ความหลากหลายของยีนไกลโคโปรตีน ดี ในไวรัสเฮอร์ปีส์ซิมเพล็กซ์ที่แยกได้จากตัวอย่างส่งตรวจ

นางสาวศิริรัตน์ แนมขุนทด

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GENETIC VARIATION OF GLYCOPROTEIN D WITHIN HERPES SIMPLEX VIRUS ISOLATED FROM CLINICAL SPECIMENS

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ศิริรัตน์ แนมขุนทด : ความหลากหลายของยีนไกลโคโปรตีน ดี ในไวรัสเฮอร์ปีส์ซิมเพล็กซ์ที่แยกได้จากตัวอย่างส่งตรวจ. (GENETIC VARIATION OF GLYCOPROTEIN D WITHIN HERPES SIMPLEX VIRUS ISOLATED FROM CLINICAL SPECIMENS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ภาวพันธ์ ภัทรโกศล, 79 หน้า.

ไวรัสเฮอร์ปีส์ซิมเพล็กซ์ (Herpes simplex virus:HSV) พบแพร่กระจายอยู่ทั่วโลก จัดอยู่ใน family *Herpesviridae* และ subfamily *alphaherpesvirinae* แบ่งออกเป็น 2 ซีโรทัยป์ คือ เฮอร์ปีส์ซิมเพล็กซ์ ทัยป์ 1 (HSV-1) และเฮอร์ปีส์ซิมเพล็กซ์ ทัยป์ 2 (HSV-2) การเข้าสู่เซลล์ของไวรัสจำเป็นต้องอาศัยไกลโคโปรตีน ดี ของไวรัสจับกับ ตัวรับบนผนังเซลล์ เพื่อกระตุ้นการเข้าสู่เซลล์ของไวรัสโดยการหลอมรวมเอนเวลลอบกับผนังเซลล์ นอกจากนี้ไกลโคโปรตีน ดี ยังเป็นตัวกระตุ้นให้ร่างกายสร้าง neutralizing antibody ดังนั้น การกลายพันธุ์ของไกลโคโปรตีน ดี จึงมีผลกระทบต่อความสามารถในการเจริญเติบโตของไวรัสในเซลล์ต่าง ๆ ซึ่งอาจมีผลต่อพยาธิสภาพของเซลล์และพยาธิกำเนิดของโรคด้วย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมของไกลโคโปรตีน ดี ของไวรัสเฮอร์ปีส์ซิมเพล็กซ์

ทำการคัดเลือกตัวอย่างส่งตรวจระหว่างปี ค.ศ.1999 - 2010 โดยการสุ่มตัวอย่างที่ให้การแยกเพาะเชื้อไวรัสเฮอร์ปีส์ซิมเพล็กซ์ให้ผลบวก จำนวน 256 ตัวอย่าง มาทั้งหมด 100 ตัวอย่าง นำมาเพาะแยกเชื้อได้เพียง 70 ตัวอย่าง และจำแนกซีโรทัยป์ด้วยวิธี Real time PCR พบว่าเป็น HSV-1 37 ตัวอย่าง และ HSV-2 33 ตัวอย่าง หลังจากนั้นจึงทำการเพิ่มปริมาณยีนไกลโคโปรตีน ดีและตรวจหาลำดับเบส เพื่อนำผลการวิเคราะห์ลำดับนิวคลีโอไทด์ เปรียบเทียบกับข้อมูลที่มีรายงานใน GenBank ผลการวิเคราะห์พบว่ารูปแบบไกลโคโปรตีน ดี ขอ งตัวอย่าง HSV-1 มีความแตกต่างจากไวรัสมาตรฐานคือ HSV-1 (KOS) 13 แบบ ในขณะที่ HSV-2 มีความแตกต่างจากไวรัสมาตรฐานคือ HSV-2 (HG52) อยู่ 9 แบบ ความแตกต่างที่เกิดขึ้นเกิดจากกลไกการกลายพันธุ์เป็นหลัก มีเพียง 6 ตำแหน่งในลำดับนิวคลีโอไทป์ที่มีการเปลี่ยนแปลงกรดอะมิโน เมื่อสร้างแผนภูมิต้นไม้เพื่อหาความสัมพันธ์ พบว่าไกลโคโปรตีน ดี ของ HSV-1 และ HSV-2 มีความแตกต่างกันอย่างชัดเจน แต่ความแตกต่างภายในทัยป์เดียวกันมีน้อยมาก

จากการศึกษาวิจัยในครั้งนี้สรุปได้ว่า ยีน ไกลโคโปรตีน ดี ของ ไวรัสเฮอร์ปีส์ซิมเพล็กซ์ที่แยกได้จากตัวอย่างส่งตรวจมีการเปลี่ยนแปลงต่ำมาก ความหลากหลายพบในทัยป์ 1 มีมากกว่าทัยป์ 2 และการเปลี่ยนแปลงที่พบส่วนใหญ่เกิดแบบ synonymous point mutation ดังนั้น ไกลโคโปรตีน ดี จึงเป็นโปรตีนที่ดีในการนำมาผลิตวัคซีน

สาขาวิชา...จุลชีววิทยาทางการแพทย์..ลายมือชื่อนิสิต.....

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SIRIRAT NAEMKHUNTHOT : GENETIC VARIATION OF GLYCOPROTEIN D WITHIN HERPES SIMPLEX VIRUS ISOLATED FROM CLINICAL SPECIMENS. ADVISOR : ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., 79 pp.

Herpes simplex virus (HSV) distributing worldwide, belongs to family *Herpesviridae*, subfamily *alphaherpesvirinae*. There are 2 serotypes, i.e., HSV-1 and HSV-2. The initial infection starts by using glycoprotein D (gD) contact to host cell surface receptors resulting to viral entry by membrane fusion. Moreover, gD can induce the production of neutralizing antibody. Therefore, mutations of HSV gD are critical for viral growth in cells which might affect cell pathology and pathogenesis of diseases. The objective of this study is to investigate the variations of HSV gD.

One hundred samples were selected by using systemic sampling from 256 clinical specimens during the year of 1999 – 2010. Only 70 samples were successfully isolated. They were typed by Real time PCR revealing 37 were HSV-1 and 33 were HSV-2. After that, gD gene was amplified and DNA sequencing. The results were compared to the sequences previously reported in GenBank. From nucleotide sequencing data, HSV-1 gD of 13 patterns were different from HSV-1(KOS) reference strain. While HSV-2 gD showed 9 patterns differed from HSV-2 (HG52) reference strain. Mutation was the major mechanism of variation. Only 6 positions had non-synonymous amino acid changes. After phylogenetic tree was constructed, the variations of HSV-1 gD and HSV-2 gD were distinctively separated. However, intratypic variation was very low.

In conclusion, HSV gD from clinical specimens had low variations. HSV-1 gD had more divergent than HSV-2. The major cause of variation was synonymous point mutation. Therefore, HSV gD is a good candidate for vaccine development.

Field of Study : Medical Microbiology Student's Signature .....

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ABBREVIATIONS

aa	Amino acid
bp	base pair
ºC	Degree Celsius
CPE	Cytopathic effect
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxside
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
E (β)	Early
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
gH	Glycoprotein H
gI	Glycoprotein I
gJ	Glycoprotein J
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein M
GM	Growth medium
gN	Glycoprotein N
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HSPGs	Heparan sulfate proteoglycans
HSV	Herpes simplex virus

HVEM	Herpesvirus Entry Mediator
ICP	Infected cell protein
IE (α)	Immediate early
L (γ)	Late
M	Molar
MOI	Multiplicity of infection
MM	Maintenance medium
µg	Microgram
µl	Microlitre
ml	Millilitre
ng	Nanogram
nm	Nanometre
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFU	Plaque performing unit
pg	Picogram
RFLP	Restriction fragment length polymorphism
rpm	Round per minute
TBE	Tris-borate ethylenediamine tetraacetic acid
UL	Unique long
US	Unique short

CHAPTER I

INTRODUCTION

Herpes simplex virus (HSV) belongs to family *Herpesviridae*, subfamily *alphaherpesvirinae*. There are 2 serotypes, i.e., HSV-1 and HSV-2. The viruses distribute worldwide (1). This virus initiates infection by invading epithelial cells of several organs such as oral, genital mucosa, cornea and skin (2). HSV-1 usually associated with the region mainly above waist. HSV-2, in contrast, is mostly found at sites below the waist (3-5). After primary infection, the virus remains latent in the sensory ganglia. Reactivation of the virus can be a result of a variety of environment stimuli, physical stress, which subsequently leads to virus replication in primary lesion (6).

HSV is a large enveloped double stranded DNA virus, with molecular weight of 80-150 x 10^6 Dalton. The genome 150 kilobasepairs (kb) is localized in central core which is enclosed in icosahedral 162 capsomers. Between the outer lipid envelope containing glycoproteins and the capsid, a tegument layer composed of viral proteins is located. HSV envelope contains at least 11 membrane glycopoteins (gB, gC, gD, gE, gG, gH, gI, gL, gM, gJ and gN) and 5 of these are essential for entry to cells. The initial binding to cell can be mediated by glycoprotein B (gB) or gC with cell surface receptor, i.e, heparan sulfate proteoglycans (HSPGs) without viral entry into cell (7). Glycoprotein D (gD) is an essential enveloped glycoprotein for virus entry. It binds to one of several cell surface receptors such as HVEM (8), nectin-1 (9-10), nectin-2 (11) and modified heparan sulfate (3-O-S HS) (12-13) and enters the cell by membrane fusion. gD binding to any one of these cell receptors triggers fusion of the viral envelope with a cell membrane and this membrane fusion requires the action of gB and gH-gL heterodimers as well as gD and gD receptor (14). The target cells of HSV thus include neuron. In addition, immune cells such as monocytes, lymphocytes and natural killer cells have shown to be the targets as well (7, 15-16). Glycoprotein D also induces the neutralizing antibody production (17).

Glycoprotein D of HSV-1 is translated as a 394 amino acid (aa) polypeptide, that composed of N-terminal 25 aa, mature glycoprotein is 316 aa, membrane spanning

domain 23 aa and cytoplasmic tail 30 aa. This polypeptides are added to O-linked glycans. Glycoprotein D of HSV-2 is shorter in mature glycoprotein by 1 aa (2). Therefore mutations in HSV-gD are critical for structure and functions of the virus. Many previous studies indicated that mutations of gD aa in essential portion by substitution, insertion or deletion can abrogate physical and function interactions with cell surface receptors. This might affect the pathogenesis of the diseases. In Thailand, intratypic variation of both HSV-1 and HSV-2 had been reported using several techniques such as monoclonal antibodies specific to gD (18), Restriction fragment length polymorphism (RFLP) (19) and Restriction endonuclease assay. However, no information of intragenotypic variations using DNA sequencing has been reported. Here, in this study, the genetic variations of gD sequences from HSV-1 and HSV-2 Thai isolates during 10 years are explored.

CHAPTER II

OBJECTIVES

Objectives of this study

1. To study nucleic acid sequences of gD of HSV isolates

2. To develop phylogenetic tree of HSV based on gD encoding sequences

CHAPTER III

LITERATURE REVIEWS

General background

The word 'to creep' has been described to herpes name in Greek and roman time that its ability to give reactive eruptions.

Herpesviridae is a large family and contains of over 100 members and dissimilated in animals. Herpesviruses are usually restricted to single species in natural infection while there are pervasive in vertebrates. In nature, inter species transmission of herpesvirus, which may be rare, can cause serve disease (20). Only 8 herpesviruses have been found in human (Table 1) and classified to 3 groups (subfamily) by molecular phylogenetic analysis (Figure 1) (1).

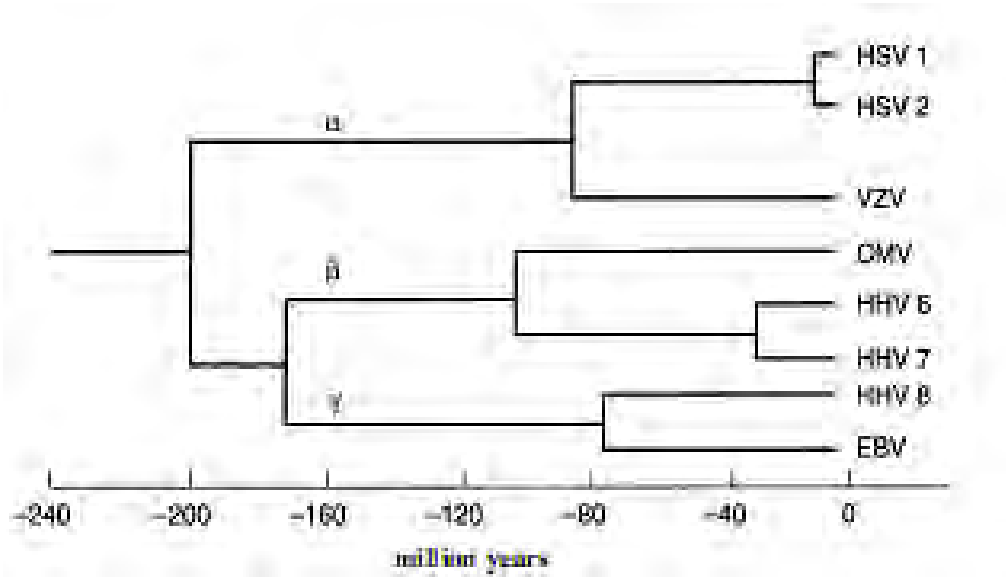


Figure 1. Phylogenetic tree of the 8 human herpesviruses (1)

Table 1 Classification of human herpesviruses (21-22)

Subfamily	Genus (-virus)	Members	Nomenclature	diseases
Alphaherpesvirinae	Simplex	HSV-1	Human herpesvirus 1	Oral herpes
		HSV-2	Human herpesvirus 2	Genital herpes
	Varicello	VZV	Human herpesvirus 3	Chickenpox (varicella)
Bataherpesvirinae	Cytomegalo	HCMV	Human herpesvirus 5	Congenital infection, mononucleosis
	Reseolo	HHV-6	Human herpesvirus 6	Exathema subitum (roseola)
		HHV-7	Human herpesvirus 7	
Grammaherpesvirinae	Lymphocrypto	EBV	Human herpesvirus 4	Infectious momonucleosis (cancer)
	Rhadino	HHV-8	Human herpesvirus 8	Kaposi's sarcoma

At least 400 million years, herpesviruses are large and double-stranded DNA, which diverse family of enveloped virus (23). The herpes virion is 120 – 200 nm in diameter (20) and genome is a size range of 124 – 241 kb (24-25). The molecular weight of virion is 80 – 150 x 10^6 dalton and G+C content ranges from 32 to 70 % (26). The number of genes encoded by the genome range from 150 – 200 genes, where HSV-1 and HSV-2 encode at least 74 genes (27). The viral structure is similar for all herpesviruses, although they are separated by electron microscope (EM). The virion consists of a core containing double-strand DNA genome in approximately 100 – 110 nm diameter that is surrounded by tegument or matrix. The component is encased in lipid bilayer composed of viral glycoproteins (Figure 2.)

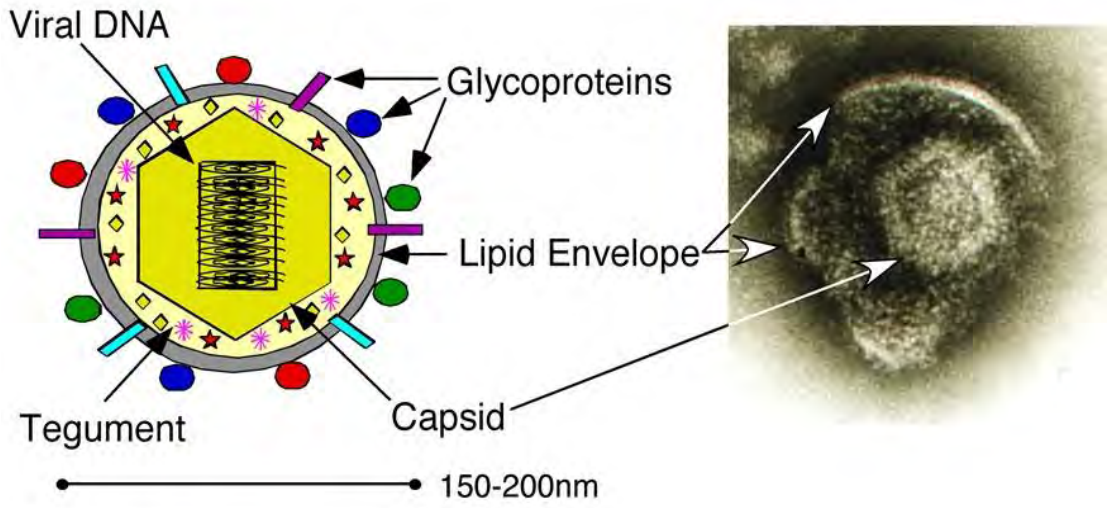


Figure 2. HSV virion structure and genome organization. The HSV virion is comprised of 4 major features: (i) the viral double-stranded DNA, packaged as a tightly wrapped spool; (ii) an icosahedral capsid shell; (iii) a tegument layer containing numerous viral proteins; and (iv) a lipid membrane envelope studded with viral glycoproteins. The major structural components of the virion can be seen in an EM photograph (right) where the viral envelope is ruptured and folded back.

