HUMAN HERPESVIRUS 6 DIAGNOSTICS: DEVELOPING BETTER TESTS AND UNDERSTANDING ANTIVIRAL RESISTANCE

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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Dedicated in memory of my favorite Uncle Ross

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AIDS	acquired immunodeficiency syndrome
ATCC	American Tissue Culture Collection
bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
CD4 / CD46	cluster determinate 4 / 46
CDC	Centres for Disease Control
cDNA	complementary deoxyribose nucleic acid
CDO	cidofovir
CIP	calf intestinal phosphatase
CPE	cytopathic effect
CSF	cerebral spinal fluid
DNA	deoxyribose nucleic acid
dNTP	deoxynucleotide phophate
E. *coli*	*Escherichia coli*
EBV	Epstein-Barr virus
EDTA	ethylene diaminetetracetic acid
ELISA	enzyme linked immunosorbent assay
E.R.	emergency room
EtBr	ethidium bromide
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
xg	times gravity
GCV	ganciclovir
GCV^R	ganciclovir resistance
HCMV	human cytomegalovirus
HHV-6A/B	human herpesvirus 6A/B
HHV-7	human herpesvirus 7
HHV-8	human herpesvirus 8
HIV	human immunodeficiency virus
HSV 1/2	herpes simplex virus 1/2
IFA	immunofluorescent assay
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
kb	kilobase (10^3 bp)
krpm	kilo-revolutions per minute (10^3 rpm)
kV	kilovolt (10^3 Volts)
L	litre
M	molar
MCS	multiple cloning site
µF	microfarad
mg	milligram (10^{-3} g)

μg	microgram (10^{-6} g)
min	minute(s)
mL	millilitre (10^{-3} L)
μl	microlitre (10^{-6} L)
mM	millimolar (10^{-3} M)
μM	micromolar (10^{-6} M)
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NIH	National Institute of Health
nm	nanometer (10^{-9} meter)
NML	National Microbiology Laboratories
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
PFA	foscarnet
PK	protein kinase
RNA	ribose nucleic acid
rpm	revolutions per minute
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
U	unit
VTM	viral transport medium
v/v	volume per volume
VZV	varicella zoster virus
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-ß-D-galactoside

ABSTRACT

In the *Herpesviridae* family there are 9 viruses of medical significance to humans. The closely related human herpesviruses 6A, 6B and 7 (HHV-6A, 6B, and 7) are associated with roseola, a common febrile illness of infants. Following primary infection these viruses remain latent in their hosts. In immunocompromised individuals these viruses can reactivate and cause significant morbidity, alone or in concert with human cytomegalovirus (HCMV). Reactivation disease is often severe enough that antiviral therapy (generally ganciclovir) is sometimes be used to control the infection.

Interpreting diagnostic results for HHV-6/7 is troubled by the lack of thoroughly evaluated laboratory tests, an unclear disease profile, and the high seroprevalence and latency associated with these viruses. Reactivation disease has the additional diagnostic dilemma of understanding and diagnosing antiviral resistance. Ganciclovir resistance in HCMV is well documented and has been shown to result from mutations in the viral polymerase or protein kinase genes. Both genes have homologues in HHV-6/7.

To address the issues seen in diagnosing HHV-6/7 infections, a study was set up at the Winnipeg Children's Hospital. The disease profile of primary infection was studied in order to better define antigen and antibody profiles for HHV-6/7, with the long-term goal of developing a rapid-point-of-care diagnostic test. To better understand ganciclovir resistance in HHV-6, recombinant baculoviruses were created containing the protein kinase gene with mutations homologous to those seen in resistant isolates of HCMV. The effect of these mutations on ganciclovir susceptibility was studied.

Although the HHV-6/7 study did not accomplish its initial goals, a new LightCycler real-time PCR assay was developed to differentiate and quantify HHV-6A,

6B, and 7, and important points regarding the study were brought up which will aid future HHV-6/7 studies. Although it would appear that whole blood might be a potential specimen for use with a rapid diagnostic point-of-care test, further investigation is required. Homologous mutations to those from ganciclovir resistant HCMV isolates in the HHV-6 protein kinase were shown to confer ganciclovir resistance on recombinant baculoviruses. Through characterization of these mutations, we have improved the diagnosis of ganciclovir resistant HHV-6.

INTRODUCTION

To date approximately 130 herpesviruses have been identified in nature. In fact they probably infect every vertebrate species. Nine members of the *Herpesviridae* have been isolated from and commonly infect humans [herpes simplex virus 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and human herpesviruses 6A, 6B, 7, and 8 (HHV-6A, HHV-6B, HHV-7, and HHV-8)]. Medically speaking, the human herpesviruses are an important group. Most members of the family cause significant morbidity and even mortality through a wide variety of clinical syndromes ranging from rashes, cold sores and other skin conditions, mononucleosis and chicken pox to more severe illnesses such as encephalitis, meningitis, congenital disease, some forms of cancers and transplant disease.

Herpes Virology

Virion structure (reviewed in White & Fenner 1994)

Herpesviruses are large viruses, generally between 120-200 nm in diameter, with a characteristic virion architecture consisting of over 30 proteins (approximately 6 in the nucleocapsid, 10-20 in the tegument, and 10 in the envelope). Typically a herpes virion is composed of a core containing a large (125 – 229 kbp), linear, double stranded DNA genome enclosed in an icosahedral capsid of approximately 100-110 nm in diameter. The icosahedral capsid is made of 162 capsomers (150 hexamers and 12 pentamers) which completely surrounds the DNA containing core, creating a solid protein structure known as the nucleocapsid. A globular, unstructured layer, known as the tegument, surrounds the

capsid and anchors it to the fragile outer lipid envelope. The envelope is mainly composed of glycoproteins, many of which project outwards as peplomers. The surface exposed peplomers are targeted by host immune systems. Different herpesviruses have distinct envelope glycoproteins. However, closely related herpesviruses (for example HHV-6A and 6B) possess some shared antigens which results in immune cross reactivity.

Genome structure

Herpesviruses are genetically highly conserved. All herpesviruses contain a core group of herpes genes. Sub-family and genus specific genes are also present in the herpes genome accounting for the differences seen amongst these viruses. Their genomes can be organized according to their structure with respect to two regions, unique long (U_L) and unique short (U_S), created by the presence of terminal and internal repeats. The number and orientation of the repeats enables one virus to produce 0 (HHV-6), 2 (VZV) or 4 different isomeric forms (HCMV) each in equimolar amounts. Five different arrangements of repeats are known. The significance of the repeats is still unknown.

Lifecycle

The lifecycle of herpesviruses are tightly regulated. Transcription and translation proceed through 3 characteristic phases, immediate early, early and late, where the gene products of each regulate the other phases. Among other proteins and gene products, the genome of all herpesviruses encodes a large assortment of enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing. Host cell nuclear functions are impaired as viral replication advances. In the cell nucleus viral DNA is synthesized and

nucleocapsids are formed before gaining the envelope while budding from the nuclear membrane. Progeny viruses are transported through the cytoplasm to the outer membrane where they are released resulting in cell lysis. An important biological property of all herpesviruses is latency. All known herpesviruses can lay dormant in host cells for long periods of time and reactivate to cause lytic infections. During latency the viral genomes form closed circular molecules only allowing expression of a limited number of specific genes.

Classification

The variability seen in the biological properties of herpesviruses allow for further subdivision of the *Herpesviridae*. Based on differences in host ranges, lifecycle kinetics, site of latency, and tissue culture characteristics, herpesviruses are divided into three subfamilies; *Alphaherpesvirinae* , including HSV 1, 2 and VZV; *Betaherpesvirinae*, including HCMV, HHV-6A, B and 7; and *Gammaherpesvirinae*, including EBV and HHV-8. This classification system is not based on sequence homology. However advances in sequencing technology have shown that members in each subfamily are genetically closer to other members within the same subfamily.

Human Herpesviruses 6 and 7

History

The combination of powerful new molecular biological methods and the AIDS pandemic has directly contributed to the identification of many pathogens including HHV-6 and 7. Between 1986 and 1994, four new human herpesviruses were discovered

(HHV-6A, 6B, 7 and 8), the first since EBV which was discovered over 20 years earlier. These newly discovered herpesviruses had likely interacted with humans for millennia. Initially named human B-lymphotropic virus, or HBLV, HHV-6 was discovered in 1986 by Salahuddin (Salahuddin *et al.* 1986). Using improved culturing techniques Salahuddin's group isolated HHV-6 from interleukin-2 stimulated peripheral blood mononuclear cells (PBMCs) of AIDS patients. Other groups began isolating the same virus from numerous patients, but it was not until the discovery of the Z-29 strain of HHV-6 that it was realized that there were actually two very closely related viruses, designated HHV-6A and HHV-6B (Lopez *et al.* 1988; reviewed in Ablashi *et al.* 1993). With new life in herpesvirology, vigorous research went into the newly discovered HHV-6A and B. A surprising result of one group's efforts was the discovery of yet another new human herpesvirus, HHV-7. HHV-7 was discovered by Frenkel's group while performing experiments on HHV-6 replication. Uninfected control cells showed signs of characteristic cytopathic effect, which, upon further investigation, yielded evidence of a new herpesvirus (Frenkel *et al.* 1990).

Roseolovirus genus

Combined, HHV-6A, 6B and 7 constitute the *Roseolovirus* genus in the *Betaherpesvirinae* subfamily along with HCMV. Although HHV-6A and B are closely related, many studies have shown that they belong in two distinct groups. Early studies focusing on properties of HHV-6 were limited by few clinical isolates. These studies concluded minor differences existed among isolates, but nothing supporting the notion of two separate groups (Joesphs *et al.* 1988; Kikuta *et al.* 1989). As more HHV-6 isolates

became available, more extensive studies proved the existence of two distinct groups, HHV-6A and B. Differences in reactivity to monoclonal antibodies, restriction endonuclease patterns, nucleotide sequences, seroepidemiology, disease associations, *in vitro* cell tropisms, and effects on T-cell markers all routinely classified HHV-6 isolates into one of the two groups (Aubin *et al.* 1991; 1993; Dewhurst *et al.* 1992; 1993; Furukawa *et al.* 1994; Schirmer *et al.* 1991; Yadav *et al.* 1991). Although mixed infections have been found on occasion in humans (Ablashi *et al.* 1991; Cone *et al.* 1996), no hybrids of the two viruses have been found suggesting reassortment does not occur. Within the last few years the genomes of the two variants of HHV-6 have been sequenced and compared. The data supports the earlier experiments finding 115 potential open reading frames (ORFs) some of which are variant specific. Most proteins coded by shared ORFs in the two variants show greater then 94% amino acid similarity with only 12 (mostly immediate early and regulatory proteins) sharing less then 70% similarity (Isegawa *et al.* 1999). HHV-7 shares a high degree of genetic similarity with HHV-6, having only a few differences in the protein coding regions accounting for the subtle differences between the viruses (Nicholas 1996). All three viruses exhibit a low degree of intra-variant divergence (0.1 to 1%) and are highly genetically conserved.

Seroprevalence

HHV-6A and B are highly prevalent worldwide. In fact, seroprevalence of HHV-6 is greater than 90% in most populations as determined by serological assays that are unable to differentiate the two variants (Braun *et al.* 1997). Careful examination of age groups revealed the presence of maternal antibodies in infants up to 5-6 months of age.

After 6 months the protective maternal antibodies wane, leaving the child susceptible to HHV-6. Seroprevalance to HHV-6 increases in this age group with most children having antibodies to HHV-6 by 13 months of age, and virtually all infants positive by age 2 or 3 (Okuno *et al.* 1989). Using antibodies specific for HHV-6A or B it has been estimated that fewer than 20% of people have significantly different titers to either virus (Chandran *et al.* 1992; Yadav *et al.* 1991).

Seroepidemiological studies estimate the seroprevalence of HHV-7 is approximately 75% worldwide, with individual study results varying from 60 to 90% (Yamanishi 2001). Age grouped serological studies (similar to those carried out for HHV-6) showed after HHV-7 specific maternal antibodies wane, seroprevalence to HHV-7 increases. This change in seroprevalence occurs at a slower rate for HHV-7, indicating it is generally acquired after HHV-6 (Clark *et al.*1993).

Transmission

The current state of knowledge with respects to transmission of HHV-6B and HHV-7 suggests the viruses are readily transmitted in saliva and oral fluids. Unexplained by this is that infectious HHV-7 is more prevalent in saliva than is HHV-6B, yet HHV-6B is almost always acquired before HHV-7. Other possible routes of transmission include congenital transmission or germ line integration of viral DNA. Both require further study. The transmission route of HHV-6A is unknown (Pellett & Tipples 2003).

Clinical Aspects

HHV-6 and 7 have been investigated as potential etiological agents for several clinically significant illnesses including malignancies, multiple sclerosis, chronic fatigue syndrome and pityriasis rosea. Proof of association with these diseases is difficult to acquire due to their ubiquitous nature. As of yet, HHV-6A is an orphan virus with no specific disease associated with it (except for the extremely rare incidence of HHV-6A roseola). HHV-7 is often misclassified as an orphan virus because it has not been associated with a medical condition of its own, however it is the cause of a small percentage of roseola cases. Illness associated with HHV-6/7 falls into one of two categories, primary infection (in infancy) or reactivation (in immunocompromised individuals). Diagnostic approaches for these two clinical categories differ due to the different challenges raised in each scenario.

Primary infection

In 1988 Yamanishi's group demonstrated that HHV-6 was the causative agent of exanthem subitum, also known as roseola or sixth disease (Yamanishi *et al.* 1988). It was later shown that roseola is almost exclusively caused by HHV-6 variant B and only in rare cases by HHV-6A (Hidaka *et al.* 1997). Primary infection with HHV-7 has also been linked with roseola with current estimates suggesting it is the cause of 5-10% of roseola cases (Tanaka *et al.* 1994).

Roseola

Roseola is a common febrile illness encountered in early childhood, generally between the ages of three months to three years. Classic roseola is characterized by a rapid onset of fever (39-40°C) lasting for 3 to 5 days followed by a maculopapular rash for 1 to 3 days that coincides with a decrease in temperature. The duration of roseola is between 2 and 7 days before resolving spontaneously without complications. This pattern of symptoms is seen in an estimated 30% of children. Primary infection with HHV-6B or 7 can also present as fever without rash, rash without fever, or with more severe symptoms, such as seizures and respiratory distress. It has been suggested that HHV-7 is associated with more severe forms of roseola such as those presenting with seizures (Caserta *et al.* 1998). There is no evidence for seasonality for HHV-6/7 infections (Nakamura *et al.* 1988). Primary infection in adulthood is extremely rare, but more severe (Akashi *et al.* 1993).

Disease profile

The disease profile, with respect to virological course and host immune response, has been studied although some aspects remain unclear. There appears to be an initial incubation period of between 5 to 15 days before symptom onset (Okuno *et al.* 1991). During and even preceding the acute febrile stage, the virus is present and readily culturable from the patient's lymphocytes (Okada *et al.* 1993; Yoshikawa *et al.* 1993). As the rash appears and the fever diminishes, viremia decreases and is usually undetectable by the time the illness resolves (Asano *et al.* 1991). At some point the virus targets the salivary glands resulting in secretion in the saliva (Kidd *et al.* 1998). The time at which

this occurs remains unclear although it has been suggested it occurs after acute presentation (Clark *et al.* 1997). The host immune response to the viral infection occurs quickly. IgM antibody, indicative of a recent infection, is detectable early, peaks within 2 to 3 weeks and persists for approximately 2 months. However, an IgM response is detectable in 5% of healthy individuals at any one time, possibly indicative of periodic HHV-6 reactivation (Cermelli & Jacobson 2000). IgG is detected as early as 7 days after symptom onset, increases for at least 3 weeks and generally remains detectable for long periods of time (Ueda *et al.* 1989; Kondo *et al.* 1990). Neutralizing IgA has not been detected (Suga *et al.* 1992).

Tissue tropism

HHV-6 utilizes the CD46 cell receptor to infect cells. HHV-6B is easily detectable in a wide range of specimens including lymphocytes, brain and tonsil biopsies, and sporadically in saliva. HHV-6A is detected less frequently although it possesses a greater neurotropism then HHV-6B (Hall *et al.* 1998). PBMCs are the most likely site of latency for HHV-6.

HHV-7 can be detected in salivary glands, PBMCs, cervix, lung, skin and mammary gland, including breast milk samples, and to a lesser extent in liver, kidney and tonsil samples (Kempf *et al.* 1998). Unlike HHV-6, HHV-7 is rarely detected in the brain and is constitutively found in saliva. HHV-7 uses CD4 as its cell receptor although other unknown receptors are likely involved in the infection of non-lymphocytes. PBMCs are the most likely site of HHV-7 latency.

HHV-6/7 infect the same cells as HIV. Interactions between HIV and these viruses have been documented, although neither HHV-6 nor HHV-7 play a role in AIDS progression (Pellett & Tipples 2003).

Diagnostics

The highly prevalent and closely related HHV-6A, 6B and 7 present unique challenges to differentially diagnosing current from past infections with either one, or combinations of the three. An Expert Working Group on HHV6/7 infections outlined what they believe are the major problems in diagnosing HHV6/7 infections (Tipples 2000). These are:

1. The risk of cross-reaction between the two closely related viruses using serological methods.
2. The difficulty in the interpretation of laboratory results when dealing with reactivation disease.
3. The lack of thoroughly evaluated HHV-6/7 diagnostic methods.
4. The lack of clearly defined HHV-6/7 disease antigen and antibody profiles.
5. The long turn around times for lab test results.

