoms of enterovirus infections are delayed, suggesting that seeding occured later in association with the major viremia (89).

4. Clinical manifestations

Most of enterovirus infections produce subclinical manifestations. The clinical expressions, when they do occur range from severe neurological symptoms and permanent paralysis, sometimes fatal, to minor undifferentiated febrile illness. All enteroviruses can cause CNS infection, those neurological diseases is presented as aseptic meningitis, poliomyelitis, and encephalitis. Enteroviruses can also cause

extraneuronal diseases such as myocarditis and pleurodymia from coxsackie B virus infection, acute hemorrhagic conjunctivitis, also hand foot and mouth disease from coxsackie A infection and mild upper respiratory tract infection from many enteroviruses. Enterovirus encephalitis results from direct virus invasion of the brain parenchyma with cytolytic infection of neurons (primary encephalitis). While the post infectious or parainfectious encephalitis is thought to be immune mediated and is referred to as a secondary encephalitis. The illness usually begins like aseptic meningitis, with a prodrome of fever, myalgias and upper respiratory symptoms. Onset in CNS signs and symptoms are often abrupt, with confusion, weakness, lethargy, drowsiness, or irritability. Progression to coma of generalized seizures may occur.

Since enteroviral encephalitis is regarded as part of the spectrum of aseptic meningitis, in many cases the decision to diagnose aseptic meningitis or encephalitis is somewhat arbitrary, based on those signs and symptoms the physician feels are the most important. The major virus types associated with CNS disease among coxsackie viruses are B1 to B6, A7 to A9. In addition, echoviruses 4,6,9,11,14,16,25,30 and 31 have repeatedly been associated with meningitis; type 3, 18 and 19 have also been responsible for some outbreak of this syndrome. Other echovirus types including 2 and 5 have been associated with meningitis only in sporadic cases. With echoviruses 6 and 9, muscle weakness and mild transient paralysis have been observed; echovirus 9 has been recovered in high titer from the medulla of a fatal cases. Among the newer enteroviruses, type 70, the agent of acute hemorrhagic conjunctivitis (AHC), in rare instances has been involved in neurological complications including poliomyelitis like illness(93). Enterovirus 71 has been associated with meningitis and severe CNS

disease and with a variant of other with clinical syndromes. However with a number of enteroviruses, there is a risk of serious neurologic sequalae among infants infected during the first year of lives (94).

5. Laboratory diagnosis

5.1 Virus isolation

Isolation of enteroviruses using cell culture is the gold standard for laboratory diagnosis. The usual specimens are stools, rectal swabs and throat swabs. In addition, enteroviruses are often isolated from CSF in cases of aseptic meningitis due to coxsackievirus or echoviruses, but seldom if this syndrome is due to a poliovirus.

The success in isolating a virus depend on the selection, collection and transportation of clinical samples. Whenever possible, specimens should be obtained at early illness. The specimen should be collected at the bedside, placed in virus transport medium, and taken directly to virology laboratory on wet ice. Repeated freezing-thawing cycles of specimens should be avoided, as decline in virus titer is inevitable. The specimens are generally inoculated into primary monkey kidney (PMK), MRC-5, RD and Vero cell culture, and if available, suckling mice. Primary cultures are excellent " broad spectrum " cell types supporting the growth of polioviruses, coxsackieviruses A7,9 and 16, coxsackieviruses B and echoviruses excepting type 21. The uses of several different cell lines increase the frequency of isolation rates (95). The infected cell culture is recognized by CPE characterized as cell shrinking with nuclear pyknosis, refractility and degeneration. The isolation of enterovirus usually takes 1 to7 days, and the successful isolation of the virus from CSF takes 4 to 8 days (10, 90). The isolation technique is improved by the spin amplification, shell vial technique which reduce significantly the time of detection of

enterovirus in cell culture (96-97). After isolation of an enterovirus, its type identification is performed conventionally by neutralization; and this process, unfortunately, frequently takes a long time.

5.2 Virus antigen detection.

The absence of a common antigen has hampered the development of immunoassays limited to particular subgroup of enterovirus serotypes, e.g., the group B coxsackie viruses that share a common antigen. Recent reports of polyclonal and monoclonal antibodies that cross react with multiple enteroviruses serotypes are promising, but further testing is required to determine the clinical relevance of those observations. Utility of these reagents for direct detection of enteroviruses in clinical specimens has not been demonstrated (98).

5.3 Virus antibody detection and measurement.

Serological diagnosis of enterovirus infection is made by comparing the antibody titer in acute and convalescent phase serum specimens as an indication of the infection with that agent. Although HI and CF take only short time to be performed, they are impractical in searching for the cause of a specific infection because there are no common group antigens or the lack of a single common antigen. The identification of particular illness might require the performance of more than 60 individual antigen. However, serodiagnosis may be useful if the prevalence of specific enteroviruses in a community is known. In this suitation, it is relatively easy to look for antibody titer changes to a selected number of viral types. Recently, ELISA tests have been developed for detecting coxsackie B virus-specific, or enterovirus 70-spicific IgM (99-100). In contrast, these tests for IgM detection may be relatively sensitive they

are not purely serotype-specific, since 10 to 70 % of sera may exhibit cross-reactive responses, presumably due to infection with other enteroviruses (90).

In addition, serodiagnosis of enterovirus infection should be interpreted with precaution because most of the infections are asymptomatic, and late convalescent carrier state is common. The seroconversion to an enterovirus may leave nothing to do with the disease symptom.

5.4 Virus genome detection.

Several reports have described RT/PCR methods to detect enterovirus RNA in clinical specimens (101-105). RT/PCR has been proved to be the most useful rapid diagnostic test for the direct identification of enterovirus genome in CSF of patients with meningitis (105-107). Three general strategies of enterovirus RT/PCR have been employed: 1) " universal " detection of many or all serotypes; 2) " serotype-specific " or " group specific " detection of a limit number of serotypes; and 3) " strain-specific" detection of variation within a single serotype . A potentially important application of group-specific enterovirus RT/PCR, is the discrimination of polioviruses from the nonpolio enteroviruses in clinical specimens. Strain-specific PCR is used mainly for studying of the genetic shifts and drifts of an individual strain, and for the study of specific molecular virulence determinants.

The universal detection is based on the fact that several regions of the 5' end of the enterovirus genome are highly conserved among different enterovirus serotypes (9, 105, 108); thus a single pair of oligonucleotide primer can effectively amplify the majority of the unknown enterovirus genomes. Such RT/PCR assays have been applied, on a limited scale, to the diagnosis of enteroviral CNS diseases (104). RT/PCR assay of CSF

could diagnose enteroviral meningitis in 6.7 to 55.5 % of the cases and in 66 % during the large community outbreak.

CHAPTER IV

MATERIALS AND METHODS

1. Subjects

The present study was conducted on a total of 168 pediatric cases aged between 1 month to 12 years who were hospitalized to the Infectious Unit of Pediatric Ward, Department of Pediatric, Faculty of Medicine Siriraj Hospital with the symptoms and signs of CNS infection. The subjects included 36 cases of viral encephalitis, 23 cases of CNS infections (post encephalitis and meningitis), 61 cases of other diseases (febrile convulsion, epilepsy, viral infections and acute disseminated encephalitis) and 43 cases of unknown diagnosis.

Inclusion criteria for viral encephalitis cases are those who presented with symptoms of fever, alteration of consciousness, and /or focal signs such as seizure; and they would be excluded if the etiology had been established to be due to others.

2. Duration of study

This study was carried out during February 1996 to October 1998.

3. Diagnostic tests for viral etiologic agents.

Viral etiologic agents were determined by the following schemes.

1. Diagnosis of JEV infection was determined by RT/PCR for presence of JEV RNA in CSF, and of antibodies to JE and dengue viruses by HI test in paired sera, and by ELISA for specific IgG and IgM both in sera and CSF.

2. Diagnosis of HSV infection was determined by nested-PCR for presence of HSV DNA in CSF, and by ELISA for HSV specific IgG and IgM in sera and by determination for index ratio of CSF specific IgG : serum specific IgG (antibody specific index ; ASI).
3. Diagnosis of HHV-6 infection was determined by nested-PCR for presence of HHV-6 DNA in CSF, and because of the limitation of unavailable test kit for serology in CSF, only ELISA for HHV-6 IgG and IgM in sera were tested.
4. Diagnosis of enterovirus infection was determined by RT/PCR for presence enterovirus RNA in CSF, and also by ELISA for enterovirus IgG, IgM and IgA in sera.

4. Specimen collection

Clinical specimens used in this study were CSF and paired blood.

Approximately 0.5 to 1 ml of CSF samples were collected in a sterile and disposable vial and transported in an ice-box to the Virology Laboratory and then kept at –80°C until tested.

Three to five ml of clotted blood were collected in a sterile test tube and transported at room temperature to the Virology Laboratory. Sera were separated by centrifugation and kept at –20°C until tested. Paired sera were collected 1 to 2 weeks apart.

5. DNA preparation

There are three methods used in this study for extracting the virus DNA from CSF samples, i.e., boiling method, phenol-chloroform extraction method, and commercial kit, QIAgen.

5.1 Boiling method.

The protocol was as described by Aurelius. E. *et. al.*(6). One hundred to three hundred microliters of CSF was boiled for 15 minutes at 95°C and DNA was precipitated by adding with 2.5 volumes of 95% ethanol and kept at –70°C overnight.

The DNA precipitate was pelleted by centrifugation at 10,000 x g at 4 °C for 30 minutes, DNA pellet was dried under vacuum, and dissolved in 10 µl of distilled water before boiling at 95 °C for 10 minutes. The DNA solution was kept at –20 °C until tested by PCR.

5.2 Phenol-chloroform extraction

One hundred microlitres of CSF were mixed with 2 µl of 100 mg/ml proteinase K (Ameresco, OH, USA) and 10 µl of 10% sodium dodecyl sulphate (SDS). The mixture was incubated at 56°C for 1.5 hours, then mixed with 50 µl of Tris saturated phenol (Sigma, MO, USA.) and 50 µl of chloroform/ isoamyl alcohol (see appendix) for at least 3 minutes before spinning at 10,000 x g in a microcentrifuge for 3 minutes at room temperature. The solution in upper phase was transferred into a new 1.5 ml eppendorf tube, and mixed with an equal volume of chloroform/ isoamyl alcohol for at least 3 minutes before spinning at 10,000 x g for 3 minutes at room temperature. The upper solution was mixed thoroughly with 10 µl of 3M sodium acetate and 250 µl of ice-cold absolute ethanol then kept at -70°C overnight. The next centrifugation step was carried out at 10,000 x g for 30 minutes at 4°C, and the supernatant was discarded. Then, the DNA pellet was washed with 1 ml of 70% ethanol by centrifugation at 10,000 x g for 3 minutes at room temperature. The supernatant was discarded and the pellet was dried in an incubator at 37°C for 1 hour. The DNA pellet

dissolved in 20 µl of distilled water was mixed with 2 µl of 50 µg/ml of Rnase H. (Amresco, OH, USA.) and then kept at -20°C

5.3 Commercial Kit (QIAmp Blood kit, QIAgen, Germany)

Three steps of the purification procedure comprised adsorption to QIAamp membrane, removal of residual contaminants, and elution of the purified RNA. Briefly, 200µl of CSF were mixed with 25 µl of QIAgen protease or proteinase stock solution and 200 µl of buffer AL, and then mixed immediately by vortex mixer for 15 seconds. This solution was incubated at 70°C for 10 minutes and mixed with 210 µl of ethanol (96-100%). The mixture solution was carefully applied to the QIAamp spin column without moistening the rim, then closed the cap, and centrifuged at 6,000 x g for 1 minute. DNA is adsorbed onto the QIAamp silica membrane in QIAamp spin column during centrifugation step. The column cap was carefully opened, added with 500 µl of buffer AW and then centrifuged at 6,000 x g for 1 minute. An adsorbed DNA on the QIAamp silica was washed by 500 µl of buffer AW by centrifugation at full speed for three minutes in order to remove the residual contaminants. The DNA was eluted with 50µl of buffer AE or distilled water, preheated to 70°C and incubated at room temperature for 1 minute, and then centrifuged at 6,000 x g for 1 minute. The DNA yield was kept at –20°C.

At first, the CSF samples were processed by the boiling method but some samples became cloudy after heating. Thus, the boiling method has been replaced by the phenol-chloroform extraction method or by QIAgen protocol. The two methods of DNA purification gave similar results as tested with positive and negative clinical samples.

6. RNA preparation

RNA was extracted from CSF sample by using Trizol reagent (GIBCO BRL, NY, USA.). One hundred microlitres of CSF were mixed with 300 µl of Trizol reagent in a 1.5 ml eppendorf tube and incubated at room temperature for 5 minutes. The 170 µl of chloroform were added into the tubes. The capped tightly tubes were shaked vigorously by hand for 15 seconds and incubated at room temperature for 2-3 minutes before spinning at 12,000 x g for 15 minutes at 4°C. After centrifugation, the mixture was separated into a lower red phenol-chloroform phase, interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase of volume about 180 µl and then 1 ml of ice-cold 70% ethanol was added. The next centrifugation step was carried out at 10,000 x g for 5 minutes at 4°C, and the supernatant was discarded. Then, the RNA pellet was washed with 1 ml of 70% ethanol again and separated by centrifugation at 10,000 x g for 5 minutes at room temperature. The supernatant was discarded and the pellet was dried in an incubator at 37°C for 1 hour. The RNA pellet was dissolved in 10 µl of DEPC water and then kept at -20°C.

7. Preparation of reference viral genome

7.1 JEV RNA

JEV RNA was prepared from LLC-MK-2 cells infected with JEV Nakayama strain. The infected cell monolayers were frozen and thawed, and spun at 10,000 x g for 10 minutes. Supernatant was collected and assayed for virus units in pfu/ml, or extracted for JE RNA genome by Trizol.

7.2 HSV DNA

The confluent Vero cells monolayers were inoculated with HSV seed and incubated at 37°C until the infected cultures showed 75% cytopathic effect (CPE). Then, the culture media was discarded and the infected monolayer was rinsed twice with cold PBS. After adding with one ml of PBS, the cells were scraped out of the bottle surface with rubber policeman. The cell suspension was spinned at 268 x g for 10 minutes at 4°C and the supernatant was discarded. The cell pellets were washed once by 1 ml cold PBS, resuspended in lysing solution (see appendix), adjusted to the final concentration of approximately 1.5 x 10^4 cells/ml, and then incubated for 5 minutes at 4°C. Forty µl of 5M NaCl was added to one ml of the infected cell solution. The mixture was centrifuged immediately at 10,000 x g in a microcentrifuge for 10 minutes at 4°C in order to precipitate cellular DNA and cell debris. The pellet was discarded, and 10 µl of 100 mg/ml proteinase K and 50 µl of 10% SDS was added to the supernatant and incubated at 37°C for 1 hour. A 250 µl of was tris saturated phenol (Sigma, USA.) and 250 µl of chloroform/ isoamyl alcohol (see appendix) were added into 500 µl of cell lysate, mixed for at least 3 minutes and centrifuged at 10,000 x g in microcentrifuge for 3 minutes at room temperature. The upper solution was transferred into a 1.5 ml eppendorf tube and repeated once with the extraction by phenol chloroform/isoamyl alcohol. Thereafter, 500 µl of the upper solution were mixed with 500 µl of chloroform/ isoamyl alcohol for at least 3 minutes, and then centrifuged at 10,000 x g for 3 minutes at room temperature. The upper solution was harvested, mixed thoroughly with 50 µl of 3M sodium acetate, and 1 ml of ice cold absolute ethanol and kept at -70°C for 15 minutes or overnight. The solution was centrifuged at

10,000 x g for 30 minutes at 4°C, and the supernatant was discarded . The DNA precipitate was washed with 1 ml of 70% ethanol by centrifigation at 10,000 x g for 3 minutes at room temperature. The supernatant was discarded and the pellet was dried in an incubator at 37°C for 1 hour. The DNA pellet was resuspended in 20 µl of distilled water, mixed with 10 µl of 50 µg/ml of Rnase (Amresco, Ohio, USA.) and kept at -20°C.