Genomic DNA of Herpes simplex virus

HSV belongs to family *Herpesviridae*, subfamily *alphaherpesvirinae*. There are 2 serotypes, i.e., HSV-1 and HSV-2 (1). They have a highly host cell range, life-long infection and reproduction cell cycle. HSVs are members of a family of DNA viruses and HSV DNA is linear double-stranded (28-29). This DNA is packaged or anchoring. The end of the genome in closeness, because the DNA is rapidly circulated in the absence of protein synthesis after viral entry to nuclei (30)(30). Molecular weight of HSV approximately 96 x 10^6 dalton, the complete sequence genome is between 152 – 154 kb, with a G+C content of 68% for HSV-1 and 69% for HSV-2 (27-28, 31). The genome composes of unique long (UL) region, and unique short (US) region where comprise 82% and 18% DNA, respectively and flaked by inverted repeat region. The repeat of L component is indicated *ab* and *a'b'*, while S component is indicated *ac* and *a'c'* (32)(32). The repeat number of *a* sequence also L termini variable of HSV genome can be represented as

$$\text{“}a_L a_n b - U_L - b'a'_m c' - U_S - c a_S\text{”}$$

where a_L and a_S are terminal sequences with unique described below, a_n and a_m are a sequence directly repeated 0 or more time (n) or present more than one copy (m) (32-36). The structure of *a* sequence is highly conserved, but it consists of a variable number of repeat elements.

The repeated regions are rearranged during replication into mixture of 4 different isomers with different orientation of UL and US regions (37-38). The L and S components of HSV yielding 4 linear isomers, the isomers have been designated as P (prototype), I_L (inversion of the L component), I_S (Inversion of the S component), and I_{LS} (Inversion of L and S components) (39).

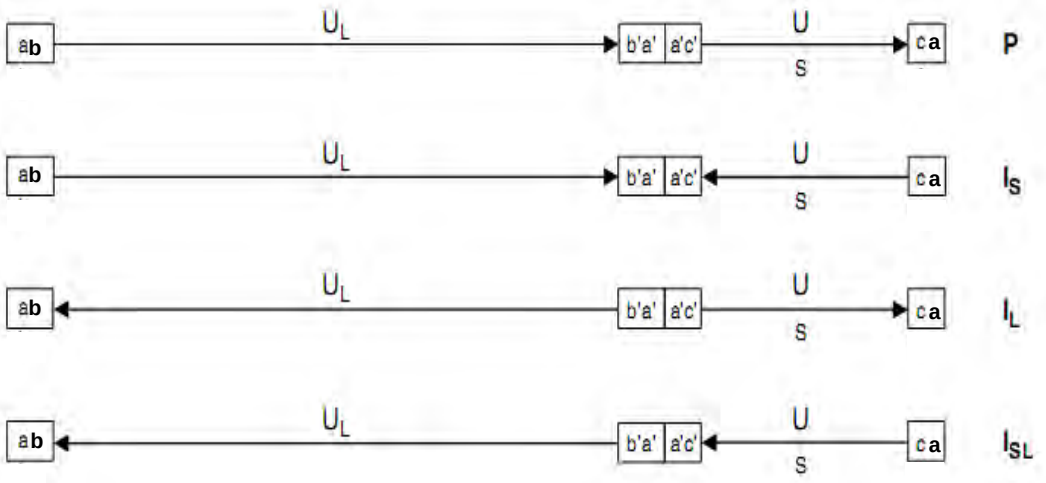


Figure 3 Diagram of HSV-1 genome. UL: unique long sequence. US: unique short sequence. a, b, c, a', b' and c' indicate the terminal repeat sequences. Arrows indicate the orientation of UL and US (40).

HSV replication cycle

HSV uses cellular transcriptional and translational machinery for expression of its genes. HSV genes expression is regulated as an ordered cascade (41). HSV-1 and HSV-2 comprise at least 74 genes which expression is tightly regulated. Three major kinetics of viral expression during productive infection, first genes expressed α (immediate-early, IE) gene. The products of immediate-early gene are regulated in activating expression of β (early, E) gene. Almost the products of early gene are enzymes and binding proteins implied in viral DNA replication. These proteins promote viral DNA replication and stimulate γ (late, L) gene expression. The late genes are expressed proteins for assembly of the progeny virions. Approximately 2 -4 hours post-infection, five (ICP0, ICP4, ICP22, ICP27 and ICP47) proteins of α genes are expressed first in absence of viral protein synthesis and regulated for expression of other viral genes. HSV IE gene does not require prior viral proteins synthesis but HSV protein brought in with the virion tegument, that HSV encodes one site specific transcriptional activator (VP16). Of these IE proteins, ICP4 and ICP27 are essential for viral DNA replication. ICP0 promotes viral infection and viral gene expression, especially at low multiplicity of infection (MOI) (42-43). Next set of HSV gene expression, between 4 – 8 hours post- infection, E genes are expressed at peak rate. These proteins are essential for replication of the viral DNA and nucleotide metabolism. Finally, L (γ) genes have been expressed into 2 groups; γ_1 is early/late or leaky late and γ_2 is late or true late genes. The γ gene 2 sets are expressed relatively early in infection and stimulated viral DNA synthesis. The initial infection, HSV must attach to cell surface receptor, it's envelope to the plasma membrane, and de-enveloped capsid transport to nuclear pores. DNA is released into nucleus of host cell, where transcription, replication of viral DNA and assembly take place (Figure 4).

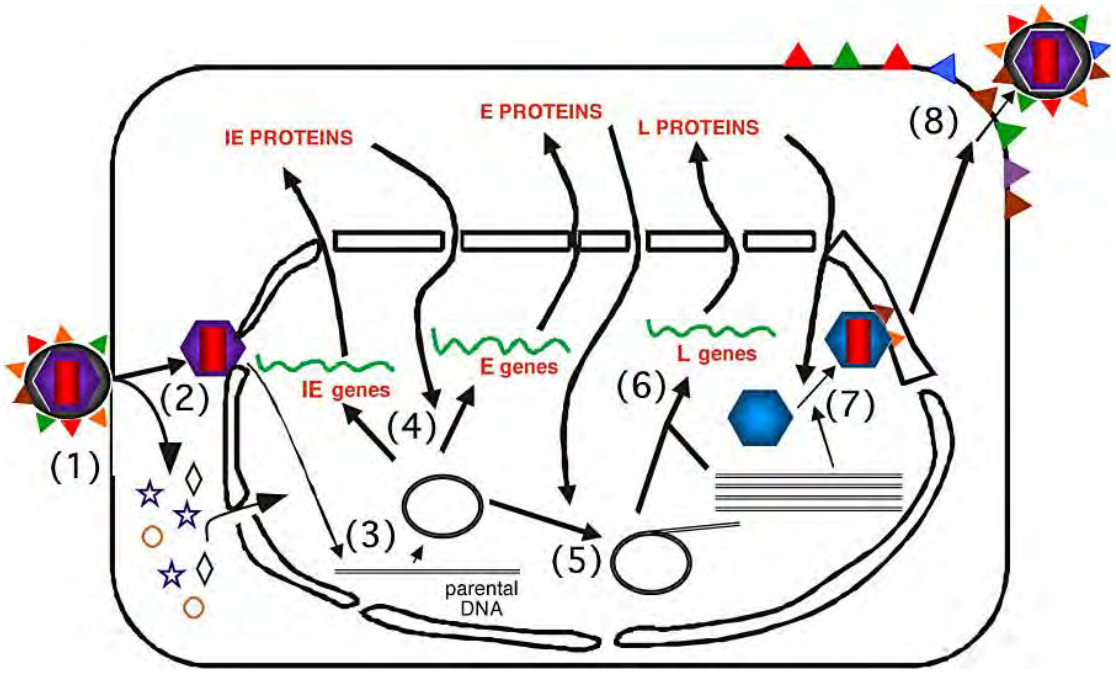


Figure 4 The cycle of productive HSV replication in a cell. The stages of HSV infection are: (1) Receptor binding and membrane fusion; (2) Release of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore; (3) Release of viral DNA into the nucleus; (4) Transcription and translation of the viral immediate early (IE) and early (E) genes; (5) viral DNA synthesis; (6) Transcription and translation of the viral late (L) genes; (7) capsid assembly and DNA packaging; and (8) egress of progeny virions (44).

HSV protein

HSV genome containing 1.6 x 10^5 base pairs (bp), encodes for approximately 100 proteins (45). Three groups of HSV proteins, i.e. α, β, and γ are synthesized from these genes (Table 2). This last gene has viral DNA replication as a strict requirement for their expression. HSV genome was shown in Figure 5.

HSV encodes at least 11 membrane glycopoteins (gB, gC, gD, gE, gG, gH, gI, gL, gM, gJ and gN) and 5 (gB, gD, gC , gH, gL) of these are essential for entry to cells. The glycoproteins are the major viral antigen expressed on the surface of both HSV virion and infected cell (46). Glycoprotein B is required for infection, which has been implicated in viral penetration and fusion (47). Egression of HSV induced fusion between virion envelope and outer nuclear membrane by gB-HSV (48). gC is a binding receptor of complement (C3b) and confers type specific antigen. gD is essentially triggered for membrane fusion (2) and inducer of neutralizing antibody and also implicates in cell adsorption (49). Whereas gE contains a receptor for the Fc protein immunoglobulins (Ig) (50) and gI was formed heterodimer with gE. gE-gI complex is important for virus egression and cell-cell spread in epithelial cells (51) and neuronal cell (52). gG is a target for antibody-mediated and uses to classification of genogroup by results in antibody response (53). gH involved in virus infection and cell fusion. It forms component of complex with gL. gH-gL heterodimer has been reported to have fusion activity (54). In addition, gK localizes to the golgi apparatus and produces egression (55-56) and it may prevent infected cell from fusion with adjacent cells. The role of gJ, gM and gN are not well characterized.

Table 2 Examples of HSV proteins and their functions (21)

Kinetics group	Name of element or proteins	Location	Functions
α (IE) gene	ICP0	α_0 (R_L)	Immediate-early transcription regulator (at low MOI)
	ICP4	α_4 (R_S)	Immediate-early transcriptional activator
	ICP22	α_{22} (U_S1)	immediate early protein, affects virus' ability to replicate in certain cells
	ICP27	α_{27} (U_L54)	Immediate-early regulatory protein, inhibits splicing
	ICP47	α_{47} (U_S12)	Immediate-early protein that inhibits MHC1 antigen presentation in human and primate cells
β (E) gene	Glycoprotein L	gL (U_L1)	Viral entry, associates with gH - polyadenylation usage changes with time
	Enzyme	U_L5, U_L8, U_L30, U_L52	Viral DNA replication
	Binding protein	U_L39	DNA replication (Ori binding protein)
	Glycoprotein B	gL (U_L27)	Glycoprotein required for virus entry
	Glycoprotein D	gD (U_S6)	Viral entry
γ (L) gene	-	U_L36, U_L39, U_L41	Protein for assembly of progeny virus and virus egress.
	Glycoprotein C	U_L44	gC mediates attachment glycosaminoglycans of heparansulphate and carried two domains involved in modulating complement activation.

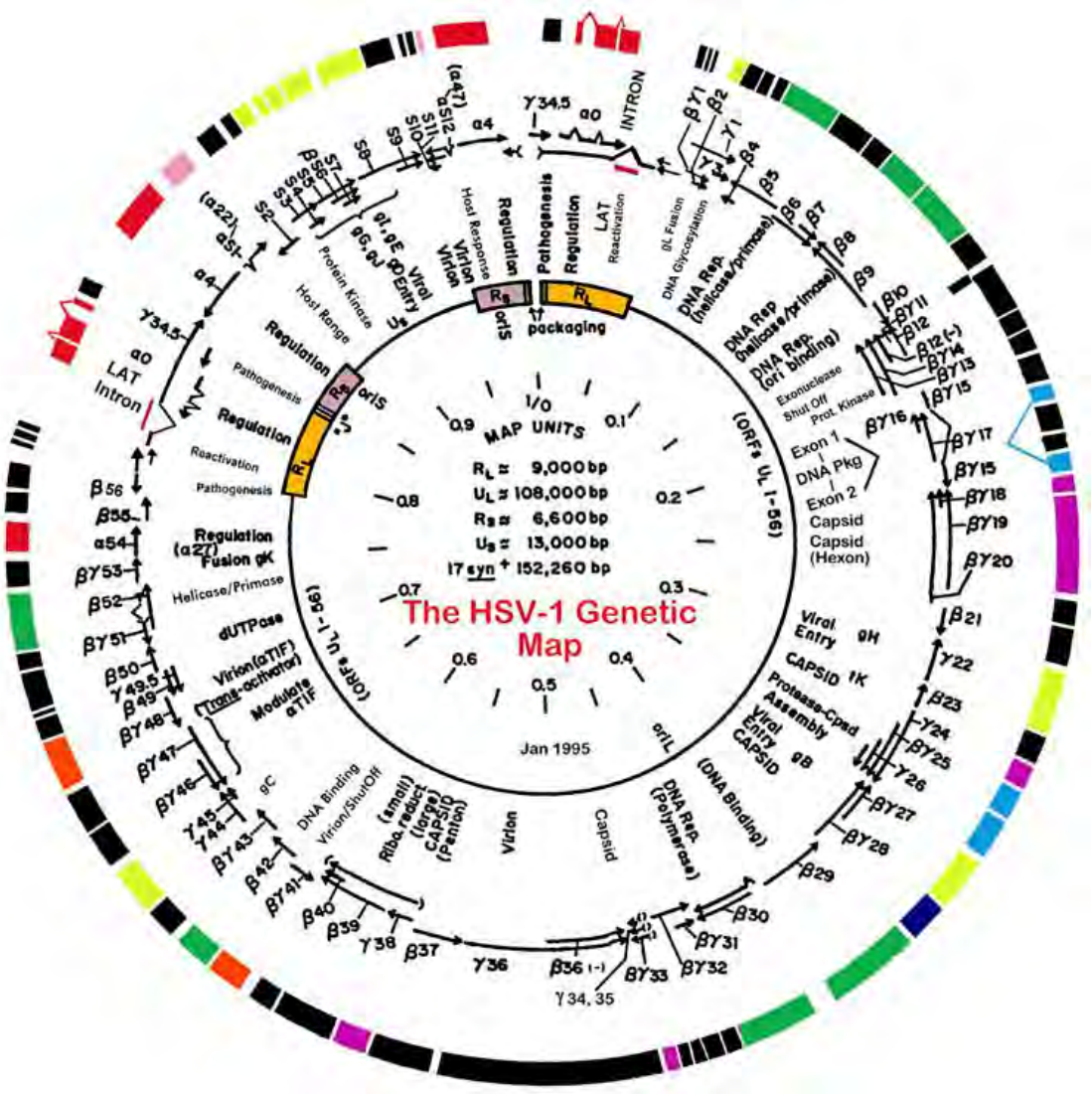


Figure 5 Functional organization of the HSV-1 genome. The circles are described from inside out. In the Circle 1: Map units kilobase pairs. Circle 1: Sequence arrangement of genome. The letters R_L and U_L identify long (L) region consisting of the unique sequence U_L flanked by inverted repeats. The letters R_S and U_S identify the corresponding sequence of the short (S) region. Ori_L and Ori_S were original of viral DNA synthesis. Circle 2: The transcriptional map of the HSV-1 genome. The map serves propose of identifying the direction of transcription. Between the circles identify protein and functions of proteins specified by open reading flames. The arrow circle are identified the open reading frame number and the class (α, β, and γ) to which they belong (21).