Laboratory diagnosis (reviewed in Pellett & Tipples 2003)

Laboratory diagnosis of primary HHV-6/7 infections is not limited by lack of techniques. Electron microscopy, virus isolation (the gold standard), blot hybridization, *in situ* hybridization, *in situ* PCR, and immunofluorescence assays (IFA) have all been used for detection of HHV-6/7 in various clinical samples. Each of the above methods has its

own limitations including cost, sensitivity, specificity, and time, which rule them out as routine diagnostic techniques for most laboratories. Polymerase chain reaction (PCR) assays, including qualitative, quantitative and reverse-transcription PCR (RT-PCR), which detect virus specific nucleic acids have proven extremely useful for detecting HHV-6/7 in a variety of clinical samples (including whole blood, serum, PBMCs, CSF, saliva, and a range of tissue biopsies). Such assays allow for easy differentiation of HHV-6A, 6B and 7 using variant specific or multiplex primers, specific hybridization probes or restriction fragment length polymorphisms. The sensitivity of PCR offers detection of HHV-6/7 even at exceptionally low virus titers. Serological assays, both enzyme-linked immunosorbent assays (ELISAs) and IFA, are also commonly used in diagnosis of HHV-6/7 infections. Current ELISA methods cannot differentiate HHV-6A specific antibodies from those specific for HHV-6B. However, the ability to detect HHV-6/7 specific IgG and IgM antibodies is useful from a diagnostic perspective. An HHV-6 ELISA is employed by Health Canada's National Microbiology Laboratory (NML) in Winnipeg for its HHV-6 reference services. Avidity assays measuring the overall affinity of IgG antibodies for antigen (low avidity IgG is indicative of a recent infection while high avidity IgG indicates a past infection) have been described (Ward *et al.* 1993). Avidity assays can also differentiate primary infections with HHV-6 or HHV-7 (Ward *et al.* 2001).

The problems with HHV-6/7 laboratory diagnostics are not in the techniques themselves, but rather in the lack of clinical evaluation of the methods. Results of laboratory diagnosis of HHV-6/7 are difficult to interpret and depend on the clinical setting of the infection (primary infection versus reactivation). Several diagnostic

approaches have been put forth for the diagnosis of primary infection with HHV-6/7. Classically detecting IgG seroconversion or significant rise in titer between acute and convalescent serum samples has been used to indicate primary infection. Generally, the presence of IgM antibodies, without IgG, is indicative of primary infection. The problem is when both IgM and IgG are positive. In infants, avidity assays can be used to diagnose primary infection. However, the diagnostic value of serology is limited in adults by two points: (i) IgG antibodies in adults are high avidity and (ii) it has been shown for HHV-6 that 5% of the population is IgM antibody positive at any time (thus affecting the negative predictive value of this assay). Molecular means of diagnosing HHV-6/7 infections have relied on a combination of samples in order to differentiate primary infection from latent virus. For example, one study suggested a PCR positive whole blood sample in conjunction with IgG negative serum indicates primary infection (Chiu *et al.* 1998). Another study has suggested a PCR positive whole blood sample with a PCR negative saliva sample is indicative of primary infection (Clark *et al.* 1997). A third study proposed performing both IgM serology as well as serum PCR in order to improve on sensitivities of either assay on its own (Bland *et al.* 1998). It is evident from the examples provided that laboratory diagnosis of HHV-6/7 needs improving.

Diagnosis in the emergency room or clinic

Due to the limitations of laboratory diagnostics (mainly the time factor) diagnosis of roseola is currently based on clinical symptoms, mainly fever and rash. Such a diagnosis is problematic for several reasons. Only 30% of primary HHV-6 infections present with the "classic" symptoms, the remaining 70% are either asymptomatic or

atypical in presentation and would be missed. Misdiagnosis of roseola as other viral rash causing infections or antibiotic allergic responses has been well documented (Tait *et al.* 1996; Black *et al.* 1996). Cases of atypical roseola, sometimes grouped into fever with no focus (or fever with no apparent cause), would potentially put the infant patient in a position where a series of tests would be run as well as unnecessary antibiotics prescribed. HHV-6 is often overlooked as a possible cause of febrile illness in young children (Ward & Gray 1994). Taking into consideration that primary infection with HHV-6 has been estimated to account for approximately half of all infants' first febrile illness and that these infections may account for between 15 and 40% of febrile admissions to pediatric emergency departments, correctly diagnosing these infections has significant benefits (Asano *et al.* 1994; Portolani *et al.* 1993). The ideal method for diagnosing HHV-6/7 primary infections would have to combine the sensitivity and specificity expected with current laboratory diagnostic methods with the ease and speed of methods utilized in today's pediatric E.R. departments. The technology already exists to achieve this and is currently being used to detect several infectious agents.

Point-of-care testing

Rapid diagnostic tests have been around in one form or another since the early 1980's. There has been a movement towards developing such point-of-care tests because of the proven usefulness in epidemiological studies, screening, diagnosis, and patient management. Several rapid tests are currently on the market for infectious agents including hepatitis B, HIV, malaria, syphilis, chlamydia, *Helicobacter pylori*, and respiratory syncytial virus to name a few. Recently a study of 13 HIV rapid test devices

was conducted looking at overall sensitivity and specificity. The majority of the tests demonstrated similar sensitivities and specificities to current laboratory methods making them an accepted alternative for diagnostic testing (Giles *et al* 1999). Aside from the speed of these tests, their advantages include ease of use and minimal need for instrumentation, making them suitable for use in busy diagnostic laboratories, E.R. departments and hospitals.

Development of a rapid, point-of-care test specific for HHV-6 would greatly reduce many of the problems associated with diagnosing roseola. Misdiagnosis of roseola would be reduced for two major reasons. First, infants presenting with atypical symptoms would be correctly diagnosed because diagnoses would no longer be based on symptoms alone and secondly, roseola would no longer be confused with other rash illnesses or drug allergies. More accurate diagnosis has numerous advantages for both the medical professionals and infant's families. Time spent in crowded ER's would decrease for infants and their families, stress on medical staff, parents, and infants would be reduced with a quick, reassuring diagnosis, confirmatory diagnostic tests and follow up exams would be reduced, and unnecessary antibiotic prescriptions would decline.

In order to develop a rapid, point-of-care, diagnostic test for roseola, many of the original problems with laboratory diagnostics still need to be addressed. A reliable marker of infection (viral antigen, viral nucleic acid or virus specific antibodies) detectable at the time of presentation in the clinic needs to be defined. Finding the appropriate marker would involve developing new and evaluating new and old laboratory diagnostic techniques. The available technology of the current point-of-care tests needs to be evaluated. The sensitivity of these tests needs to be determined so as to find a marker

for roseola that is present in suitable quantities. In short, a study to define the disease profile of roseola (primary HHV-6/7 infections) is needed.

Reactivation

In healthy immunocompetent individuals, HHV-6/7 will remain latent in lymphocytes and cause no problems. The most serious clinical syndrome associated with HHV-6 and HHV-7 is reactivation in immunocompromised individuals. The beta-herpesviruses are well known pathogens in transplant recipients and AIDS patients. HCMV causes the most severe disease in these settings and thus is studied most commonly. HHV-6 and to a lesser extent HHV-7 are now being recognized as significant opportunistic pathogens (Singh & Carrigan 1996; Ljungman 2002). As a result of the development of more sensitive diagnostic techniques more studies are focusing on HHV-6 as an opportunistic pathogen. Unlike the relatively mild primary infection roseola, infection or reactivation of HHV-6 in solid organ transplant, bone marrow transplant, or stem cell transplant patients can result in serious clinical manifestations. Clinically HHV-6 has been associated with encephalitis, interstitial pneumonia, hepatitis, bone marrow suppression, leucopenia, liver dysfunction, and neurological complications all of which cause significant morbidity and mortality (Drobyski *et al.* 1993; Carrigan *et al.* 1991; Carrigan & Knox 1995; Ljungman *et al.* 2000; Wang *et al.* 1999; Zerr *et al.* 2001). HHV-7 has been shown to play a role in post-transplant diseases, although to a much lesser extent then HHV-6 (Chan *et al.* 1997; Chapenko *et al.* 2001; Griffiths *et al.* 1999). More studies are needed to further evaluate the role of HHV-7 in this setting.

Perhaps even more important than the direct clinical symptoms seen are the indirect effects this group of viruses can have on the patient. It is well documented that HCMV disease increases the risk of other opportunistic infections in immuno-compromised individuals. Several studies have shown preventing herpesvirus infections with prophylaxis drug treatment reduces the risk of non-herpesvirus infections, like *Candida* and *Staphylococcus*, as well as lowers the risk of acute graft rejection (Lowance *et al.* 1999; Wagner *et al.* 1995). It remains unclear the effects HHV-6/7 have on other opportunistic pathogens as most of the work carried out on this subject to date has looked specifically at HCMV. Several studies have implicated HHV-6, 7, or both as a risk factor for HCMV infection (DesJardin *et al.* 1998; Kidd *et al.* 2000; Wang *et al.* 2002). Other studies have suggested the opposite in that HCMV may reactivate HHV-6, 7 or both, and that in turn may result in the symptoms of HCMV syndrome (Dockrell & Paya 2001; Lautenschlager *et al.* 2002). Either way, an interaction between the beta-herpesviruses is likely and the combined interactions result in serious complications (DesJardin *et al.* 2001).

Reactivation diseases with HHV-6/7 are much less defined when compared to primary infections, making their diagnosis more problematic. Active infections with HHV-6/7 can be diagnosed by viral isolation, a significant rise in antibody titers, detection of viral nucleic acid in plasma/serum, detection of viral specific mRNA or increased viral loads. The latter two options are perhaps the best methods: Detection of HHV-6/7 spliced mRNA is clear-cut evidence of viral replication (Norton *et al.* 1999); quantitative PCR methods have the most potential due to their ability to monitor viral loads of the individual when the proper clinical samples are tested (Ohyashiki *et al.*

2000). Choosing the most appropriate sample to test may vary according to each case because of the wide variety of clinical syndromes possible. Both PBMCs and serum have been used to some success in monitoring the presence of viral DNA in these patients (Allen *et al.* 2001; Ohyashiki *et al.* 2000). While it has been demonstrated that reactivation disease elicits an IgM antibody response (Fox *et al.* 1990), serology based diagnosis is still hindered by the limitations previously discussed in the diagnosis of primary infections section. The diagnostic issues raised by reactivation disease need to be re-evaluated in a larger study. Unfortunately one clinical study will not solve the diagnostic problems of both settings. Reactivation disease adds an additional challenge to the diagnostic dilemma of HHV-6/7, solely brought on by treating the disease itself, antiviral resistance.

Anti-viral therapy and treatment

HHV-6/7 are considered to be resistant to acycloguanosine (9-[2-hydroxyethoxymethyl] guanine, or Acyclovir), the first major drug used against herpesviruses like HSV-1 and VZV. Since the development of acyclovir in 1977 a large amount of research has gone into antiviral chemotherapeutic agents active against herpesviruses, like HCMV, which did not respond to acyclovir therapy. Ganciclovir (9-[1,3-dyhydroxy-2-propoxymethyl] guanine, or GCV), an acyclovir derivative, and Trisodium Phosphonoformate (Foscarnet or PFA) are two antiviral agents used against the beta-herpesviruses. More recently cidofovir ([S]-1-[3-hydroxy-2-phosphonylmethoxypropyl] cytosine or CDO) has been licensed for use. All three exert

their antiviral effects on the viral encoded DNA polymerase thus inhibiting viral DNA synthesis. Most work with these antivirals has been carried out with HCMV.

GCV, the first drug offering acceptable therapy for HCMV infections, is an analogue of deoxyguanosine, which must be tri-phosphorylated before it is active. The initial phosphorylation is carried out by a viral encoded protein kinase (UL97 in HCMV, U69 in HHV-6) after which the host cell kinases convert it from mono- to di- and tri-phosphate GCV. GCV-triphosphate competitively inhibits the viral polymerase causing slowing and eventual cessation of DNA synthesis (Crumpacker 1996; Field *et al.* 1983; Reid *et al.* 1988). CDO results in a similar outcome for the viral lifecycle with the only differences being it is an acyclic nucleoside phosphonate that is not dependant on phosphorylation by a viral protein kinase. Host kinases convert it to its active di-phosphate form (De Clercq 1993; Lalezari *et al.* 1995; Lalezari *et al.* 1997). PFA is a pyrophosphate analogue that is neither incorporated into the growing viral DNA chain, nor does it require activation steps by the virus or host cell. PFA blocks the pyrophosphate-binding site of the viral DNA polymerase and inhibits cleavage of pyrophosphate from deoxynucleoside triphosphates (Balfour *et al.* 1990; Chrisp & Clissold 1991).

Treatment of HHV-6/7 infections is generally reserved for serious clinical syndromes, such as transplant or AIDS patients. Primary infection is only treated with anti-viral agents in rare severe cases. Currently GCV is the drug of choice for beta-herpesviruses. PFA has been shown to have better in vitro activity, but long-term use is not recommended due to serious side effects, mainly nephrotoxicity (MacGregor *et al.* 1991). GCV has shown consistent activity against HHV-6 in PBMCs (Manichanh *et al.*

2000), however, only small scale studies have been published on GCV's clinical efficacy against HHV-6 in adults (Zerr *et al.* 2002). The efficacy of GCV treatment in children or on HHV-7 infections is less understood. Several adverse, yet reversible, side effects have been documented with prolonged GCV therapy. The main (dose-limiting) effect is bone marrow suppression leading to granulocytopenia and thrombocytopenia although neutropenia, diarrhea, nausea, neurological effects (neuropathy and paresthesias), fever, rash, and spermatogenesis (irreversible and only shown in canine studies) have been noted in a minority of cases (Flaherty & Crumpacker 1999). Despite these effects, both preemptive (meaning treatment is given when a laboratory confirms the presence of a virus with high probability of causing disease) and prophylaxis (meaning treatment is given before evidence of active viral replication in attempts to prevent infection) treatment strategies are common in transplant situations. Both are effective (as shown mostly with HCMV) but their intrinsic worth is controversial due to drug resistance (Hart & Paya 2001; Emery 2001).

Antiviral resistance

Often times when antiviral agents are used to control an infection by HHV-6 or 7, it is for an extended period of time due to the immune status of the infected individual. Long-term exposure of the beta-herpesviruses to these agents creates the opportunity for drug resistant mutants to arise. GCV resistant HCMV mutants have been well documented clinically and in the laboratory for many years (reviewed in Erice 1999). Several studies have confirmed resistant phenotypes seen are due to amino acid deletions

or substitutions in conserved regions of the viral protein kinase and/or polymerase proteins (Baldanti *et al.* 1995; Lurain *et al.* 1992; 1994; Smith *et al.* 1997).

Little work has been done on the subject of GCV resistant HHV-6 and none has been published for HHV-7. Manichanh's group cultured HHV-6 in increasing concentrations of GCV and was able to create a resistant mutant. Sequencing revealed an amino acid substitution in the protein kinase, U69 gene, homologous to a known mutation found in the HCMV protein kinase, UL97 gene. The same group went further and found the same mutation in a clinical sample from an AIDS patient on long term GCV therapy for an HCMV infection (Manichanh *et al.* 2001). Comparisons of the polymerase and protein kinase genes from HCMV (UL54 and UL97 respectively) and HHV-6B (U38 and U69 respectively) reveals a majority of documented GCV resistance causing mutations in HCMV have potential homologues in HHV-6B (see Tables 1 and 2, and Figures 1 and 2). Only a few reports are available regarding treatment of HHV-6 infections with GCV, PFA or CDO (Ljungman *et al.* 2000; Zerr *et al.* 2002). It is clear from Manichanh's work that HHV-6 is being exposed to these agents even though it is rarely the specific target of treatment. GCV resistance in HHV-6/7 is undoubtedly much higher than is currently documented. Multiple beta-herpesviruses resistant to GCV, PFA or CDO are a possibility when an individual undergoes prolonged treatment. A second concern, which has been shown with HCMV, is that mutant isolates can be resistant to multiple antiviral agents (Chou *et al.* 1997; Harada *et al.* 1997). This most often occurs when mutations arise in the viral encoded DNA polymerase with or without existing mutations in the viral protein kinase.

Table 1: Documented GCV^R protein kinase mutations in HCMV with homologues in HHV-6

HCMV mut.[a]	a.a. change	HHV-6 mut.	HCMV reference
M460V	Methionine - valine	M318V	Chou *et al.* 1995a
M460I	Methionine - isoleucine	M318I	Wolf *et al.* 1995a
A591D	Alanine - aspartic acid	A447D	Wolf *et al.* 1998
A591V	Alanine - valine	A447V	Chou *et al.* 1995b
C592G	Cysteine - glycine	C448G	Chou *et al.* 1995b
L595S	Leucine - serine	L450S	Erice *et al.* 1997
L595F	Leucine - phenylalaine	L450F	Wolf *et al.* 1995b
L595T	Leucine - threonine	L450T	Smith *et al.* 1997
L595W	Leucine - tryptophan	L459W	Chou *et al.* 1995b
C607Y	Cysteine - tyrosine	C463Y	Baldanti *et al.* 1998
A606D	Alanine - aspartic acid	A462D	Wolf *et al.* 1998
Del591-594	Deletion of alanine-alanine-cysteine-arginine	Del446-449	Chou *et al.* 1995b
Del595	Deletion of leucine	Del450	Baldanti *et al.* 1995

[a] mut = mutation. HCMV GCV^R protein kinase mutations are reviewed in Erice, 1999.