8. Polymerase chain reaction

Nested PCR was employed to amplify viral DNA. The first step involved amplification with a pair of outer primers, and then 10% of the amplified DNA product was transfered to a new reaction tube for the second round of amplification which utilizes a pair of inner primers.

9. Reverse Transcription/PCR (RT/PCR)

The RT/PCR for detection of viral RNA was performed in two reaction steps. First, the RNA was reversely transcribed into complementary DNA (cDNA) by using SuperScript ™ II RNase H⁻ Reverse Transcriptase (GIBCO BRL, New York, USA.) and followed by PCR amplification of the cDNA as described above.

10. Oligonucleotide primers

JEV primers

Oligonucleotide primers for RT/PCR and two pairs of nucleotide primers for nested PCR designed from non structural protein 3 region (NS3) of JEV genome were used in this study. The primer sequences were designed by Dr. Pilaipan Puthavathana, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University and Dr.Boonyos Ruengsakulrach, Infus Medical company, Bangkok.

(Genbank; Accession No. 331329 for NS3 JEV complete genome). The NS3 region of the JEV genome is conserved sequences for JEV strains (42). The amplified products were 506 and 292 base pairs in length for primary and nested PCR, respectively (see Table 1).

HSV primers

Two pairs of nucleotide primers designed from thymidine kinase (TK) region of HSV genome were used in this study. The primer sequences were obtained from Dr.Wasan Chantratit, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. TK gene of HSV-1 and HSV-2 are highly conserved and are common for both types of HSV(7). The outer primer pairs amplify a region of 540 base pairs, which the inner primer pairs amplify a region of 250 base pairs(see Table 2).

HHV-6 primers

HHV-6 isolates could be divided into two major variants, designated A and B (67). The nested PCR was used to amplify specific sequences in the immediate early gene locus which cover the area of DNA deletion. Variants A and B could be distinguished by the difference in size of the amplified products. The primer sequences were designed by S.Yalcin *et. al.*,(73). The amplification products for variant A and B were 193 and 421 base pairs in length, respecitively.

11. RT/PCR test system

RT/PCR for cDNA synthesis was done in a 0.5 ml polypropylene tube in a total volme of 20 µl. Five µl of the RNA extract was heated to 65°C for 3 minutes before quenching on ice, then added with 15 µl of reverse transcription reaction mixture containing 4 µl of 5X first strand buffer (GIBCO BRL, New York, USA.), 4 µl of 2.5

mM dNTPs (Pharmacia, New York, USA.) to give the final concentration 200 µM, 2 µl of 0.1 M DTT (GIBCO BRL, New York, USA.), 2 µl of 12.5 pmol/µl anti-sense primer for cDNA(final concentration was 25 pmol), 1 µl of 10 U Rnasin (GIBCO BRL, New York, USA.) 1 µl of DEPC-treated water and 1µl (200 units)of SuperScript ™ II RNase H Reverse Transcriptase (GIBCO BRL, New York, USA.). The RT reaction was performed by using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, USA.) for 42°C for 2 hours, and followed by incubation at 70°C for 15 minutes in order to inactivate reverse transcriptase activity in the reaction. The cDNA was amplified for NS3 specific sequence by using the PCR test reaction.

12. Polymerase chain reaction test system

PCR was done in a 0.5 ml polypropylene microcentrifuge tube in a total volume of 50 µl of 10x PCR buffer, 4 µl of 2.5 mM dNTPs (Pharmacia, New York, USA.) to give a final concentration of 200 µm, 3 µl of 25 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, USA. or Promega) and 2 µl of 12.5 pmol/µ l of each primer (final concentration was 25 pmol). Then, 23.5 µl of sterile deionized distilled water and 10 µl of target DNA was added in to the primary PCR reaction tube. Contents in the nested PCR reaction were as the same as those in the primary reaction tube except 28.5 µl of sterile deionized distilled water and 5 µl of target DNA from the primer PCR product was used. Preparation of these PCR reagents is shown in the appendix.

The reaction mixture was covered by 1-2 drops of sterile mineral oil to prevent evaporation during amplification. The PCR was run in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, USA.) for 30 cycles. The step involved the cycle of a 1

minute-denaturation at 94°C, a 1 minute-annealing at 50°C, and a 30 minute-elongation at 72°C followed by 28 cycles of a 1 minute-denaturation at 94°C, a 1 minute-annealing at 50°C, and a 2 minute-elongation at 72°C. After the 30 cycles, the elongation step at 72°C was extended to 7 minutes. The PCR protocol was designed by Dr. Pilaipan Puthavathana Department of Microbiology, Faculty of Medicine Siriraj Hospital.

Table 1. Oligonucleotide primers for amplification of sequence specific for JVE NS3

Primers	Nucleotide sequence	Nucleotide position in NS3 gene	Length of amplified product in base pairs
JEV			
NS3cDNA	5′ ATT CCA CCT TGG TAG CAA 3′	5526-5509	
NS3 outer 1	5′ GGC TGC AGT AAA CAT CCA GA 3′	4919-4938	
NS3 outer 2	5′ GTG ACA TCA GTC TAT GGG 3′	5424-5407	506
NS3 inner 1	5′ CTT GGC GAT GGC TCA TAC 3′	5073-5090	
NS3 inner 2	5′ GGT GCT CTC TCT GCA CTG 3′	5364-5347	292

Table 2.Oligonucleotide primers for amplification of sequence specific for HSV TK

Primer	Nucleotide sequence	Nucleotide position in TK gene	Length of amplified product in base pairs
HSV-1			
TK1 outer	5′ CGA ACA ACG AGC GAC CCT GC 3′	404-423	
TK2 outer	5′ TGA GGA GCC AGA AGC GCG TC 3′	924-943	540
TK1 inner	5′ ACG CTA CTG CGG GTT TAT AT 3′	656-677	
TK2 inner	5′ TGC CCA TTG TTA TCT GGG CG 3′	887-956	250
HSV-2			
TK1 outer	5′ CGA ACA GCG AGC GAC CCT GC 3′	222-241	
TK2 outer	5′ TGA GGA GCC AAA AGC GCG TC 3′	742-761	540
TK1 inner	5′ ACG CGA CTG CGG GTT TAT AT 3′	475-494	
TK2 inner	5′ TGC TCA TTG TTA TCT GGG CG 3′	705-724	250

Note : Only HSV 1 primers were used in the study

Table 3. Oligonucleotide primers for amplification of sequence specific for HHV-6 IE gene

Primers	Nucleotide sequence	Nucleotide position in IE gene	Length of amplified product in base pairs
HHV-6			
IE1 outer	5′ TTC TCC AGA TGT GCC AGG GAA ATC C 3′	2848-2872	
IE2 outer	5′ CAT CAT TGT TAT CGC TTT CAC TCT C 3′	3401-3377	326,554
IE1 inner	5′ AGT GAC AGA TCT GGG CGG CCC TAA TAA CTT 3′	2953-2982	
IE2 inner	5′ AGG TGC TGA GTG ATC AGT TTC ATA ACC AAA 3′	3373-3344	193*,421*

The primers used for HHV-6 cover the deleted area in IE gene and variants can be distinguished by the differences in sizes of the amplified products. The 193 bp and 421 bp fragments were the nested PCR products of variants A and B, respectively.

13. Detection of amplified product by gel electrophoresis

The amplified product from nested PCR was detected by gel electrophoresis in 2% agarose gel which was prepared by melting 2 grams of agarose powder (agarose type I-A) (Sigma, St.Louis, USA.) in 100ml of 0.5x TBE buffer (see appendix). When the gel was completely dissolved, it was cool down to temperature about 60-80 °C and poured into a gel-casting platform with comb. The thickness of gel about 3 to 5 mm. It was left for hardening at room temperature for 30 minutes and the well former was then carefully taken off. The gel and casting platform were transferred into an electrophoresis chamber, and 0.5X TBE buffer was added into the chamber to cover the gel to a depth of about 1 mm.

14. Running gel electrophoresis

Five µl of the PCR product or 0.6 µg of DNA standard size marker ØX 174 RF DNA / *Hae III* (GIBCO BRL, USA.) was mixed with one µl of gel loading dye buffer (see appendix) before loading into each well of the gel. The electrophoresis was carried out at constant voltage of 50 volts and the electric current was run off when the dye in the gel loading buffer migrated to the bottom of the gel. The gel was then stained with 5 µg/ml of ethidium bromide solution (see appendix) for 10-15 minutes and destained in distilled water for 15 minutes. The bands of the amplified DNA products in the gel were visualized on an UV transluminator (Spectronic, New York, USA.) and photographed with a polaroid camera.(Polaroid, Cambridge, USA.)

15. Typing of HSV by using restriction enzyme cleavage of the PCR product.

Preparation of purified PCR product

Mineral oil was carefully removed from the tube of the amplified DNA product, and 150 µl of sterile deionized distilled water was added. A volume of 100 µl of phenol saturated with Tris buffer (Sigma, St.Louis, USA.) plus 100 µl of chloroform/ isoamyl alcohol was mixed and allowed to stand for at least 3 minutes before spinning at 10,000 x g at room temperature. A volume of upper phase solution was transferred into a new tube and mixed with 20 µl of 3M sodium acetate. The solution of DNA product was added with 450 µl of ice cold absolute ethanol, mixed thoroughly and kept at -70°C for 15 minutes or overnight, then centrifuged at 10,000 x g for 30 minutes at 4°C and the supernatant was discarded. The DNA precipitate was washed with 1 ml of 70% of ethanol by centrifugation at 10,000 x g for 3 minutes at roomtemperature. Subsequently the supernatant was discarded and the pellet was dried in an incubator at 37°C for 1 hour. The pellet was resuspended in 20 µl of distilled water and kept at -20°C for typing by restriction endonuclease cleavage.

Restriction endonuclease digestion

The endonuclease restriction reaction mixture consisted of 1 µl of NE buffer (10X), 0.1 µl of BSA (100X) (see appendix), 0.5 U of *BStNI* enzyme (New England Biolabs, Beverly, MA,USA.), 3.4 µl of deionized water and 5 µl of purified PCR product. The reaction mixture was incubated at 60 °C for 1 hour. Finally, the expected bands was examined by electrophoresis in 2% gel (Nusieve, FMC,Rockland USA.)

HSV-1 DNA cut with *BstNI* enzyme will give 3 fragments of 63, 73, and 114 base pairs in length. While HSV-2 DNA will give 2 fracments of 76 and 174 base pairs in length.

16. Detection of Enterovirus RNA by using AMPLICOR enterovirus test kit

The Amplicor enterovirus test kit (Roche Diagnostic System, NJ, USA.) consisted of five major processes. (1) specimen preparation, (2) reverse transcription of the target RNA to generate cDNA, (3) PCR amplification of target cDNA using enterovirus specfic complementary primers, (4) hybridization of the amplified products to specific oligonucleotide probes, and (5) detection of the probe-bound amplified products by color formation.

The procedures were carried out by dispensing 400 µl of EV lysis buffer reagent containing guanidine thiocyanate (GuSCN), dithiothreitol (DTT) and glycogen into a 1.5 ml sterile tube. A volume of 100 µl of CSF specimen or 100 µl of each control was added to the tube of EV lysis buffer and incubated 10 minutes at room temperature. Then 500 µl of isopropanol was added and mixed for 5 seconds by vortex to precipitate RNA molecule. The solution was centrifuged at maximum speed for 10 minutes at room temperature and the supernatant was discarded. The pellet RNA was washed by adding with 750 µl of 70% ethanol and centrifuged at maximum speed for 10 minutes at room temperature. The supernatant was removed as much as possible. The pellet RNA was resuspended with 200 µl of EV specimen diluent and mixed by vortex for 10 seconds; and 50 µl of the RNA extract was transferred into microamp tube for amplification. Reverse transcription and PCR amplification were carried out in a single reaction by using thermostable recombinant enzyme, rTth DNA polymerase

from *Thermus thermophilus* that process efficient reverse transcriptase and DNA polymerase activities. A component of the master mix was AmpErase which contained the enzyme uracil-N-glycosylase (UNG), which recognized and catalyzed the destruction of deoxyuridine containing DNA, but not DNA containing thymidine or RNA containing uridine thus, contamination by the DNA carryover from previously experiments was prevented. Then, the amplification target was identified for sequence specific to the 5'non-coding region of approximately 750 nucleotides that was highly conserved among enteroviruses. The Amplicor Enterovirus test used the primers EV1b and EV2b to define a sequence of approximately 150 nucleotides within this highly conserved 5' region. The primers EV1b and EV2b were bound to biotin after the amplification process, the amplicons were denatured with 100 µl of denaturation solution to form single strands of which only 25 µl was added into 100 µl EV hybridization buffer in a microwell plate which coated with oligonucleotide probe EV3. The plate was sealed with a cover paper and incubated at 37 °C for 1 hour. Then, the plate was washed 5 times for removal of unbound DNA and non specific substances.

Detection of the probe bound amplified products by color formation

An avidin-horseradish peroxidase (Av-HRP) conjugate was added to the wells and incubated at 37 °C for 15 minutes. The Av-HRP bound to the biotin-labeled amplicon captured by the plate bound probe. The plate was washed 5 times again, then added with 100 µl of the working substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethybenzidine (TMB), and further incubated for 10 minutes at room temperature in the dark. The HRP catalyzed the oxidation of hydrogen peroxide in the present of TMB to form the color complex. The reaction was stopped by adding with

100µl of weak acid, and the optical density was measured at 450 nm in an automated microwell plate reader. The result was determined by comparing the absorbance values of unknown specimen to that of the cut off value. The clinical specimen with an A_{450} value equal to or greater than that of the cut off value was considered positive for the presence of enterovirus RNA. The specimen with an A_{450} result less than the cut off value should be considered a presumptive negative for enterovirus RNA.

17. Detection of JEV and dengue IgM and IgG antibodies by capture enzyme linked immunoabsorbance assay (ELISA).

In the assay for JE IgM/ IgG antibodies, the serum sample was diluted to the dilution of 1:100, while the CSF sample was diluted to dilution of 1:10 by using PBS as diluent. The test protocol was as described after Innis BL *et. al.*(41). Each 50 µl of the working controls or diluted samples were added into the wells coated with goat anti-human IgM or IgG in duplicate, then incubated at room temperature for 3 hours or at 4°C for overnight. The plate was washed with PBS-tween (PBS-T) for 6 times. Then, 50 µl of JEV antigen at dilution 50 units was added to each well of both reaction plates and incubated for at least 2 hours at room temperature or overnight at 4°C. The plate was washed for 6 times as above and 25 µl of the horseradish peroxidase(HRP) conjugated anti-flavivirus diluted to the optimal concentration with PBS containing 20% normal human serum (NHS) and 0.5% bovine albumin was then added and incubated at 35 to 37°C for 1 hour. The plate was washed 6 times again, and 100 µl of the chromogenic substrate (hydrogen peroxide and TMB) was added for approximately 30 minutes. The oxidation reaction stopped by adding with 50 µl of 4M sulfuric acid. Finally, plate was read by spectrophotometer at wavelength of 492 nm.

Interpretation of results:

1. The weak positive control (WPC) was defined as 100 units
2. Calculate a binding index: $BI = \frac{\text{OD test sample} - \text{OD neg control}}{\text{OD WPC} - \text{OD neg control}}$
3. Units = BI x 100
4. A value of ≥ 40 units is positive.
5. A ratio of anti-dengue IgM to anti-Japanese encephalitis IgM ≥ 1.0 is typical of a dengue infection. A ratio of < 1.0 is typical of a Japanese encephalitis infection. Test specificity is > 95 %. This ratio is not valid for determining if JE occurred in a dengue-immune host. Test specificity is > 95 %
6. For dengue infection, a ratio of anti-dengue IgM to IgG (if either test is ≥ 40 units) of ≥ 1.8 is typical of a primary infection. A ratio of units IgM to units IgG < 1.8 is typical of a secondary infection.