Pathology and pathogenesis

The appearance of HSV infected cells from lesions of skin and mucous membranes is similar in both Primary and reactivation of HSV infection. HSV-1 usually associated with the region mainly above waist, i.e. oral, pharynx and skin while HSV-2 is mostly at sites below the waist, i.e. sexual herpes. Human is infected by contacting with a secretion of HSV infection. Steps for infections are (i) HSV infects host cell by attachment to surface host cell receptor and penetrates capsid into cytoplasm. (ii) Viral replication starts at site of infection and invades to nerves system, (iii) this viruses hidden in dorsal root ganglia establishes latent stage (57). Generally, symptoms of disease are not apparent in primary infection but may be found in newborns, immunocompromised and immunosuppressive treatment (6, 58).

In the latency stage, the virus has no replication in ganglia. HSV comes back to the ancient site and replicates at skin or mucous membranes when reactivation of HSV was induced by physical emotion stress or nerves injury (59). Reinfection with the same strain can occur by autoinoculation at a distant site. Thus, HSV-1 could be mechanically transmitted from one site to another site as it occurs in cases of mouth-to-genital transmission (60) or intended inoculation of vesicle fluid “bolster immunity” (61).

Natural HSV infection induces specific and non specific immune defend mechanism. For both infections (primary and recurrent infection), HSV was destroyed by HSV specific humeral and cellular immunity. However, this immunity limits local viral replication, so that recurrent infections are less extensive and less severe (21).

Molecular epidemiology and classification

HSV distributes worldwide. No animal reservoirs or vectors are involved with the human viruses. Transmission is by contact with infected secretion. Epidemiology of HSV-1 and HSV-2 are difficult to perform on clinical groups alone because of frequently of asymptomatic infection and antigenic cross-reactivity between two virus types. HSV-1 and HSV-2 transmitted several routes which involve different area of body. As noted previously, there is a great deal of overlap between epidemiology and clinical manifestation of infections caused by these viruses. When Nahmias *et al*. (62), identified HSV-1 as the causative agent in most non-genital infection while HSV-2 was shown to cause the majority of genital and neonatal infection. In 1974, Heine *et al* (63) demonstrated the use of a molecular basis for observing variations in composition of virion polypeptides separated by polyacrylamide gel electrophoresis from several strains of HSV-1. Analyses pattern of HSV DNA fragment by restriction enzymes cleavage site has been used not only to distinguish between HSV-1 and HSV-2 but also for differentiation within an HSV type (34, 64-67). The differentiation markers are mainly the presence or absence of restriction endonuclease cleavage sites, rather than variation fragment lengths from variation in the copy number of short tandemly repeated sequences (68).

According to this model, genetic variation is essential for studies evolutionary HSV subtypes. The variations of gene are important for life cycle or pathogenesis of viruses. Genetic variation and classification into different genogroups have been described for herpesviruses, but data based on DNA sequencing of HSV isolated are limited. Each gene is essential for virus entry when gene mutation involved virus propagation. Previous studies, McGeogh studied evolutionary relationships of US region contains continuous of five genes (US4, US5, US6, US7 and US8) which encoding virion glycoproteins in *alphaherpesvirus* (68). In 2004, Norgerg *et.al.*, classified genetic groups and recombinant virus of HSV-1 by phylogenetic tree analysis. They studied variation in US genes that DNA sequence encoding envelope glycoproteins (gG, gI and gE) (69). And they studied divergence of clinical HSV-2 isolates and recombination in this samples (70). Detection and classification of DNA sequencing is much easier and

most sensitive in detection of minor variation between strains. Phylogenetic tree by analytical polymorphous 400 bp regions in HSV-2 was constructed (71).

Detection and classification is also important to diagnosis and care for patients. The isolation of virus using cell and occasionally embryonated egg is a slow process (72). The directly detection of viral antigens in clinical specimen can be applied from immunoassay. The access to viral culture improves accurate clinical recognition of HSV lesions (73). The clinical specimens inoculated into cell culture system, which is susceptible for demonstration of the cytopathic effect (CPE) characteristic of HSV infection. HSV grows rapidly with approximately 50% of positive culture producing detectable CPE within 24 hours, 85% within 48 hours and more than 99% within four days of inoculation of sensitive cell (74). After 24 – 48 hours incubation, the method can be used to detect HSV antigen in specimen. i.e. Fluorescent antibody (FA) straining method for typing of virus, enzyme immunoassay (EIA) which can be detect both antigen and antibody with primary HSV infection. In 1983, Kary Mullis developed PCR method for diagnostic virology. PCR has proved to be very versatile, having the ability detects DNA or RNA and to provide quantitative as well as qualitative information. The detection and identification of HSV is based on seminested PCR technique with primers followed by restriction enzyme analysis, the assay has been specifically designed clinical application. HSV species identification has been done by digesting the amplicons with endonuclease *Bam*HI and *Sma*I (75). The method for classification of HSV can be used to Restriction Fragment Length Polymorphism (RFLP). This method basis is the activity of restriction endonucleases, which are enzymes that cleave DNA at recognition sequences.

Essential gD for viral entry and fusion

HSV entry dependents on a key step: the interaction of gD with one of the HSV cellular entry receptors. After this interaction, the virus can choose between two different ways to enter the cell: fusion with the cellular membrane or penetration through an endocytic pathway (76). Several lines of evidence indicate that gD plays a critical role in viral entry.

In attachment step, HSV used gD binding to cell surface receptor before entry into host cell. Receptor of HSV was classified into 3 groups; (i) tumor necrosis factor receptor family; Herpes virus mediator (HVEM) distributes in human tissue. It was initially reported to restrict to T-lymphocyte, suggesting a role for this receptor in HSV infection of activated T lymphocytes. (ii) Immunoglobulin subfamily; nectin-1 and nectin-2 distributed in sensory neurons and muco-epithelia, including that of human and murine vagina. (iii) specific modifications in haparan sulfate (3-OS-HS) present in a number of human cell, including neuronal, endothelial cell and corneal fibroblasts. (14, 77-80).

In 1984, Watson published a 1608 bp sequence of gD gene of HSV-1 strain Patton. The gD coding sequence runs from residue 5815 to 6996 (68). The gD is a typical transmembrane glycoprotein (Figure 6A), containing hydrophobic transmembrane region (TM) near the C-terminus. Mature HSV-1 gD contains 369 aa, in N-terminal has no cysteine (Cys) residues, but in ectodomain of gD have 6 Cys residues. Cys is located in the TM of HSV-1 gD but not of HSV-2 gD. The structure of gD depends on disultfied bonds that essential for the stability of gD protein. gD function is highly dependent on its structure and stability dependent on maintenance of 3 entire disulfide bonds. gD structure showed 3 disulfide bonds in ectodomain that pairing aa positions 66 and 189, 106 and 202 and 118 and 127. The biochemical analysis of HSV-2 gD agreed with the genetic analysis of HSV-1 gD. In 1994, Chiang studied mutation of identified 4 functional regions (FR) within both proteins: region I (residues 27-43) closes to or overlaps residues that have been implicated in gD restriction of HSV entry. Region II (residues 126-161) encompasses a loop structure that stabilized by the disulfide bond between Cys3 and Cys4. Region III (residues 225-246) exhibits no complementation activity. The structural changes appeared to be minimal. And region IV (residues 277-

310) involves in gD function. It was a part of a putative stalk structure. The FR I through IV are physically near each other in the folded structure of gD (81-83).

The gD structure was directly affected into binding to cell surface receptor (Figure 6A). The resolved structure consists of 3 regions: an Ig-folded central core (residues 55-185), the N-terminus (residues 1-37), which harbors all the contact residues to HVEM, was disordered in the structure of gD alone, but formed a hairpin in the crystal of gD complexed with HEVM (84). The C-terminus folds back towards the N-terminus and forms an a-helix which provides structural support to the folded N-terminal hairpin. The crystal structure of gD in complex with nectin-1 has not yet been solved. The gD nectin 1-binding site, determined by means of insertion-deletion or substitution mutants, appears to be more widespread than the HVEM interaction region. A number of critical residues for interaction with nectin-1 was identified including amino acids R134, P221, R222, F223, D215 (85-87).

The gD genes of HSV-1 and HSV-2 encoded gD and located in US region of HSV genome. US6 sequences of HSV-1 and HSV-2 have percentage similarly of 80 (88)(71). Mutations of gD structure affected the mechanism of cell surface receptor. Substitutions in essential position of gD (Q27P, Q27R) cause lost of activity to interact with HVEM receptor. In contrast, it increased binding activity with nectin-1 receptor (80). Double or triple aa substitution at positions 215, 222 and 223 in gD binding to nectin-1 cause inability induction of cell fusion or viral entry of HSV via nectin-1 or nectin-2 (86). In 1990, McGeoch studied evolutionary relationships of US regions gene encode glycoproteins in virion of alphaherpesvirus. In this studies, within each of the US4, US5 and US6 relate sets, the aa sequences are most conserved in a region containing several residues (89). The result of mutation in gD-A3Y/Y38C positions failed to tagger fusion in absence of receptor (90). The result from previous studies may be summarized as follows; (i) substitution aa that direct contact with receptor, revealed that three (Q27A, T29A and D30A) position mutations abrogated binding of gD to HVEM, cell fusion and viral entry mediated by HVEM. And 4 position mutations (M11A, N15A, L25A and L28A) decreased binding to HVEM but only incompletely reduced cell fusion and viral entry activity (80). (ii) Random mutations of HSV-1 gD identified additional substitution and a deletion that can't contact viral entry by HVEM, without effect for entry via nectin-1 (91).

(iii) Deletion of amino acid within HVEM contact regions (aa 7-32) in HSV-1 gD abrogated physical and functional interactions with HVEM and 3-O-S HS without affect to nectin-1 (87). The mutations of essential positions in gD gene affect the structural and function of protein which involved in binding receptor, fusion and entry of virus into cells.

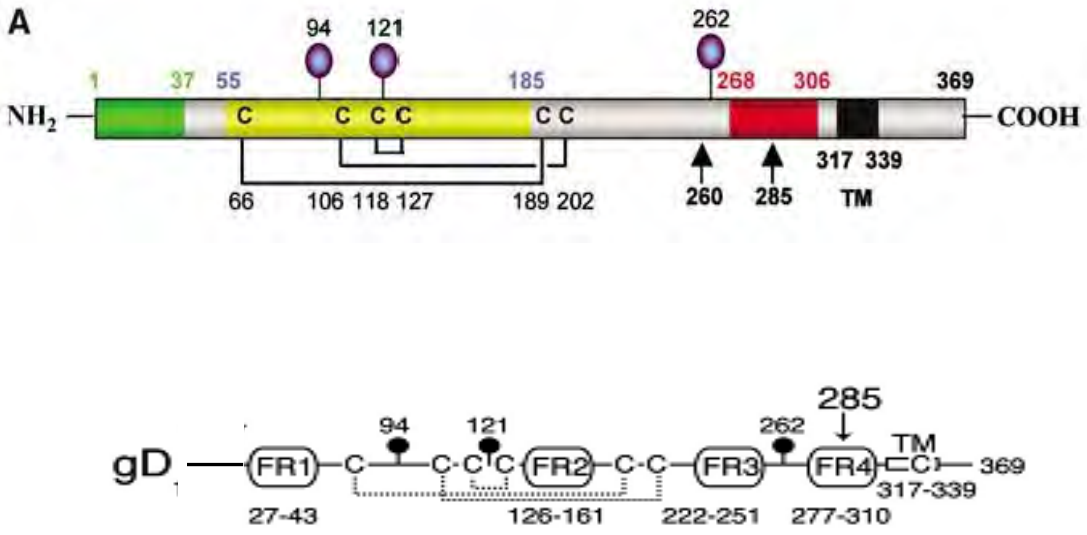


Figure 6 Schematic representation of HSV-1 gD. Disulfide bonds are shown as black lines and *N*-linked oligosaccharides as lollipops. Colors of the N-terminal region, forming the HVEM-binding hairpin in the gD285–HVEM complex, the Ig-like core, and the C-terminal region past residue 255 are represented in green, yellow, and red, respectively. Positions of important amino acids and domain boundaries are numbered according to the mature form of gD. TM: transmembrane region (84, 92).

CHAPTER IV
MATERIALS AND METHODS

PART I SPECIMENS SELECTION

Total 256 specimens with HSV positive isolation were reviewed from log book of Virology laboratory, Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand during the year of 1999 to 2010. The clinical samples were randomly selected by serially counting the number. Ten specimens were expected from each year.

Systematic sampling is often used instead of random sampling. After the required sample size has been calculated, every N^{th} record is selected from a list of population members. This sampling method is as good as the random sampling method. Systematic sampling is frequently used to select a specified number of records from a computer file. Steps for sampling follow to :

1. Calculated rang is N/n = k
2. Collected first sample by sampling 1 to k ($1 \leq r \leq k$)
3. 2^{nd} sample r+k
4. Collect other sample by r, r+k, r+2k,…r+ck

whereas N = Divide the total number of items in the population by n

n = sample size

k = this gives your interval (N/n)

r = select a random number, between 1 and k

For example, a sample size of 10 is selected from 50 clinical specimens in the Laboratory, giving an interval of 50/10 = 5. A random number between 1 and 5 is generated and comes up with 3. Starting with the 3th specimens sent to Laboratory, every 5th specimens is given a brief interview.

The clinical specimens for selection were separated into two groups according to type of specimens. One was HSV DNA specimens (obtained during 1999 to 2004, 137 specimens) and another was isolated HSV specimens (obtained during January 2006 to October 2010, 119 specimens).

PART II VIRUSES PREPARATION

1. Cell culture

Vero cell (African green monkey kidney cells) was obtained from Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand.

Vero cells were grown in T25 flask at 37 °C with growth medium [(GM; M199 medium (GIBCO, U.S.A.) supplemented with 10% fetal bovine serum, 0.01 HEPES (N-2-hydroxylethlpiperazine-N'-2-ethanesulfonic acid) (Sigma, U.S.A.) and 100 units/ml penicillin G and 100 µg/ml streptomycin (GIBCO, U.S.A)]. For preparation of viruses, M199 media containing 2% fetal bovine serum (MM; Maintenance medium) was used.

To subpassage the Vero cell, the monolayer cells was washed twice with 5 ml, phosphate buffer saline (PBS) pH 7.5 after the old culture media was discarded. One ml of trypsin-PBS (see APPENDIX II) was added and the cells were incubated at 37 °C, 1 to 2 minutes. After removing trypsin-PBS, the culture flask was knocked on palm until the cells were detached, followed by adding 5 ml GM. The monolayer cells were subcultured at 3 or 4-day intervals with splitting ratio of 1:3.

2. Viruses

2.1 Standard herpes simplex virus : HSV-1 strain KOS and HSV-2 strain HG52 were provided by Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand.

2.2 Clinical isolates : A total of 119 specimens (obtained during January 2006 to October 2010) were from Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital. The clinical specimens were isolated for HSV.

3. Virus isolation

Vero cell suspension containing 1.5 – 2.0 x 10^5 cell/ml was plated in each well of a 24-well tissue culture plate (NUNC, Denmark). After confluent monolayer cells were formed, they were washed twice with PBS, pH 7.5. Then, 200 µl of selected specimens was added in each well. After viral adsorption for 1 hour at 37 °C, non-adsorbed virus

was washed off with PBS, pH 7.5 and 1 ml of MM was added. The infected cells were incubated at 37 °C for 24 to 48 hours or until the cytopathic effect (CPE) was observed more than 75%. The cells were frozen and thawed for 3 to 4 times and centrifuged at 1500 rpm for 10 minutes. The virus in supernatant was aliquoted and kept -70 °C until use. However, when the infected cells were not observed CPE within 48 hours in first passage, they were collected for supernatant as described above and re-inoculated again for 2-3 passages to increase yield of viruses.

4. Virus propagation

Vero cell monolayer, 1.5 - 2.0 x 10^5 cells, were infected with either standard virus or clinical isolates at approximate multiplicity of infection (MOI) 1.0 plaque forming unit per cell (PFU/ml). After inoculation, the cells were incubated for 1 hour at 37 °C and washed once with PBS, pH 7.5 (see APPENDIX II) and added MM. The infected cells were incubated at 37 °C until the cytopathic effect (CPE) was observed more than 75%. The cells were frozen and thawed for 3 to 4 times and centrifuged at 1500 rpm for 10 minutes. The virus in supernatant was aliquot and kept -70 °C until use.

5. Virus titration

The virus was titrated in Vero cell and titer was revealed as PFU. In brief, the volume of 50 µl of each of the 10-fold serial dilutions in M199 medium containing 2% fetal bovine serum was added in wells, followed by 50 µl suspended Vero cell, 3x10^4 cells/well and incubated at 37 °C for 3 hours. Then, 100 µl overlay medium (see APPENDIX II) was added to each well. The overlay medium was discarded after 4 or 5 days and the cells were stained with 1% crystal violet in 10% formaldehyde for 30 minutes. The plate was washed, air-dried and the number of plaque was counted. Each dilution was done in quadruplicate.