Table 2: Documented GCV^R polymerase mutations in HCMV with homologues in HHV-6

HCMV mutation	Amino acid change	+/- P.K.[a] mutation	HHV-6 mutation	HCMV reference
N408D	asparagine – aspartic acid	-	N364D	Smith *et al.* 1997
F412C	phenylalanine – cysteine	+	F368C	Chou *et al.* 1997
D413E	aspartic acid – glutamic acid	+	D369E	Erice *et al.* 1997
L501F	leucine – phenylalanine	+	L441F	Harada *et al.* 1997
L501I	leucine – isoleucine	+	L441I	Smith *et al.* 1997
T503I	threonine – isoleucine	+	T443I	Smith *et al.* 1997
K513R	lysine – arginine	+	K453R	Smith *et al.* 1997
K513E	lysine – glutamic acid	+	K453E	Smith *et al.* 1998
P522A	proline – alanine	+	P462A	Erice *et al.* 1997
T700A	threonine – alanine	-	T555A	Erice *et al.* 1997
L802M	leucine – methionine	+	L657M	Chou *et al.* 1997
A809V	alanine – valine	+	A664V	Chou *et al.* 1998
G841A	glysine – alanine	+	G696A	Erice *et al.* 1997

[a] P.K. = protein kinase. HCMV GCV^R polymerase mutations are reviewed in Erice, 1999.

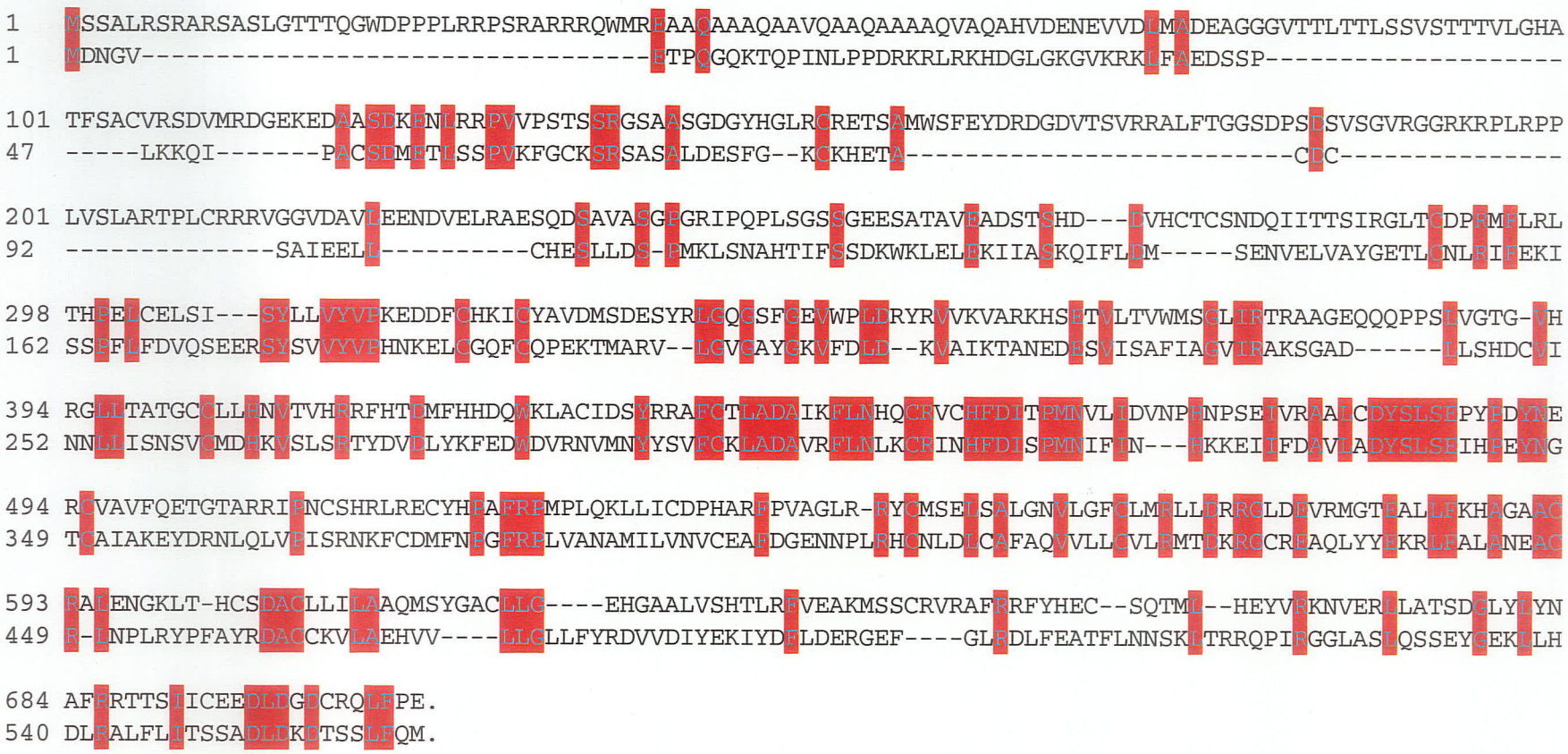


Figure 1. Alignments of the HCMV UL97 protein kinase and the HHV-6B U69 protein kinase. Upper sequence is HCMV, lower sequence is HHV-6B. Regions in red indicate homology between the two proteins

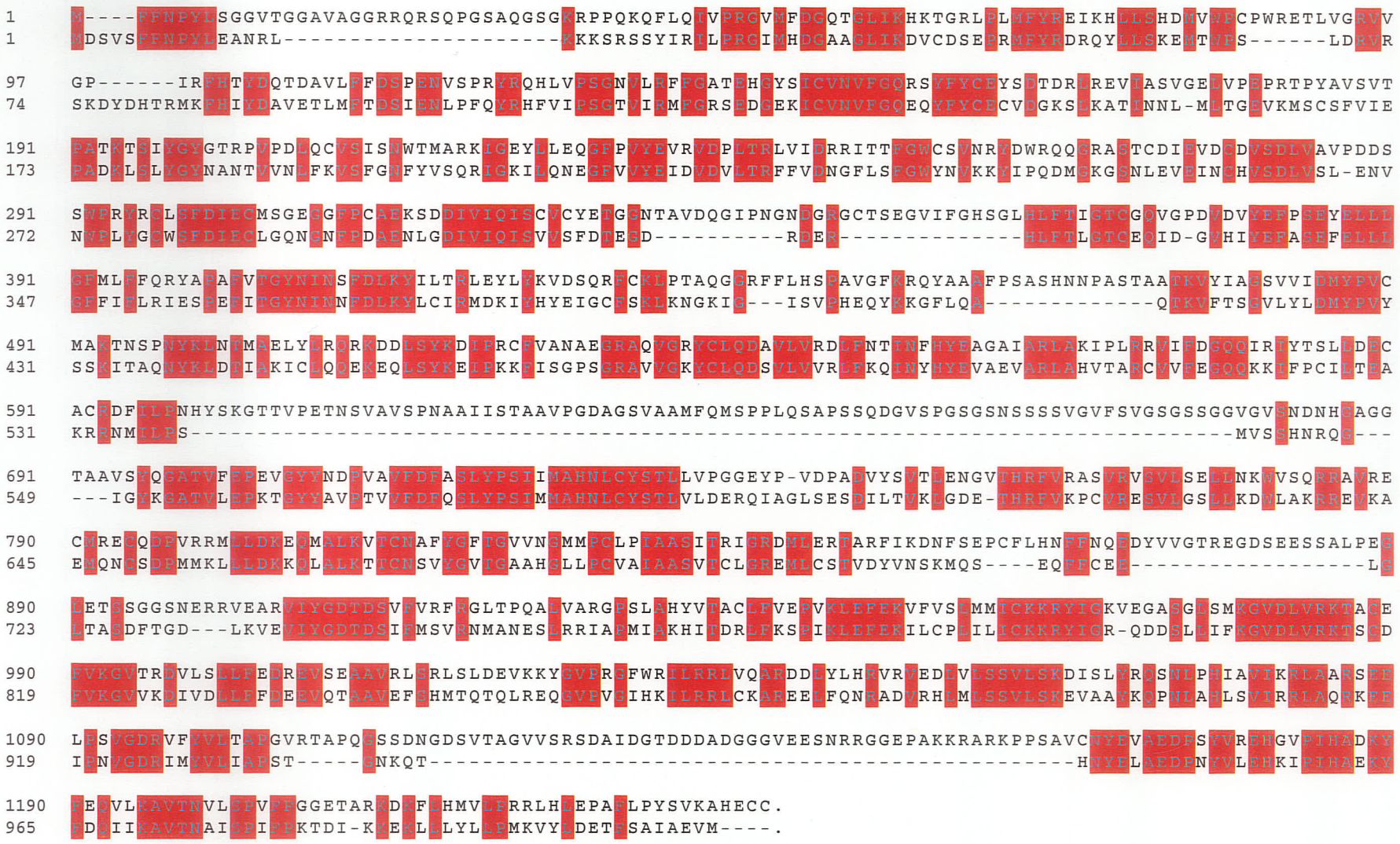


Figure 2. Alignment of the HCMV polymerase (UL54) and the HHV-6B polymerase (U38) proteins. Upper sequence is HCMV, lower sequence is HHV-6B. Regions in red indicate homology between the two proteins

Diagnosing GCV^R HHV-6/7 should be as easy as sequencing areas within which GCV resistance causing mutations most often arise. Before this is done however, GCV resistance causing mutations must first be documented and described both clinically and in the laboratory.

Objectives

1. To design a study and develop the laboratory methods necessary to define the primary disease profile (roseola) of HHV-6/7 in order to identify the most appropriate specimen (with respect to a marker of infection), which could be used in the development of a rapid point-of-care test for HHV-6/7 diagnostics.

2. To define ganciclovir resistance conferring mutations in the HHV-6 U69 protein kinase.

Hypothesis

1. A marker of primary HHV-6/7 infection (viral antigen, nucleic acid or specific antibody), present at the time of acute disease presentation, would be suitable for detection by a point-of-care diagnostic test.

2. Mutations in the HHV-6 U69 protein kinase homologous to documented ganciclovir resistance causing mutations in the HCMV UL97 protein kinase will confer ganciclovir resistance upon HHV-6.

MATERIAL AND METHODS

1. HHV-6/7 Study

The study received ethical approval from the University of Manitoba biomedical research ethics board (ethics number B2001:056) in March 2001 with protocol modifications approved in October 2001 and a request for study extension approved in March 2002. In addition, Dr. Milt Tenenbein, Director of the Pediatric E.R. Department, of the Winnipeg Children's Hospital, approved the protocol. The study ran from December 2001 until November 2002.

1.1. Target population and enrollment criteria

Children presenting to the Winnipeg Children's Hospital Emergency Department or fast track clinic were enrolled in the study according to the criteria outlined in Table 3. Children with seizures, or rashes other than those described were included.

Enrollment of patients occurred directly in the E.R. and was done as quickly as possible to reduce the stress on the infant, the parent, and the medical staff. Eligible children were initially identified by E.R. nurses or physicians who asked parents for permission to be approached regarding the study. The study staff discussed enrollment with the parents only if they were willing and after the medical staff had assessed the patient's condition and ruled out exclusion criteria. Signed, informed consent was obtained at this time and the first samples were collected. Day 0 was defined as the day of symptom onset (fever or rash).

Table 3: Inclusion and exclusion criteria for enrolling infants in the HHV-6/7 study

Inclusion criteria	Exclusion criteria
Male or female child between 3 months and 3 years of age	Bacterial illness including purulent otitis media, sepsis, meningitis, bone/joint infection, and cellulitis
Residing in the city of Winnipeg, Manitoba	Obvious viral infections including varicella, bronchiolitis, or enteroviral illness
Presenting with a documented fever $\geq$ 38.5°C or	Suspected tuberculosis or blastomycoses
History of fever and undiagnosed rash or	Congenital or acquired immune defiencies
Undiagnosed rash without fever	Malignancies
Signed informed consent by parent or legal guardian	

1.2. Study procedure

Participation in the study lasted for 21 days. Two blood samples were collected, an acute sample upon enrollment and a convalescent sample approximately 21 days later. Acute blood samples were collected by venipuncture into anticoagulant EDTA vacutainers (VWR #36-7661) using a butterfly needle apparatus. 3mL of blood was collected from each child and immediately refrigerated until further processing. Convalescent blood samples were collected by a research nurse during a home visit 3 weeks after enrollment. A total of approximately 1mL of blood was collected into 2 EDTA microtainers (VWR #365973) by way of a finger poke.

Saliva samples were collected on enrollment, the next day and every second day thereafter for 3 weeks. Parents collected all saliva samples, after instruction by study staff, using previously proven methods (Vyse *et al.* 1997). Briefly, Dacron tipped, plastic handled swabs (VWR #10805-165) were used to gently message the gums of the child for thirty seconds to one minute. The swabs were then placed in 1.5mL screw cap tubes containing 500µl of viral transport medium, VTM, (29.5g/L Tryptose phosphate broth pH 7.3, 10.9g/L Na_2HP0_4, 0.8g/L NaH_2PO_4 H_20, 5g/L Gelatin, 0.083g/L Gentamicin, 67 units/L Penicillin G, 67 units/L Nystatin) provided by Cadham Provincial Laboratory. The swab handles were snapped off and the tubes sealed and refrigerated for up to 21 days until collected by the research nurse during the home visit.

Two oral fluid samples were also collected, corresponding to the acute and convalescent blood samples, using OraSure™ oral specimen collection devices (Epitope Inc) suited for collection of salivary antibodies (Thieme *et al.* 1992). The collection pad was placed in the child's mouth, between the lower cheek and gum for 2 minutes, before

being placed in the specimen vial containing transport medium. In addition, parents filled out a patient diary everyday in which they recorded the child's temperature (taken orally with a thermometer which was provided), presence of rash, medications taken, and any other notes they felt important. At the time of the home visit, the research nurse picked up the saliva samples and diaries.

HHV-6/7 negative children enrolled in the study provided age-matched controls for the study.

1.3. Sample processing

1.3.1. Blood samples

All blood samples were processed within 24 hours of collection. 200µl of whole blood was removed and immediately stored at -80°C for DNA extraction. The remaining blood was centrifuged for 15 minutes at 600 xg, plasma removed and stored at -80°C (for antibody detection) and the remaining blood reconstituted with an equal volume of phosphate buffered saline, PBS (138mM NaCl, 1.5mM KH_2PO_4, 8mM $NaHPO_4$, 3mM KCl, pH 7.4). The lymphocytes were separated and washed using Ficol-Paque (Amersham Pharmacia Biotech #17-0840-03) as outlined in the manufacturer's instructions. Two aliquots of 10^6 lymphocytes, counted on a hemocytometer, were stored at -80°C for DNA and RNA extractions. Due to the reduced volume of the convalescent sample, whole blood and lymphocytes were not processed; only plasma was separated and stored.

1.3.2. Saliva samples

Following collection of all the saliva samples, the tubes were centrifuged at 20,000 xg for 5 minutes in a micro-centrifuge. 300µl of VTM was removed and stored at -80°C for later use while the cell pellet was re-suspended in the remaining 200µl and stored at -80°C until DNA was extracted. The OraSure™ collection devices were processed and stored according to manufacturer's instructions. Briefly, the samples were centrifuged individually through the supplied filters for 15 minutes at 600 xg and the filtrate was stored at -80°C.

Prior to the study, preliminary experiments were performed to confirm the integrity of HHV-6 in the saliva swabs after 21 days at 4°C. Healthy adults (co-workers who were PCR positive for HHV-6 DNA in their saliva that day) were asked to provide 6 saliva swabs taken one after the other, in a method identical to that used in the study. The swabs were placed in the refrigerator and each week, one sample was extracted for total DNA (see nucleic acid extractions). After 4 weeks HHV-6/7 PCR was performed (see quantitative PCR) on the extracted DNA and analyzed by agarose gel electrophoresis. To ensure the virus secretion occurred throughout the day, the same healthy adults were asked to provide 6 saliva samples from one day. These samples were processed and analyzed as above.

1.3.3. Nucleic acid extractions

Total DNA was extracted using QIAamp® DNA mini blood kits (Qiagen # 51106) from acute lymphocytes and whole blood, and saliva samples according to the manufacturer's spin protocol. Total RNA was extracted from acute lymphocytes using

RNA mini kits (Qiagen #74104), again following manufacturer's spin protocol outlined in the product insert.

1.4. Antibody detection

HHV-6 specific IgG and IgM were detected in acute and convalescent serum samples using an enzyme linked immunosorbent assay (Parker *et al.* 1993). 96 well microtiter plates were coated with a crude cell lysate of HHV-6A infected HSB-2 cells on the top half of the plate and control, uninfected, HSB-2 cell lysate on the bottom half (courtesy of Michael Gray, NML). Briefly, serum samples, or appropriate controls, were diluted 1:100 to a final volume of 500µl in PBS with 0.05%Tween 20 (PBST) and 1% BSA. Samples for IgM antibody detection were incubated for 15 minutes at room temperature with RF absorbent (Dade Behring # OUCG 15) according to the product insert. 100µl of each sample was added in duplicate to appropriately labeled wells of the plates and incubated for 1 hour at 37°C in a humidified container. Plates were washed 3 times with PBST before adding 100µl of secondary antibody, anti-human IgG or IgM antibody conjugated to alkaline phosphatase (Jackson immuno-research laboratories #s 109-035-088 and 309-035-043 respectively) diluted (according to lot number) in PBST with 1% BSA. Plates were again incubated for 1 hour at 37°C followed by 3 more PBST washes as above. 100µl of alkaline phosphate substrate, 4-Nitrophenyl phosphate (Sigma # 104-105), prepared in substrate buffer (9.7% v/v diethanolamine, 200mg/L NaH_3, 100mg/L $MgCl_2 \cdot 6H_2O$, pH 9.8) was added to each well and incubated at room temperature in the dark for 30 minutes before addition of 50µl stopping reagent (3M NaOH) to each well. The optical density was measured as the absorbance at 405nm minus 650nm. Absorbance

due to HHV-6 specific antibodies was calculated by subtracting the absorbance of HSB-2 control wells from the HHV-6/HSB-2 wells for each sample and compared to previously determined cut offs (varies with plate lots) to determine qualitative results (positive, negative or equivocal).