18. Detection of HSV IgG and IgM antibodies in serum by ELISA

Specific HSV-1 IgG and IgM antibodies were determined by indirect ELISA using commercial kits.(CSF Combi-kit ELISA, Genzyme Virotech GmbH, Russelsheim, Germany). The test was performed according to the protocol described in the kit instruction. One hundred microliters of the test serum at dilution of 1:100 was added into the coated well of the plate and incubated at 37°C for 30 minutes. The microwells were washed 4 times with washing solution. Then, 100 µl of the ready to use HRP conjugated anti-human IgG or IgM was added into each microwell, then covered and incubated at 37°C for 30 minutes. All microwells were washed 4 times again. The one hundred microliters of ready to use TMB substrate was added into each

microwell, and incubated at 37°C for 30 minutes in the dark. The reaction stopped by adding with 50 µl of citrate stopping solution. Measurement of color intensity was done by spectrophotometer at the wavelength of 450/620 nm. The optical density value was directly related to the concentration of specific antibody present in the test serum. The test control set included positive control serum, negative control serum, cut off control serum and blank control wells. Criteria to interprete the result followed after the kit instruction.

19. Detection of HSV IgG antibodies in serum-CSF pairs by ELISA

HSV IgG in serum-CSF pairs was determined by indirect ELISA commercial kit. (CSF Combi- kit ELISA, Genzyme Virotech GmbH, Russelsheim, Germany). CSF at dilution of 1:2 and serum at dilution of 1:401 were used in the test. The IgG Ab standard sera for quantifying virus specific antibody concentrations (ready to use; 100 aMU, 25 aMU, 6.2 aMU, 1.5 aMU) (MU = arbitrary measurement unit) were provided in the kit in order to set a reference curve. Then, HSV-1 ELISA for specific IgG and IgM antibodies in human serum and IgG in CSF were performed as described above.

The optical density (OD.) value of the test serum and CSF were extrapolated with the reference curve in order to obtained the MU values of IgG concentration. Calculation for CSF: serum IgG ratio was expressed in term of antibody specific index (ASI) and criteria for interpretation of the result followed that of the kit instruction.

AI values lower than 0.6 are theoretically not possible and normally point out of an analytical mistake, and the values higher than 1.5 was suggestive of CNS infection.

However, high AI value alone without corresponding clinical reference does not allow a definite conclusion for an acute phase of an infectious CNS-illness.

20. Detection of HHV-6 specific IgG and IgM antibody by ELISA

HHV–6 IgG and IgM in sera were detected by ELISA test kit (Panbio, East Brisbane, Australia). In the assay, the serum sample was diluted with serum diluent to the dilution of 1:100 for HHV-6 IgG detection. And serum sample was diluted with serum diluent to the dilution of 1:10 then, diluted to 1:100 dilution by serum absorbent again for HHV-6 IgM detection. Each 100 µl of the negative, positive and cut off calibrator sera or the diluted test sample were added into microwell test strips which coated with HHV–6 antigen, then incubated at 37°C for 20 minutes.The test plate was washed six times and 100 µl of horseradish peroxidase(HRP) conjugated anti-human IgG or conjugated anti-human IgM was added to each well. The plate was incubated at 37°C for 20 minutes, then, was washed for six times again before adding with 100 microliters of the TMB chromogenic substrate, and incubated for 10 minutes at room temperature. One hundred microliters of the stop solution was added to all wells. Finally, the plate was read by spectrophotometer at the wavelength of 450 nm. The color intensity was directly related to the concentration of the HHV-6 IgG or IgM antibodies in the test sample.

Interpretation for positive and negative result was based on the kit instruction.

21. Detection of enterovirus specific IgG, IgM and IgA antibodies by ELISA

Enterovirus ELISA test kit (Genzyme Virotech GmbH, Germany) consisting of the test strip (Enterovirus antigen) as well as the reference strip (control antigen) was used to detect specific IgG , IgM and IgA antibodies.

The test was carried out by pipetting 100 µl each of ready to use dilution buffer (Blank), positive IgG, IgM, IgA, negative and cut off controls as well as diluted patient sera of dilution 1:100 into microwell of each strip. All strips were incubated at 37°C for 30 minutes then washed 4 times by washing solution. Either of anti-human IgG, or IgM, or IgA conjugated enzyme (ready to use) was added into each well and incubated at 37°C for 30 minutes. Then all strips were washed 4 times by washing solution again before adding one hundred µl of the chromogenic substrate (TMB) and incubated at 37°C for 30 minutes. The further steps were repeated and the interpretation of the results was as described for HHV-6 ELISA.

22. Hemagglutination inhibition (HI) test

The method of HI test for JEV and dengue antibody detection was modified after Clarke and Casals, 1958 (38). Principle of the test system was based on the fact that hemagglutination of goose erythrocytes by JEV hemagglutinin antigen can be inhibited by presence of the specific antibody.

JEV and dengue antigens

JEV and dengue antigens were purchased from the National Institute of Health, Thailand. The viruses were grown in mouse brain and the hemagglutinin antigen was purified by sucrose-acetone extraction.

Treatment of serum from non-specific inhibitor

The method of acetone extraction was used for treatment of non-specific inhibitor in the test sera. Briefly, 100 µl of serum in a glass tube was inactivated at 56°C for 30 minutes. Then, 10 ml of chilled acetone was added, mixed vigorously and spun in a refrigerated centrifuge at 268 x g for 10 minutes. The supernatant was discarded, and

the precipitate was washed once with 10 ml of chilled acetone. After spinning at 268 x g for 10 minutes, supernatant was discarded and the precipitate adhering on the surface of the glass tube was air dried overnight at 37°C. The dry precipitate was dissolved with 1 ml of 0.4% bovine albumin in borate saline pH 9 and subjected to further treatment of nonspecific agglutinator.

Treatment of nonspecific agglutinator

After treatment of nonspecific inhibitor, the serum was added with 50 ul of 50% goose red blood cells and mixed at intervals for 20 minutes at 4°C before spinning at 268 x g for 10 minutes. The supernatant was harvested as the treated serum at dilution of 1:10 and ready for HI test.

Virus titration

The hemagglutinin antigens were titrated for hemagglutination units in 96 wells, U-shaped microtiter plates. Each 25 µl of 0.4% bovine albumin in borate saline pH 9.0 was added into each well of the plate. Then, 25 µl of solution of the undiluted antigens was added into the first well of the column, and mixed thoroughly before transferring 25 µl of the solution to the second well. The antigen was further diluted in a two-folded serial dilution manner. After that 50 µl of 0.25% goose erythrocytes suspended in borate saline buffer at pH of 6.2 or 6.4 or 6.6 were added into every well containing dengue 1 or dengue 2 or JEV antigen respectively, then, followed by an incubation at 4°C for 1 hour. The control wells included in every test run consisted of a well containing 50 µl of buffer and 50 µl of 0.25% goose red cells. All reaction wells were performed in duplicate.

The highest dilution that gave complete agglutination of goose red blood cells is defined as one hemagglutination unit. Working concentration of the hemagglutination antigen in HI test was 4-8 units.

Antibody titration of the test sera

The test was performed in a 96 wells-V shaped microtiter plate as followed. A 25 µl of 0.4% bovine albumin in borate saline was added into each well of the test plate, and followed by an adding of a 25 µl of the test serum into the first well only. The test serum in the first well was now at the dilution of 1:20. Two-folded serial dilution was performed starting from the dilution of 1:20 to 1:20480. Twenty-five microliters of the antigen at concentration of 4 hemagglutination units were added to every well, then, mixed and incubated for 2 hours at 4°C. After that 50 µl of 0.25% goose erythrocyte suspension was added to every well and incubated for 1 hours at 37°C before reading the result. HI titer was determined from the highest serum dilution which completely inhibited hemagglutination.

Three kinds of controls were included in each run as follows.

1. Serum control well: This well consisted of 25 µl of the test serum at dilution of 1:20, 25 µl of buffer and 50 µl of goose red blood cells. This control well aimed to demonstrate that the test serum was completely got rid of non-specific agglutinator,thus, hemagglutination reaction should not be observed.

2. Cell control: This well consisted of 50 µl of goose red blood cell suspension and 50 µl of buffer. This control aimed to show a good condition of red blood cells which should appear as round red button at the bottom of the well.

3. Back titration: These wells consisted of 25 µl of antigen each at concentration of 4,2,1 and 0.5 HA units, 25 µl of buffer and 50 µl of goose red blood cells. This control set aimed to show that the test antigen was used at the exact concentration of 4 HA units, so the wells presumed to contain 4,2 and 1 HA units of the antigen should show complete hemagglutination, whereas the well with 0.5 HA unit should show partial hemagglutination.

Criteria to diagnose JEV encephlitis

Refer to criteria established by Dr. Bruce Innis (41), only specimens of CSF with ≥ 40 units of anti JEV IgM should be classified as coming from a patient with "Japanese encephalitis." Cases with only serum positive for anti-JEV IgM should be diagnosed "recent JEV infection." In addition, we suggested that cases with only paired sera showed a four-folded rise of HI antibody titer should be also classified as " current JEV infection."

CHAPTER V

RESULTS

Laboratory investigation for JEV encephalitis/JEV infection

Laboratory diagnosis of JEV encephalitis and JEV infection was performed by 1) detection of JEV RNA in CSF by RT/PCR; 2) ELISA for specific IgG and IgM in both CSF and sera, and 3) HI test for a four-folded rise of antibody titer in paired sera. Criteria for the diagnosis was based on what has been described in materials and methods.

Detection of JEV RNA by RT-nested PCR

Detection of JEV RNA was carried out by synthesis of cDNA from RNA genome by reverse transcription followed by amplification of cDNA by nested PCR. Oligonucleotide primers for reverse transcription and nested PCR were designed from non structural protein 3 region (NS3) of JEV genome were. The NS3 region of the JEV genome is conserved among JEV strains. The amplified products of primary and nested PCR are of 506 bp and 292 bp in length, respectively.

Determination for sensitivity of RT/PCR in the detection of JEV genome.

JEV Nakayama strain obtained from Dr. Boonyos Raengsakulrach, Infus Medical Company, Bangkok was used as the reference control. JEV at titer of $4x\ 10^6$ pfu/ml was serially ten-fold diluted from the stock solution to the dilution of

10^{-7}, and 300 µl of each dilution was extracted with Trizol reagent (GIBCO BRL, New York USA.). Five µl of the extracted JEV RNA genome was used as the template to synthesize cDNA, and then 10 µl of each cDNA dilution were amplified by PCR. The result showed that primary PCR could amplify JEV cDNA from the undiluted solution up to the dilution of 10^{-2} ($3x10^5$ to $3x10^3$ PFU) as showed in Figure 1, and the nested PCR could do so from the undiluted solution up to the dilution of 10^{-5} ($3x10^5$ to 3 pfu). The nested PCR was performed by using 10% (1:10) of the primary amplified DNA product. Then, the sensitivity of nested PCR was 0.3 pfu per nested PCR reaction as showed in Figure 2.

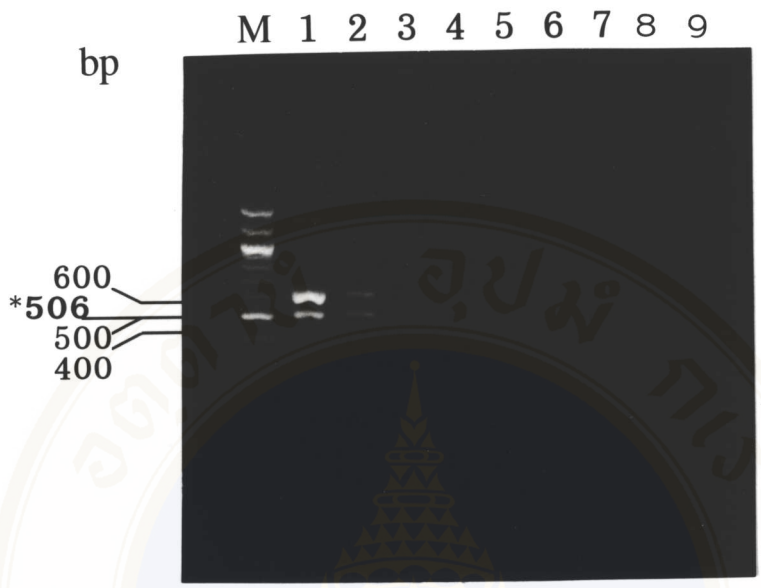

Figure 1. Gel electrophoresis showing the primary PCR product of size 506 base pairs as various dilutions of JEV cDNA were amplified.

Lane M 100 bp ladder DNA marker

Lanes 1-8 cDNA synthesized from undiluted to dilutions of 10^{-7} of JEV RNA ($3x10^{5}$ to $3x10^{-2}$ pfu)

Lane 9 Blank control

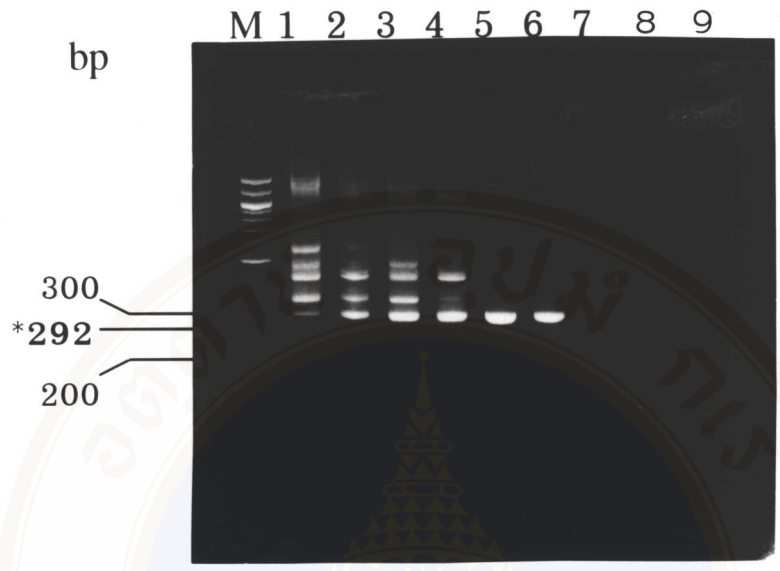

Figure 2. Gel electrophoresis showing the nested PCR products of size 292 base pairs as primary PCR products of various dilutions were amplified.

Lane M 100 bp ladder DNA marker

Lanes 1-8 primary PCR products of cDNA obtained from a serial ten-folded dilutions of JEV RNA ($3x10^5$to $3x10^{-2}$ pfu)

Lane 9 Blank control

Detection of JEV RNA in CSF by RT-PCR

RNA was extracted from a total of 14 CSF samples obtained from patients who were diagnosed viral encephalitis and 11 cases with other diagnosis. This RNA was used as template to synthesized cDNA which will be a template for further DNA amplification. Reference JE RNA controls were included in every test run. (Figure 3)

The result showed that no JEV RNA was detected from all 25 CSF samples tested, (table 4).