Viral plaque titration was a quantitative measurement of virus activity. To determine the viral titer (PFU/ml), the number of plaques in each well was counted and calculated using the formula below.

PFU/ml = plaques/(D x V)

Where D = Dilution factor

V = Volume of diluted virus added to well

PART III PREPARATION OF VIRAL DNA

1. Preparation of viral DNA by NucleoSpin® Kit

The viral DNA was directly extracted from 200 µl of viral seed from step of propagation virus. The method of extraction followed the commercial kit's procedure (NucleoSpin kit, MACHEAREY-NAGEL, Germany). Briefly, 200 µl of viral seed and the solution, 25 µl proteinase K and 200 µl buffer B3 were added and mixed by vortexing 15 seconds. After lysis for 10 – 15 minutes at 70 °C, DNA was precipitated by adding 210 µl absolute ethanol to the solution and vortexed vigorously. This solution was transferred to NucleoSpin® column tube and centrifuged for 1 minute at 11000 rpm. After discarding the flow-through, the column was placed back into a new collection tube. Then, NucleoSpin® column was washed with 500 µl buffer BW and moved the column into centrifuge and spined at 11000 rpm for 1 minute, follow by adding 600 µl buffer B5 to wash the column and centrifuged at 11000 rpm for 1 minute. The column tube containing viral DNA was removed to a new collection tube and silica membrane was dried by centrifuging the column again. After column was transferred into 1.5 microcentrifuge tube, the viral DNA was eluted with 50 µl pre-warmed buffer BE (70 °C). Then, the column was incubated at room temperature and centrifuged at 11000 rpm for 1 minute. The eluted containing viral DNA was kept at -20 °C until use.

2. Quantitation of extracted viral DNA

The amount of viral DNA was measured by Quant-iT DNA assay kit (Invitrogen, New York). In brief, preparation of working solution by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer was done. 200 µl of working solution are required for each sample and standard. For the standard tubes, working solutions were aliquoted 190 µl into 0.5 microtube (Bioscience Inc., U.S.A.) and the standard from kit was added 10 µl into tubes with working solution. But sample tubes was used 198 µl of working solution and the DNA sample adding to working solution 2 µl. Then, all tubes were mixed by vortex for 2-3 seconds. After the standard and samples tubes were incubated at room temperature for 2 minutes, calibration of Qubit® fluorometer by inserting standards into its, followed by read all samples was done. Concentration of DNA in eluate was

measured by fluorescence emitted from DNA reacted to Quant-iT™ reagent. Fluorescence was determined by using Quant-iT™ Fluorometer (Invitrogen, New York, U.S.A.). Calculation of DNA concentration was compared with standard curve.

3. Typing of HSV by Real time PCR

Typing of HSV by real time PCR was rapid detection and high sensitivity. The evaluation of Real time PCR procedure is based on the Light cycler technique and program. Briefly, 5 µl of DNA was used for real time amplification in a final 20 µl reaction volume, using 1x FastStart DNA Master^{PLUS} SYBER Green I (Roche, Germany) 4 µl per reaction, 5µM primer concentrations of each primer and water PCR grade to final volume. The capillary tube was taken into LightCycler instrument (Roach, U.S.A.) and was amplified by incubation at 95 °C for 10 minutes followed by 45 cycles of 95 °C for 10 seconds, 55 °C for 10 seconds, 72 °C for 10 seconds and followed by denaturing the amplification products by slow increase of temperature (0.1 °C/s) up to 99 °C. The sequences of gD primer were gD-R: 5'GTAAAACAAGGGCTGGTGCGAGG3' gD F–5'CCAAACTGGCACATCCCGTCG3' (Invitrogen, U.S.A.). Negative (distilled water) and positive (HSV-1 strain KOS DNA and HSV-2 strain HG52 DNA) controls were included in each run.

The results were determination of the temperature of denaturing amplified product called melting temperature (T_m). This T_m depended on percentages of G+C content in the fragment amplified products. The property of the system was the ability to differentiate HSV-1 and HSV-2 in a reaction and the melting temperature of gD fragments amplified in each type was measured within each narrow temperature range. The T_m values were determined with the software provided by manufacturer.

4. Amplification of HSV gD by PCR

Primers specific to HSV gD region were designed to bracket a conserved HSV gD region, based on sequence and alignment of reference strains. Our specific primer set was purchased from Invitrogen, U.S.A.(Table 3). In brief, each reaction was performed in microtube of a total volume 50 µl. The reaction contained 10 µl 5X PCR buffer (FINNZYMES, Finland), 5 µl of dNTPs (2mM), 1.5 µl of DMSO (FINNZYMES,

Finland), 0.25 μM of primer, 0.2 μl of tag polymerase (FINNZYMES, Finland) and PCR grade distilled water to a volume of 45 μl and 5 μl of viral DNA (1 pg/μl) was added. The gD gene was synthesized by 98 °C for 1 minute a cycle following to 40 step cycles of 98 °C for 30 seconds, 55 °C for 30 seconds and 72 °C 1 minute, finally a cycle of 5 minutes. Negative (distilled water) and positive (HSV-1 strain KOS DNA and HSV-2 strain HG52 DNA) controls were included in each run. The PCR product was detected by 1.5% agarose gel electrophoresis stained with ethidium bromide. The amplified HSV-1 gD and HSV-2 gD product size are 853 and 925 bp, respectively.

Table 3 gD primer sequences

Serotype	Primers	Sequence (5'-3')	PCR product (base pairs)
HSV-1	gD1-P33	5' TTCTGGCTGCGTGGCGTTGG 3'	853
	gD1-P866	5' GGGGGCCGTGATTTTGTTTG 3'	
HSV-2	gD2-P58	5' CTCCGCGTCGTCTGCGCCAAATACG 3'	925
	gD2-P982	5' CGACGTCCTGGATCGACGGGATGTG 3'	

PART IV HSV GLYCOPROTEIN D SEQENCING

1. Purification of PCR product

HSV-1 gD and HSV-2 gD products were purified by PCR clean-up gel NucleoSpin® extract II kit (MACHEREY-NAGEL, Germany). Briefly, 2 volume of buffer NT was added into 1 volume of sample. The mixture was incubated at 50 °C for 10 to 15 minutes. Then, it was transferred to NucleoSpin® extract II column and centrifuged at 11000 rpm for 1 minute. The flow-through was discarded. The column was put into new collection tube and was washed with 600 µl buffer NT3 at 11000 rpm for 1 minute. To dry membrane, the column was centrifuged. Finally, the column was transferred into a 1.5 microcentrifuge tube and the viral DNA was eluted with 25 µl distill water PCR grade after incubating at room temperature for 1 minute and centrifuging at 12000 rpm. The eluted containing viral DNA was kept at -20 °C until use. The concentration of purified DNA was measured by Qubit® fluorometer.

2. Sequencing and data analysis

The purified product was transported to Macrogen Inc (Korea) for sequencing. Alignments of gD sequence were produced and edited by ClustalW (version 1.82). Phylogenic tree of sequencing bases was constructed by MEGA version 4 program (93-94), calculated bootstrapping to 100 replications by neighbor – joining (NJ) method. References of HSV-1 gD and HSV-2 gD nucleotide sequences restricted from GenBank as indicated in Table 4 were used to compare with our data. The DNA sequence of pseudorabies virus (PRV) or suid herpesvirus-1 (SHV-1) Accession no. BK001744 was used as outgroup.

Table 4 Herpes simplex virus reference strains in GenBank

Serotype	Area	Country	strain	Accession no.
HSV-1	America	USA	F	AF293614
			H129	GU734772
	Europe	Sweden	KOSc	EF157319
			KOSc(AC3,AC6)	EF157320
			KOSc(gC-39-R6)	EF157321
		UK	17	FJ593289
	Asia	Korea	KHS1	AF487902.1
			KHS2	AF487902.2
		Thailand	-	AY155225
HSV-2	America	USA	WTWT1A	U12183.1
			MMA	U12182.1
			MS	EU445527.1
			16293	AY779754.1
			11449	AY779753.1
	Europe	Sweden	333	EU018091.1
			333 (AC8)	EU018125.1
		UK	HG52	NC_001798
	Asia	Iran	Isolated Iranian	AY517492.1

CHAPTER V

RESULTS

1. Selection of Clinical specimens

Total 256 specimens with HSV positive isolation were reviewed from log book of Virology laboratory, Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand during the year of 1999 to 2010. There are 2 groups of specimens. Total 137 specimens from 1999 to 2004 were already extracted as DNA whereas another 119 specimens from 2006 to 2010 were original clinical samples. Among 137 HSV DNA positive specimens, 60 specimens were systemic sampling and only 39 specimens were successfully amplified (Table 5). For 119 clinical specimens, they were re-isolated and only 31 specimens were able to isolate (Table 6). In 2005, no positive clinical specimens were found. Only low passage (< 4 passages) for viral propagation was used to further DNA amplification. Thus, total samples in this study were 70 samples.

Table 5 Clinical HSV DNA specimens during 1999 to 2004

Years	Number of selected specimens	Number of successive specimens
1999	10	6
2000	10	7
2001	10	7
2002	10	4
2003	10	10
2004	10	5

Table 6 Clinical HSV specimens during 2006 to 2010

Years	Number of clinical specimens	Number of isolated specimens		Number of successive specimens
		positive	negative	
2006	31	9	22	9
2007	45	10	35	10
2008	27	5	22	5
2009	9	3	6	3
2010	7	4	3	4

2. HSV typing by Real time PCR using T_m

The DNA concentrations of 70 clinical samples were range from 0.56 to 60.0 µg/ml. Real time PCR method was rapid identification of HSV-1 and HSV-2. In Figure 7, quantification of HSV-1 strain KOS DNA ranging from 0.01 pg to 0.1 ng was demonstrated. Determination T_m of gD amplification product of standard HSV-1 and HSV-2 were shown in figure 8. The specific T_m signal of standard HSV-1 (KOS) was in range from 90.83 to 91.56 °C and standard HSV-2 (HG52) was 92.89 to 93.76 °C. However, low DNA concentration (< 0.1 pg) showed poor signal for melting temperature. Both HSV-1 and HSV-2 amplified products were clearly differentiated. The typing result of 70 clinical specimens revealed that 37 specimens were HSV-1 and 33 specimens were HSV-2 (Table 7 and Table 8).

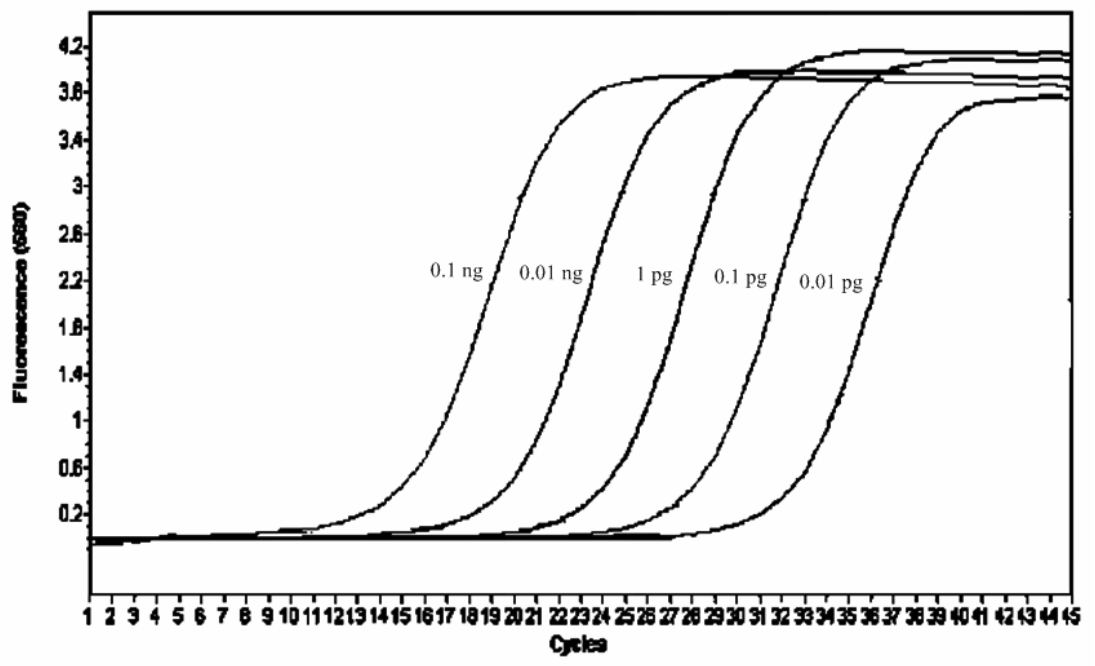


Figure 7 The standard HSV strain KOS at various DNA concentration (0.1 ng to 0.01 pg) determinated by Real time PCR

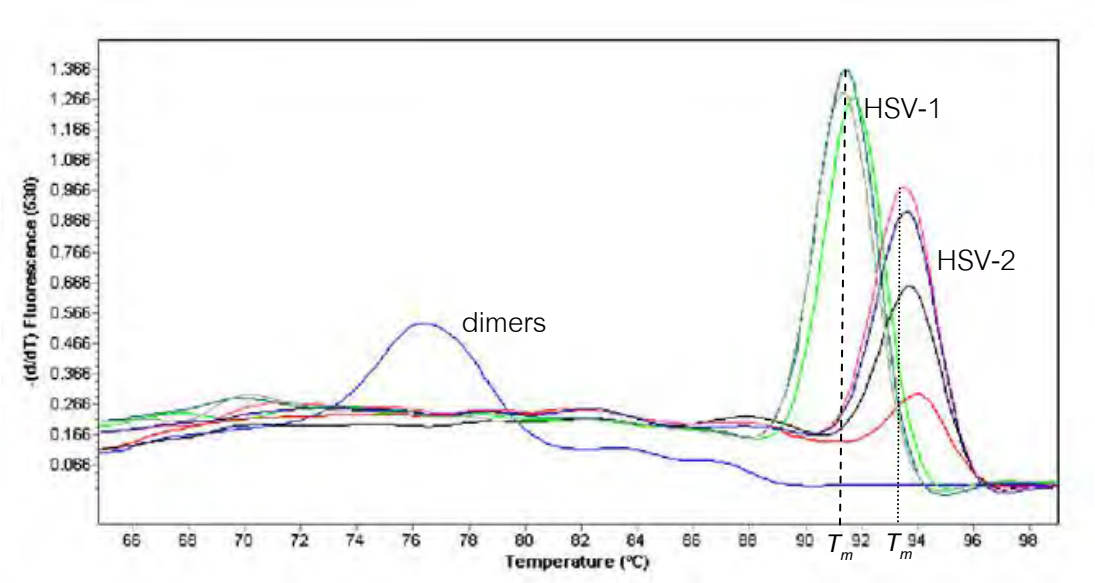


Figure 8 Melting curves of HSV-1 and HSV-2 amplification fragments generated by Real time PCR.

Table 7 Characteristics of HSV-1 and HSV-2 DNA specimens.

No.	Isolated strain	Type	No. of passages (*)	DNA concentration (μg/ml)	T_m (°C)
1	MVCU10/99	1	3	36.1	91.30
2	MVCU11/99	2	3	31.2	93.67
3	MVCU15/99	2	3	9.05	93.17
4	MVCU16/99	1	3	35.0	91.26
5	MVCU18/99	1	3	14.9	91.35
6	MVCU19/99	2	3	12.2	92.32
7	MVCU20/99	1	3	33.2	91.24
8	MVCU01/00	2	3	45.2	92.40
9	MVCU02/00	2	3	12.2	92.64
10	MVCU09/00	1	3	32.2	90.57
11	MVCU17/00	2	3	17.7	92.44
12	MVCU19/00	2	3	15.4	92.50
13	MVCU20/00	1	3	60.0	90.36
14	MVCU22/00	2	3	52.0	92.14
15	MVCU03/01	1	3	35.6	90.93
16	MVCU09/01	2	3	43.1	93.34
17	MVCU11/01	1	1	17.9	90.60
18	MVCU21/01	1	1	11.5	90.22
19	MVCU33/01	2	3	44.0	93.16
20	MVCU36/01	1	2	10.0	91.35
21	MVCU39/01	1	1	15.9	90.72
22	MVCU05/02	1	3	4.08	90.98
23	MVCU17/02	2	3	1.79	93.11
24	MVCU23/02	1	3	3.87	90.74
25	MVCU29/02	1	3	3.13	91.35

Table 7 (continue)

No.	Isolated strain	Type	No. of passages (*)	DNA concentration (µg/ml)	T_m (°C)
26	MVCU04/03	2	2	17.8	92.54
27	MVCU05/03	1	2	22.2	90.27
28	MVCU09/03	2	2	16.9	92.40
29	MVCU18/03	1	2	32.9	90.25
30	MVCU21/03	2	2	20.4	92.29
31	MVCU25/03	2	2	8.79	92.28
32	MVCU35/03	2	2	24.7	92.44
33	MVCU38/03	1	2	2.96	90.32
34	MVCU41/03	2	2	36.0	92.41
35	MVCU03/04	1	2	7.84	90.20
36	MVCU13/04	1	2	25.4	90.71
37	MVCU38/04	2	2	14.5	93.07
38	MVCU48/04	2	2	19.9	92.96
39	MVCU61/04	1	2	38.4	91.20

* Visaprom, S (19)

Table 8 Characteristics of HSV-1 and HSV-2 clinical specimens.