1.5. Virus detection

1.5.1. Qualitative PCR

DNA from all saliva, whole blood and lymphocyte samples were tested for the presence of HHV-6 or 7 specific DNA using a previously described multiplex PCR method (Johnson *et al*, 2000) with minor modifications. Briefly, final concentrations of 3mM $MgCl_2$, 1x PCR buffer (Qiagen), 0.2mM dNTPs (Invitrogen #10297-018), 0.2µM of each primer (VZV-P1 and VZV-P2 targeting the polymerase gene, see appendix 1 for primer sequences) and 2.5U Hotstar Taq (Qiagen #203205) were mixed together with 25µl water and 10µl template DNA for a total volume of 50µl. PCR was performed on MJ Research thermocyclers with cycling parameters as follows; 95°C/15min., followed by 40 cycles of 95°C/30s, 47°C/30s, 72°C/1min. with a final incubation of 72°C/5min. Post PCR samples were analyzed by electrophoresis of 10µl of product through a 1.5% agarose gel stained with ethidium bromide (EtBr, Fisher #BP1302-10) and visualization on a BIORAD gel doc apparatus. Positive samples were then digested (10µl PCR product, 1.5µl enzyme buffer, 1µl restriction enzyme and 2.5µl H_2O) with *BstUI* (NEB #RO518L) or *Bam HI* (Gibco # 15201-031) and run on an EtBr stained, 1.5% agarose gel according to the published method to identify the species of herpesvirus.

1.5.1.1. Sensitivity and specificity

Prior to testing study samples, the sensitivity and specificity of primers VZV-P1 and P2 were assessed. Serial dilutions of purified viral DNA (see preparation of standards) containing known genomic equivalents of HHV-6A, 6B and 7 were subjected to PCR as outlined above in order to determine the lower limits of detection for each virus. The specificity of the primers was assessed by running samples positive for other potential rash causing agents. These included RNA viruses (4 echovirus (E9) isolates, West Nile virus, dengue virus (serotype 2), measles virus, rubella virus, enterovirus EV 71, coxsackie viruses A-16 and B-4), DNA viruses (parvovirus B19, herpes simplex virus 1 and 2, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, human herpesvirus 8), and the bacterium Leptospira, which often presents clinically with mild, viral like symptoms. The echo, coxsackie and enteroviruses, along with appropriate confirmatory PCR primers were provided by Dr. Michael Carpenter (NML). West Nile and dengue viruses and Leptospira, along with confirmatory PCR primers were provided by Dr. Michael Drebot (NML). HHV-8 and parvovirus B19, along with B19 PCR primers were provided by Dr. Runtao He (NML). HSV 1 and 2 and the CMV cosmid library were provided by Dr. Alberto Severini (NML). A rubella isolate was provided by Brian Klisko (Cadham Provincial Laboratory). Each virus was extracted for nucleic acids (DNA or RNA) using methods outlined above. RNA was reverse transcribed to cDNA. Briefly, 5µl (corresponding to 5-10µg) of RNA was mixed with 5µl of RNase/DNase free water (Gibco #10977-015) and 1µl of random primers (Gibco #48190-011) and incubated for 10 minutes at 70°C. 8µl of RT master mix consisting of 4µl of 5x buffer and 2µl of 0.1M DTT (Invitrogen # Y00147), 1µl of 10mM dNTP mixture, and 1µl of RNase inhibitor

(Gibco #15518-012) was added to the RNA/primer mix and incubated for 10 minutes at room temperature followed by 2 minutes at 42°C. 1µl of Super Script II reverse transcriptase (Invitrogen #18064-014) was added and incubated for 50 minutes at 42°C followed by 15 minutes at 70°C. The extracted DNA, RNA and cDNA was then used as template for qualitative PCR with VZV-P1 and P2 primers along with their appropriate confirmatory primers to ensure presence of the specific agents.

The ability of the VZV P1/P2 primers to detect mixed infections with HHV-6A, 6B or 7 was also evaluated by mixing pure viral DNA at various ratios, performing PCR as outlined above.

1.5.2. Quantitative PCR

Quantitative PCR was conducted on samples testing positive by the qualitative PCR described in 1.5.1. A LightCycler based, real time PCR assay was developed for use with the VZV P1 and P2 primers. Fluorescent hybridization probes were designed to bind inside the 533 bp polymerase gene fragment generated. Two sets of probes were made, H6FL/H6LC (LCRed640) and H7FL/H7LC (LCRed705), see appendix 1, (TIB MolBiol reference #000106371) to differentiate HHV-6 and HHV-7. Further, the H6FL/H6LC probes were designed to differentiate HHV-6A from 6B by binding over a single base mismatch between the two viruses. Real time PCR was carried out using a LightCycler FastStart DNA Master Hybridization Probes kit (Roche #3003248) with each reaction having final concentrations of 3mM $MgCl_2$, 1µM of each primer (VZV-P1 and VZV-P2), 0.2µM LC probe, 0.4µM FL probe mixed with 2µl of master mix, 5.7µl sterile water and 2µl template. Cycling conditions are outlined in detail in appendix 2. PCR products were

quantified by comparison to a standard curve generated from purified viral DNA with known genomic equivalents (see 1.5.2.2)

1.5.2.1. Quantitative PCR sensitivity, specificity, and reproducibility

The sensitivity of this assay was determined by running serial dilutions of purified viral DNA with known genomic equivalents. Both sets of hybridization probes were tested against HHV-6A, 6B, 7 and VZV (the only viruses amplified by the primers) to determine their specificity. The reproducibility of the assay was tested by running each standard in 4-6 replicates and determining the variance in the crossing points for each set of standards (Technical Note No. LC 12/2000, Roche Molecular Biochemicals).

1.5.2.2. Preparation of standards for quantitative PCR

The herpesvirus nucleocapsids were isolated from HHV-6A, 6B or 7 tissue culture infected cells using published methods with a few modifications (Martin *et al.* 1982; and Straus *et al*, 1981). Briefly, 10^7 infected cells were pelleted at 500 xg for 5 minutes and re-suspended in 6mL lysis buffer (0.5% deoxycholate, 0.5% NP-40, 30mM Tris pH 7.5, 5mM $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 125mM KCl, 0.5mM EDTA, 3.6mM $CaCl_2$, 6mM 2-mercaptoethanol) and sonicated (Branson Sonifier 250) on ice at 50% power for 3 sets of 10, 1 second bursts. Cell lysis was confirmed by light microscopy on a hemocytometer. 6µl (600-1500U) of DNase I (Invitrogen #18047-019) and 6µl of 10mg/mL RNase A (Sigma #R5500) was added and the mixture was incubated at 30°C for 90 minutes with 3-6µl of DNase I added every 30 minutes. 6mL of 1,1,2-trichloro-trifluoroethane (Sigma # 27036-9) was added and mixed vigorously before centrifugation at 800 xg for 10 minutes. The top fraction was removed and ultracentrifuged (Beakmen Optima XL-100K) through a 5 to 40% glycerol gradient in lysis buffer at 15,000 xg (35

krpm) for 1 hour at 4°C. The pellet was re-suspended in 2mL 2x STEP buffer (2% SDS, 0.1M Tris pH 7.5, 20mM EDTA), added to 1mL 2x STEP buffer with 3mg/mL proteinase K (Gibco #25530-049) and incubated for 1-2 hours at 55°C. The DNA was extracted from the nucleocapsid preparation using a QIAamp® DNA blood maxi kit (Qiagen #51192) following manufacturer's instructions. Purity of the viral DNA was confirmed by checking for contaminating DNA by PCR with primers BG1/2 (see appendix 1 for primer sequences), specific for human beta-globin cellular house keeping genes, using procedures outlined above (see 1.5.1). The absorbance at 260nm was measured and the viral genome copy number was determined (outlined in Appendix 3).

1.6. Contamination prevention

Care was taken to reduce the possibility of contaminating the study samples during sample preparations. Blood separation occurred in bio-safety cabinets located in a clean room four floors away from the main laboratory. All sample extractions took place in a clean pre-PCR sample extraction room again in biosafety cabinets. A three-room approach was taken in the set up and execution of each PCR reaction. Master mix was prepared in one room, taken to the second, the sample extraction, room where aliquots of master mix were distributed and test sample templates were added before entering the final room where positive controls were added and thermocycling took place. Each room used in the sample preparation procedures was physically separated and was operated under strict clean room guidelines.

1.7. Virus propagation

1.7.1. HHV-6A

HHV-6A strain GS (gift from Dr. Phil Pellett, CDC) was propagated at 37°C, 5% CO_2 in the T-cell line HSB-2 (ATCC #CCL-120.1) in IMDM media (Sigma #I-2501) supplemented with 5% fetal bovine serum, FBS, (Cansera #CS-C08-500) and 1% penicillin-streptomycin (Wisent #30-002-CI). Infected cells were co-cultured with uninfected HSB-2 cells at a ratio between 1:20 to 1:25. Every 2 to 3 days the cells were pelleted at 500 xg for 5 minutes and re-suspended in fresh media. The infection took between 5 and 7 days. When approximately 80% of the cells showed cytopathic effect, CPE, (enlarged cells) the cells were either harvested for nucleocapsid extractions or co-cultured again.

1.7.2. HHV-6B

HHV-6B strain Z29 (Lopez *et al.* 1988; gift from Dr. Phil Pellett, CDC) was propagated at 37°C, 5% CO_2 in the T-cell line Molt-3 (gift from Dr. Phil Pellett, CDC, NIH lineage not ATCC) in RPMI 1640 media (Gibco #31800-071) supplemented with 5% FBS, 1% penicillin-streptomycin, and 200mM L-glutamine (Wisent #26-005-CI). Infected cells were co-cultured with uninfected Molt-3 cells at a 1:1 ratio, at an overall cell concentration of 0.5 $x10^6$ cells/mL. Cells were either harvested or co-cultured again when approximately 80% of the cells showed CPE, generally 3-4 days post infection.

1.7.3. HHV-7

HHV-7 strain SB (Black *et al.* 1993; ATCC # VR-1384) was propagated at 37°C, 5% CO_2 in the T-cell line Sup-T1 (ATCC #CRL-1942) in RPMI 1640 media supplemented with 5% FBS and 1% penicillin-streptomycin. Infected cells were co-

cultured at a ratio of 1:20 with uninfected cells. The media was changed every 2-3 days during the 5-7 day infection. When approximately 80% of the cells showed CPE, the cells were either harvested, or passaged into uninfected cells.

1.8. Sensitivity of RSV rapid point of care test

The limits of detection of the technology used in rapid, point of care diagnostic kits was tested using Abbott's Test Pack Immediate Care Diagnostics RSV kits (Abbott Laboratories). The RSV kits are solid phase enzyme immunoassays that detect the presence of respiratory syncytial virus antigen. Titered RSV stocks (gift from Dr. Natalie Bastein, NML) were serial diluted in sterile PBS and tested using the RSV kits following manufacturer's instructions.

2. Ganciclovir Resistance

2.1. Case study (patient's clinical history)

Early in 2002 an infant was born in distress at the Hospital for Sick Children in Toronto. The newborn presented with seizures refractory to treatment, foetal tracing suggested acute and chronic hypoxia, although biochemical tests were normal. HHV-6B was demonstrated in CSF and serum using the qualitative PCR assay outlined above. Persistence of viral DNA was observed in spite of 21 days of GCV treatment. Pre and post GCV treatment serum samples were obtained from the infant along with a serum sample from the mother collected at the same time as the infant's pre-treatment sample. Viral isolation was not attempted.

2.2. HHV-6 U38 and U69 sequencing

The HHV-6 polymerase (U38) and protein kinase (U69) genes from the infants pre and post therapy serum samples as well as the mother's serum sample were PCR amplified and sequenced using primers POL A, ABIS, A1, A2, A3, A4, A5, A-MOR, B, B1, B2, B3, B4, and B-MOR and GCVK A1, A2-B, A3, A4, B1-B, B2 and B3 (see appendix 1 for primer sequences) on ABI 3100 and 377xl sequencers using Big Dye Terminator version 3.0 Cycle Sequencing kits (ABI # 439-0246), using previously described methods (Manichanh *et al.* 2001).

2.3. HHV-6B U69 cloning

Methodologies utilized in the cloning procedures all followed standard protocols (Sambrook *et al.* 1989) except where otherwise specifically referenced. The U69 protein kinase gene from HHV-6B (strain Z29) was cloned into the pBlueBacHis2C vector (Invitrogen #V375-20) such that the protein produced would be fused to a small Xpress™ epitope using previously described primers (see appendix 1) IP1-BamHI and IP2-HindIII (Ansari and Emery, 1999).

2.3.1. U69 protein kinase insert preparation

DNA was extracted from an early passage of tissue culture propagated HHV-6B using QIAamp DNA mini kits. PCR was performed with final concentrations of 1.5mM $MgCl_2$ (Gibco), 0.2mM dNTPs, 1x PCR buffer (Gibco), 0.2μM of each of the cloning primers (IP1-BamHI and IP2-HindIII), 5U of Platinum Taq (Gibco #10966-034), 79μl water, and 2μl DNA template. The fragment was amplified in an MJ Research thermocycler with an initial incubation of 95°C/3min., followed by 30 cycles of

95°C/30s, 55°C/30s, 72°C/2min. with a final extension of 72°C/7min. Following PCR, 10µl of product was analyzed on a 1% agarose gel stained with EtBr to confirm correct band size (1.7 kb). The remaining product was cleaned up using QIAquick® gel extraction kits (Qiagen #28704) according to manufacturer's guidelines for PCR clean up. 500ng of purified PCR product was digested with 10U of *Bam HI*, re-purified, and digested with 10U of *Hind III* (Gibco # 15207-020). The appropriately digested insert was gel purified through a 1.5% low melt agarose gel and extracted using QIAquick gel extraction kits according to manufacturer's instructions.

2.3.2. Vector preparation

500ng of pBlueBacHis2C was digested with *Bam HI* and *Hind III* (creating linear vector DNA in the range of 4.8 kb) and cleaned up in the same manner as the U69 protein kinase insert. Following gel purification the vector was de-phosphorylated by adding de-phosphorylation buffer to a final concentration of 1x and 0.2µl calf intestinal phosphatase, CIP (Roche # 713 023). The mixture was incubated at 37°C for 15 minutes at which point an additional 0.2µl CIP was added and incubated for a further 45 minutes. To remove the CIP, the mixture was extracted using the PCR clean up protocol with QIAquick columns.

2.3.3. Ligations

Ligations were performed at a 3:1 insert to vector ratio in 15µl total volume. 75ng of appropriately digested insert was mixed with 25ng of digested (corrections for molecular weight differences of insert and vector were not done), de-phosphorylated vector and added to 1x final concentration ligation buffer, water, and 1µl T4 DNA ligase (Invitrogen 15224-025). Ligation reactions were carried out overnight at 14°C.

2.3.4. Preparation of electrocompetent *E. coli*

100mL of LB medium was inoculated with 1mL of an overnight culture of DH5α *E. coli* cells. The cells were incubated at 37°C with shaking at 220rpm until achieving an OD_{600} of 0.5 +/- 0.03 (typically 3-4 hours) at which point the flask was chilled on ice for 20 minutes. Cells were pelleted by centrifugation at 1,200 xg for 10 minutes at 4°C. The supernatant was decanted, the cells resuspended in 1 volume ice cold 10% glycerol in pure water and chilled again on ice for 20 minutes. A total of three glycerol washes were carried out, resuspending the cells in 10mL the second time and 400µl the third (personal communication with Dr. R.D. Gietz, modified from Chung *et al.*1989). From this, 10, 40µl aliquots of electrocompetent cells were snap frozen on dry ice, stored at -70°C and only thawed, on ice, immediately before use.

2.3.5. Transformation of competent *E.coli* cells

1.5µl of the overnight ligation mixture was added to 40µl of electrocompetent DH5α *E.coli* cells and electroporated using a BIORAD gene pulser II at 1.25kV, with 25µF capacitance and 400 ohms resistance (Dower *et al.* 1988). Immediately after electroporation, 1mL of S.O.C. media (Sigma # S-1797) was added and then incubated at 37°C with shaking at 220rpm for 1 hour. 200µl of the cultures were plated on LB agar plates containing 50µg carbenicillin (Sigma # C1389) and incubated at 37°C for 16-24 hours. From each plate 5-10 colonies were picked and grown overnight at 37°C in 5mL LB broth supplemented with 50µg/mL ampicillin.

2.3.6. Plasmid isolations

Plasmids from 5mL overnight cultures of *E.coli* cells in selective medium were isolated using QIAprep® spin mini prep kits (Qiagen # 27104) according to manufacturer's instructions.