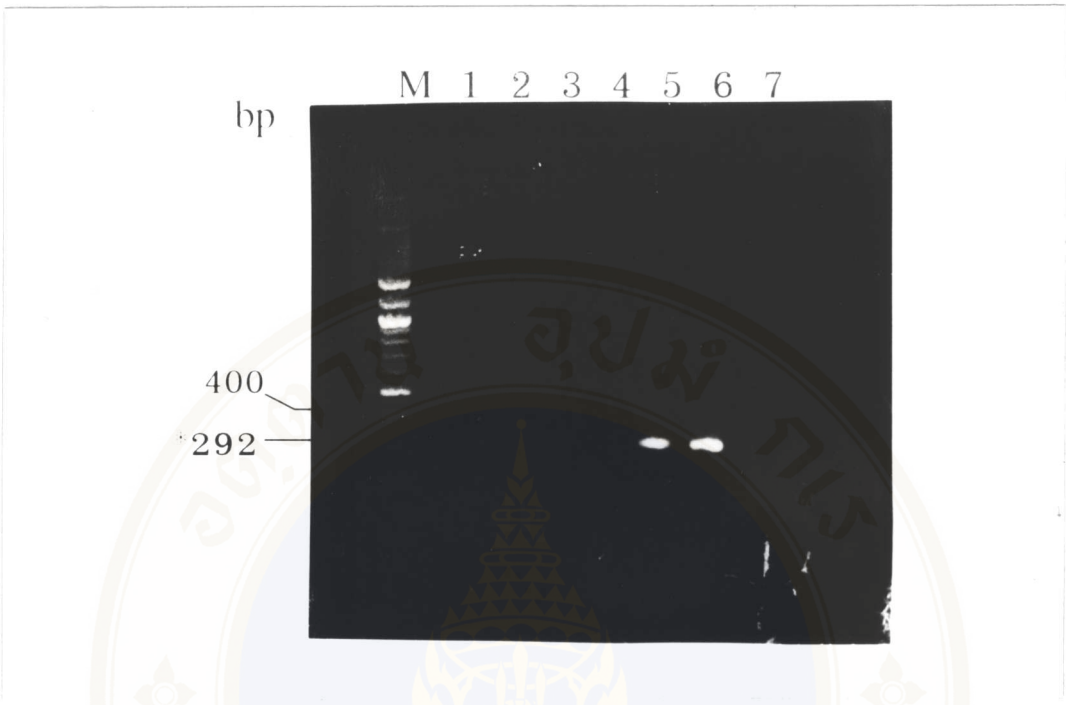

Figure 3. Gel electrophoresis showing the nested PCR product of size 292 base pairs as clinical specimens of CSF were amplified.

Lane M	100 bp ladder DNA marker
Lanes 1-4	CSF samples
Lanes 5 & 6	JEV cDNA - weak positive control
Lane 7	Blank control

Detection of JEV antibody in serum samples by HI and ELISA.

In order to diagnoses JEV infection by HI and ELISA, only cases possessed paired sera were included. Therefore, a total of 68 patient with different diagnosis were investigated. A four-folded rise in antibody titer was observed in 7 patients, while ELISA IgM was detected in 10 paired sera. It happened that positive serological test by HI and ELISA IgM was found not only in viral encephalitis cases but also in two patients with other diagnosis (Table 4).

Table 4. Investigation for JEV infection in the study cases by RT/PCR, ELISA IgG, and IgM and HI

Diagnosis	No.of paired sera in case study	No. of cases with						
		RT/PCR	ELISA IgG > 40 units in			ELISA IgM > 40 units in			> 4 folded rise of HI Ab in paired sera
			CSF	Acute	Conv.	CSF	Acute	Conv.	
Viral encephalitis	27	0/14	7/23	5/27	7/27	6/23	8/27	8/27	5 cases
		0.00%	30.43%	18.52%	25.93%	26.09%	29.63%	29.63%	18.52%
CNS infection	12	0/6	0/9	0/12	0/12	0/9	0/12	0/12	0 case
		0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0%
Other diseases	20	0/2	0/14	1/20	1/20	0/14	1/20	1/20	1 case
		0.00%	0.00%	5.00%	5.00%	0.00%	5.00%	5.00%	5.00%
Unknown diagnosis	9	0/3	0/7	0/9	1/9	0/7	0/9	1/9	1 case
		0.00%	0.00%	0.00%	11.11%	0.00%	0.00%	11.11%	11%
Total	68	0/25	7/53	6/68	9/68	6/53	9/68	10/68	7 case
		0.00%	13.21%	8.82%	13.24%	11.32%	13.24%	14.71%	10.29%

Comparison on the diagnosis of JEV encephalitis / JEV infection by HI and ELISA

Among 27 cases of viral encephalitis, there were 5(18.52%) cases who showed a four-folded rise of HI antibody titer in paired sera. However, ELISA IgM ≥40 units was detected in sera from 8 (29.63%) viral encephalitis cases. In another word there were 3 more cases whose sera had ELISA IgM ≥40 units but a four-folded rise in antibody titer was not observed.

There were 6 cases who had JE ELISA IgM ≥40 units in CSF; and also ELISA IgM ≥40 units in sera, however only 4 of them developed a rise in HI antibody titer in paired sera. In addition there was one case of viral encephalitis whose sera showed a rise in antibody titer and presence of ELISA IgM ≥40 units but his CSF did not possess such units of ELISA IgM. Investigation for presence of ELISA IgM in CSF was not performed in 3 cases according to lack of clinical samples.

These findings demonstrated that detection of ELISA IgM is more sensitive than HI test in the diagnosis of JEV encephalitis / JEV infection.

HI and ELISA result of all positive cases are show in Table 5.

Table 5 Comparison on the diagnosis of JEV encephalitis / JEV infection by HI and ELISA

Code	Diagnosis	Date at onset (day)	Specimens	HI	JE ELISA	
					IgM	IgG
MCh.	Viral encephalitis	-	Acute serum 25 May	1:20	156	57
			Conv.serum 29 May	1:80	86	4
			CSF		163	41
PPl.	Viral encephalitis	3	Acute serum 11 Sep	1:20	110	21
		17	Conv.serum 25 Sep	1:20	147	33
			CSF		199	1
SK.	Viral encephalitis	7	Acute serum 9 Apr	1:80	96	173
		19	Conv.serum 21 Apr	1:640	108	159
			CSF		166	267
PW.	Viral encephalitis	8	Acute serum 18 Apr	<1:20	15	20
		15	Conv.serum 25 Apr	1:160	93	210
			CSF		6	13
CT.	Viral encephalitis	-	Acute serum 1 Jul	<1:20	155	20
			Conv.serum 14 Jul	1:40	177	21
			CSF		190	40
RT.	Viral encephalitis	-	Acute serum 8 Jul	1:320	140	89
			Conv.serum 16 Jul	1:640	133	90
			CSF		197	181
SM.	Viral encephalitis	-	Acute serum 20 Jul	1:160	186	76
			Conv.serum 24 Jul	1:160	160	107
			CSF		189	125
Kpo.	Viral encephalitis	1	Acute serum 24 Jul	<1:20	69	32
		16	Conv.serum 9 Aug	1:40	42	8
			CSF		ND	ND
TO.	Other disease	-	Acute serum 2 Apr	1:80	57	101
			Conv.serum 29 Apr	1:2560	53	95
			CSF		ND	ND
ChB.	Unknown diagnosis	3	Acute serum 9 Feb	<1:20	18	11
		21	Conv.serum 27 Feb	1:2560	43	119
			CSF		ND	ND

Dengue hemorrhagic fever in cases early presented as viral encephalitis

Among 27 viral encephalitis cases, there were 5 cases who presented with signs suggestive of viral encephalitis at early onset of illness but progressed to frank dengue hemorrhagic fever later. A four folded rise or high HI antibody titer and presence of ELISA IgM ≥40 units were found in sera of these patients. Nevertheless, both dengue and JE IgM ≥40 units was not found in their CSF (Table 6).

Summarization of laboratory investigations for JEV encephalitis, JEV infection and dengue infection in clinically diagnosed viral encephalitis cases are show in Table7.

Based on the criteria as described in Materials and Methods, we could diagnose JEV encephalitis, JEV infection and dengue infection in 6, 4 and 5 cases, respectively.

Table 6 Dengue hemorrhagic fever in cases early presented as viral encephal as investigated by HI and ELISA

Code	Date at onset (day)	Specimen	HI		EIA	
			D-1	D-2	IgM	IgG
KB.	-	Acute serum 28 Aug	1:40	1:40	3	0
		Conv. Serum 25 Dec	1:640	1:640	67	283
		CSF			6	155
NT.	-	Acute serum 23 Oct	1:320	1:80	90	239
		Conv. Serum 8 Nov	1:640	1:320	79	234
		CSF			ND	ND
Nta	-	Acute serum 3 Nov	1:640	1:640	72	329
		Conv. Serum 15 Nov	1:640	1:640	107	203
		CSF			0	49
TT.	20	Acute serum 8 Aug	1:40	1:40	104	17
	42	Conv. Serum 9 Sep	1:80	1:40	77	0
		CSF			ND	ND
PS.	1	Acute serum 31 Oct	1:20	1:20	5	21
	8	Conv. Serum 6 Nov	1:20	1:20	133	9
		CSF			0	1

Table 7 Summarization on the laboratory diagnosis of JEV encephalitis, JEV infection and dengue infection by HI and ELISA

Code	Diagnosis	Specimen	JEV antibody by			Dengue virus antibody by				Conclusion
			HI	ELISA IgG	ELISA IgM	HI D-1	HI D-2	ELISA IgG	ELISA IgM	
MCh.	Viral encephalitis	Acute serum	1:20	57	156	<1:20	1:20	0	14	JEV encephalitis
		Conv. Serum	1:80	4	86	<1:20	1:20	0	2	
		CSF		41	163			0	11	
PPl.	Viral encephalitis	Acute serum	1:20	21	110	<1:20	<1:20	4	8	JEV encephalitis
		Conv. Serum	1:20	33	147	<1:20	<1:20	4	6	
		CSF		1	199			0	13	
SK.	Viral encephalitis	Acute serum	1:80	173	96	1:160	1:160	240	18	JEV encephalitis
		Conv. Serum	1:640	159	108	1:160	1:160	249	12	
		CSF		267	166			358	23	
PW.	Viral encephalitis	Acute serum	<1:20	20	15	<1:20	<1:20	13	4	JEV infection
		Conv. Serum	1:640	210	93	1:160	1:80	242	19	
		CSF		13	6			22	3	
CT.	Viral encephalitis	Acute serum	<1:20	20	155	<1:20	<1:20	0	10	JEV encephalitis
		Conv. Serum	1:40	21	177	<1:20	<1:20	0	10	
		CSF		40	190			0	18	

Table 7 continued

Code	Diagnosis	Specimen	JEV antibody by			Dengue virus antibody by				Conclusion
			HI	ELISA IgG	ELISA IgM	HI D-1	HI D-2	ELISA IgG	ELISA IgM	
RT.	Viral encephalitis	Acute serum	1:320	89	140	1:160	1:320	80	7	JEV encephalitis
		Conv. Serum	1:640	90	133	1:320	1:320	108	11	
		CSF		181	197			219	16	
SM.	Viral encephalitis	Acute serum	1:160	76	186	<1:20	<1:20	16	7	JEV encephalitis
		Conv. Serum	1:160	107	160	<1:20	<1:20	7	3	
		CSF		125	189			15	7	
KPo.	Viral encephalitis	Acute serum	<1:20	32	69	<1:20	<1:20	0	38	JEV infection
		Conv. Serum	1:40	8	42	1:80	1:80	0	17	
		CSF		ND	ND			ND	ND	
TO.	Other disease	Acute serum	1:80	101	57	1:80	1:80	33	23	JEV infection
		Conv. Serum	1:2560	95	53	1:2560	1:10240	115	66	
		CSF		ND	ND			ND	ND	
ChB.	Unknown diagnosis	Acute serum	<1:20	11	18	<1:20	<1:20	0	27	JEV infection
		Conv. Serum	1:2560	119	43	1:2560	1:2560	261	33	
		CSF		ND	ND			ND	ND	

Table 7 continued

Code	Diagnosis	Specimen	JEV antibody by			Dengue virus antibody by				Conclusion
			HI	ELISA IgG	ELISA IgM	HI D-1	HI D-2	ELISA IgG	ELISA IgM	
KB.	Dengue hemorrhagic fever	Acute serum	1:80	18	22	1:40	1:40	0	3	Secondary dengue infection
		Conv. Serum	1:1280	128	39	1:640	1:640	283	67	
		CSF		80	32			155	6	
NT.	Dengue hemorrhagic fever with Bell palsy	Acute serum	1:80	207	32	1:320	1:80	239	90	Secondary dengue infection
		Conv. Serum	1:320	194	26	1:640	1:320	234	79	
		CSF		ND	ND			ND	ND	
NTa.	Dengue hemorrhagic fever and salmonella infection	Acute serum	1:640	166	69	1:640	1:640	329	72	Secondary dengue infection
		Conv. Serum	1:640	89	15	1:640	1:640	203	107	
		CSF		48	0			49	0	
TT.	Dengue hemorrhagic fever	Acute serum	1:20	16	14	1:40	1:40	17	104	Primary dengue infection
		Conv. Serum	1:20	0	0	1:80	1:40	0	77	
		CSF		0	0			7	9	
PS.	Dengue hemorrhagic fever	Acute serum	1:20	23	10	1:20	1:20	29	5	Primary dengue infection
		Conv. Serum	1:20	13	28	1:20	1:20	9	133	
		CSF		0	4			1	0	

Correlation between JEV and dengue HI antibodies

A total of 68 paired sera were investigated for JEV, dengue 1 and dengue 2 antibody by HI test and the results are showed in Figures 4 and 5. Usually positive correlation between levels of HI antibody to JEV and dengue 1 and dengue 2 were observed ($r_1 = 0.7$ and $r_2 = 0.7$), which suggested high cross reactivity between viruses belonging to the same genus. (Spearman *P* value = < 0.05, no significant)

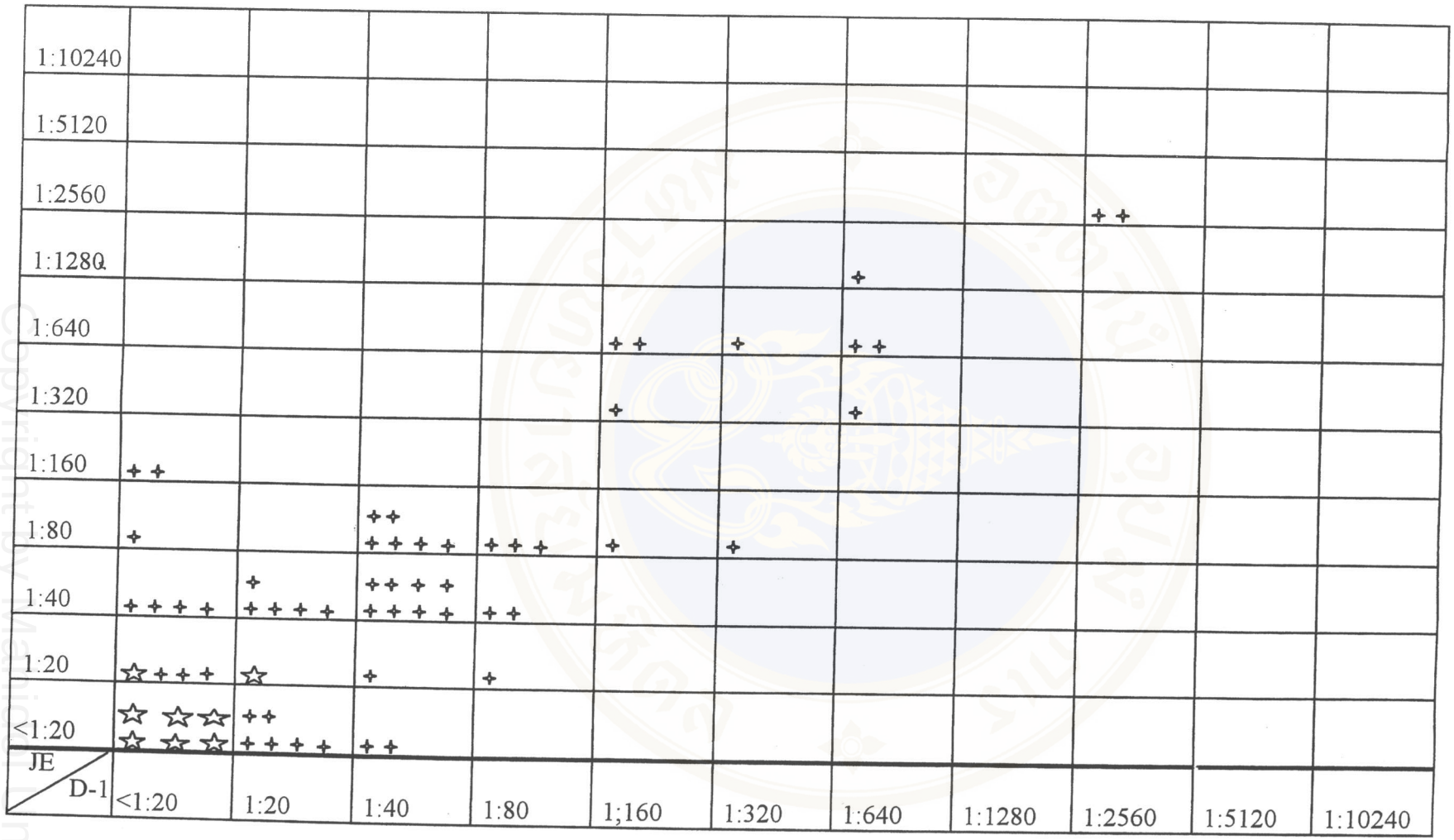

Figure 4. Correlation between JEV and dengue type 1 virus HI antibody titer ☆ = 10 ✧ = 1