No.	Isolated strain	Type	No. of passages	Plaque titration assay (PFU/ml)	DNA concentration (μg/ml)	T_m (°C)
1	MVCU02/06	2	3	1.4×10^7	14.4	92.42
2	MVCU04/06	2	2	1.5×10^2	6.5	92.85
3	MVCU07/06	1	2	6.0×10^7	16.7	90.39
4	MVCU12/06	1	2	4.5×10^6	2.00	90.22
5	MVCU13/06	1	3	6.5×10^6	1.56	90.24
6	MVCU19/06	2	3	4.0×10^4	3.30	92.75
7	MVCU22/06	1	2	1.2×10^6	5.36	90.83
8	MVCU24/06	1	3	8.5×10^6	5.56	90.64
9	MVCU26/06	1	2	4.0×10^7	7.73	90.46
10	MVCU03/07	1	2	1.1×10^4	3.45	90.37
11	MVCU06/07	1	2	9.0×10^6	3.12	90.29
12	MVCU07/07	2	2	8.0×10^5	2.56	92.39
13	MVCU09/07	1	2	2.1×10^8	7.86	90.71
14	MVCU15/07	2	2	6.5×10^4	1.48	92.61
15	MVCU16/07	1	2	1.1×10^7	13.7	90.26
16	MVCU21/07	2	2	3.0×10^4	2.09	92.55
17	MVCU27/07	1	2	5.0×10^7	10.4	90.44
18	MVCU35/07	2	2	1.1×10^4	3.06	92.54
19	MVCU43/07	2	2	9.5×10^3	5.03	92.89
20	MVCU01/08	2	2	5.0×10^3	15.3	92.69
21	MVCU10/08	2	3	3.5×10^3	10.0	92.46
22	MVCU18/08	1	2	2.8×10^5	20.3	90.46
23	MVCU19/08	2	3	5.5×10^2	0.84	92.81

Table 8 (continue)

No.	Strain	Type	passages	Plaque reduce assay	DNA concentration (µg/ml)	T_m (°C)
24	MVCU21/08	1	1	2.0×10^7	32.8	91.31
25	MVCU03/09	1	1	4.0×10^5	8.27	90.29
26	MVCU04/09	1	1	8.0×10^4	7.59	92.39
27	MVCU06/09	1	1	9.0×10^4	4.88	90.66
28	MVCU01/10	2	3	2.4×10^4	1.05	92.91
29	MVCU02/10	1	2	8.0×10^5	0.56	90.19
30	MVCU03/10	1	3	2.7×10^8	1.73	90.36
31	MVCU06/10	2	2	3.0×10^4	4.80	92.84

3. Sensitivity of HSV PCR

To determine the sensitivity of PCR assay, serial dilutions of standard HSV-1 (KOS) and standard HSV-2 (HG52) DNA were used. The result showed that PCR assay can detect HSV-1 DNA and HSV-2 DNA at less than or equal to 0.1 pg (Figure 9, 10).

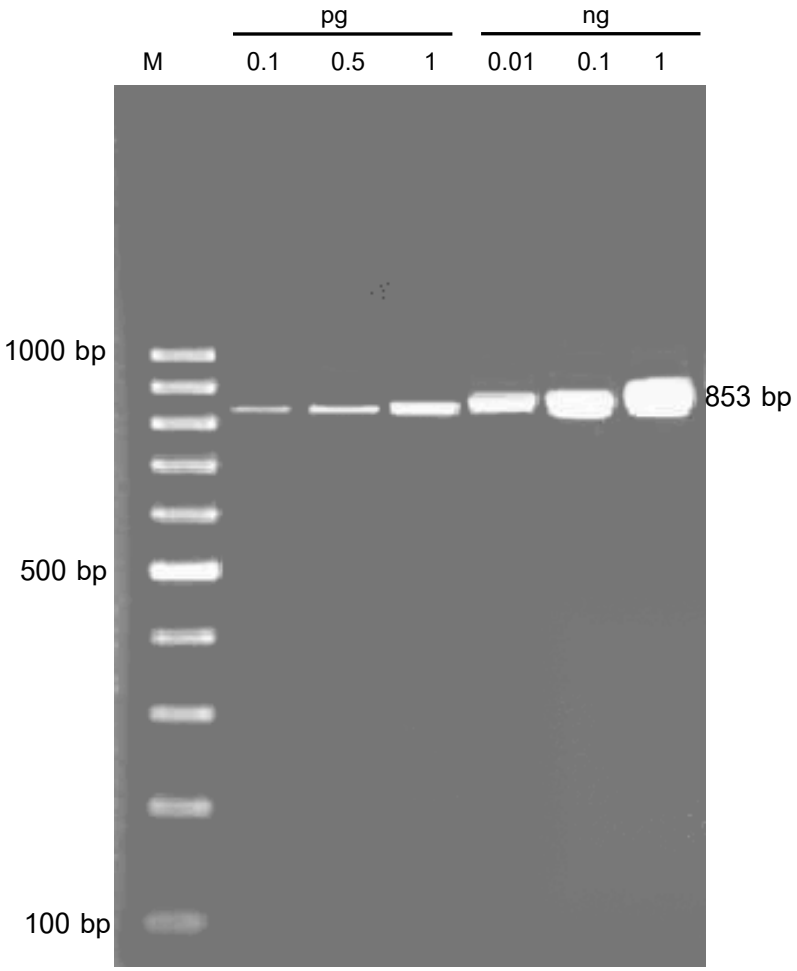


Figure 9 Sensitivity of standard HSV-1 strain KOS by PCR

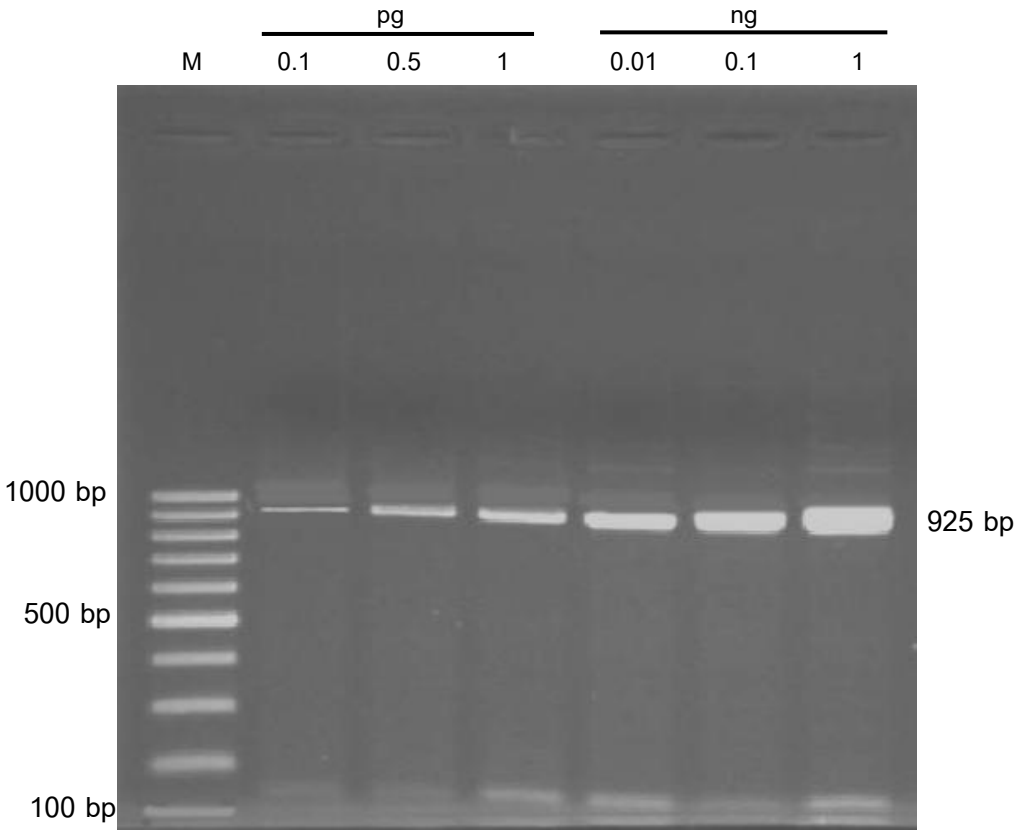


Figure 10 Sensitivity of standard HSV-2 strain HG52 by PCR

After measuring the concentration of DNA and HSV typing, all of them were further amplified HSV-1 gD gene and detected the product by 1.5% agarose gel electrophoresis (Figure 11). Although equal DNA concentration (5 pg) of each sample was used, 2 samples (Figure 11, lane 9 and lane 1) showed very faint band of amplified product comparing to lane 12; standard HSV-1 (KOS) and others. The same observation was also demonstrated in HSV-2 samples as in Figure 12. Increase of DNA template did not help.

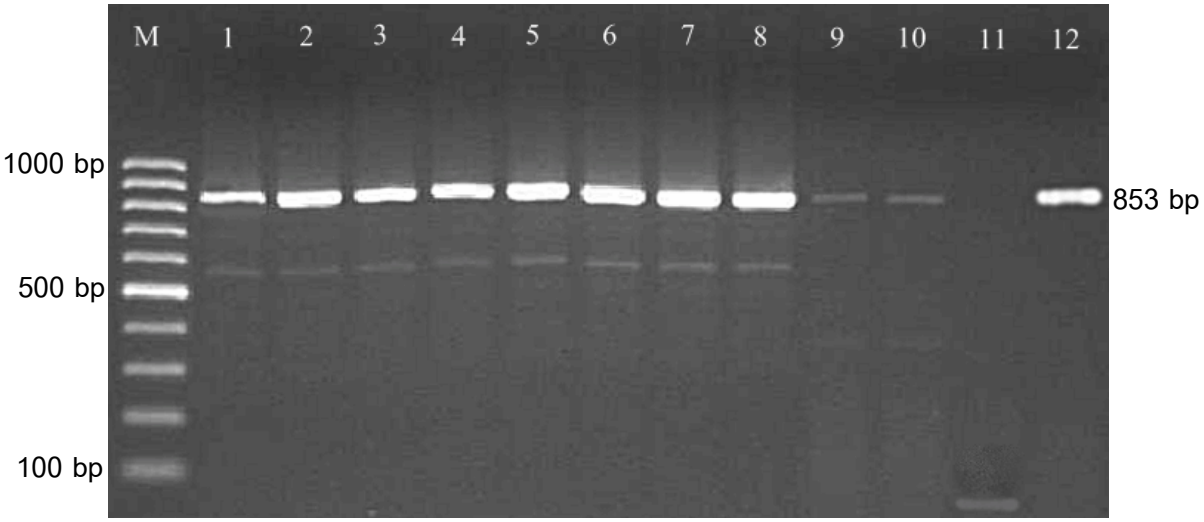


Figure 11 Amplification of HSV-1 gD from HSV-1 isolates. M; 100 bp ladder marker, lane 1-10; clinical specimens, lane 11; Distilled water, as negative control and lane 12; standard HSV-1 strain KOS DNA.

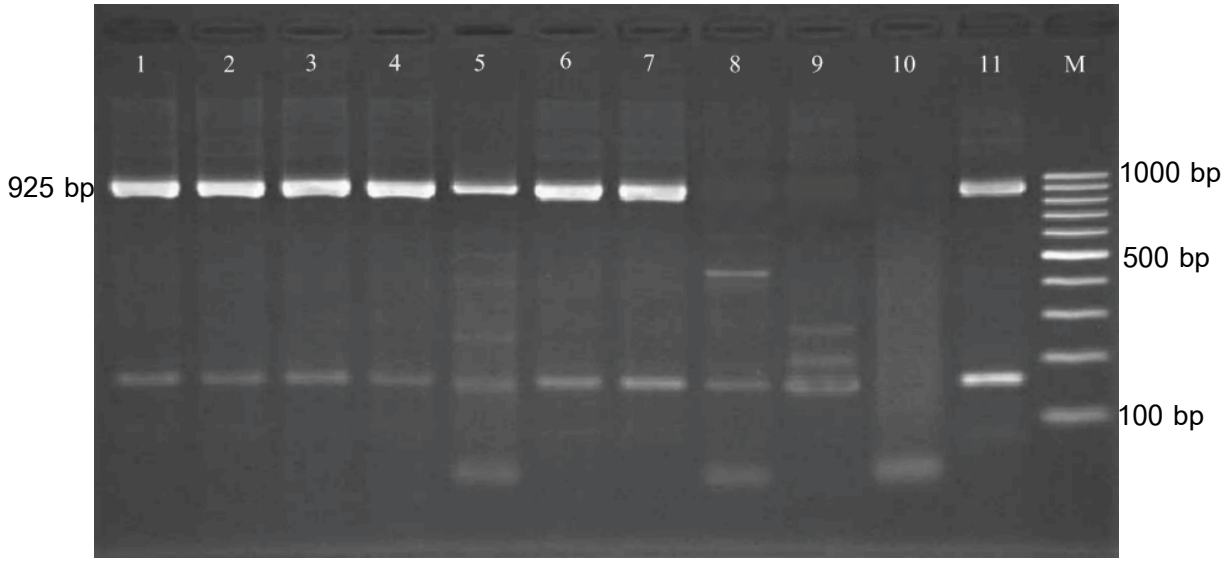
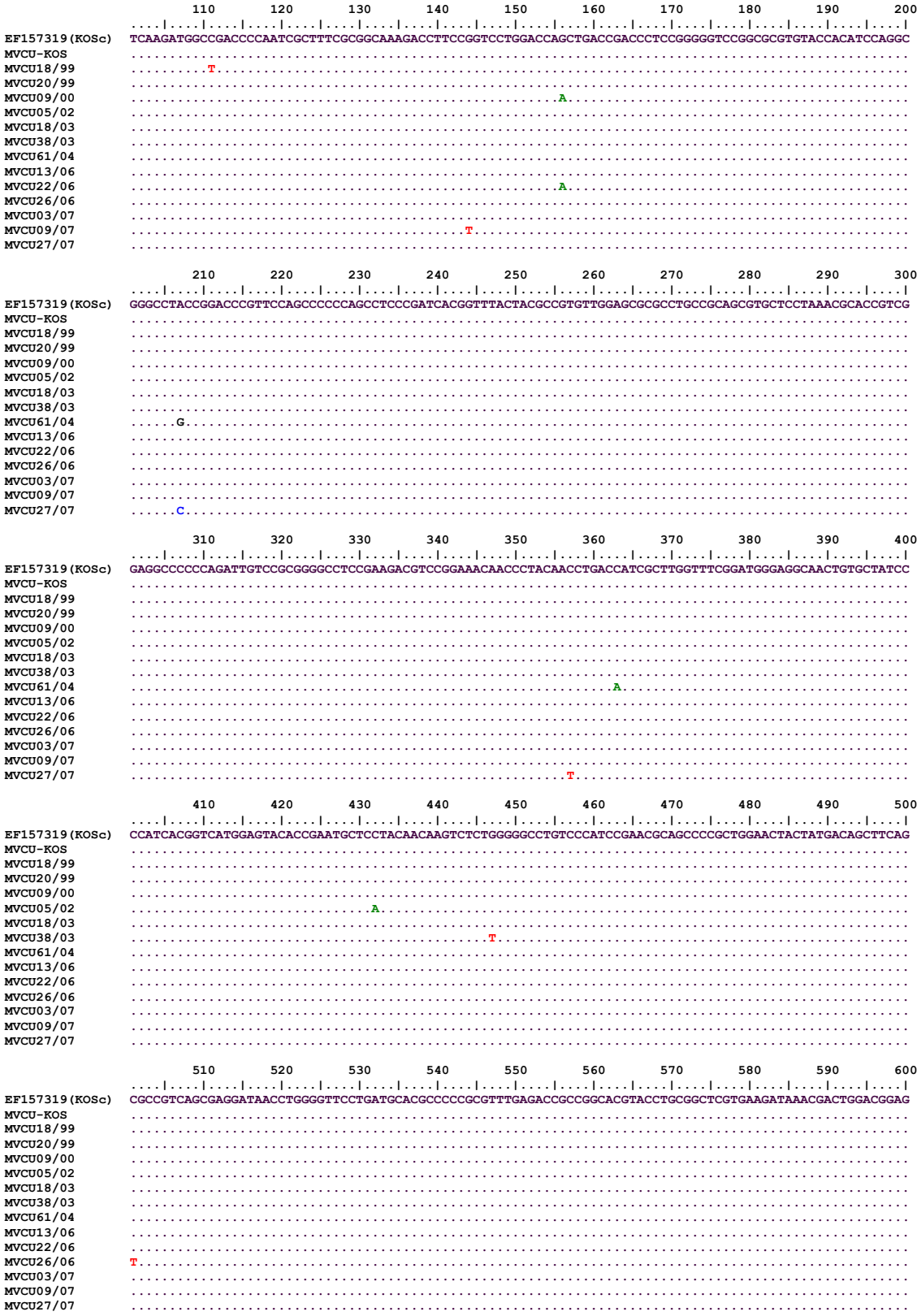


Figure 12 Amplification of HSV-2 gD from HSV-2 isolates. M; 100 bp ladder marker, lane 1-9; clinical specimens, lane 10; Distilled water, as negative control and lane 11; standard HSV-2 strain HG52 DNA.