2.3.7. Screening of recombinant plasmid

Plasmids containing the U69 protein kinase insert were confirmed three ways. First plasmids were re-digested with *Bam HI* and *Hind III* and electrophoresed through a 1.5% agarose EtBr stained gel to confirm the presence of the insert and vector bands. Second, PCR was performed (as outlined in U69 insert preparation section) across the multiple cloning site, MCS, of the plasmid using primers Bac F and Bac R (pBlueBacHis2C instruction manual, see appendix 1). 10µl of product was visualized on a 1.5% agarose gel stained with EtBr to confirm the presence of the correct band size (approximately 2 kb with insert or 356bp without) and the remaining 90µl were processed through a PCR clean up column and the U69 insert sequenced as outlined above. The resulting pBBH-U69-wt construct was compared to published wild-type sequences to verify no mutations were induced in the process of cloning.

2.4. Mutagenesis

A PCR based site-directed mutagenesis strategy was used to create five mutant constructs from the U69-wt-pBBH construct. Five mutant PCR primers were designed (U69-L213I, U69-M318V, U69-C448G, U69-L450S and U69-C463Y) each matching the wild-type U69 sequence exactly except for one base in the middle of the primer which was changed to induce the mutation at the amino acid indicated. Four more primers were

designed (*BsmI* F, *Pml* F, *Pml* R, and *Hind III* R) which flank unique restriction sites in the wild-type U69 sequence. Two rounds of PCR were used to generate a mutated fragment with unique restriction sites on both ends (Figure 3). The first round of PCR used a mutagenic primer and the appropriate reverse primer (Table 4) to create a small fragment containing the desired mutation. 100ng of the U69-wt-pBBH construct was used as template with 0.2μM of each primer, 1.5x PFU Turbo buffer (Stratagene), 2mM dNTPs, and 2.5U of PFU Turbo (Stratagene #600252). The overall volume was brought to 100μl using purified water. Fragments were amplified, after an initial incubation of 95°C/3min, with 18 cycles of 95°C/1min, 55°C/1min, and 72°C/2min, followed by a final extension of 72°C/10min. Products were gel purified in a 1.5% low melt agarose gel using QIAquick® gel extraction kits (Qiagen #28704). The second round of PCR used 25μl of the small, mutated fragment generated in the first round as the reverse primer with the appropriate forward primer and followed the same reaction and thermocycling conditions as the first round. The final product was again gel purified and double digested, first with *Pml I* (NEB #RO532S) and *Bsm I* (NEB #RO134S) or *Hind III*. Fragments were ligated back into the appropriately digested pBBH2-U69-wt construct, used to transform electrocompetent DH5α cells, and plasmids isolated all as described previously. Mutations were confirmed by re-sequencing the area synthesized in the mutagenesis procedures.

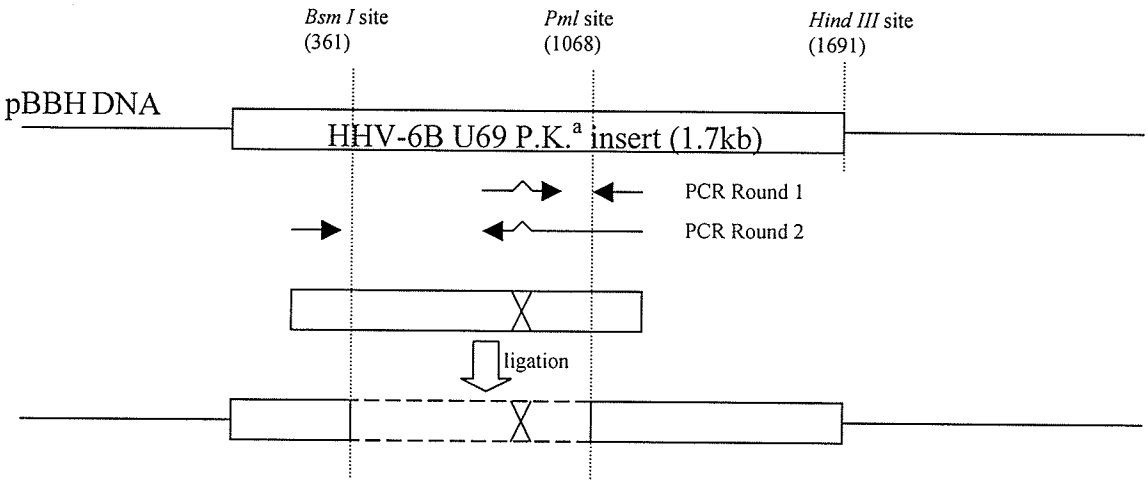


Figure 3. Basic strategy for PCR based site-directed mutagenesis. [a] P.K. = protein kinase. The numbers in brackets represent site locations in bases within the 1.7 kb HHV-6 U69 P.K. insert. The first round of PCR created a fragment (between 153 and 465 bp) containing the desired point mutation. The mutated fragment was used as reverse primer in the second round of PCR creating a section of the U69 gene, containing the appropriate point mutation, and flanked by two unique restriction sites allowing easy ligation into the pBBH-U69-wt construct.

Table 4: PCR based site-directed mutagenesis

PCR Round 1			PCR Round 2			
Forward Primer	Reverse Primer	Fragment Generated	Forward Primer	Reverse Primer	Fragment Generated	R.E. digests (pre-ligation)
U69-L213I	*PmlI* Rev	L213I-465	*BsmI* For	L213I-465	Mut213-801	*BsmI* + *PmlI*
U69-M318V	*PmlI* Rev	M318V-153	*BsmI* For	M318-153	Mut318-801	*BsmI* + *PmlI*
U69-C448G	*HindIII* Rev	C448G-432	*PmlI* For	C448G-432	Mut448-878	*PmlI* + *HindIII*
U69-L450S	*HindIII* Rev	L450S-423	*PmlI* For	L450S-423	Mut450-878	*PmlI* + *HindIII*
U69-C463Y	*HindIII* Rev	C463Y-384	*PmlI* For	C463Y-384	Mut463-878	*PmlI* + *HindIII*

For primer sequences see Appendix 1

2.5. Creating recombinant baculoviruses

2.5.1. Cell propagation

The insect ovary cell line *Spodogtera frugiperda* (Sf-9, Invitrogen # 11496-015) was propagated in complete Sf-900 media (Sf-900 media supplemented with 5% FBS and 0.5% penicillin-streptomycin, Gibco #10902-088). Cultures were maintained at a concentration of 1 x10^6 cells/mL, in spinner flasks incubated at 28°C. Viability of Sf-9 cells was determined using a trypan blue exclusion assay where 400µl of trypan blue was added to 100µl of cell suspension, mixed, 8.8µl loaded into a hemocytometer and examined by light microscopy. The total cell count was determined and the percentage of dead cells (blue in color) counted to estimate overall viability of the cells.

2.5.2. Transfections

Recombinant baculoviruses were created by co-transfecting Sf-9 cells with Bac-N-Blue™ DNA (Invitrogen # K855-01) and one of the constructs in the pBlueBacHis 2C baculovirus transfer vector (created above). 500ng of Bac-N-Blue™ DNA was mixed with 1µg of construct DNA and diluted in 100µl of Sf-900 media. In a separate tube, 9µl of CellFECTIN™ reagent (Gibco #10362-010) was diluted in 100µl Sf-900 media. The two solutions were mixed and incubated at room temperature for 45 minutes after which 800µl Sf-900 media was added. 6 well plates were seeded at approximately 40% confluency with Sf-9 cells having greater then 98% viability. After adhesion, the cells were washed once with Sf-900 media and aspirated twice to remove all the media. 1mL of transfection mixture was added to the wells and incubated for 5 hours at 28°C. For each transfection well, a mock-transfected control

well (cells only) was incubated with 1mL of Sf-900 media. The transfection mixtures were aspirated and to each well 2mL of complete Sf-900 media was added. Plates were incubated for 72 hours at 28°C at which point the supernatant (containing the baculovirus) was removed and stored at 4°C for later use in plaque purifications.

2.5.3. Plaque purifications

Six well plates were seeded to approximately 50% confluency with Sf-9 cells having greater then 98% viability. Cells were allowed to adhere to the wells (approximately 30 minutes) and were washed once with 2mL of Sf-900 media. 10^{-1} and 10^{-2} dilutions of the original transfection stocks were made in complete Sf-900 media and 400µl of each added to the freshly washed and aspirated cells. Each dilution was done in triplicate. The plates were incubated for 1 hour at room temperature with gentle rocking every 15-20 minutes before the supernatant was removed from the cells and 2mL of overlay (3 parts Sf-900 media supplemented with 10% FBS, 1% penicillin-streptomycin and 200µg/mL X-gal mixed with 1 part 2% w/v low temperature melting agarose (SeaKem ME, BMA #50010) in ultra pure H_2O and equilibrated to 40°C) added. Plates were allowed to cool before incubation at 28°C in a humidified, sealed container for 5-7 days. For each recombinant virus, 5 blue plaques (indicating the presence of a functional Lac Z gene, suggesting homologous recombination occurred) were picked, using a sterile pasteur pipette, and each used to infect 2 x10^6 Sf-9 cells, with greater then 98% viability, in 6 well plates. 2.5mL of complete media was added and the plates incubated at 28°C. On day 3, 0.5mL of supernatant was removed and screened for recombinant virus. The

remaining 2mL of supernatant was collected on day 7 and stored at 4°C. These were the P1 stocks.

2.5.4. Screening recombinant viruses

2.5.4.1. Sequence confirmation

200µl of the day 3 supernatant was extracted for DNA using Qiagen DNA columns according to manufacturer's instructions. PCR was performed using the Bac F and Bac R primers as outlined above (see 2.3.7.). Recombinant viruses containing the insert were identified and again sequenced to ensure no undesired mutations were introduced.

2.5.4.2. Protein confirmation

Recombinant U69 protein kinase expression was confirmed by immunofluorescent staining of cells infected with each of the recombinant baculoviruses. Briefly, approximately $2x10^6$ cells (1.5 days post infection) were washed three times in PBS pH7.4, and re-suspended in PBS at $1x10^4$ cells /µl. 10µl of this was spread onto a cover slip and allowed to dry (approximately 30 minutes) before they were fixed with ice cold methanol for 6 minutes. Cover slips were re-hydrated with three 5 minute PBS washes and blocked using PBS supplemented with 1% BSA. The primary antibody (Invitrogen # R910-25) was diluted at 1:1000 in PBS 1% BSA. The primary antibody was a monoclonal directed against the Xpress™ epitope fused to the recombinant protein kinase (see appendix 4). Cover slips were probed for 60 minutes at room temperature with 200µl of the primary antibody before being washed three times with PBS 0.1% BSA. The FITC-conjugated secondary antibody (Jackson Laboratories 115-095-003) was diluted 1:400 in PBS 1% BSA.

The cover slips were probed with 200µl of the secondary antibody for 60 minutes, in the dark, at room temperature. The cover slips were washed twice in PBS 0.1% BSA and once with PBS and mounted (cell side down) in 5µl glycerol on light microscopy slides. Slides were viewed on an Olympus IX70 confocal microscope.

2.5.5. Generating high titer stocks

To generate a large volume of high titer viral stocks, two rounds of viral amplification were carried out, producing passage 2 and 3 (P2 and P3) viral stocks. In both rounds flasks were seeded to 50% confluency with Sf-9 cells having greater than 98% viability. After adhering for 30 minutes the cells were washed once for 10 minutes with serum free Sf-900 media. The wash media was removed and the cells infected. P2 viral stocks were generated in T-25 flasks. Cells were infected with 400µl of P1 stocks for 1 hour at 28°C after which 2mL of complete Sf-900 media was added and the flasks incubated for 72 hours at 28°C. Supernatant was collected and stored at 4°C. P3 viral stocks were generated in T-150 flasks infected, as just outlined, with 1.5mL of P2 viral stocks diluted to 5mL in serum free Sf-900 media. After the 1 hour incubation 15mL of complete Sf-900 media was added and the flasks incubated for 72 hours. Supernatant was harvested and stored at 4°C.

2.5.6. Determining viral titers

Plaque assays were carried out to determine titers of the viral stocks. Briefly, stocks to be titered were 10-fold serially diluted down to 10^{-8} in complete Sf-900 media. 6 well plates were seeded to 60% confluency with Sf-9 cells and washed as outlined above. Wells were infected in duplicate with 200µl for each dilution and incubated at 28°C for 1 hour with gentle rocking every 15 minutes. The virus stock

was removed and replaced with 2mL of overlay (equilibrated to 40°C) consisting of Sf-900 media, supplemented with 10% FBS and 1% penicillin-streptomycin, mixed 3:1 with sterile 2% low temperature melting agarose in ultra pure water. After the overlay hardened, plates were incubated at 28°C for 5-7 days before staining with 2mL of 0.1% neutral red (sigma #N2889) in ultra pure water over night. Plaques were tallied on a light table.

2.6. Drug susceptibility experiments

2.6.1. Drug treatments

6 well plates were seeded with $1x10^6$ Sf-9 cells having greater then 98% viability. The monolayer was washed once and infected at an M.O.I. of 0.3 with 200µl of one of the recombinant baculoviruses. The viruses were allowed to attach for 1 hour at 28°C with gentle rocking every 15 minutes. The infection medium was removed and replaced with 1.5mL of complete Sf-900 media supplemented with ganciclovir to a final concentration of 0.5mM, 0.75mM, 1.0mM, or 1.25mM. As controls a mock infected well and an infected well with no ganciclovir were included in each plate. The virus containing supernatant was harvested after 72 hours incubation at 28°C.

2.6.2. Effects of the ganciclovir

2.6.2.1. Plaque reduction assays

Virus titers in the supernatant following the drug treatments were determined via plaque assays as outlined above (see 2.5.6.).

RESULTS

HHV-6/7 Study:

Method Validation

Antibody detection

The ELISA methods used in this study were previously validated for use on serum samples by technicians (Michael Gray and Michael Garbutt) in the National Microbiology Laboratory. In order to use the methods appropriately on saliva samples we needed to surpass a critical number of samples in order to properly validate the test for the new sample. The samples collected in the study were far from the amount needed to achieve this. Analysis of salivary antibodies could therefore not be performed.

Qualitative PCR

10-fold dilutions of quantified, pure viral DNA standards demonstrated the PCR assay was able to reproducibly detect a lower limit of 10 viral copies for HHV-6A, HHV-6B, and HHV-7 (Figure 4A). The only other rash causing agent to test positive with the qualitative PCR assay was, as expected, VZV. The rest of the agents tested were positive when tested with the appropriate confirmatory primers and negative with the multiplex assay, indicating a high degree of specificity. The 4 viruses amplified with the primers (HHV-6A, 6B, 7 and VZV) were reliably differentiated with *Bam HI* and *Bst UI* digestion (Figure 4B). When DNA from HHV-6B and HHV-7 were mixed, both were amplified equally and easily distinguishable with restriction enzyme analysis (Figure 4C).

Quantitative PCR

The sensitivity of the LightCycler based real-time assay matched that of the qualitative PCR assay (Figure 5). Due to increased variability of the crossing point at the lowest dilution (10 viral DNA copies) the assay could accurately quantify between 100 and 1,000,000 viral DNA copies. The two sets of hybridization probes (H6 and H7) allowed for differentiation of HHV-6A, 6B and 7 using melting point analysis (Figure 6). Neither set of hybridization probes cross-reacted with the other viruses or VZV. Both sets of probes were highly reproducible with the crossing points for each set of standards varying from 0.5% to 3.2% (compared to 0.4% to 2.7% for Roche's published LightCycler assays) (Figure 7). The quantitative assay was also able to reliably detect 2 fold differences in viral DNA concentration (Figure 7).

Preparation of PCR standards

The nucleocapsid isolations and extractions reliably yielded pure viral DNA as determined by the absence of cellular DNA in PCR analysis specific for human house keeping genes. 10^7 tissue culture infected cells resulted in 1 to 1.5mL of 10^8-10^9 viral DNA copies/mL

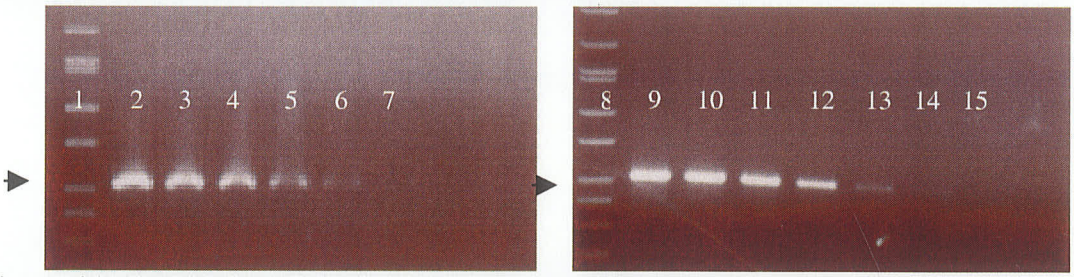


Figure 4A. Sensitivity of qualitative HHV-6/7 PCR. Presence of the 534 bp band of the polymerase gene of HHV-6B and HHV-7 demonstrates the assay can detect as low as 10 viral copies. Lanes 1, 8 = 100 bp molecular weight markers. Lane 2 through 6 = HHV-6B 100,000 to 10 DNA copies respectively. Lanes 9 through 14 = HHV-7 1,000,000 to 10 DNA copies respectively. Lanes 7 and 15 = no template controls. ▶ indicates 500 bp.