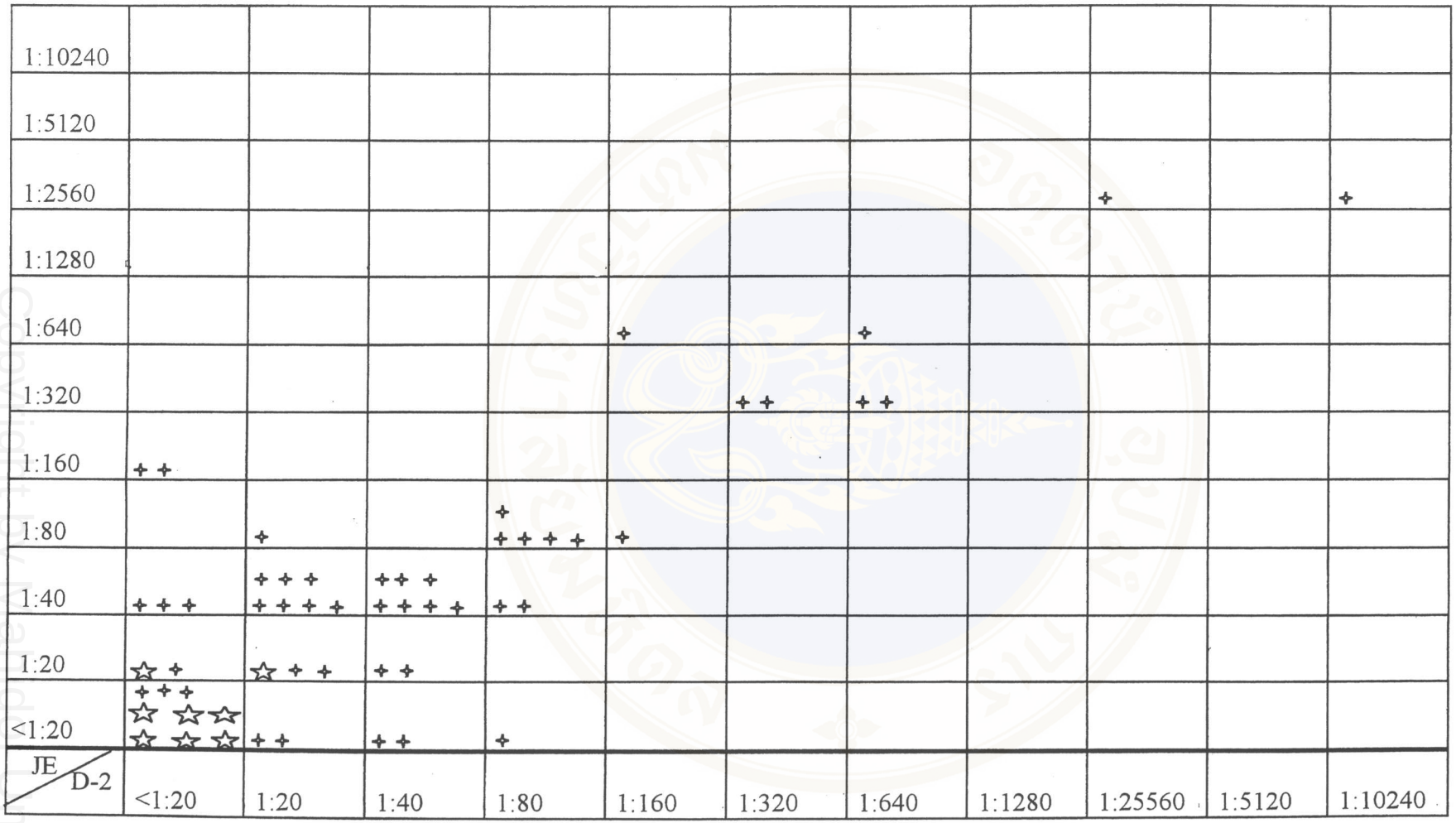

Figure 5. Correlation between JEV and dengue type 2 virus HI antibody titers ☆ = 10 ✧ = 1

Laboratory investigation for HSV-1 and HSV-2 infection

Laboratory diagnosis of HSV CNS infection was performed by detection of viral genome in CSF, and by determination of ASI (ratio of CSF : serum IgG) and HSV IgM in serum.

Detection of HSV-I and HSV-II DNA by nested PCR

HSV-1 and HSV-2 DNA was amplified by nested PCR which amplified a region in the TK gene of HSV genome. Sequence of oligonucleotide primers was shown in table 1. These oligonucleotide primers could amplify both HSV-1 and HSV-2 DNA and gave the products of the same fragment size. The primary PCR products were of 540 bp in length, while the nested PCR products were of 250 bp. The results from previous work performed by Mr. Navin Horthongkham showed that sensitivity of this nested PCR system was 4.3 molecules of HSV genome per one reaction.

2. Detection of HSV DNA from CSF by nested PCR

DNA was extracted from 163 CSF samples collected from 36 patients who were diagnosed encephalitis and 127 cases with other diseases. This target DNA was used for amplification and HSV DNA extracted from the Vero infected cells as the reference DNA controls were included in every test run. As various clinical samples and the controls were tested, the HSV nested PCR products are shown in Figure 6.

HSV DNA was found in 4(11.11%) of 36 samples collected at acute phase of illness from pediatric who diagnosed viral encephalitis. In overall 17 (10.43%) of 163 CSF samples were positive for HSV DNA (Table 8).

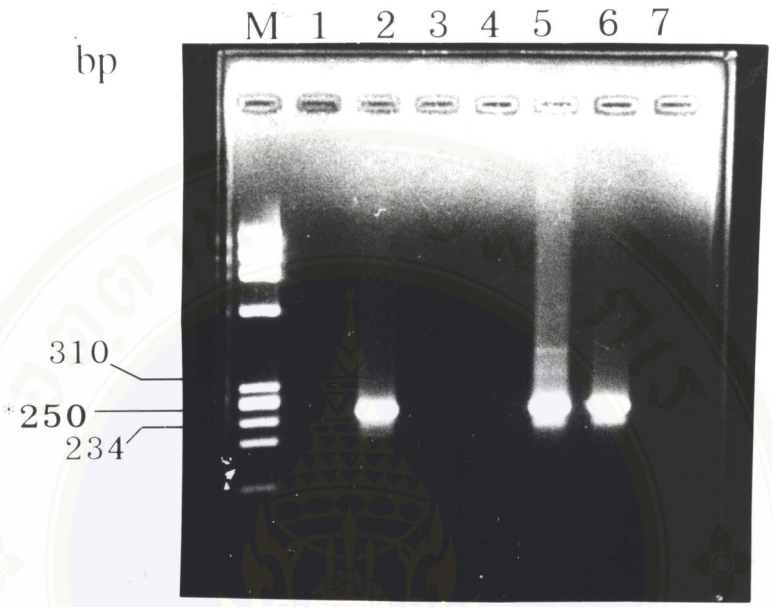

Figure 6. Gel electrophoresis showing the nested PCR products of size 250 base pairs as HSV DNA were amplified.

Lane M	ØX 174 RF DNA/*Hae III* standard marker
Lanes 1,3 & 4	HSV negative CSF samples
Lane 2	HSV positive CSF samples
Lane 5	HSV DNA - strong positive
Lane 6	HSV DNA - weak positive
Lane 7	Blank control

HSV typing by restriction endonuclease cleavage

HSV DNA amplified products were purified and concentrated by phenol-chloroform extraction. Then, digested by restriction endonuclease enzyme *BStN I.* HSV-I amplified product cleaved with this enzyme yielded three fragments of size 63, 73, and 114 bp, while HSV-II amplified product cut with the same enzyme yielded 2 fragments of size 76 and 174 bp as shown in Figure 7.

Of 17 nested PCR products obtained, 11 of them were digested by *BStN I.*, and all of them were HSV-1.

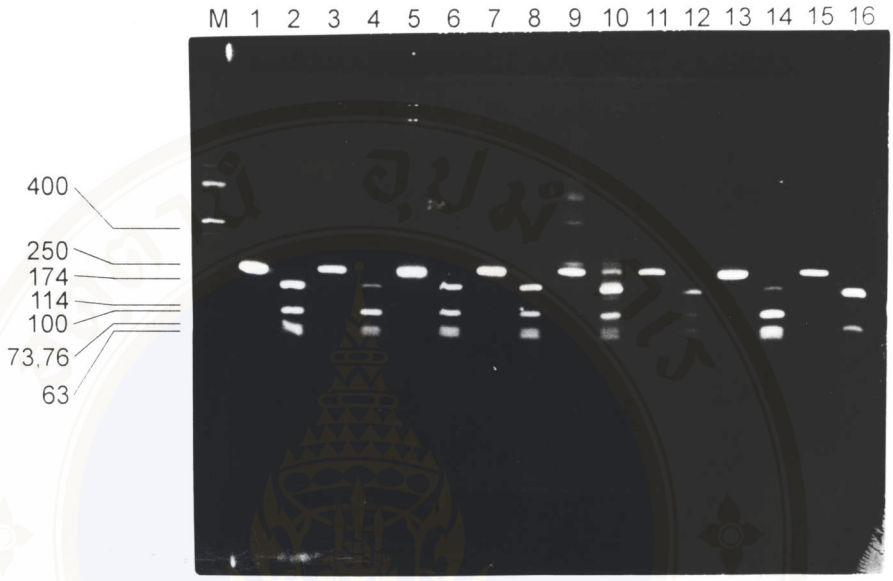

Figure 7. Cleavage of HSV PCR products by *BStNI* enzyme.

Lane M	ØX 174 RF DNA/ *HaeIII* standard marker
Lanes 1,3,5,7,9 & 11	Uncut PCR products from HSV-1 positive CSF samples
Lanes 2,4,6,8,10 & 12	Cut PCR products from HSV-1 positive CSF samples
Lanes 13 & 14	Uncut and cut PCR products of reference HSV-1 DNA
Lanes 15 and 16	Uncut and cut PCR products of reference HSV-2 DNA

Investigation for HSV IgG and IgM in patients' sera by ELISA

Investigation by ELISA in paired sera showed that HSV IgG was present in 32.79%, while ELISA for HSV IgM was performed in 9.84% of 61 cases studied. The results of an investigation in either acute or convalescent serum was the same both IgG and IgM assay (Table 8).

Investigation for HSV antibody specific index

Acute serum and CSF sample collected on the same day during acute phase of the disease were tested for presence of HSV specific IgG. Ratio between HSV IgG antibody present in CSF and serum samples was determined according to the instruction attached to the commercial kit and expressed as antibody specific index (ASI). ASI values greater than 1.5 is suggestive for HSV CNS infection.

Investigation on 41 pairs of CSF-acute serum showed that 8(19.51%) cases were positive for HSV infection. ASI values suggestive for HSV infection were also demonstrated in 6 cases with other diagnosis (4 CNS infections and 2 other diseases) as showed in Table 8.

Summarization on investigation for HSV infection in viral encephalitis cases

The diagnosis of HSV infection was relied on the investigations by PCR or ASI. We could diagnose 6(16.67%) of HSV infection in 36 viral encephalitis cases. Four of them were diagnosed by PCR and two cases were diagnosed by ASI (Table 8). Nevertheless, PCR and ASI had diagnosed HSV infection in patients with other diseases. The results of different methods of investigation in each case are showed in Table 9.

Table 8. Summarization of the investigation for HSV infection by PCR, ELISA IgG, ELISA IgM and ASI

Diagnosis	No. of cases positive by (%)			
	PCR	ELISA IgG	ELISA IgM	ASI
Viral encephalitis	4/36(11.11%)	9/26(34.62%)	1/26(3.85%)	2/16(12.50%)
CNS infections	2/23(8.69%)	5/11(45.45%)	3/11(27.27%)	4/7(57.14%)
Other diseases	11/61(18.03%)	5/16(31.25%)	2/16(12.50%)	2/12(16.67%)
Unknown diagnosis	0/43(0.00%)	1/8(12.50%)	0/8(0.00%)	0/6(0.00%)
Total	17/163(10.43%)	20/61(32.79%)	6/61(9.83%)	8/41(19.51%)

Table 9. Summarization on laboratory investigation in all HSV infected cases.

Code	Date of onset	PCR	Acute serum	Conv. serum	ASI	ELISA IgG		ELISA IgM		Remark
						A	C	A	C	
Viral encephalitis										
VB.	3 days	+	2 July	-	ND	-	ND	-	ND	
VP.	1 day	+	14 May	28 May	-	+	+	+	+	
PKi.	Not known	+	13 May	-	ND	-	ND	-	ND	
JP.	Not known	+	-	-	ND	ND	ND	ND	ND	
ChD.	2 days	-	1 June	3 June	1.9	-	-	-	-	
PW.	8 days	-	18 Apr	25 Apr	2.2	+	+	-	-	JEV infection HHV-6 IgM+ve
CNS infection										
SD.	1 day	+	20 Nov	9 Dec	ND	+	+	-	-	
PJ.	Not known	+	10 Feb	14 Mar	ND	-	-	-	-	
RC.	Not known	-	27 May	9 June	1.7	-	-	+	+	
PKh.	2 days	-	7 Mar	29 May	1.6	+	+	+	+	
PH.	9 days	-	27 Oct	13 Dec	2.8	+	+	+	+	
SSr.	Not known	-	30 Sep	6 Oct	3.3	+	+	+	+	
Other diseases										
PKl.	Not known	+	29 May	-	ND	-	ND	-	ND	
SHl.	Not known	+	10 Apr	-	ND	-	ND	+	ND	
KK.	Not known	+	16 Feb	-	ND	ND	ND	ND	ND	
PP.	Not known	+	4 Mar	-	ND	+	ND	-	ND	
WK.	7 days	+	20 May	6 June	ND	-	-	-	-	
SP.	1 day	+	2 Apr	-	ND	-	ND	-	ND	
AP.	Not known	+	19 Dec	31 Dec	ND	-	-	-	-	
ChK.	1 day	+	15 Dec	-	ND	-	ND	-	ND	
NP.	2 days	+	3 May	-	ND	-	ND	-	ND	
PB.	Not known	+	6 Aug	20 Aug	ND	-	-	-	-	
SHo.	Not known	+	26 Apr	8 May	ND	-	-	-	-	
TD.	8 days	-	15 Oct	22 Dec	1.6	+	+	-	-	Enterovirus IgM & IgA +ve
SPa.	Not known	-	8 May	22 May	1.9	-	-	-	-	

Criteria for HSV IgG and IgM antibody testing

< 9.0 UNITS = NEGATIVE (-)

9-11 UNITS = GRAY ZONE (±)

> 11 UNITS = POSITIVE (+)

Criteria for HSV antibody specific index (ASI)

ASI > 1.5 is considered as HSV CNS infection

Amplification of HHV-6 DNA by nested PCR

This method was carried out to amplify a region in the immediate early gene of HHV-6 genome. Two major variants, of HHV-6 isolates designated A and B could be distinguished by the difference in size of the amplified products. The primary PCR products were of 321 and 554 bp, while those of the nested PCR were of 193 and 421 bp for variants A and B, respectively.