4. Sequence variations of gD gene

4.1 Variations of HSV-1 gD gene : The result from 768 bp of HSV-1 gD sequencing indicated that standard HSV-1 (MVCU KOS) was similar to the reference strain KOSc accession no. EF157319 obtained from GenBank. The majority of HSV-1 clinical isolates (24/37, 64.86%) were the same as the reference KOSc. 13 HSV-1 clinical isolates were differentiated by comparing with reference strain (Figure 13). However, there were 2 isolates (MVCU20/99 and 22/06) have a distinct nucleotide at position beyond 768 bp. altogether the similarity among HSV-1 isolates was 99.8%. Among 13 distinct patterns presented in Table 9, the exchanged nucleotide sequences resulted in amino acid change. Mutation by base substitution was prone to be a main mechanism of gD variation. Although mutation occurred in gD gene, most of them encoded synonymous amino acid. Only 3 strains (MVCU20/99, MVCU03/07 and MVCU27/07) showed nucleotide differentiation which resulted in non-synonymous aa substitution.

4.2 Variations of HSV-2 gD gene : The comparison between HSV-2 (HG52) reference strain from GenBank accession no. NC_001798 and standard HSV-2 (MVCU_HG52) showed no difference. 13 out of 33 (42.86%) HSV-2 clinical isolates were similar to the reference strain. The nucleotide similarity among HSV-2 isolates was 99.5%. The majority of HSV-2, 20 clinical isolates differed from the reference strain (Figure 14). There were 9 distinct patterns showed in Table 10. The 9 of 20 (45%) sequences were classified as pattern 1 which differed in nucleotide position 506. 3 sequences were classified as pattern 2 which differed in nucleotide position 735. 2 sequences were differed in position 549 were classified as pattern 3. However, the sequences of 3 isolates (MVCU17/00, 19/06 and 07/07) were exchanged more than 2 positions (Table 10). Altogether among 14 exchanged positions, only 3 positions resulted in non-synonymous aa substitution.


110 120 130 140 150 160 170 180 190 200
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MVCU-KOS
MVCU18/99 T
MVCU20/99
MVCU09/00 A
MVCU05/02
MVCU18/03
MVCU38/03
MVCU61/04
MVCU13/06
MVCU22/06 A
MVCU26/06
MVCU03/07
MVCU09/07 T
MVCU27/07
210 220 230 240 250 260 270 280 290 300
EF157319(KOSc) GGGCCTACCGGACCCGTTCCAGCCCCCCAGCCTCCCGATCACGGTTTACTACGCCGTGTTGGAGCGCGCCTGCCGCAGCGTGCTCCTAAACGCACCGTCG
MVCU-KOS
MVCU18/99
MVCU20/99
MVCU09/00
MVCU05/02
MVCU18/03
MVCU38/03
MVCU61/04 G
MVCU13/06
MVCU22/06
MVCU26/06
MVCU03/07
MVCU09/07
MVCU27/07 C
310 320 330 340 350 360 370 380 390 400
EF157319(KOSc) GAGGCCCCCCAGATTGTCCGCGGGGCCTCCGAAGACGTCCGGAAACAACCCTACAACCTGACCATCGCTTGGTTTCGGATGGGAGGCAACTGTGCTATCC
MVCU-KOS
MVCU18/99
MVCU20/99
MVCU09/00
MVCU05/02
MVCU18/03
MVCU38/03
MVCU61/04 A
MVCU13/06
MVCU22/06
MVCU26/06
MVCU03/07
MVCU09/07
MVCU27/07 T
410 420 430 440 450 460 470 480 490 500
EF157319(KOSc) CCATCACGGTCATGGAGTACACCGAATGCTCCTACAACAAGTCTCTGGGGGCCTGTCCCATCCGAACGCAGCCCCGCTGGAACTACTATGACAGCTTCAG
MVCU-KOS
MVCU18/99
MVCU20/99
MVCU09/00
MVCU05/02 A
MVCU18/03
MVCU38/03 T
MVCU61/04
MVCU13/06
MVCU22/06
MVCU26/06
MVCU03/07
MVCU09/07
MVCU27/07
510 520 530 540 550 560 570 580 590 600
EF157319(KOSc) CGCCGTCAGCGAGGATAACCTGGGGTTCCTGATGCACGCCCCCGCGTTTGAGACCGCCGGCACGTACCTGCGGCTCGTGAAGATAAACGACTGGACGGAG
MVCU-KOS
MVCU18/99
MVCU20/99
MVCU09/00
MVCU05/02
MVCU18/03
MVCU38/03
MVCU61/04
MVCU13/06
MVCU22/06
MVCU26/06 T
MVCU03/07
MVCU09/07
MVCU27/07

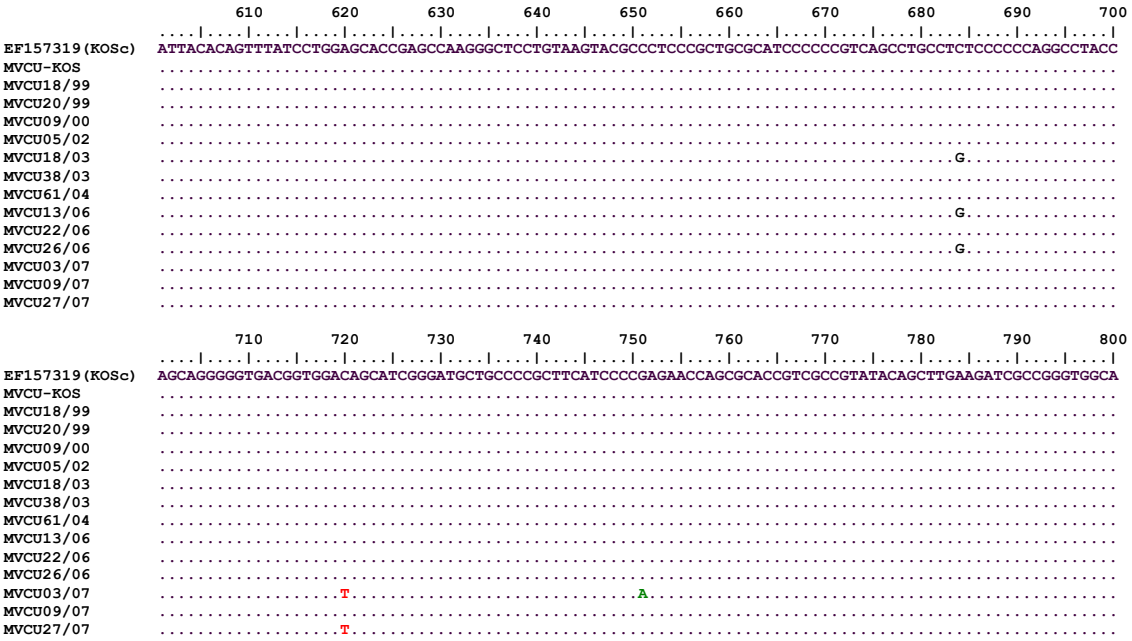

Figure 13 Variations of HSV-1 gD sequence comparison between reference GenBank (EF 157319), standard HSV-1 (MVCU_KOS) and HSV-1 clinical specimens. Alphabet position in clinical specimens sequence showed the mutation of nucleotide in this sequence. Figure showed nucleotide position starting from 100 to 800.

Table 9 Variations in HSV-1 gD nucleotides of 13 clinical isolates compared with KOSc (GenBank)

No. Pattern	Specimen no.	Nucleotides position	Ref. codon	Isolated codon	amino acid	amino acid position	Note
1	MVCU18/99	111	GCC	GC**T**	Ala	37	synonymous
2	MVCU20/99	854	ACC	A**A**C	Thr285Asn	285	Non-synonymous
3	MVCU09/00	156	CAG	CA**A**	Gln	52	synonymous
4	MVCU05/02	435	TCC	TC**A**	Ser	144	synonymous
5	MVCU18/03	684	CTC	CT**G**	Leu	228	synonymous
6	MVCU38/03	447	CTG	CT**T**	Leu	149	synonymous
7	MVCU61/04	207	CTA	CT**G**	Leu	69	synonymous
		363	ACC	AC**A**	Thr	121	synonymous
8	MVCU13/06	684	CTC	CT**G**	Leu	228	synonymous
9	MVCU22/06	156	CAG	CA**A**	Gln	52	synonymous
		849	TCC	TC**T**	Ser	283	synonymous
10	MVCU26/06	501	AGC	AG**T**	Ser	167	synonymous
		684	CTC	CT**G**	Leu	228	synonymous
11	MVCU03/07	720	GAC	GA**T**	Asp	240	synonymous
		751	GAG	**A**AG	Glu252Lys	252	Non-synonymous
12	MVCU09/07	144	CCG	CC**T**	Pro	48	synonymous
13	MVCU27/07	207	CTA	CT**G**	Leu	69	synonymous
		357	ACC	AT**C**	Asn119Ile	119	Non-synonymous
		720	GAC	GA**T**	Asp	240	synonymous

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                         210       220       230       240       250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_001798(HG52)     GAGCCTGGAGGACCCGTTCCAGCCCCCCAGCATCCCGATCACTGTGTACTACGCAGTGCTGGAACGTGCCTGCCGCAGCGTGCTCCTACATGCCCCATCG
MVCU-HG52           ..........................................................................................................
MVCU11/99           ..........................................................................................................
MVCU15/99           ..........................................................................................................
MVCU01/00           ..........................................................................................................
MVCU02/00           ..........................................................................................................
MVCU17/00           ......................A...................................................................................
MVCU22/00           ..........................................................................................................
MVCU04/03           ..........................................................................................................
MVCU25/03           ..........................................................................................................
MVCU35/03           ..........................................................................................................
MVCU02/06           ..........................................................................................................
MVCU04/06           ..........................................................................................................
MVCU19/06           ..........................................................................................................
MVCU07/07           ..........................................................................................................
MVCU15/07           ..........................................................................................................
MVCU21/07           ..........................................................................................................
MVCU35/07           ..........................................................................................................
MVCU10/08           ..........................................................................................................
MVCU19/08           ..........................................................................................................
MVCU01/10           ............................T.............................................................................
MVCU06/10           ..........................................................................................................

                         310       320       330       340       350       360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_001798(HG52)     GAGGCCCCCCAGATCGTGCGCGGGGCTTCGGACGAGGCCCGAAAGCACACGTACAACCTGACCATCGCCTGGTATCGCATGGGAGACAATTGCGCTATCC
MVCU-HG52           ..........................................................................................................
MVCU11/99           ..........................................................................................................
MVCU15/99           ..........................................................................................................
MVCU01/00           ..........................................................................................................
MVCU02/00           ..........................................................................................................
MVCU17/00           ..........................................................................................................
MVCU22/00           ..........................................................................................................
MVCU04/03           ..........................................................................................................
MVCU25/03           ..........................................................................................................
MVCU35/03           ..........................................................................................................
MVCU02/06           ..........................................................................................................
MVCU04/06           ..........................................................................................................
MVCU19/06           ..........................C.............................................................G.............
MVCU07/07           ..........................................................................................................
MVCU15/07           ..........................................................................................................
MVCU21/07           ..........................................................................................................
MVCU35/07           ..........................................................................................................
MVCU10/08           ..........................................................................................................
MVCU19/08           ..........................................................................................................
MVCU01/10           ..........................................................................................................
MVCU06/10           ..........................................................................................................

                         410       420       430       440       450       460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_001798(HG52)     CCATCACGGTTATGGAATACACCGAGTGCCCCTACAACAAGTCGTTGGGGGTCTGCCCCATCCGAACGCAGCCCCGCTGGAGCTACTATGACAGCTTTAG
MVCU-HG52           ..........................................................................................................
MVCU11/99           ..........................................................................................................
MVCU15/99           ..........................................................................................................
MVCU01/00           ..........................................................................................................
MVCU02/00           ..........................................................................................................
MVCU17/00           ..................................................A.......................................................
MVCU22/00           ..........................................................................................................
MVCU04/03           ..........................................................................................................
MVCU25/03           ..........................................................................................................
MVCU35/03           ......................A...................................................................................
MVCU02/06           ..........................................................................................................
MVCU04/06           ..........................................................................................................
MVCU19/06           ..........................................................................................................
MVCU07/07           ..........................................................................................G.............
MVCU15/07           ..........................................................................................................
MVCU21/07           ..........................................................................................................
MVCU35/07           ..........................................................................................................
MVCU10/08           ..........................................................................................................
MVCU19/08           ..........................................................................................................
MVCU01/10           ..........................................................................................................
MVCU06/10           ..........................................................................................................