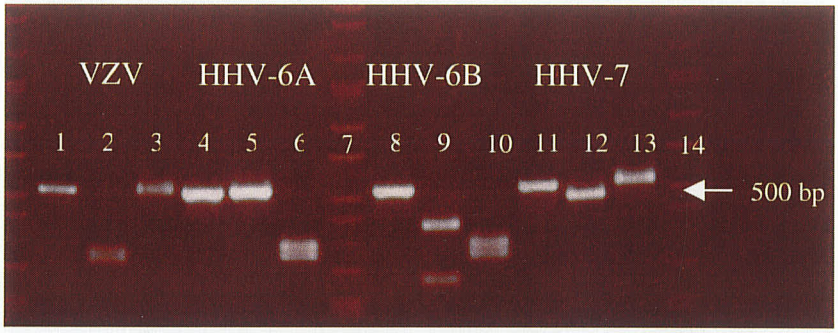


Figure 4B. Restriction digests differentiating VZV, HHV-6A, HHV-6B and HHV-7. For each virus: Lanes 1, 4, 8, 11 = uncut; Lanes 2, 5, 9, 11 = *Bst UI* digest; Lanes 3, 6, 10, 13 = *Bam HI* digest; Lanes 7, 14 = 100 b.p. molecular weight markers

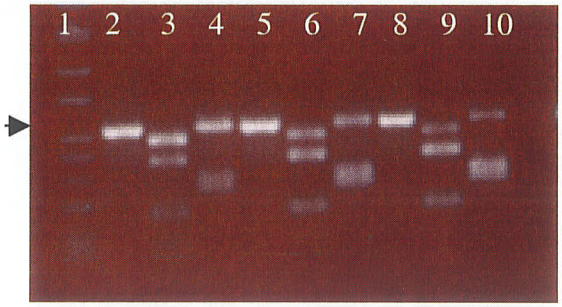


Figure 4C. Co-amplification of HHV-6B and HHV-7. Digestions of mixed HHV-6B and 7 mixed infections. Lane 1 is 100 b.p. molecular weight markers. ▶ = 500 bp
Lanes 2-4 are mixed 1:5, lanes 5-7 are 1:1 and 8-10 are 5:1 with HHV-6B - HHV-7 respectively. (Lanes 2, 5, 8 = uncut. Lanes 3, 6, 9 = *Bst UI* digets. Lanes 4, 7, 10 = *Bam HI* digests).

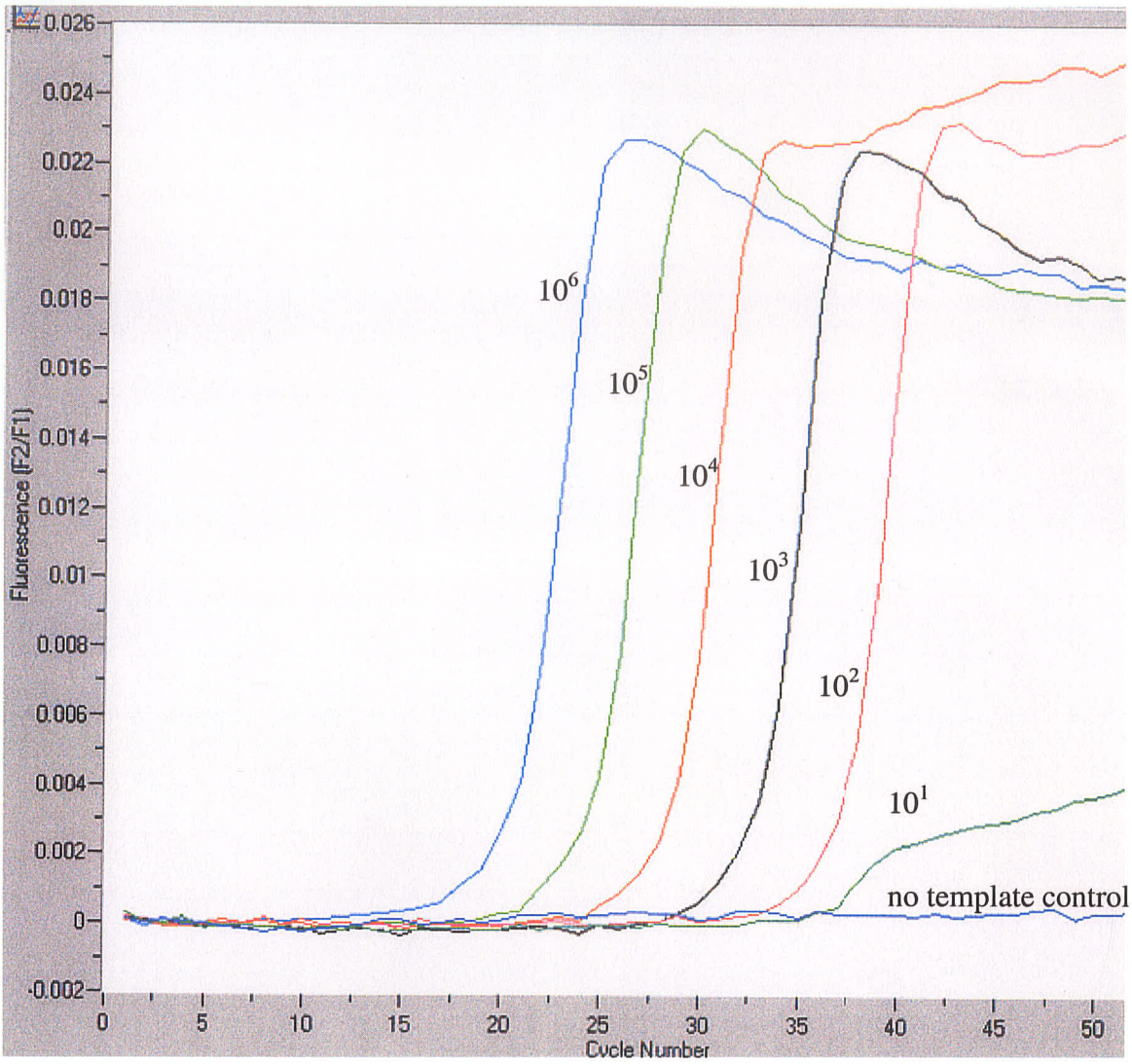


Figure 5. Standard curve of purified HHV-6B DNA. Quantification on the LightCycler was achieved by running pure viral DNA standards, created by extracting DNA from the nucleocapsid preparations, along side of every sample.

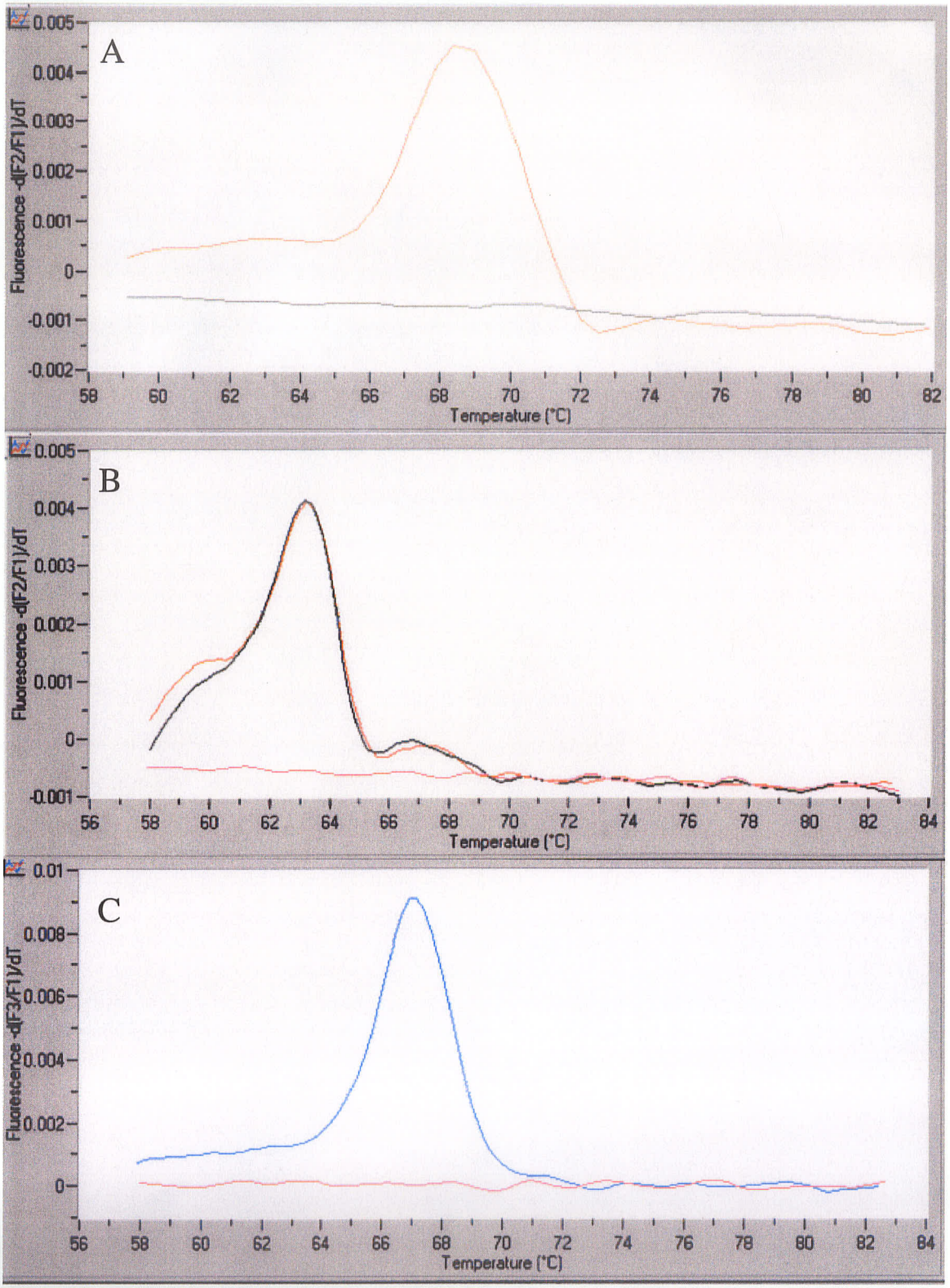


Figure 6. Real-time differentiation of HHV-6A, 6B, and 7. HHV-6A (A) and HHV-6B (B) detected with hybridization probes H6 FL/LC read on filter F2/F1. Melting points = 68.5 and 63°C respectively. HHV-7 (C) detected with hybridization probes H7 FL/LC read on filter F3/F1. Melting point = 67°C.

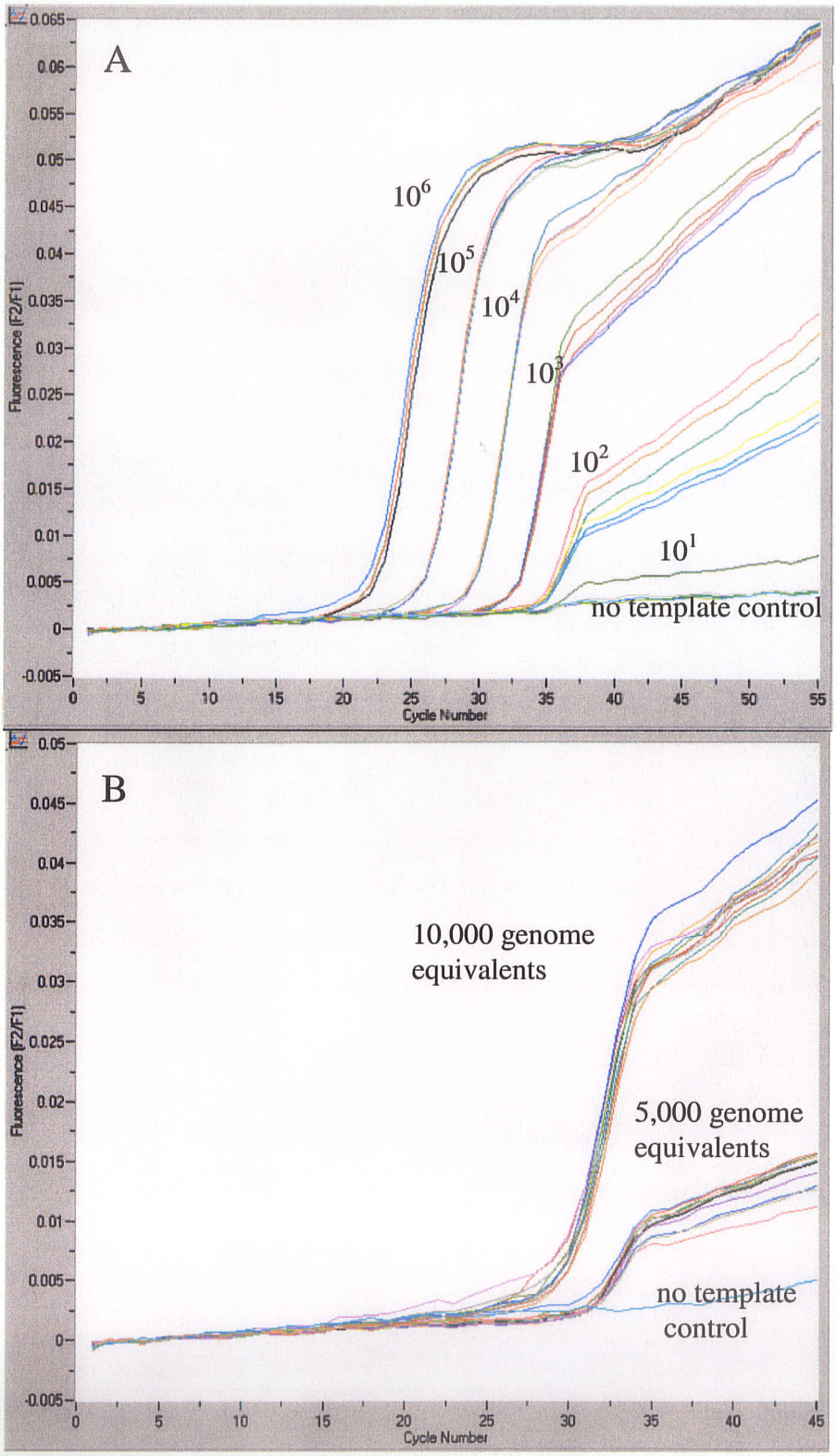


Figure 7. Reproducibility of the quantitative HHV-6/7 PCR assay. (A) Reproducibility of the standard curve showing the dynamic range of quantification between 10^2 and 10^6 viral copies. (B) Detecting 2-fold differences in input viral DNA samples.

HHV-6/7 Study Results

Enrollment

Over the enrollment period lasting just short of one year, December 2001 through November 2002, approximately 20 infants were identified to the study staff as potential participants for the roseola study with a total of 5 enrolling in the study.

Collection

Acute sample collection was done in hospital and provided the study with a sufficient quantity of high quality samples. The acute blood sample consistently yielded 200µl of whole blood, 500µl plasma, 2 aliquots of lymphocytes at 10^6 cells, and an extra aliquot of lymphocytes. Acute saliva swabs by the parents (in the hospital) yielded good quality saliva specimens as determined by PCR analysis with house keeping and virus specific primers. Acute Orasure™ saliva samples were not always collected due to restlessness of the infant after collection of blood. Specimens following the acute samples were not as consistent in regards to quality, volume, or timing. The finger poke method of collecting blood did not result in high volumes. Initially we aimed for an equivalent volume to that of the acute sample, however, the volume of convalescent blood collected rarely exceeded a 200-300µl. One convalescent blood coagulated before delivery to the laboratory (faulty collection tubes) resulting in only 4 paired serum samples collected. The schedule for collecting saliva swabs at home by the parents was generally not followed. Only one parent collected the complete set of samples with the average of the remaining four parents collecting 3-4 (or approximately one third) saliva samples over

the 3 weeks. The samples at home did not yield as high of DNA concentrations as those collected in the hospital.

Preliminary experiments confirmed virus specific DNA did not decrease substantially in saliva swabs stored at 4°C for up to 1 month as determined with quantitative PCR (data not shown). A second set of experiments showed that virus specific DNA was detected in all 6 samples collected at various time points throughout the day (data not shown).

Patient samples (Figure 8 and Table 5)

Patient 001

Symptoms: Patient 001 was a 7 month old infant who presented to the pediatric E.R. with classical symptoms of roseola. The infant had a three day history of fever with a highest recorder temperature of 38.9 °C. Upon presentation to the E.R. the infant had the characteristic rash of roseola all over the body which lasted for three days.

Laboratory results: Serology on the acute and convalescent serum samples demonstrated the presence of IgM in the acute sample and IgG seroconversion from equivocal in the acute to positive in the convalescent sample. Qualitative PCR performed on the acute whole blood and PBMCs showed the presence of HHV-6B (although the PBMCs were only weakly positive). LightCycler PCR confirmed the presence of HHV-6B and determined the viral load in the whole blood to be 7 $x10^3$ genomes / mL. The viral load in the PBMCs was below the dynamic range for the LightCycler assay thus it could not be quantified. Presence of HHV-6B in the saliva samples colleted from the infant, both in the E.R. and at home, was demonstrated by both qualitative and quantitative PCR analysis. The viral load was determined to be 7.5 $x10^3$ genomes / swab.

Diagnosis: The laboratory evidence (IgM positive, IgG seroconversion and PCR detection of HHV-6B in the blood) supports the E.R. diagnosis of roseola for this infant.

Patient 002

Symptoms: Patient 002 was a 7 month old who presented to the pediatric E.R. with a three day history of fever. The peak temperature recorder was 38.8 °C. The infant never acquired a rash.