Sensitivity in the detection of HHV-6 genome by nested PCR

The sensitivity of PCR method was determined by using purified HHV-6 DNA type A and type B kindly provided by Associated Professor Uraiwan Kositanont, Department of Microbiology, Faculty of Medicine, Siriraj Hospital as the standard control. Both types of HHV-6 DNA each at concentration of 100 ng/ml was determined for copy number of HHV-6 genomes by basing on its molecular weight and Avogadro number as follows

Virus	%GC	Length(Kb)	Molecular weight
HHV-6	43	167	113.0×10^6

Molecular weight for an A-T base pair = 669.4 g/mol

Molecular weight for a G-C base pair = 686.4 g/mol

[A = 347.2, T = 322.2, C = 323.2, G = 363.2]

Molecular weight of the whole genome =

(G-C content x G-C molecular weight x lengt) +

(A-T content x A-T molecular weight x length)

Molecular weight of HHV-6 genome = $(0.43 \times 686.4 \times 167 \times 10^3) + (0.57 \times 669.4 \times 167 \times 10^3)$

$= 113.0 \times 10^6$

$$\text{Number of molecules in Xg of DNA} = \frac{\text{Avogardro constant x Xg}}{\text{Molecular Weight of genome}}$$

Number of molecules of HHV-6 genome present in 100 ng[100×10^{-9} grams]

$$= \frac{6.023 \times 10^{23} \times 100 \times 10^{-9}}{113.0 \times 10^6}$$

$= 5.33 \times 10^{10}$ molecules/ml

The stock of HHV-6 genome at concentration of 5.33×10^{10} molecules/ml was ten-fold diluted from 10^{-1} to 10^{-10}, and 10 µl of each dilution were amplified by PCR. The result showed that primary PCR test system could amplify HHV-6 DNA type A (U 1102) at the dilution of 10^{-1} up to the highest dilution of 10^{-4} as showed in Figure 8, and the nested PCR could do so from the dilution of 10^{-1} up to the highest dilution of 10^{-9} as showed in Figure 9. Thus, sensitivity of the nested PCR was at the dilution of 10^{-9}. In another word, the sensitivity of nested PCR for amplification of HHV-6 type A was 0.5 copies in a 10µl of one nested PCR. as showed in Figure 9.

Sensitivity of the PCR test system for HHV-6 DNA type B (HST) was also determined in the similar manner, and it was found to be at the dilution of 10^{-4} and10^{-8} for a primary and nested PCR, respectively. And thus, sensitivity of nested PCR for amplification of HHV-6 type A was 5.3 copied per reaction. The results are showed in Figure 10 and 11.

Figure 8. Gel electrophoresis showing the primary PCR product of size 326 base pairs as various dilutions of purified HHV-6 type A DNA were amplified.

Lane M ØX 174 RF DNA/ *Hae III* standard maker

Lanes 1 to 9 Serial ten-folded dilutions of HHV-6 DNA starting from 10^{-1} to 10^{-9}

Lane 10 Blank control

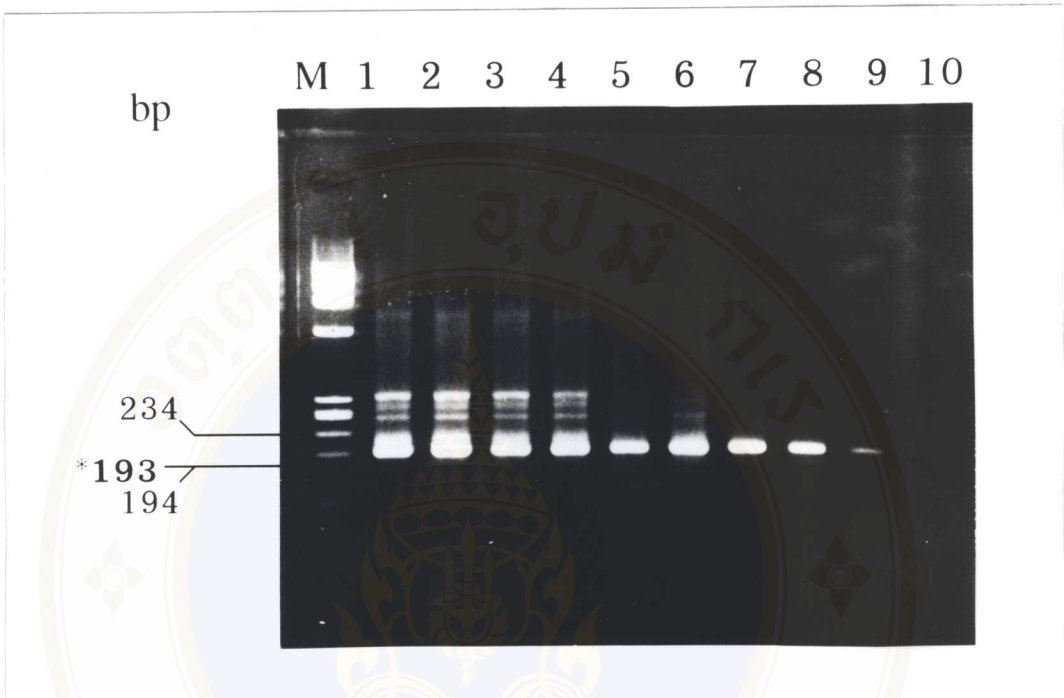

Figure 9. Gel electrophoresis showing the nested PCR product of size 193 base pairs as primary PCR products of HHV-6 type A DNA were amplified.

Lane M ØX 174 RF DNA/ *Hae III* standard marker

Lanes 1 - 9 Serial ten-folded dilution of HHV-6 DNA 10^{-1} to 10^{-9}

Lane 10 Blank control

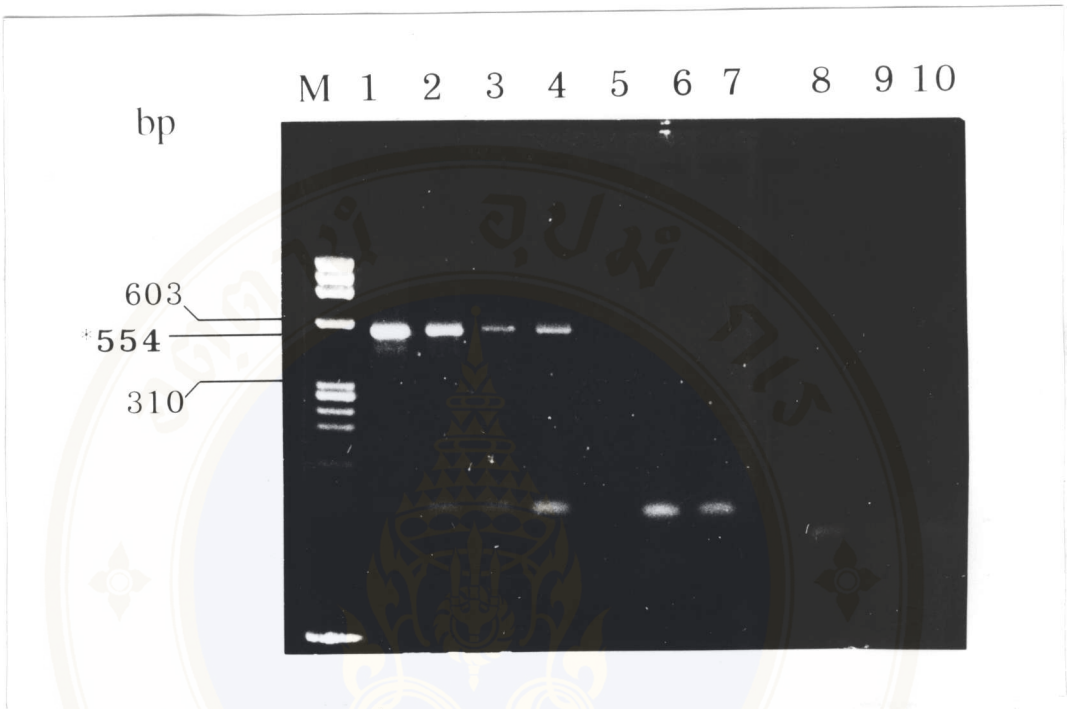

Figure 10. Gel electrophoresis showing the primary PCR product of size 554 base pairs as various dilutions of purified HHV-6 type B DNA were amplified.

Lane M ØX 174 RF DNA/ *Hae III* standard marker

Lanes 1-9 Serial ten-folded dilutions of HHV-6 DNA starting from 10^{-1} to 10^{-9}

Lane 10 Blank control

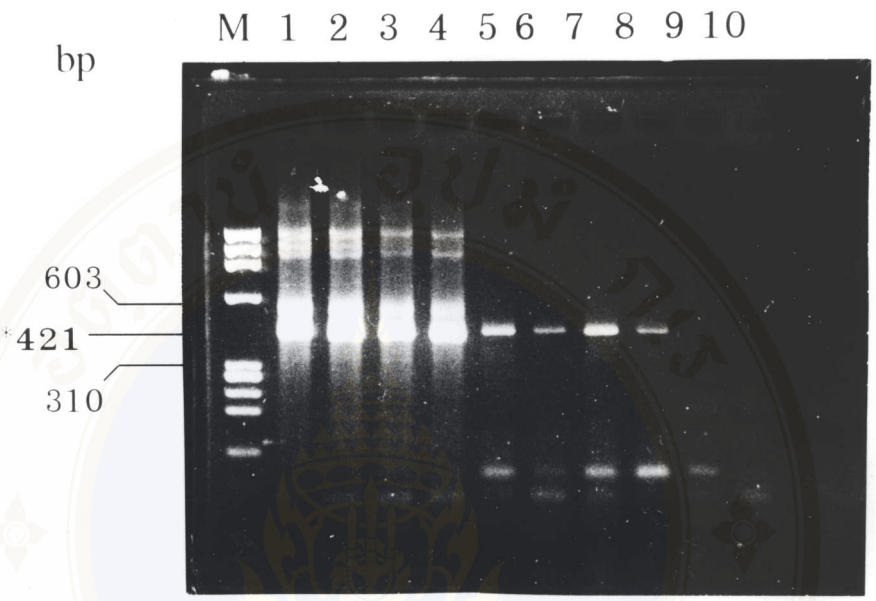

Figure 11. Gel electrophoresis showing the nested PCR product of size 421 base pairs as primary PCR products of HHV-6 type B were amplified.

Lane M ØX 174 RF DNA/ *Hae III* standard marker

Lanes 1 to9 Serial ten-folded dilution of HHV-6 DNA starting from 10^{-1} to 10^{-9}

Lane 10 Blank control

The specificity of HHV-6 oligonucleotide primers was confirmed as they could not amplify the DNA extracts from CMV infected human foreskin fibroblast, B-95-8 EBV lymphoblastoid cells, adenovirus infected HEp-2 cells, and uninfected Vero cells. As various clinical samples and various controls were tested, the HHV-6 nested PCR products are showed in Figure 12.

Detection of HHV-6 DNA in CSF by nested PCR

DNA was extracted from CSF 36 samples obtained from patients with viral encephalitis and 107 cases with other diagnosis. This target DNA was used in the amplification for HHV-6 DNA, the reference DNA control was included in every test run.

The result showed that HHV-6 DNA was found in three (8.33%) cases with viral encephalitis (Table 10).

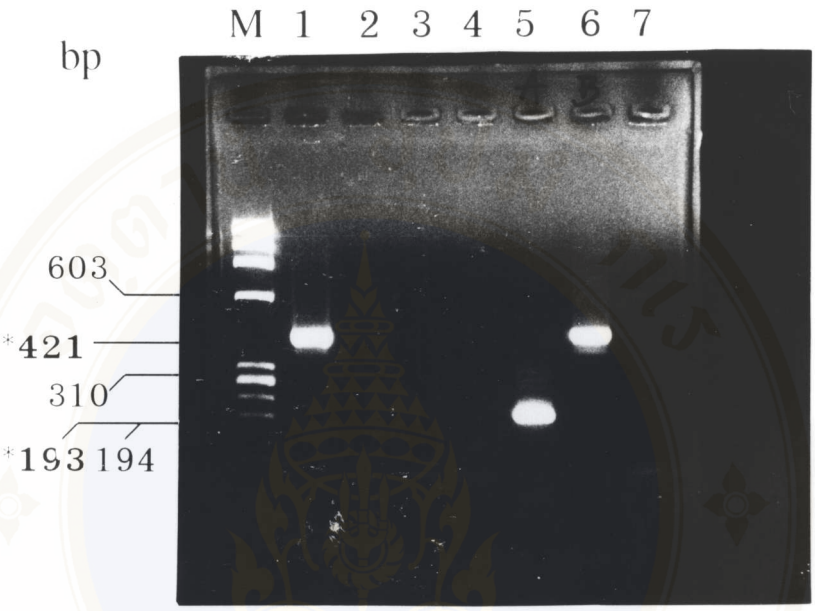

Figure 12. Gel electrophoresis showing the nested PCR product of size 421 and 193 base pairs as purified HHV-6 DNA in clinical samples were amplified.

Lane M ØX 174 RF DNA/ *Hae III* standard marker

Lane 1 HHV-6B positive clinical sample

Lanes 2 - 4 HHV-6 negative clinical samples

Lane 5 HHV-6A positive control

Lane 6 HHV-6B positive control

Lane 7 Blank control

Investigation for HHV-6 specific IgG and IgM by ELISA

Investigation for HHV-6 IgG showed positive finding in 42(68.85 %) of 61 serum samples collected at either acute or convalescent phase of the disease. While the investigation for HHV-6 IgM showed the positive rate of 10(16.39%). The result for each disease diagnosis is showed in Table 10.

Summarization on laboratory investigation for HHV-6 infection

A total of 36 CSF samples of viral encephalitis cases were investigated by PCR and 61 paired sera were investigated by ELISA IgG and IgM. Three (8.33%) of patients had HHV-6 DNA in CSF; while ELISA IgG was present in16/26(61.54%), and ELISA IgM was 5/26(19.23%) of the sera studied. However, HHV-6 IgM was also found in 5/35(14.29 %) of the patients with other disease diagnosis (Table 10).

Without presence of viral markers in CSF, the definite diagnosis of HHV-6 encephalitis could not be obtained. We investigated the role of HHV-6 in viral encephalitis by basing on level of specific IgM in sera alone. Based on the criteria of the kit instruction, sera with specific IgM between 10-20 units is considered low positive, and > 20 units is considered high positive. We, then choose the levels of > 20 units as the criteria to diagnose presumably HHV-6 encephalitis. Nevertheless, among all five viral encephalitis cases who possessed specific IgM, non had the levels >20 units. Thus, we concluded that no HHV-6 encephalitis was found in our study by detection to HHV-6 IgM.

The result of different methods in the investigation in each viral encephalitis case who possessed HHV-6 DNA or HHV-6 IgM are shown in Table 11.

Table 10. Summarization on laboratory investigation of HHV-6 infection by PCR, ELISA IgG and IgM.

Diagnosis	No. of cases positive by (%)		
	PCR	ELISA IgG	ELISA IgM
Viral encephalitis	3/36(8.33%)	16/26(61.54%)	5/26(19.23%)
CNS infections	0/19(0.00%)	9/11(81.18%)	3/11(27.27%)
Other diseases	0/49(0.00%)	11/15(73.33%)	1/15(6.67%)
Unknown diagnosis	0/38(0.00%)	6/9(66.67%)	1/9(11.11%)
Total	3/143(2.11%)	42/61(68.85%)	10/61(16.39%)

Table 11. Laboratory investigators for HHV-6 infection in viral encephalitis cases.