                         510       520       530       540       550       560       570       580       590       600
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_001798(HG52)     CGCCGTCAGCGAGGATAACCTGGGATTCCTGATGCACGCCCCCGCCTTCGAGACCGCGGGTACGTACCTGCGGCTAGTGAAGATAAACGACTGGACGGAG
MVCU-HG52           ..........................................................................................................
MVCU11/99           .....C....................................................................................................
MVCU15/99           ..........................................................................................................
MVCU01/00           .....C....................................................................................................
MVCU02/00           .....C....................................................................................................
MVCU17/00           ..........................................................................................................
MVCU22/00           .....C....................................................................................................
MVCU04/03           ..........................................................................................................
MVCU25/03           ..........................................................................................................
MVCU35/03           ..........................................................................................................
MVCU02/06           ..................................................T.......................................................
MVCU04/06           .....C....................................................................................................
MVCU19/06           ..........................................................................................................
MVCU07/07           T.........................................................T................................................
MVCU15/07           .....C....................................................................................................
MVCU21/07           .....C....................................................................................................
MVCU35/07           .....C....................................................................................................
MVCU10/08           .....C....................................................................................................
MVCU19/08           ..........................................................................................................
MVCU01/10           ..........................................................................................................
MVCU06/10           ..................................................T.......................................................
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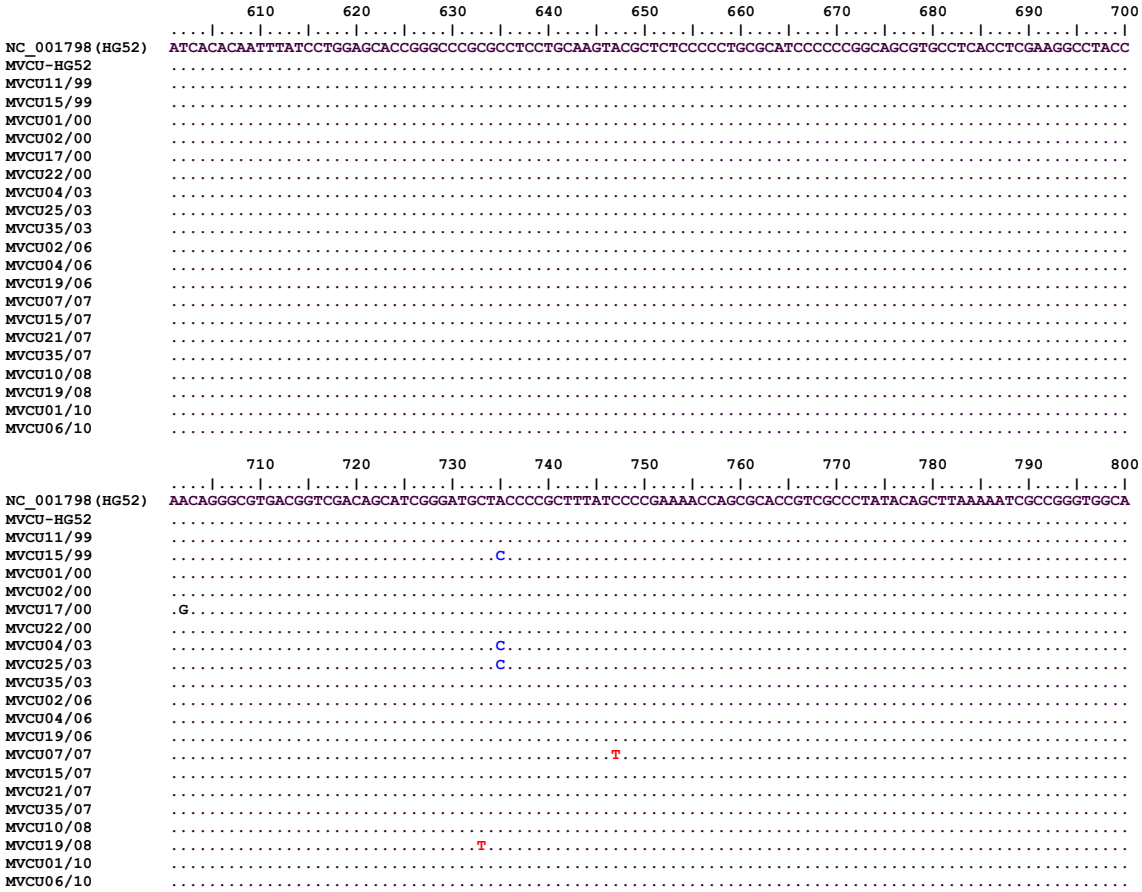


Figure 14 Variations of HSV-2 gD sequence comparison between reference GenBank (NC_001798), standard HSV-2 (MVCU_HG52) and HSV-2 clinical specimens. Alphabet position in clinical specimens sequence that showed the mutation of nucleotide in this sequence. Figure showed nucleotide position starting from 200 to 800.

Table 10 Variations in HSV-2 gD nucleotides of 20 clinical isolates compared with HG52 (GenBank)

No. Pattern	Specimen no.	Nucleotides position	Ref. codon	Isolated codon	amino acid	aa position	Note
1	MVCU11/99 MVCU01/00 MVCU02/00 MVCU22/00 MVCU04/06 MVCU15/07 MVCU21/07 MVCU35/07 MVCU10/08	506	GTC	G**C**C	Val169Ala	169	Non-synonymous
2	MVCU15/99 MVCU04/03 MVCU25/03	735	CTA	CT**C**	Leu	245	synonymous
3	MVCU02/06 MVCU06/10	549	TTC	TT**T**	Phe	183	synonymous
4	MVCU35/03	424	GAG	**A**AG	Glu142Lys	142	Non-synonymous
5	MVCU19/08	733	CTA	**T**TA	Leu	245	synonymous
6	MVCU01/10	228	CCC	CC**T**	Pro	76	synonymous
7	MVCU19/06	327	CTT	CT**C**	Leu	109	synonymous
		384	GGA	GG**G**	Gly	128	synonymous
8	MVCU17/00	222	CAG	CA**A**	Gln	74	synonymous
		450	GGG	GG**A**	Gly	150	synonymous
		702	CAA	CA**G**	Gln	234	synonymous
9	MVCU07/07	481	AGC	**G**GC	Ser161Gly	161	Non-synonymous
		501	AGC	AG**T**	Ser	167	synonymous
		549	TTC	TT**T**	Phe	183	synonymous
		747	ATC	AT**T**	Ile	239	synonymous

Table 11 Number of mutations in gD sequences

Type	Total pattern *	Single mutation (% of pattern)	Double mutation (% of pattern)	3 – 4 mutations (% of pattern)
HSV-1	13	8	4	1
		(61.54%)	(30.77%)	(7.69%)
HSV-2	9	6	1	2
		(66.67%)	(11.11%)	(22.22%)

* Reference type does not include

4.3 Number of variations of gD HSV : In the Table 11, HSV-1 had 14 patterns and HSV-2 had 10 patterns which included the similarity pattern of reference strain. HSV-1 isolates were more divergence than HSV-2 isolates. Single mutation was more than 60% revealing that point mutation is a major mechanism.

5. Phylogenetic analysis of gD

5.1 Analytical of HSV gD

Phylogenetic analysis of 691 bp from 74 nucleotide sequences (70 clinical specimens, 2 standard strains and 2 reference strains from GenBank) comparing to PRV gD sequence was demonstrated in Figure 15. The HSV-1 and HSV-2 gD sequences were distinctively different as shown by bootstrapping value of 99%.

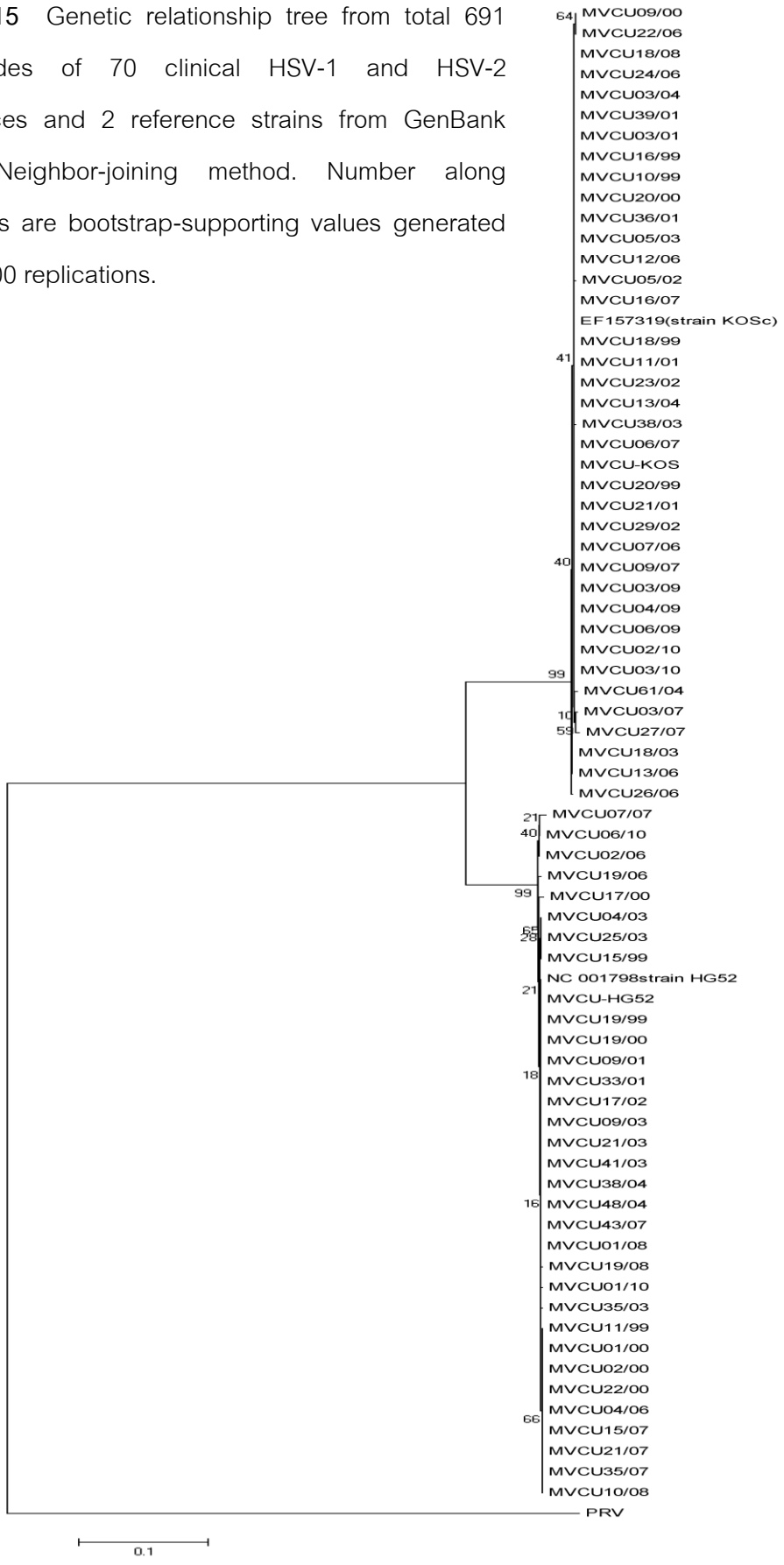
5.2 Analytical of HSV-1 gD

After alignment of *gD* sequences with selected 768 bp from 37 HSV-1 clinical isolates with standard HSV-1 (KOS) and other reference strains (Table 4) from GenBank, a phylogenetic tree was produced by neighbor-joining (NJ) method and visualized by MEGA, version 4 (Figure 16). Our clinical specimens distributed in a single group with 8 separated branches. Among those branches, only 2 branches (MVCU09/00, 22/06 and MVCU18/03, 13/06, 26/06) had bootstrap-supporting value over 50%. Most of our HSV-1 isolates (30/37, 81.08%) are closed to HSV-1 isolated from Europe areas except a branch of MVCU18/03, 13/06, 26/06 (3/37, 8.11%) is closed to HSV-1 isolated from U.S.A.

5.3 Analytical of HSV-2 gD

The NJ tree analysis of 800 nucleotides of 33 HSV-2 gD sequences comparing with HSV-2 reference strain from GenBank (Table 4) was shown in Figure 17. The result revealed 2 main separated branches but it seemed to be within 1 group, due to 1 branch had bootstrap supporting value only 30% while another branch had 64% bootstrap supporting value. The distribution of our HSV-2 gD sequences were similar to other strains worldwide. However we notice that 9 from 33 isolates (27.27%) were cluster in a unique branch with bootstrapping value 65%.

Figure 15 Genetic relationship tree from total 691 nucleotides of 70 clinical HSV-1 and HSV-2 sequences and 2 reference strains from GenBank using Neighbor-joining method. Number along branches are bootstrap-supporting values generated after 1000 replications.



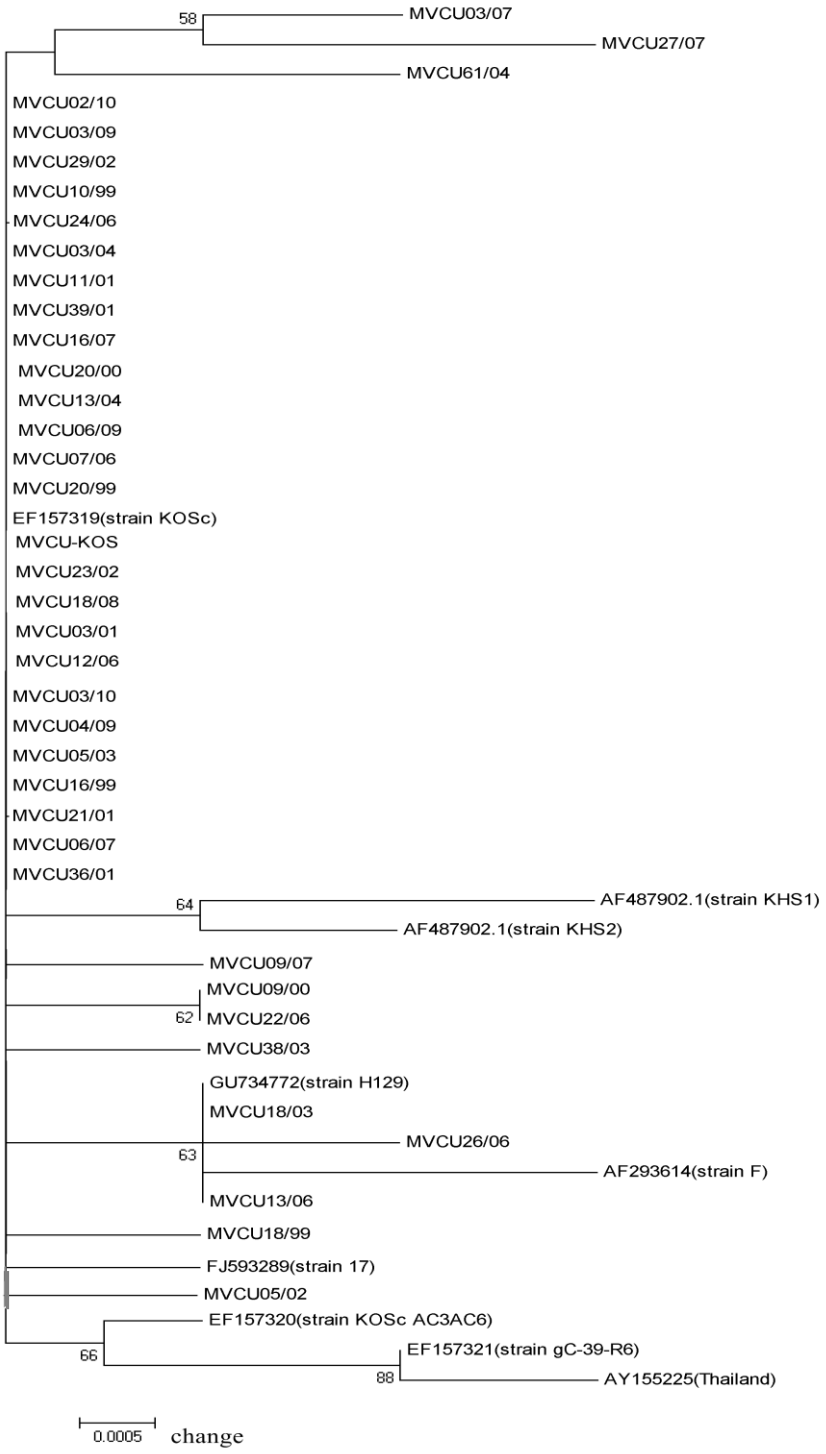


Figure 16 Genetic relationship tree from total 768 nucleotides of 37 clinical HSV-1 sequences and 10 reference strains from GenBank using Neighbor-joining method. Number along branches are bootstrap-supporting values generated after 1000 replications, The bootstrap values less than 50% are not shown on the tree.