Laboratory results: The convalescent blood sample from patient 002 coagulated before arrival to the laboratory thus it could not be used for serology. The acute serum sample was negative for IgM and was positive for HHV-6 specific IgG antibodies. Qualitative PCR performed on the acute whole blood and PBMC samples was negative for the presence of HHV-6A, 6B, and 7. Quantitative (LightCycler) PCR was not performed on these blood samples. Qualitative PCR demonstrated the presence of HHV-6B in the saliva samples collected (both in the E.R. and at home) from this infant. LightCycler PCR confirmed the presence of HHV-6B and quantified the average viral load in the saliva at 8.4×10^3 genomes / swab (with a range from 2.2×10^2 to 2.8×10^4 genomes / swab).

Diagnosis: The E.R. did not diagnose this infant with roseola. Laboratory evidence supports this diagnosis and indicates the infant had acquired HHV-6B previously (IgG positive and HHV-6B in the saliva). Patient 002 was later diagnosed with bacteremia and was placed on appropriate antibiotic therapy.

Patient 003

Symptoms: Patient 003 was a 17 month old who presented to the pediatric E.R. with symptoms indicative of classic roseola. The infant had a three day history of fever, with a peak recorder temperature of 38.4 °C. Upon enrollment the infant had the characteristic roseola maculopapular rash which lasted for two days. Earlier in the week of enrollment, during the febrile stage, patient 003 was diagnosed by a physician as having an ear infection and sent home.

Laboratory results: Serology on the acute and convalescent serum samples was IgM positive and showed IgG seroconversion for HHV-6 specific antibodies. Qualitative PCR performed on the acute whole blood and PBMCs showed the presence of HHV-6B (although the PBMCs were only weakly positive). Quantitative PCR confirmed the presence of HHV-6B and concluded the viral load in the whole blood to be 7.5×10^3 genomes / mL. The viral load in the PBMCs was below the dynamic range for the LightCycler assay and thus could not be quantified. Saliva samples obtained in the E.R. and at home were positive for HHV-6B by qualitative and quantitative PCR analysis. The viral load in the saliva was quantified at 7.5×10^3 genomes / swab.

Diagnosis: The clinical diagnosis of roseola is supported by the laboratory findings (IgM positive, IgG seroconversion and PCR detection of HHV-6B in the blood).

Patient 004

Symptoms: Patent 004 was a 15 month old who presented with classic roseola symptoms. There was a three day history of fever (peak temperature not recorded) and a rash was present at the time of presentation in the E.R., which lasted for three days.

Laboratory results: Serology on both the acute and convalescent serum samples was negative for the presence of HHV-6 specific IgM or IgG antibodies. Qualitative PCR performed on the acute whole blood and PBMC samples and every saliva sample taken was negative for the presence of HHV-6A, 6B, and 7.

Diagnosis: This infant was diagnosed in the E.R. with roseola. Laboratory findings do not support the diagnosis of roseola and indicate the infant has not previously been exposed to HHV-6 or 7 (no detectable serum antibodies or virus in any sample).

Patient 005

Symptoms: Patient 005 was a 24 month old who presented to the pediatric E.R. with a three day history of fever (unrecorded). Just prior to presentation the infant underwent a seizure. The infant never developed a rash.

Laboratory results: Serology performed on both the acute and convalescent sera were negative for the presence of HHV-6 specific antibodies. Qualitative PCR performed on the acute whole blood and PBMC samples as well as all the saliva samples were negative for the presence of HHV-6A, 6B, and 7.

Diagnosis: This infant was not diagnosed with roseola. Laboratory findings support this diagnosis. In fact, molecular and serological evidence indicates this two year old has never been infected by HHV-6 (lack of specific serum antibodies, and no detectable virus in blood or saliva). Patient 005 underwent further testing, the results of which the study staff did not receive.

Table 5: Summary of results from the infants enrolled in the HHV-6/7 study

			Serology				PCR (acute samples only)						
			Acute		Convalescent		Qualitative			Quantitative			
patient	age[a]	E.R. diag.[b]	IgM	IgG	IgM	IgG	WB[c]	PBMC[d]	Sal[e]	WB	PBMC	Sal	Lab diag.
001	7	roseola	+	+/-[f]	+/-	+	+	w.+[g]	+	$7x10^3$	n.q.[h]	$7.5x10^3$	roseola
002	7	f.n.f.[i]	-	+	n.d.[j]	n.d.	-	-	+	nd	nd	$8.4x10^3$	p. inf.[k]
003	17	roseola	+	+/-	+	+	+	w.+	+	$7.5x10^3$	n.q.	$7.5x10^3$	roseola
004	15	roseola	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.i.[l]
005	24	f.n.f.	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.i.

[a] age = age in months; [b] diag = diagnosis; [c] WB = whole blood; [d] PBMC = peripheral blood mononuclear cells; [e] Sal = saliva; [f] +/- = equivocal; [g] w.+ = weakly positive; [h] n.q. = non quantifiable; [i] f.n.f. = fever no focus; [j] n.d. = not determined; [k] p.inf. =past infection; [l] n.i. = never infected (not roseola, not past infected).

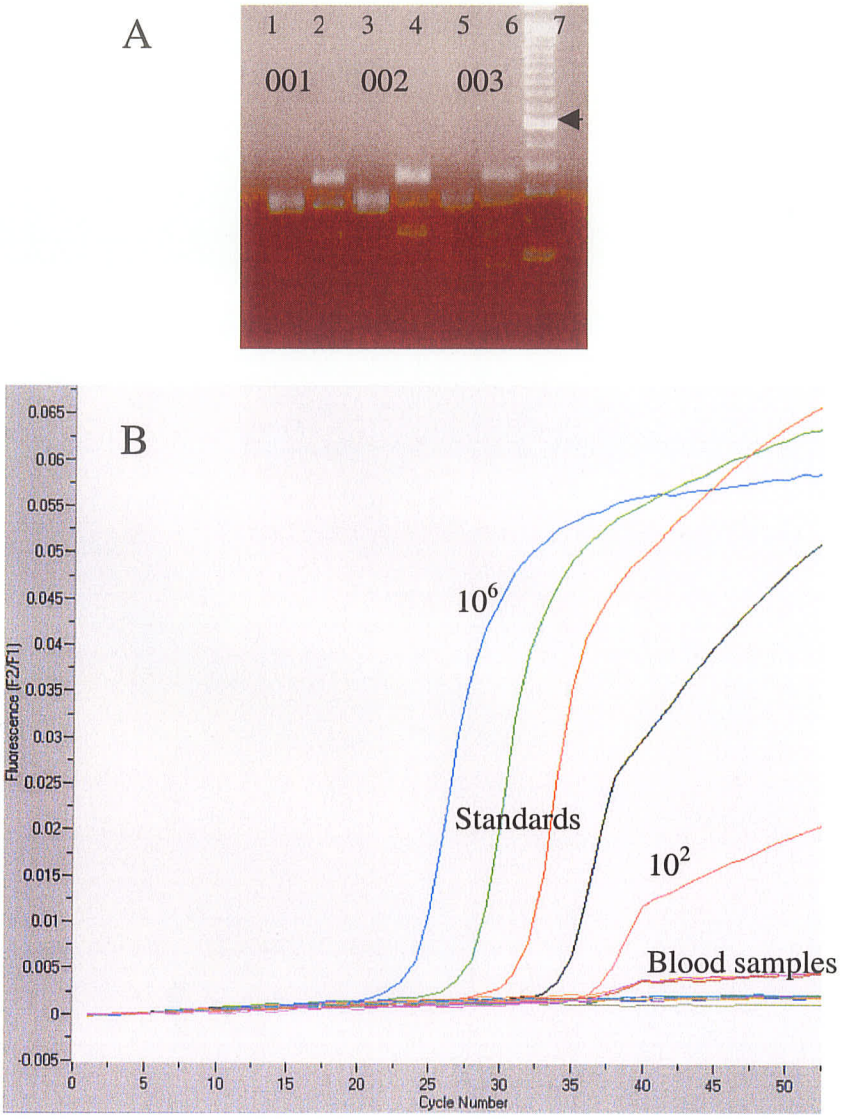


Figure 8. PCR analysis of samples from infants enrolled in the HHV-6/7 study. (A) Restriction enzyme analysis of post PCR saliva samples from infants 001, 002 and 003. Odd lanes = *Bam HI* digestion. Even lanes = *Bst UI* digestion. Lane 7 = 100 bp molecular weight markers. ◀ = 500 bp
(B) Quantification of blood samples from patients 001 and 003.

Sensitivity of rapid point of care tests

In order to determine the sensitivity of the technology used in current point-of-care tests, the lower limit of detection of such a test was determined. 10-fold serial dilutions of the quantified RSV stocks demonstrated Abbott's Test Pack: Immediate Care Diagnostics RSV kits were able to detect virus down to between 1 $x10^4$ and 1.5 $x10^4$ plaque forming units per sample.

Ganciclovir Resistance:

U38 and U69 sequencing (case study)

The sequence of the HHV-6 U38 (polymerase) gene from the mother's and infant's samples matched the published wild-type sequence (strain Z29, gene bank accession #AF157706) exactly. Sequencing performed on the HHV-6 U69 protein kinase gene of both the pre and post GCV therapy samples from the infant revealed the presence of a nucleotide transversion (specifically an c636 a base change) resulting in a L213I amino acid substitution.

HHV-6B U69 cloning

The U69 protein kinase gene from HHV-6B was cloned into the baculovirus transfer vector pBlueBacHis2c creating the pBBH2-U69-wt construct. Following transformation of *E.coli* with pBBH2-U69-wt, all plasmids isolated demonstrated the presence of the U69 protein kinase insert (1.7 kb) and vector (4.8 kb) band upon re-digestion with *Bam HI* and *Hind III*. PCR across the MCS yielded a single band of approximately 2 kb corresponding to the presence of the U69 insert. Subsequent

sequencing determined that no mutations were introduced during the process of amplifying and cloning the gene.

Site directed mutagenesis

The mutagenesis procedures resulted in 5 mutant constructs (pBBH2-U69-L213I, pBBH2-U69-M318V, pBBH2-U69-C448G, pBBH2-U69-L450S and pBBH2-U69-C463Y). Sequencing confirmed the presence of the desired mutation.

Generation of recombinant baculoviruses

On average the transfections yielded a titer of approximately 10^3 virus particles / mL. The pBBH2-U69-wt construct along with 4 of the 5 mutant U69 protein kinase constructs (pBBH-U69-L213I, pBBH-U69-M318V, pBBH-U69-C448G and pBBH-U69-C463Y) recombined such that all the plaques from the purification step were blue (indicating all of the recombination events were homologous). The transfections resulted in the creation of recombinant baculoviruses BV-U69-wt, BV-U69-L213I, BV-U69-M318V, BV-U69-C448G, and BV-U69-C463Y. Several plaques from each were picked and processed (PCR and sequencing) to ensure the presence of the correct insert. Plaque purification of the L450S transfection yielded no blue plaques. Transfection and plaque purification were carried out twice for the pBBH2-U69-L450S construct. In both cases no blue plaques were seen and further analysis ruled out the presence of the U69-L450S protein kinase or any other insert. BV-U69-L450S was therefore not created.

After two rounds of viral amplification with each of the 5 recombinant baculoviruses and one native virus (AcMNPV with no insert), plaque assays revealed the viral titers for each exceeded 10^8 viral particles / mL.

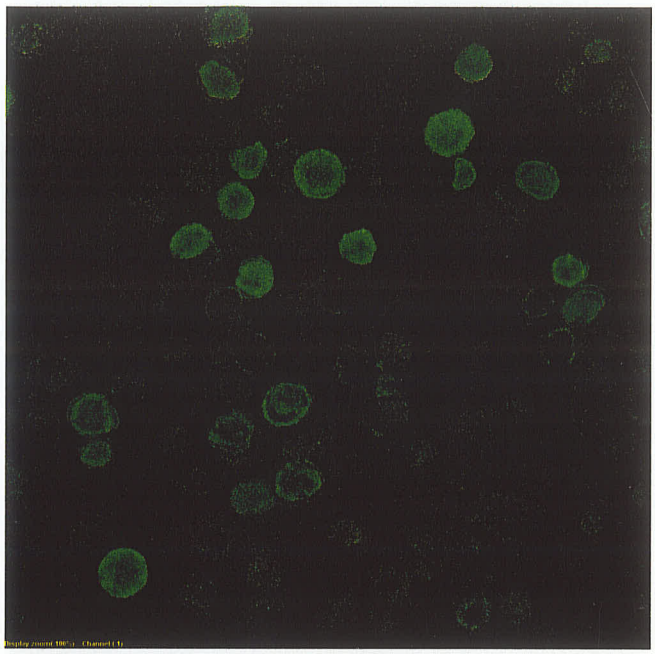
Protein confirmation

Staining Sf-9 cells (with a monoclonal antibody against the Xpress™ epitope) infected with the recombinant baculoviruses indirectly demonstrated the presence of the expressed recombinant protein (U69 protein kinase). Sf-9 cells that remained uninfected were not stained by the antibodies (Figure 9)

Ganciclovir (GCV) susceptibility

The titers of the recombinant baculoviruses BV-U69-wt and BV-U69-L213I (containing the wild-type and L213I mutant protein kinase inserts) were drastically affected by the presence of GCV (Figure 10). The 50% inhibitory concentration (IC_{50}) for each was approximately 0.6 to 0.625mM GCV. The titers of the native virus, AcMNPV (no insert), BV-U69-M318V, BV-U69-C448G, and BV-U69-C463Y (containing the M318V, C448G, and C463Y mutant protein kinase inserts) were unaffected by the presence of GCV (Figure 10). The IC_{50} for these viruses was between 1.85 and 2.0mM GCV.

A



B

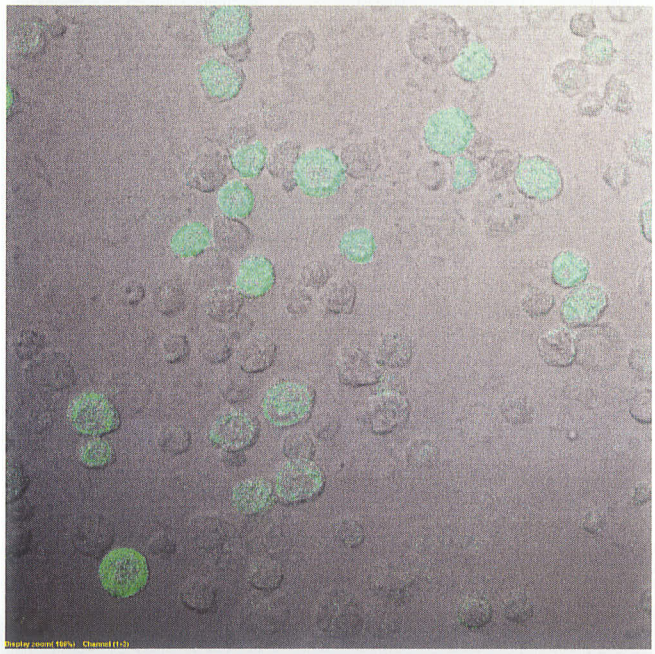


Figure 9. Confocal imaging of Sf-9 cells infected with recombinant baculoviruses. Florescent cells indicate the presence of the recombinant protein production in baculovirus infected cells. Cells not infected do not demonstrate the presence of the protein. (A) Cells viewed under a fluorescent microscope. (B) The same cells viewed under a light microscope with the fluorescent cells superimposed.

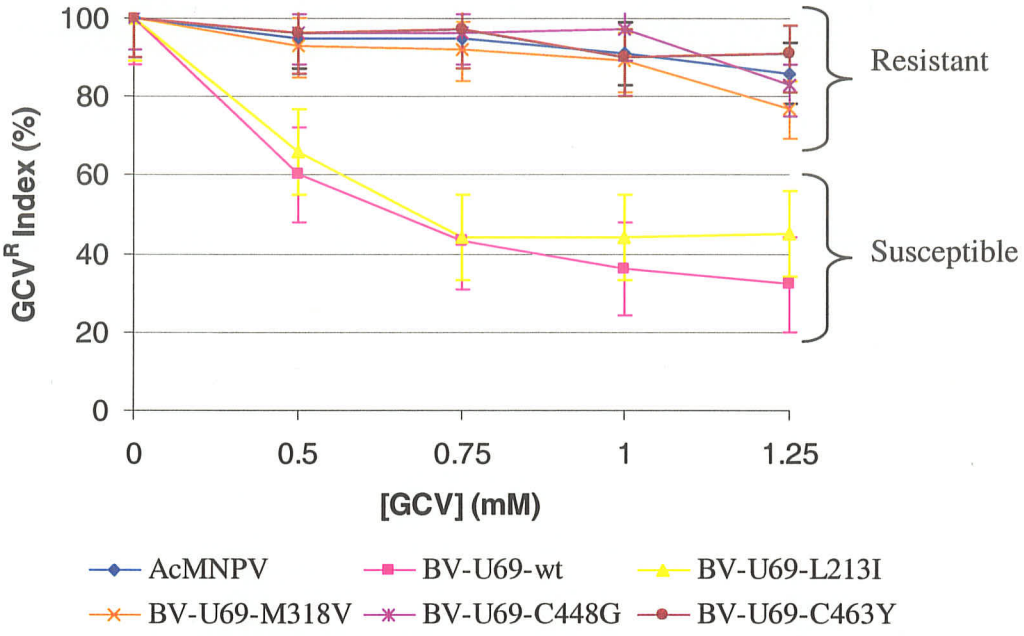


Figure 10. Effects of GCV on HHV-6 U69 protein kinase recombinant baculovirus titers.
GCV^R Index (%) = treatment titer / non-treatment titer

DISCUSSION

HHV-6/7 Study:

Method validation

The collection methods used in the study performed well. The strength of the study would have improved if the viral load in the convalescent blood sample could have been determined as it had been done in the acute sample. This was not possible due to the drastically reduced amounts blood collected by finger poke. It would be beneficial to change the method of collection of the convalescent blood sample to venipuncture. However, this would likely have a negative impact on enrollment and/or follow-through with the whole study.