Viral encephalitis

Code	Date of onset	PCR	Acute serum	Conv. serum	ELISA IgG		ELISA IgM		Remark
					A	C	A	C	
KKa.	Not known	+	1 Aug	-	ND	ND	ND	ND	
TN.	2 days	+	10 Nov	17 Nov	20.2	21.0	-	-	
AM.	3 days	+	16 June	-	ND	ND	ND	ND	
PPr.	3 days	-	11 Sep	25 Sep	17.5	19.8	6.8	10.7	JEV encephalitis and Enterovirus IgM +ve
Kbu.	Not known	-	24 Apr	4 May	7.1	12.9	10.5	15.5	Enterovirus IgM +ve
SK.	7 days	-	9 Apr	21 Apr	13.4	12.7	10.5	15.5	JEV encephalitis
PW.	8 days	-	18 Apr	25 Apr	20.4	18.7	14.1	17.0	JEV infection and HSV ASI +ve
SSa.	2 days	-	24 Jan	31 Jan	27.0	27.8	11.8	10.3	

CNS infection

SSr.	Not known	-	30 Sep	5 oct	16.4	13.3	11.5	12.0	HSV ASI & IgM+VE
PH.	9 days	-	27 Oct	13 Dec	13.2	14.7	22.9	19.2	Enterovirus IgM +ve HSV ASI&IgM +ve
PKe.	Not known	-	16 Mar	21 Apr	13.3	11.8	10.5	15.5	Enterovirus IgM +ve

Other disease

JS.	Not known	-	31 July	6 Aug	20.7	15.4	10.9	13.6	

Unknown diagnosis

WCh.	14 days	-	27 May	4 June	12.0	11.3	15.4	12.4	Enterovirus IgA +ve

Criteria for HHV-6 IgG and IgM

< 10 PANBIO UNITS = NEGATIVE

10-20 PANBIO UNITS = LOW POSITIVE

> 20 PANBIO UNITS = POSITIVE

Laboratory investigation for enterovirus infection

Laboratory methods to diagnose enterovirus associated diseases include the detection of enterovirus RNA in CSF and of specific IgG, IgM and IgA in paired sera by ELISA.

Detection of enterovirus RNA by RT/PCR

RNA sequence conserved for the genus *Enterovirus* was detected in by using Amplicor reagent kit which was based on RT/PCR system in principle. Only of (3.1%) of 32 CSF samples studied was positive for enterovirus RNA. On the other hand only 1 (5.88 %) of 17 viral encephalitis had enterovirus RNA in CSF(Table 12).

Investigation for enterovirus IgG, IgM and IgA by ELISA

Paired sera were investigated for presence of enterovirus IgG, IgM and IgA antibodies; and they were found in 15(40.54%), 18(50.00%) and 12(41.38%) of 29 the samples studied, respectively. Only enterovirus IgA, but not IgM and IgG, was detected in the patient who had enterovirus RNA in her CSF. The result is showed in Table 12.

Summarization on laboratory investigation for enterovirus infection

Only presence of specific IgM and IgA in serum without positive viral marker in CSF, the definite diagnosis for enterovirus encephalitis could not be accomplished. We, thus, suggested the etiologic role of enterovirus in viral encephalitis by basing on levels of specific IgM and /or IgA in sera. According to the criteria of the test kit, the levels of specific IgG or IgM or IgA $\geq$ 11 units is considered to be positive. We also found that the case with presence of enterovirus RNA in CSF had specific IgA $\geq$ 15 units, while that of the specific IgM was < 10 units. We also found 9(42.86%) and 8 (47.06%) of viral encephalitis cases who possessed positive IgM and IgA in sera, respectively. However two were definitely diagnosed JEV encephalitis and JEV encephalitis with HHV-6 IgM positive, one case of JEV infection, one case of dengue infection and the other HHV-6 IgM positive. These two cases possessed very high levels of enterovirus of IgA (26.26 and 29.20units), respectively. We therefore conclude that test for serum specific IgM and IgA and unreliable to diagnose or associate the role of enterovirus in viral encephalitis(Table 13).

Table 12. Summarization on laboratory investigation of enterovirus infection by RT/PCR, and ELISA IgG, IgM and IgA

Diagnosis	No. of cases positive by %			
	RT/PCR	ELISA IgG	ELISA IgM	ELISA IgA
Viral encephalitis	1/17(5.88%)	10/21(47.62%)	9/21(42.86%)	8/17(47.06%)
CNS infection	0/7(0.00%)	2/7(28.57%)	3/6(50.00%)	0/4(0.00%)
Other diseases	0/5(0.00%)	2/5(40.00%)	3/5(60.00%)	1/4(25.00%)
Unknown diagnosis	0/3(0.00%)	1/4(25.00%)	3/4(75.00%)	3/4(75.00%)
Total	1/32(3.13%)	15/37(40.54%)	18/36(50.00%)	12/29(41.38%)

Table 13. Laboratory investigators for enterovirus infection in viral encephalitis cases

Code	Date of onset	RT/ PCR	Acute serum	Conv. serum	ELISA IgG		ELISA IgM		ELISA IgA		Remark
					A	C	A	C	A	C	
UP.	6 days	+	2 Dec	12 Dec	4.2	3.7	10.1	9.6	21.1	15.6	
PPl.	3 days	-	11 Sep	25 Sep	10.2	9.6	ND	13.8	ND	2.5	JEVencephalitis HHV-6 IgM+ve
RS.	Not known	-	5 Mar	14 Mar	13.1	13.6	32.1	16.7	45.3	39.3	
LT.	3 days	-	26 May	5 June	11.7	12.5	14.7	19.8	13.6	16.0	
MCh.	Not known	-	25 May	29 May	9.1	9.3	ND	19.4	ND	26.3	JEVencephalitis
WS.	7 days	ND	4 Nov	15 Nov	12.3	ND	19.0	19.1	9.3	9.8	
PS.	1 days	-	31 Oct	6 Nov	7.2	9.6	ND	14.0	ND	ND	Dengue
ChC.	Not known	-	28 Feb	12 Mar	13.6	14.3	ND	10.7	14.5	13.1	
KBu.	Not known	-	24 Apr	4 May	12.9	13.2	13.2	14.0	7.8	8.4	HHV-6 IgM+ve
KPo.	1 days	-	14 July	8 Aug	11.2	13.1	11.3	13.0	27.6	29.2	JEV infection
TP.	3 days	ND	13 Mar	25 Mar	1.9	2.7	ND	7.8	ND	12.3	
VP.	1 day	-	14 May	28 May	9.4	9.4	ND	ND	ND	22.2	HSV PCR & IgM +ve
CNS infection											
PKe.	Not known	-	16 Mar	21 Apr	10.2	9.6	10.8	11.4	9.8	8.7	HHV-6 IgM+ve
PKh.	2 days	-	5 Feb	29 May	4.3	4.9	11.7	11.1	5.6	6.2	HSV ASI & IgM +ve
PH.	9 days	-	27 Oct	13 Dec	3.4	3.2	10.4	12.2	9.6	10.2	HSV ASI& IgM+ve, HHV-6 IgM+ve
Other diseases											
APo.	Not known	-	14 May	28 May	10.2	9.8	10.6	10.3	4.6	5.1	
JY.	Not known	-	21 Apr	7 May	6.6	8.9	10.8	11.1	7.7	7.8	
TD.	8 days	-	15 Oct	22 Dec	10.1	11.1	11.0	17.6	27.0	30.1	HSV ASI+ve

Table 13. (cont.)

Unknown diagnosis

Code	Date of onset	RT/ PCR	Acute serum	Conv. serum	ELISA IgG		ELISA IgM		ELISA IgA		Remark
					A	C	A	C	A	C	
VV.	Not known	-	5 Sep	8 Sep	7.8	9.3	11.4	10.8	9.8	11.5	
PT.	Not Known	-	17 Dec	21 Dec	9.8	10.1	10.7	10.3	10.8	12.2	
WCh.	14 days	ND	27 May	4 June	5.4	8.9	9.5	10.2	8.8	11.4	HHV-6 IgM+ve

Criteria for Enterovirus IgG, IgM and IgA antibody testing

< 9.0 UNITS = NEGATIVE (-)

9-11 UNITS = GRAY ZONE (±)

> 11 UNITS = POSITIVE (+)

Viral etiologic agents in cases with clinically diagnosed acute viral encephalitis

With the limitation on volume of CSF or serum obtained, we could not investigated for all viral agents in clinical sample. The prevalence of viral etiologic agents in our subjects thus base on summation of percent of positive rate for each agent as showed in Table 14.

Table 14. Viral etiologic agents in cases with clinical diagnosis of acute viral encephalitis : JEV, dengue, HSV, HHV-6 and enterovirus.

Viruses	No. positive cases / No. tested	No. positive (%)
JEV	6/23	26.09
Dengue	5/36	13.88
HSV	6/36	16.67
HHV-6	3/36	8.33
Enterovirus	1/32	3.13
Total No. of viruses found/ Total viral encephalitis cases	25*/36	69.44%

***Two cases of mumps, one case of varicella-zoster and one case of rabies encephalitis as diagnosed by Laboratory of Immunology and Virology were added up.**

CHAPTER VI

DISCUSSION

The present study aimed to determine the prevalence of viral etiology in pediatric patients who were clinically diagnosed acute viral encephalitis. However among 163 subjects, there were only 36 viral encephalitis cases obtained irrespective of incomplete specimens being sent for laboratory investigation. These events rendered a difficulty for an interpretation of the laboratory result; in addition, the data obtained by basing on small number of subjects may not represent the true prevalence of the population. The detection of viral genome in CSF, and of specific IgG and IgM in CSF and of specific IgG, IgM and IgA in sera were performed in order to diagnose viral etiology in this study.

RT/PCR has been developed with an enormously potential in the diagnosis of viral CNS infections because of its high specificity and sensitivity and the speed with which the results can be obtained. Our RT/PCR system was performed using the primers designed from the NS 3 nonstructural gene in order to detect JEV RNA in CSF samples. The sensitivity of this RT/PCR was 0.3 PFU/reaction tube as Nakayama strain was used as the reference virus. Twenty-five CSF clinical samples were investigated by this RT/PCR system, but no JEV RNA was detected in all of the

samples tests. Somehow, some of this CSF samples were JEV IgM positive by ELISA capture test. An experiment to exclude the inhibitor effect present in these CSF samples on the RT/PCR system included the titration of Nakayama RNA suspended in normal CSF in order to determine an endpoint dilution which gave positive RT/PCR product. The result was then compared to the endpoint dilution as Nakayama RNA was suspended in distilled water; and presence of the RT/PCR inhibitor in CSF was not observed (unpublished data).

Failure to detect JEV RNA in CSF which were positive for JEV IgM should not be explained by insensitivity of the RT/PCR system, because we were able to detect JEV RNA at the sensitivity of 0.3 PFU/reaction; and it should not be due to the degradation of viral RNA in CSF samples because CSF were kept frozen at –70°C until tested. It was likely that JEV encephalitis were not always accompanied by the presence of the organism in CSF. Our finding was also confirmed by previously investigators (109-110).

All of 68 paired sera and 53 CSF samples from subjects with different clinical diagnosis were studied in parallel by HI and ELISA, and the results were summarized in Tables 4, 5 and 7. A four folded rise in HI antibody titer in paired was shown in 7 of 68 cases, while ELISA IgM was detected in sera of 10 cases. Cases positive by HI were also positive by ELISA IgM. In another word, HI failed to diagnose JEV infection in three cases. On the other hand, 6 of 53 CSF samples were positive for ELISA IgM > 40 units and together with presence of ratio between JEV over dengue IgM higher than one. These six cases were laboratory diagnose JEV encephalitis. Seroconversion by HI test or presence of ELISA IgM in serum were also seen in these six cases. Totally, JEV encephalitis was accounted for 6(26.09%) of 23 viral

encephalitis cases studied. There was one case who showed ELISA IgM in serum together with JEV seroconversion, but his CSF possessed JEV IgM less than 40 units. CSF sample collected on day 1 to 8 after onset of illness may be to early for IgM detection. Nevertheless, definite diagnosis as JEV encephalitis can not be given to this case, and also including the other three cases who developed a four-folded rise of antibody in paired sera or presence of ELISA IgM in serum, but their CSF was not sent for laboratory investigation.

Actually, extraneural JEV infection will develop a four-folded rise of antibody or higher level of JEV IgM $\geq$ 40 units in sera, but there will be no local synthesis of JEV IgM in CSF. Asymptomatic JEV infection rate is also high. One JEV encephalitis case usually occurred among 300 cases of JEV infection (37-41). Therefore, the diagnosis of JEV encephalitis can not be as certain in cases whose CSF was not investigated or does not confirmed CNS infection. Nevertheless, our study on the detection rate of 26% of JEV encephalitis was similar to what had been reported by Tisayakorn U. and Nimmannit S. in 1985(111).

It was interesting to discover five cases who early presented with signs and symptoms which lead to the clinical diagnosis of viral encephalitis, but the symptoms progressed to frank DHF later. ELISA dengue IgM $\geq$ 40 units was seen in all five cases, but a four-folded rise in HI antibody titer appeared in only two of them. CSF samples from four cases was examined for dengue IgM but none had demonstrated that dengue caused CNS infection. However dengue encephalopathy as an unusual manifestation occurred in DHF were previously reported (112-113).

Referred to the cross reactivity among flavivirus genus, patients who were investigated for JEV encephalitis would be determined for HI antibodies against JEV

antigen and also dengue 1 and dengue 2 antigens. The results in Table 7 showed that cross reactivity was not only seen in HI test but also in ELISA. However, specific antibody to the causative agent was higher than that of the non-causative one. These phenomenon lead to the establishment of the criteria to differentiate between JEV and dengue infection that : a ratio of anti-dengue IgM to anti-JEV IgM > 1 is typical of a dengue infection; ; and a ratio of <1 is typical of JEV infection. In addition, sequence of dengue infection can be determined by ELISA basing on the criteria that a ratio of anti-dengue IgM to IgG (if either test is ≥40 units) of > 1.8 is typical of primary infection and a ratio of < 1.8 is typical of secondary infection. Our study had detected two cases of primary and three cases of secondary infections.(Table 7) It has been previously shown that anti-dengue antibody was broader in specificity than anti-JEV antibody(41). This study also showed a high cross reactivity between dengue and JEV by HI in Figures 4 and 5. Level of antibody titers to both dengue 1 (r =0.7) and dengue 2 (r = 0.7)(Spearman *P* value = < 0.05, no significant)

Concerning the examination for HSV encephalitis by nested PCR and ASI, the prevalence of 6(16.67%) of 36 viral encephalitis cases were found (Table 8). HSV typing was performed and all were belonging to type 1. We also found HSV infection in several cases with other diseases.

Consider the laboratory method for determining ASI, we found that its calculation system is too complicate for general routine use and to draw a standard curve has to be very accurate since the ASI values obtained were dependent on the precision of the curve.

Isolation of the HSV and histopathology in brain biopsy had been used as the gold standard method to diagnose HSV encephalitis for several years (50-53,60-61).

However, the method to obtain the specimens is quite invasion. This approach and laboratory diagnosis is then replaced by PCR. Which is accounted as a new gold standard method at present. However, by its high sensitivity, it is difficult to find the confirmation test for PCR. Since most of our HSV infected cases were diagnosed by PCR alone without confirmation by serological result, validity of PCR was doubtful. It should be kept in mind that HSV present in CSF may derive from the infected brain tissue or may result from HSV reactivation according to the other disease stimulation. Quantitation for number of genome copies may differentiate these two points of view by basing on the fact that HSV genome copies as a result of reactivation should be lower in number. HSV DNA could be detected as early as at one day after onset of illness; and if the first CSF samples from cases with highly suspicious HSV encephalitis was negative, the further samples should be reexamined (58,61). It was also noted that PCR result could be negative in patients already receiving acyclovir (6). HSV DNA usually disappeared from CSF within 14 days, but may sometimes persist for up to 27 days (6,58).