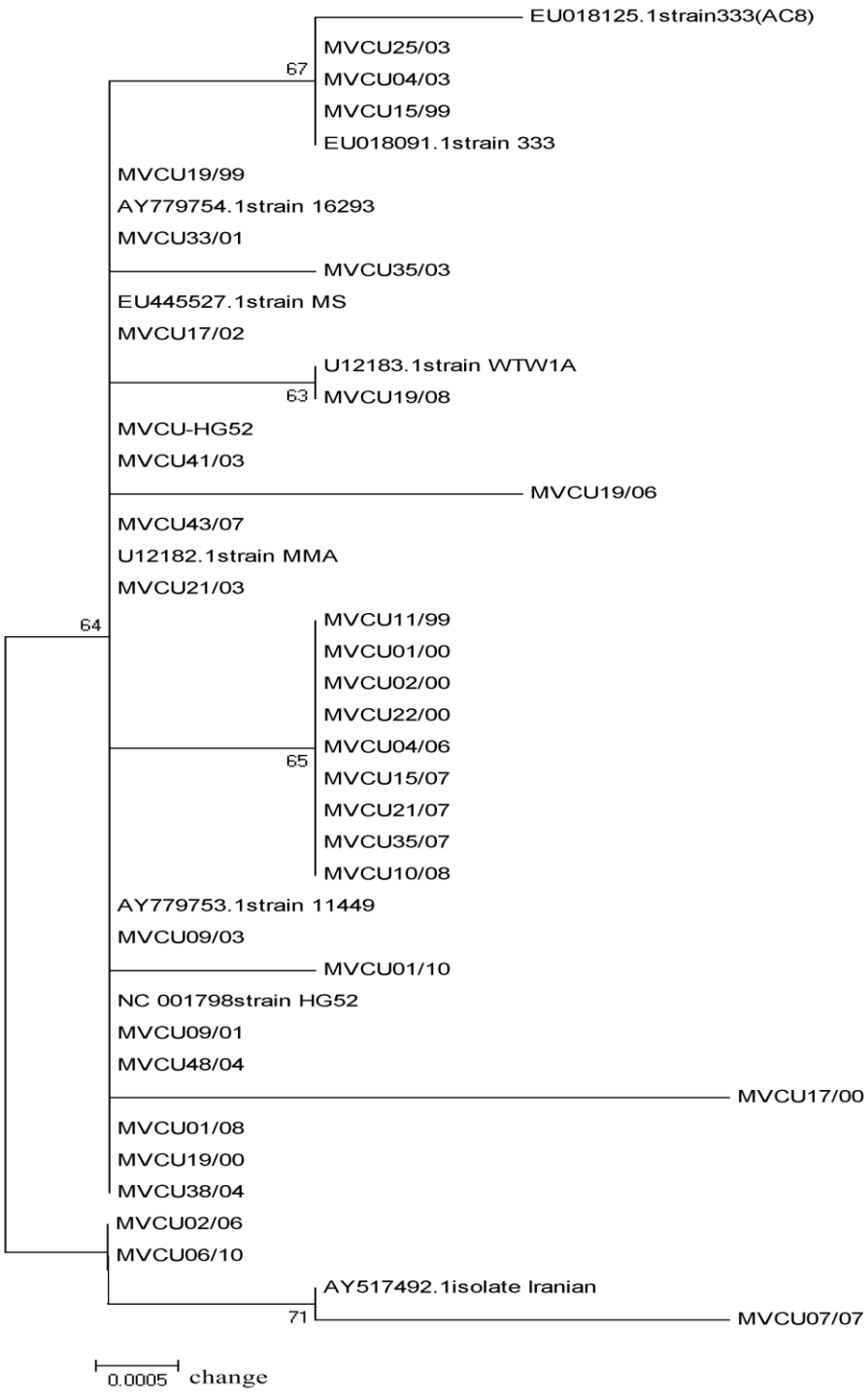


Figure 17 Genetic relationship tree from total 800 nucleotides of 33 clinical HSV-2 sequences and 9 reference strains from GenBank using Neighbor-joining method. Number along branches are bootstrap-supporting values generated after 1000 replications, The bootstrap values less than 50% are not shown on the tree.

CHAPTER VI

DISCUSSION

HSV-1 and HSV-2 are extremely spread worldwide in human. They are able to replicate in many types of cell with rapidly growth and highly cytolysis (57, 95). The process of infection starts when virion binds to cell surface receptor. gD is known to be essential determinant of cell recognition for HSV entry (10, 76, 92, 96). Entry receptors of HSV including human and animal have 3 classes, i.e., HVEM, nectin and HS-3-OS, that distributed in several cell types (8, 80, 86-87, 90-91). HVEM appears mostly abundant in haematopoietic cells and lymphoid tissues (16, 97). Nectin-1 is broadly expressed in numerous human tissues including epithelial, fibroblast, endothelial and neuroblastoma cells (9-10, 78, 98) while nectin-2 serves as receptors for unrestricted HSV-1 mutants (99-100) but not for wild-type HSV-1 and HSV-2 (72). In this present studies, we interested in gD genetic variations of Thai HSV clinical isolates. In 1991, Bhattarakosol, P., *et. al*. demonstrated intratypic variations in neutralizing epitopes among HSV-2 isolates using monoclonal antibodies (18). However, no previous studies reported about genetic variation of HSV gD, although several studies demonstrated that mutations of gD affected the viral entry and growth efficiency especially at position of gD binding surface receptor (FR I - IV) (21, 87, 101). The changing of gD sequence effected to binding site such as the gD mutation in FRI can not binding HVEM but it can infected cell and propagated progeny. Beside those FR I to IV, the proper folding of gD ectodomain may also play role in step of viral entry. Manoj, S., *et al*. in 2004 demonstrated that deletion of HVEM binding site in gD did not inhibit viral entry indicating that HSV can use other receptors on host cell surface membrane (86).

Molecular diversity of HSV was previously reported by using restriction endonuclease enzyme detection (19, 67, 102-105). The results showed that HSV nucleotide divergence between continents was consistent with the ages of human population (106). Using DNA sequencing technology, HSV phylogenetic tree was constructed mostly based on gB gI gG and gE sequences instead of whole genome

(53, 69-70, 89). Since gD plays important role in viral entry, replication, cell pathology and induces neutralizing antibody, we therefore interest to investigate gD variations.

In present study, we selected HSV clinical samples from Virology laboratory, Division of Virology, Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand within 10 years (A.D. 1999 - 2010) in order to increase proportion in mutation of gD sequence since Sakaoka reported that the mutation rate of HSV DNA genome was very low, $3.5x10^{-8}$ mutations/site/year (67). Systemic sampling was performed in 256 specimens to 100. Only 70 samples were successfully recruited. 37 specimens were HSV-1 and 33 specimens were HSV-2 by Real time PCR typing. The principle of Real time PCR typing is based on T_m determination of gD ectodomain (nucleotide position is between 949 and 1160, locating at C-terminal region). From our results, the T_m range of HSV-1 clinical isolates was 90.20 – 91.35 °C while that of HSV-2 clinical isolates was 92.14 – 93.67 °C. The T_m range of both HSV hinted that gD ectodomain had very few variations. The variations of each standard PCR product testing by SYBER Green Real time PCR were swing in run amplification. This appearance is possibly due to small variations in volume of each reagents such as DNA template, Mg^{2+} and also condition in each running. Observation of gD PCR product after amplification (Figure 11 and 12) indicated that although equal amount of DNA template was used the band of amplicon showed unequal in thickness. This might be the variations in DNA measurement caused by fluorometer.

HSV gD sequencing was done in all 70 samples. The designed primers start from nucleotide position 33 (HSV-1) and 58 (HSV-2) equivalent to aa position 11 and 18 to nucleotide position 866 (HSV-1) and 982 (HSV-2) equivalent to aa position 289 and 327 respectively. However, the data of gD sequences obtained from Macrogen Inc., was unable to analyze complete the expected gD sequences. The sequences of FRI position that contacted HVEM (1-37 aa, N-terminal) (2, 101) and C-terminal (260-396 aa) with contacting complex glycoproteins when entry into cell (9, 92) were lost. However, the gD sequences remained comparable to other parts of the gD reference strains, such

as FR II and III partitions, binding regions of nectin-1 and nectin-2 locating at the gD ectodomain.

The results of gD nucleotide sequences of HSV-1 and HSV-2 indicated that these two viruses were distinctively separated (Figure 15) and the divergence of HSV-1 gD was higher than HSV-2 gD (Figure 16 and 17). Among Thai HSV-1 clinical isolates, 81.08% were similar to the HSV-1 (KOS) reference strain reported in GenBank since 1985 (68, 89). This observation implied that the gD of circulating HSV viruses are highly conserved throughout 26 years. In addition, HSV-1 (KOS) may be the first strain spread in Thailand. In contrast, Thai HSV-2 isolates showed characteristic similar to other HSV-2 strains around the world. Our results were corresponded to our previous report in 2005 using RFLP, which indicated that HSV-1 isolates were more divergence than HSV-2 isolates similar to Sakaoka's studies (67, 105). Although those reports determined the whole genome sequence, only gD sequence seemed to be adequate to see the variations.

After phylogenetic tree was analyzed, the variations of HSV-2 gD were more than HSV-1 possibly due to high frequently reactivation of HSV-2. As a result of primary HSV-1 infection usually occurs in young children and most often asymptomatic. Recurrences of one per year or less were found in 61% (106). For HSV-2 are usually acquired sexual contact and more than 50% of primary infection present clinical symptom. Several studies have reported a HSV-2 frequency of nearly 90% of patients having one or more recurrences per year, 38% more than 6 recurrences, and 20% more than 10 recurrences (107-109). Besides host factors, difference of gD genetic variations among HSV-2 might be one factor causing difference in HSV-2 reactivation frequency.

From comparing HSV gD sequences, it revealed that most of nucleotide sequences were substituted in point mutation pattern. Thus, mutation by nucleotide substitution was prone to be a main mechanism of HSV gD variations. All those point mutation positions happened in region where did not involve in viral binding receptor. Although mutations occurred in gD gene, most of them encoded synonymous amino acid. Only 6 positions (HSV-1; 3 positions and HSV-2, 3 positions) of 6 patterns were aa

exchanged within the same group except two. This phenomenon did not cause any effect to the functions of gD especially entry step confirming its intrigrity.

The mutation positions of Thai HSV gD clinical isolates and both standard HSV-1 (KOS) and HSV-2 (HG52) including some reference strains obtained from GenBank were compared (Figure 13 and 14). Most exchanged positions were in ectodomain of gD containing FR II and III. The nucleotide positions 142, 161 and 169 of HSV-2 gD (Table 10) were exchanged in nucleotide positions which resulted to aa substitution but previous studies, functional region II (residues 126-161) compresses a loop structure of gD and interesting that several MAb resistant mutation selected with group immunoglobulin antibodies lie within or just adjacent to region II (81). And nucleotide position 119, 252 and 285 of gD HSV-1 (Table 9) were exchanged in nucleotide positions which resulted to aa substitution but not similar with position in FR I and FR II in ectodomain. Thus, HSV genome was strongly stable especially genes encode proteins involving in viral replication functions. Amino acid change in Thai isolates was mainly synonymous substitution which might not interfere the structure of gD protein. The mutation in previously studies of specific position of gD ectodomain that contacting to nectin-1 and nectin-2 were not specified. Substitutions in our sequences were transition pattern more than transversion pattern and the most of aa substitution were synonymous. Moreover, our study did not found variations in essential positions for binding receptor in gD sequence but showed strong gD ectodomain sequence. From previously report showed HSV gD involves in production of HSV neutralizing antibody (49) therefore conserving gD structure within HSV type is a good opportunity in using gD as a candidate protein for vaccine production (110-111).

In summary, this study showed that glycoprotein D gene sequences from 70 clinical HSV isolates during the year of 1999 to 2010 were highly conserved. Some point mutations were showed as a few variations between groups (Figure 15 and 16). Only 6 isolates had non-synonymous aa changes in the area out off the receptor binding site positions. Our information confirmed that very low mutation occurred in DNA containing virus (112). If it did, it seemed not to affect the structure and functions of receptor

binding sites. The strictly conserve in this area believed to be for viral survival. Finally, HSV gD is a good candidate for vaccine development.

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APPENDICES

APPENDIX A

Reagents, material and instruments

1. Reagents and material

Absolute ethanol	(Merck, Germany)
Agarose	(Bio Basic, U.S.A)
Boric acid	(Bio Basic, U.S.A)
100 bp DNA Ladder	(Fermentous,)
dNTPs	(Fermentous,)
Ethidium bromide	(Bio-Rad, U.S.A.)
FastStart DNA Master^{PLUS} SYBER Green I	(Roche, Germany)
Fetal bovine serum	(PAA, USA)
Filter tip	(Sorenson, U.S.A.)
Gum tragacanth	(Sigma, U.S.A.)
M199 medium	(GIBCO, U.S.A.)
Micro-centrifuge tube	(Bioscience Inc., U.S.A.)
$NaHCO_3$	(Bio Basic, U.S.A)
NaCl	(Merck, Germany)
N-2-hydroxylethlpiperazine-N'-2-ethanesulfonic acid	(Sigma, U.S.A.)
Penicillin G and 100 µg/ml streptomycin	(GIBCO, U.S.A.)
Quant-iT DNA assay kit	(Invitrogen, U.S.A.)
Trypsin	(Merck, Germany)
Tris-base	(Bio Basic, U.S.A)
Flat 24 and 96 well plate	(NUNC, Denmark)

2. Instruments

Autoclave	(Tomy, Japan)
DNA thermocycle	(BioER Technology, U.S.A)
Chemi doc	(Merck, Germany)
Electrophoresis chamber	(CBS, U.S.A)
LC Carousel Centrifuge 2.0	(Roach, U.S.A.)
Microcentifuge (model : Force1418)	(Edison, U.S.A)
Mixer –vertex	(Scientific industrial, U.S.A)
pH meter	(Accmet Basic, Singapore)
Power supply (model :1000/500)	(Bio Rad, U.S.A)
Qubit® fluorometer	(Invitrogen, U.S.A.)
Real time PCR thermocycle	(Roach, U.S.A.)
Water bath	(Julabo, Germany)

APPENDIX B

REAGENT PREPARATION

Reagents and Media for Cell Culture

1. **2X Medium M199**

M199 with Earle's salts, with L-glutamine, without $NaHCO_3$	10 g
Sterilized deionized distilled water	500 ml

Filtration and stored at 4 °C

2. **1M HEPES**

HEPES (N-2-hydroxyethyl-piperamine-N'-2-ethan sulfonic acid)	23.83 g
Deionized distilled water	100 ml

Sterilized by autoclaving 121 °C 15 minutes

3. **10% $NaHCO_3$**

$NaHCO_3$	10 g
Deionized distilled water	100 ml

Sterilized by autoclaving 121 °C 15 minutes

4. **10% M199 medium (GM)**

2X M199 Earle's salts, with L-glutamine, without $NaHCO_3$	50 ml
Fetal bovine serum	10 ml
1M HEPES	1 ml
Penicillin/Streptomycin antibiotic (10^5 unit/ml)	1 ml
Sterilized deionized distilled water	37 ml
10% $NaHCO_3$ adjusted to pH 7.4	1 ml

5. **2% M199 medium (MM)**

2X M199 Earle's salts, with L-glutamine, without $NaHCO_3$	50 ml
Fetal bovine serum	2 ml
1M HEPES	1 ml
Penicillin/Streptomycin antibiotic (10^5 unit/ml)	0.1 ml
Sterilized deionized distilled water	45 ml
10% $NaHCO_3$ adjusted to pH 7.4	1 ml

6. **10X PBS (Phosphate buffer saline)**

NaCl	40 g
KCl	1 g
$NaHPO_4$	5.75 g
KH_2PO_4	1 g
Deionized distilled water	1000 ml

Adjust pH to 7.5

Sterilized by autoclaving 121 °C 15 minutes

7. **1X PBS**

10X stock PBS	100 ml
Deionized distilled water	900 ml

8. **10X Trypsin**

Trypsin	0.5 g
EDTA	0.2 g
NaCl	9 g
Deionized distilled water	100 ml

Sterilized by filtration and kept at -20 °C

9. 1X Trypsin

10X stock Trypsin	10 ml
Sterilized deionized distilled water	90 ml

kept at 4 °C

10. Plaque overlay medium

Solution A

10X M199 Earle's salts, with L-glutamine, without $NaHCO_3$	20 ml
Fetal bovine serume	20 ml
1M HEPES	2 ml
Penicillin/Streptomycin antibiotic (10^5 unit/ml)	0.2 ml
Sterilized deionized distilled water	42 ml
10% $NaHCO_3$ adjusted to pH 7.4	3 ml

Solution B

Gum tragacanth	1.6 g
Deionized distilled water	100 ml

Sterilized by autoclaving 121 °C 15 minutes

Working solution

The solution A and solution B were mixed at a ratio 1 : 1 before use.

Reagent for Electrophoresis

1. **10X Tris-borate buffer (TBE)**

Tris-base	60.50 g
Boric acid	30.85 g
$Na_2EDTA \cdot 2H_2O$	3.72 g
Distilled water	1000 ml

Sterilized by autoclaving 121 °C 15 minutes, kept at room temperature

2. **Ethidium bromide (10mg/ml)**

Ethidium bromide	1 g
Sterilized distilled water	100 ml

3. **Loading dye**

Bromphenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	30 ml
Distilled water	70 ml

4. **1.5% Agarose gel**

Agarose	0.325 g
0.5X TBE buffer	25 ml

APPENDIX C

One and three letter symbols for the amino acid

Amino acid	One letter symbol	Three letter symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagines	N	Asn
Aspartic acid	D	Ast
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

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Presentation: Molecular variation of herpes simplex virus type 1 glycoprotein D (HSV-1 gD) gene from clinical specimens of Thai patients within 10 years. Poster presentation in " Joint Conference in Medical Sciences 2011. Bangkok Thailand

Publication: Sirirat Naemkhunthot, Parvapan Bhattarakosol. Phylogenic analysis of herpes simplex virus type 1 glycoprotein D (HSV-1 gD) gene from clinical specimens during 1999 -2003 Poster presentation in The 12th Graduate Research Conference, Khon Kaen University (Thailand), Jan 2011: 995-1000.