HHV-6 encephalitis is rare in immune mature host. And if occurs, HHV-6 was more frequency observed in CSF than HHV-6B (74-76). Thus, HHV-6 A has greater neurotropism. Our result could detect HHV-6 B DNA in 3(8.33%) of 36 viral encephalitis cases studies but all of them were HHV-6B. We had tried to exclude the false positive finding by repeating the test for three times with three different methods of DNA extraction, and the results were also the same.

Presence of HHV-6 IgM in different groups of subjects could not conclude its role in the disease production. Our data showed low level of HHV-6 IgM in 5 sera of 26 viral encephalitis cases (Table 10). HHV-6 IgM was also present in sera from

patients with other diagnosis. It is generally accepted that HHV-6 infection is universal and starts since early lives. HHV-6 persists throughout lives after primary infection (80,82,84). This may explain the presence of HHV-6 IgM at low to moderate levels in our subjects; and thus lead to our conclusion that presence of HHV-6 IgM in these subjects was not diagnostic for viral encephalitis.

Isolation of enterovirus from CSF sample by using cell culture method remains the gold standard for diagnosis. The success to virus isolation requires more than one type of cell culture system. Moreover, some enterovirus serotypes, particularly within the coxsakievirus group A do not grow in cell culture and suckling mouse inoculation is needed. Virus identification is also time-consumed. These are the pitfall of the virus isolation method to diagnose enterovirus infection. By the way that an enterovirus infection induces both serotype specific and heterotypic antibodies, common antigens have been prepared for detecting enterovirus infection. Our study used commercial kit that employed enteroviral antigen which are denatured by heat. Nevertheless, the kit does not inform that which serotype it can or cannot detect. In addition, most enterovirus infection is asymptomatic, and the infection is universal. Interpretation for enterovirus diseases should be also handled with care. Our study found enterovirus IgA or IgM in cases with JEV encephalitis, JEV, VZV, HSV, and dengue infections and also in cases possessed HHV-6 IgM (Table 13)

Direct detection of entervirus genome in CSF specimen is the most promising diagnostic tool among several laboratory methods. Several laboratories reported the use of general primer derived from the 5' non coding region to detect universal enteroviruses(95,103,104). Among CNS diseases, enterovirus is the most common cause of meningitis, while encephalitis occurs in a lesser extent. Our data had detected

enterovirus RNA only in one viral encephalitis cases that accounted for only 3.13% of the subject studies. With limited use of serodiagnosis, the true prevalence of enteroviral encephalitis may be underdetermined.

Our study showed that JEV and HSV encephalitis are common in Thailand. JEV encephalitis is a preventable disease since an effective vaccine is available. In contrast, HSV has no protective vaccine, but HSV encephalitis can be treated with anti-herpes drugs. Thus, the precise diagnosis of HSV encephalitis is needed. In such a way that viral encephalitis can be caused by several virus infections, and the clinical symptoms caused by those viruses could not be differentiated: in addition, CSF samples are usually, obtained in a small volume test. These pitfalls rendered the need to develop multiplex PCR in which several viral agents can be investigated in one reaction tube. However, It should be kept in mind that PCRs are susceptible to contamination with extraneous DNA fragments that can be amplified and carried over through successive amplification rounds. Moreover, PCR may yield false positive result cause by nonspecific amplification of unrelated nucleic acid sequences. The lack of gold standard against which to validate the PCR result makes the interpretation of positive PCR tests extremely difficult. With accounting to these disadvantages, our study still showed that the genome detection methods of PCR or RT/PCR was superior to the antibody detection methods for the diagnosis of HSV, HHV-6 and enterovirus infections. While IgM antibody detection in CSF and sera was superior to genome detection in the diagnosis of JEV encephalitis.

CHAPTER VII

CONCLUSION

The present study reported the prevalence of Japanese encephalitis virus (JEV), herpes simplex virus(HSV), human herpes virus-6 (HHV-6) and enterovirus infections in pediatric patients who were admitted to the Department of Pediatrics, Faculty of Medicine Siriraj Hospital with sign and symptoms suspected of acute viral encephalitis/ meningoencephalitis during the study period between February 1996 and October 1998. The methods of investigation included 1) the detection of HSV and HHV-6 DNA genomes in CSF by PCR, and of JEV and enterovirus RNA genomes by RT/PCR; 2) determination for ratio of CSF : Serum HSV IgG as expressed in term of antibody specific index (ASI) by ELISA; 3) detection of IgG and IgM to JEV and dengue virus antigens in CSF and paired sera; 4) detection for a four-folded rise of hemagglutination inhibiting(HI) antibodies to JEV and dengue virus type 1 and 2 in paired sera; and 5) detection of specific antibodies in paired sera, i.e., HSV IgG and IgM and HHV-6 IgG and IgM, and enterovirus IgG, IgM and IgA.

According to the limited amount of clinical samples obtained, one specimen could not be investigated for all viral agents. The prevalence of 6(26.09%) Japanese encephalitis, 6(16.67%) HSV encephalitis, 3(8.33%) HHV-6 encephalits and 1(3.13%) enterovirus encephalitis were reported by our study. Furthermore, we had diagnosed five cases of dengue infection, two cases of mumps, one case of VZV and one case of

rabies were diagnosed by the routine laboratories. These finding were added up; and we herein concluded that 25(69.44%) of 36 acute viral encephalitis cases were confirmed for viral etiologies. Regarding the laboratory methods performed, the diagnosis of viral encephalitis could not be achieved without investigation of the CSF samples, either by detection of viral genomes or of the specific antibodies. Nevertheless, we could not detect JEV RNA in any of the CNS specimens tested. The diagnosis of JEV encephalitis in our study was based solely on presence of ELISA JEV IgM in the CSF: and 4 cases who showed a four-folded rise of HI antibody titer, but CSF was not available for investigation were diagnosed JEV infection. We could diagnosed HSV encephalitis both by the detection of the viral genome in CSF and by possessing ASI values of ≥ 1.5. Unfortunately, no reagent for detection of HHV-6 and enterovirus antibodies in CSF is available. A four-folded rise of HI antibody titer to JEV, and the presence of specific IgM to HSV and HHV-6 and of IgM and IgA to enterovirus were found in some cases, but these findings could not lead to the disease diagnosis. The present study then, concluded that PCR and RT/PCR are very useful tools in the diagnosis of HSV, HHV-6 and enterovirus infections in CNS. And since the disease can be caused with many viral agents, multiplex PCR should be further developed in order to reduce materials and time consumed and most of all when the specimens obtained are of very minute amount. On another hand, The IgM antibody detection in CSF and sera either with or without the HI test for a four folded rise in antibody titer in paired sera are still of value in the diagnosis of JEV encephalitis and JEV and dengue infections.

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APPENDIX

1.Media and reagents for cell culture

1.1 Growth media

Minimum Essential Medium 10X	10	ml
Deionized double distilled water	90	ml
Fetal calf serum	10	ml
1 M HEPES	1	ml
Penicillin (20,000 µg/ml)	0.5	ml
Gentamycin (4,000 µg/ml)	0.5	ml
Fungizone (1,000 µg/ml)	0.1	ml

Adjust to pH 7.2-7.4 with 5% $NaHCO_3$

1.2 Maintenance media

MEM 10X	10	ml
Deionized double distilled water	90	ml
Fetal calf serum	2	ml
1 M HEPES	1	ml
Penicillin (20,000 µg/ml)	0.5	ml
Gentamycin (4,000 µg/ml)	0.5	ml
Fungizone (1,000 µg/ml)	0.1	ml

Adjust to pH 7.2-7.4 with 5% $NaHCO_3$

1.3 Phosphate buffer saline Ca^{2+} and Mg^{2+} Free (1X), pH 7.5

This solution is used for preparing Trypsin solution and for cell washing

before trypsinization.

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.12	g
Na_2HPO_4 (anhydrous)	0.91	g

Adjust volume by addition of deionized distilled water to 1,000 ml

Autoclave at 121 °C with pressure 15 lb/square inch for 15 minutes,then

store at 4 °C.

1.4 0.25% Trypsin (10X)

Trypsin powder (1:300)[Gibco, BRL, USA.]	25	g
PBS 1X, pH 7.5	1,000	ml

The solution is passed through ash-free filter paper, then sterilized by milipore filtration and store at -20 °C

1.5 Versene(EDTA) solution 1:500 (10X)

Versene (sodium EDTA)	5	g
PBS 1x, pH 7.5	2,500	ml

Sterilize by autoclaving at 121 °C under pressure of 15 lb/square inch and store at 4 °C

1.6 Trypsin- Versene solution.

0.25% Trypsin (10X)	100	ml
Versene (10X)	100	ml
PBS 1X, pH 7.5	800	ml
Penicillin (20,000 µg/ml)	1	ml
Gentamycin (4,000 µg/ml)	1	ml
Fungizone (1,000µg/ml)	0.2	ml

1.7 1M HEPES buffer

HEPES (N-2-hydroxyethylpiperazine-2-ethane sulfonic acid)	253.3	ml
Deionized distilled water	1,000	ml

Sterilize by milipore filtration and store at 4 °C.

2. Reagents for DNA extraction

2.1 Lysing solution

This solution is used for lysing infected cell culture before DNA extraction.

Triton X-100	0.25	ml
1M EDTA	1	ml
1M Tris HCl	1	ml
Adjust volume by addition of distilled water to	100	ml

Sterilize by autoclave at 121 °C with pressure 15 lb/square inch, for 15 minutes, store for 4 °C.

2.2 Chloroform / isoamyl alcohol (24:1)

Chloroform	48	ml
Isoamyl alcohol	2	ml

2.3 Phenol / chloroform / isoamyl alcohol

Mix equal volume of phenol and of chloroform / isoamyl alcohol just before use.

2.4 3M sodium acetate, pH 5.2

Sodium acetate. H_2O	408.1	g
Distilled water	750	ml

Adjust with glacial acetic acid to obtain pH 5.2, then add distilled water to a total of 1,000 ml.

Sterilize by autoclaving at 121 °C with pressure 15 lb/squre inch, for 15 minutes.

2.5 5M NaCl

NaCl	292.2	g
Distilled water	1,000	ml

Sterilize by autoclave at 121°C with pressure 15 lb/squre inch, for 15 minutes.

3. Reagents for RNA extraction

3.1 Trizol reagent (Gibco BRL, USA)

4. Reagents for PCR technique

4.1 Lysis buffer

1M KCl	5	ml
1M Tris HCl (pH 8.3)	1	ml
1M MgC_{l2}	0.25	ml
NP-40	0.45	ml
Tween-20	0.45	ml
Distilled water	100	ml

Sterilize by autoclaving at 121 °C with pressure 15 lb/squre inch, for 15 minutes, and store at 4 °C.

4.2 Protinase K (10 mg/ml)

Prptinase K	10	mg
10 mM Tris HCl (pH 7.5)	1	ml

To prepare working solution: 8 µl of protinase K solution, are added to 1 ml of lysis buffer, just before use

4.3 Deoxynucleotide triphosphate (dNTPs) mixture

Each dATP, dGTP, dCTP, and dTTP is supplied in a vial of 100 concentration

4.3.1 Preparation of 10 mM stock solution of each dNTP

dNTP	100	µl
Sterile double deionized distilled water	900	µl

4.3.2 Preparation of working solution

10 mM dATP	100	µl
10 mM dGTP	100	µl
10 mM dCTP	100	µl
10 mM dTTP	100	µl

4 µl of the working solution are used in one PCR reaction.

4.4 NTE buffer

NaCl	100	mM
Tris-Cl pH 7.4	10	mM
EDTA	1	mM

Sterile by milipore filtration.

4.5 Taq DNA polymerase (500 units) [Promega, GIBCO BRL,New York, USA.]

0.5 µl (2.5 units) of Taq DNA polymerase are used in one PCR reaction.

4.6 AmpliTaq DNA polymerase (250 units) [Perkin Elmer, USA.]

1.0 µl (2.5 units) of AmpliTaq DNA polymerase are used in one PCR reaction.

4.7 Loading buffer (6X)

Bromophenol blue	100	mg
Sucrose	20	g
TBE buffer (0.5X)	50	ml

4.8 TBE buffer (10X)

Tris base	108	g
Boric acid	55	g
0.5 EDTA (pH 8.0)	40	ml

4.9 Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g
Distilled water	100	ml

Store in a dark bottle at room temperature.

4.10 Agarose gel for electrophoresis

Agarose type I-A (Sigma, USA.)	0.5	g
TBE buffer (0.5X)	25	ml

Melt and warm before pouring in a gel cast with comb of 17 teeths.

5. Reagents for RT-PCR technique.

5.1 0.1% DEPC [Diethyl pyrocarbonate]- treated water

DEPC	1	ml
Deionized distilled water	1,000	ml

Mix and cover with parafin then incubate the solution at 37 °C for at least 12 hours and autoclave 2 times and keep at 4 °C

5.2 75% Ethanol

Absolute ethanol	75	ml
Sterile distilled water	25	ml

Mix and keep at –20 °C

5.3 2M Sodium acetate pH 4.0

Sodium acetate [CH_3COONa]	8.2	g
Sterile distilled water	20	ml

Adjust pH by using glacial acetic acid until the pH 4.0

and add sterile distilled water to 50 ml

6. Reagent for ELISA

6.1 0.006 M carbonate-bicarbonate buffer pH 9.0

Solution A	Na_2CO_3	0.64 g/L
Solution B	$NaHCO_3$	0.50 g/L

Slowly add solution B to solution A to give pH 9.0

6.2 10X PBS

NaCl	160.0	g
KCl	4.0	g
$NaH_2PO_4 H_2O$	2.8	g
Sodium phosphate, dibasic, anhydrous	20.0	g
Distilled water to	2000.0	ml

Adjust pH to 7.4 by adding NaOH

6.3 0.05% Tween in PBS

1X PBS	1000.0 ml
Tween 20	0.5 ml

6.4 0.1 M citrate phosphate buffer pH 5.0

Solution A 0.1 citric acid	19.21 g/L
Solution B 0.2 sodium phosphate dibasic anhydrous	28.40 g/L

Mix 24.3 ml of solution A with 255.7 ml of solution B and make up to 90 ml. Adjust pH to 5.0 by adding solution A or solution B; now bring to final of volume 100 ml

7. Reagent for HI test

7.1Borate Saline pH 9

1.5 M Sodium chloride

NaCl	87.675 g
Distilled H_2O	1,000 ml

2.0 M Dibasic sodium phosphate

Na_2HPO_4 (anhydrous)	283.96 g
Distilled H_2O	1,000 ml

2.0 M monobasic sodium phosphate

$NaH_2PO_4 H_2O$	276.02 g
Distilled H_2O	1,000 ml

Virus-adjusting diluent for addition of cell suspensions

Stock A

0.15 M NaCl - 0.2 M Na_2HPO_4

1.5 M NaCl	100	ml
2.0 M Na_2HPO_4	100	ml
Distilled H_2O	800	ml

Stock B

0.15 M NaCl - 0.2 M NaH_2PO_4

1.5 M NaCl	100	ml
2.0 M $NaH_2PO_4 H_2O$	100	ml
Distilled H_2O	800	m

Adjusting diluents of addition of cell suspensions

Final pH*	Stock A	Stock B
5.75	3.0	97.0
6.0	12.5	87.5
6.2	22.5	78.0
6.4	32.0	68.0
6.6	45.0	55.0
6.8	55.0	45.0
7.0	64.0	36.0
7.2	72.0	28.0
7.4	79.0	21.0

* An indicate pH is obtained by mixing equal volumes of borate saline, and the adjusting diluent solutions. Store at 4°C.

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