The swab method of saliva collection proved to be effective. Viral loads detected in the sample taken in hospital were comparable to those stored for up to 21 days at 4°C. Quantitative PCR analysis showed the viral DNA was not degraded. The only direct problem of the method was the inability to quantify viral load against volume of saliva. The collection strategy with respect to home collection of saliva seemed to fail with these 5 patients. Relying on parents to remember to take the swabs every second day, and, more importantly, the quality of samples collected by parents was problematic. PCR analysis with primers for house keeping genes demonstrated some swabs contained little to no human DNA indicating a poor quality swab. However, neither is cause to abandon the technique. If the study had reached its target size (approximately 300 infants) there would have been enough quality swabs covering each of the days over the three weeks to prove the presence or absence of virus. Alternative methods using strips of filter paper to collect saliva from individuals have been described with comparable results as our

method. One study found this to be an efficient and easy method of collecting saliva which allowed for trouble free DNA extraction and later detection of HHV-6 and 7 specific DNA (Zerr *et al.* 2000).

The samples collected using OraSure™ saliva collection devices were not analyzed because the ELISA method could not be evaluated for detecting salivary antibodies. Therefore, it is impossible to comment on the samples collected. This method of sample collection may not be suitable for infants though, due to the time needed to collect a complete sample. Infants often became restless before the sample was even half collected.

Both qualitative and quantitative PCR methods developed and/or adapted for use in this study were effective. The previously described qualitative method proved to be as sensitive (detected down to 10 genomes) as reported by Johnson *et al.* (2000). This multiplex PCR method is advantageous because it allows for the detection of more than one potential causative agent. Its high specificity and sensitivity make it a useful diagnostic tool. To adapt the qualitative PCR assay to be quantitative, we designed hybridization probes which bound to the amplicon generated by the multiplex primers (Johnson *et al.* 2000). The result was an equally sensitive (reliable detection of 10 genomes) and specific real-time PCR assay on the LightCycler which allowed quantification of the viral target. Quantification was achieved by comparing the crossing point (a point set automatically by the LightCycler which defines a sample positive when the signal generated by the probes exceeds it) of samples to those generated by the standards. The last few years have seen an explosion of publications describing real-time PCR methods. By December 2002 there were at least 15 publications detailing real-time

PCR techniques for the detection of human herpesviruses on the LightCycler. Several have been published for HHV-6 or 7 (Aritaki *et al.* 2001; Fernandez *et al.* 2002; Gautheret-Dejean *et al.* 2002; Locatelli *et al.* 2000; Ohyashiki *et al.* 2001) but none that differentiate HHV-6A, 6B and 7. The real-time PCR assay we developed allows for that differentiation. By using two sets of probes, we easily differentiated HHV-6 from HHV-7. Melting point analysis was used to differentiate HHV-6A from HHV-6B. Because the probes bound over a single base mismatch, they denatured from the viral template at different temperatures. Since a signal from the probes is only detectable when they are bound to the viral template (and hence in close proximity), we were able to differentiate HHV-6A from HHV-6B by monitoring the temperature at which the probes denatured from the template. Another improvement with this assay is in the DNA standards used. Unlike most assays described to date (which use a plasmid containing the target sequences), purified viral DNA was used to create the standard curve. The nucleocapsid preparations (although more time consuming to prepare) provide a standard identical to the target sample. The amplification efficiency of the standards and the samples are closer than if a plasmid standard was used, thus improving quantification (Niesters 2001; MacKay *et al.* 2002). Plasmid DNA tends to have a higher amplification efficiency which could skew the standard curve and result in lower apparent viral loads (amplification efficiencies of standards and samples should be virtually equal to one another). Although the real-time PCR assay performs well in the laboratory further evaluation using a large clinical panel is needed to fully evaluate this diagnostic method for HHV-6/7.

For the roseola study, a case of primary infection was defined by seroconversion between the acute and convalescent serum samples. An obvious problem with the study

design was the inability to detect seroconversion for HHV-7. Although, it is possible that strong immune responses against HHV-7 may be weakly detected (due to cross reaction with HHV-6) using the HHV-6 ELISA. Good serological methods, for example assays based on using purified HHV-6A, 6B, or 7 recombinant antigens, would allow for serological differentiation of HHV-6A, 6B and 7. Although the development of such recombinant antigen ELISAs are not in the scope of this master's project, the Viral Exanthemata program at the National Microbiology Laboratory is working towards development of such assays.

Patient results

Of the five infants enrolled in the study two had primary infections with HHV-6B (as determined by HHV-6 specific antibody seroconversion), two were uninfected and susceptible to infection with HHV-6/7 (as determined by the absence of HHV-6 specific antibodies) and one had previously been infected with HHV-6B (as determined by the presence of serum IgG specific for HHV-6 and HHV-6B in the saliva).

The two infants with primary HHV-6B infections (001 and 003) demonstrated the presence of virus in their blood and saliva upon presentation in the E.R. The finding of HHV-6B in their saliva is contrary to other studies which have suggested the absence of HHV-6 in the saliva early in infection (Clark *et al.* 1997). Quantitative LightCycler PCR confirmed the presence of HHV-6B in the saliva at 7.5 $x10^3$ genome / swab. Other studies have shown healthy adults have on average 4.8 $x10^3$ DNA equivalents of HHV-6 / mL of saliva (Fujiwara *et al.* 2000). Unfortunately the method of saliva collection

employed in this study does not allow for precise measurement of the amount of saliva obtained from the infant.

Blood samples, whole blood and PBMCs, from the infants with laboratory confirmed HHV-6 primary infection were also, as expected, positive for HHV-6B (although surprisingly the PBMCs were only weakly PCR positive). LightCycler analysis confirmed the presence of HHV-6B in the whole blood (on average 7.25 $x10^3$ genome / mL). Past studies have shown viral levels of 2.4 $x10^4$ genomes per million PBMCs during primary infection (Clark *et al.* 1997). It is difficult to directly compare these concentrations since the unit denominators are different (per mL and per 10^6 PBMC). The fact that we were unable to quantify the amount of virus in the infants PBMCs is most likely due to our extraction procedures. Although one million PBMCs is the lowest concentration of cells suggested for extraction using Qiagen DNA columns, high levels of viral DNA are expected. It is possible that our initial quantification of the PBMCs on a hemocytometer was inaccurate. One way around this would be to extract as many PBMCs as the sample provides, then quantify (using real-time PCR and hybridization probes) a human house keeping gene against which the samples could be standardized. This method has been shown to work well (Ohyashiki *et al.* 2000). Standardization of results in this manner also allows for easier data analysis and comparison, a point lacking in our techniques as seen in viral quantification in saliva and whole blood. Serum PCR was not attempted in this study because it is an unlikely specimen for a rapid point-of-care test (due to the requirement of processing unlike with whole blood or saliva). In addition, the remaining sera and samples from the study were to become a panel of

diagnostic specimens used to evaluate other laboratory diagnostic tests, and therefore sample volumes were precious.

The three patients who were not undergoing primary infection with HHV-6/7 had no virus detectable in their whole blood or PBMC samples. For two of the infants (004 and 005) this was expected because they had no HHV-6 specific antibodies in their serum (suggesting they had not yet been infected by the virus). Saliva samples from these two infants were also negative. The lack of detectable virus in the infant who had been previously infected (002) was unexpected, although not impossible. Past studies have detected 1.6 $x10^3$ HHV-6 genomes per million PBMCs of individuals with past infection (Clark *et al.* 1997). Thus, we had expected to detect the virus but at lower levels than those undergoing primary infection. HHV-6B was easily detectable in every saliva sample taken from this infant. This was not surprising since saliva is believed to be the transmission route of HHV-6B (the virus is frequently detected in saliva of healthy adults, years after primary infection). Real-time PCR confirmed the presence of HHV-6B in the saliva samples of patients with evidence of past HHV-6 infections at levels marginally higher than the infants undergoing primary infection (8.4 $x10^3$ genomes / swab compared to 7.5 $x10^3$ genomes / swab when undergoing primary infection), although we don't have a sufficient sample size to make conclusions regarding the statistical significance.

HHV-6/7 study summary

Enrollment in the study was much lower than anticipated. Original estimates were that 3-5 infants per night fitting the inclusion/exclusion criteria would be seen in the

pediatric E.R. If even the lower estimate was true there should have been just over 1000 potential infants over the duration of our study. However, 20 infants, representing 2% of the projected population, were identified to the study staff as fitting the enrollment standards. Of the 20 identified, 5 infants (or 25%) took part; showing parents were enthusiastic and willing to enroll their infants in this study. Initial estimates were that between a quarter to a third of parents would be willing to participate.

The disappointing enrollment is due in part to at least three factors. The first is the title given to the study. In the E.R. this was known as the "roseola study". To most people, medical staff included, the medical condition known as roseola is synonymous with fever and rash. However, 30% of roseola cases present with classic roseola symptomology; the remainder are atypical presentation or asymptomatic. Although the focus of the study was roseola, by labeling it "the roseola study" the prospective enrollment population was biased towards fever and rash. In doing so the study potentially lost a large portion of the target population (and because these were the cases often misdiagnosed they represented a very important component of the study).

A second factor in the poor enrollment may have been the exclusion criteria. It is possible that in the E.R. setting, attending medical staff were more willing to provide a presumptive diagnosis of bacterial associated illness, draw the appropriate specimen from the infant (for laboratory confirmation), prescribe antibiotics and send the infant home (ironically a point this study ultimately hoped to reduce). Asking medical staff to differentiate between bacterial and viral causes of fever or rash cases may have contributed to the low number of eligible infants seen each night. It was never the intention of the exclusion criteria to rule out everything except those cases which had a

high probability of being primary HHV-6/7 infections. For example, inflammation and redness in the ears (indicative of an ear infection) seemed to be a common cause of exclusion (as many as 7 omitted in one night). It was only intended that those with purulent (a sign of a bacterial caused ear infection) otitis media be excluded; however in actuality, a red ear drum inevitably meant the study staff would not be informed about the infant. The inclusion criteria were broad enough that most if not all infants having a primary infection with HHV-6/7 would have been ruled in.

A third factor is to ensure E.R. staff are fully involved in planning and development of the project from the beginning to ensure better awareness of the clinical study once initiated.

It is impossible to draw conclusions from a study with a sample population of 5. However, there are some points worth thinking about. Detection of HHV-6B in the saliva of both primary infected and past infected infants (at virtually the same levels) suggests it is an unsuitable specimen for use in a HHV-6/7 diagnostic test. One potential target specimen for a rapid point-of-care test may be whole blood. In previously infected individuals, the virus level in PBMCs appears to be very low. During an infection viral levels should rise significantly. If the sensitivity of a test was in between these levels one could use whole blood (perhaps in the form of a finger or heel poke) to determine infection. PBMCs alone may work but whole blood offers two advantages in it requires no processing (like the separation of PBMCs) and the viral levels would be higher since virus in the serum of infected individuals would also be present. Although our data is insufficient to make any definitive conclusions in this regard, it is certainly worth considering for further study.

A second important point illustrated in this study is that misdiagnosis of HHV-6/7 primary infections is ongoing. Of the five infants enrolled, three were diagnosed with roseola based strictly on age and symptoms. One of these infants did not seroconvert nor have any molecular evidence of HHV-6/7. This indicates the infant was misdiagnosed with roseola. In a second case, one of the infants with a laboratory confirmed case of roseola was diagnosed with an ear infection earlier in the week of enrollment. The apparent concurrent ear infection was diagnosed but the roseola diagnosis was missed. Although the sample numbers are low, it still shows diagnosis of HHV-6/7 in infants needs improvements.

There is a current push by health organizations to evaluate the potential of oral fluids to replace more invasive samples, like blood, for diagnosis, epidemiology and evaluating immune levels (Nokes *et al.* 2001). As far back as the early 1990's groups in the United Kingdom were studying the use of salivary antibodies in diagnosing rubella, measles, and mumps (Ramsay *et al.* 1998; Perry *et al.* 1993). ELISA techniques have been utilized in detecting salivary antibodies to other herpesviruses (Sarid *et al.* 2001). Unfortunately with the lack of samples seen in this study analysis of salivary antibodies could not be done. Although it appears saliva is unlikely to be used as a sample in PCR diagnostic approaches, the use of salivary antibodies as a marker of primary infection requires further investigation.

To make inferences into the potential design of a rapid point-of-care test, the available technology needed to be evaluated. Abbott's RSV rapid test is a frequently used point-of-care test. Evaluation of the Abbott RSV test using titered tissue culture grown RSV stocks demonstrated the kit has a lower limit of detection in the range of 10^4 viral

particles. According to the samples tested in this study, the current technology for point-of-care tests is not sensitive enough for detection of HHV-6/7. However, due to the small study population, this needs further investigation and as such a point-of-care test for HHV-6/7 should not dismissed.

The goal of the HHV-6/7 study was to develop and evaluate laboratory methods in order to define a marker of primary infection suitable for use with a rapid diagnostic test. Several diagnostic methods were developed and evaluated in the laboratory. In the end none of them were fully evaluated. With only five patients enrolled, and of those only two positives for primary infection with HHV-6B, we cannot comment on the disease profile of primary infection. Without the clinical samples we cannot fully evaluate the diagnostic methods developed. We therefore cannot definitively say which, if any, are the most suitable HHV-6/7 markers for use in a rapid point-of-care diagnostic test.

GCV Resistance in HHV-6B:

Study results

GCV^R mutations in the HCMV UL97 protein kinase have historically been studied using a recombinant vaccinia virus system (Metzger *et al.* 1994; Zimmermann *et al.* 1997). Experiments with recombinant vaccinia viruses have shown questionable efficacy in studying the HHV-6 U69 protein kinase interactions with GCV (De Bolle *et al.* 2002). Despite use in studying the HCMV UL97 protein kinase, it is compromised by the finding that GCV tri-phosphate has a weak inhibitory effect on the vaccinia virus polymerase (St. Clair *et al.* 1980). The HHV-6 U69 protein kinase is 10-fold less

effective at phosphorylating GCV when compared to its HCMV homologue (De Bolle *et al.* 2002). This observation may explain why the vaccinia virus system is not practical for the study of HHV-6 U69 protein kinase. The baculovirus system has also been used to study the HCMV UL97 protein kinase (Baek *et al.* 2002b.).

The baculovirus system performed well for the evaluation of potential GCV^R mutations in the HHV-6B U69 protein kinase. Comparison of the titers of recombinant virus, containing wild-type or mutated forms of the HHV-6B U69 protein kinase, grown in the presence of GCV demonstrated that the M318V, C448G and C463Y mutations confer GCV^R while wild-type and the L213I mutation retains GCV susceptibility. The susceptibility of BV-U69-L213I (essentially a wild-type U69 protein kinase due to the polymorphic nature of amino acid 213) to GCV demonstrates that introduction of non GCV^R mutations into the system does not affect the recombinant protein function resulting in a false GCV^R effect. Each of the recombinant baculoviruses produced wild-type or mutated proteins that were stably expressed (in that they did not lose the mutations or entire U69 inserts). Overall, the baculovirus method is well suited for studying protein kinase mutations and their effect on GCV susceptibility.

Flow cytometry based evaluation is fast becoming the method of choice for evaluating antiviral susceptibility in part due to decreases in time and labor (reviewed in McSharry 1999). This method was attempted (data not shown) with the baculovirus system however, due to antibodies against the U69 protein kinase and baculovirus antigens not being available this method was not pursued. A second method, which we did not attempt in this study, used to speed up results of antiviral testing is real-time PCR. Both methods have been utilized for studying the effects antiviral on HHV-6 (Amjad *et*

al. 2001; Stranska *et al.* 2002). Although plaque reduction assays (the gold standard) were used in these experiments, future work should consider both of these methods to determine viral titers.

HHV-6 GCV resistance summary

This is only the second report dealing with GCV^R in HHV-6B (Manichanh *et al.* 2001). Using the baculovirus system three GCV^R mutations were identified in the HHV-6B U69 protein kinase (one previously documented in HHV-6, M318V, and two new GCV^R mutations, C448G and C463Y). All three mutations have homologues in HCMV which have been well documented to cause GCV^R (reviewed in Erice 1999). This confirms our hypothesis that mutations in the HHV-6 U69 protein kinase homologous to those documented in the HCMV UL97 protein kinase confer GCV^R.

It is not surprising that the mutations resulted in decreased susceptibility to GCV. The M318V mutation has been previously documented to cause GCV^R in HHV-6 (Ansari & Emery 1999). It is homologous to a common HCMV GCV^R mutation (M460V) which is located in a conserved region of the protein required for its kinase activity (Michel *et al.* 1999). The C448G mutation in HHV-6 lies in the middle of an ACR motif. The homologous motif in HCMV been shown to be essential for GCV phosphorylation (Sullivan *et al.* 1992). Although the region around C463Y GCV^R mutation is not as well characterized, the mutation has been seen in at least six clinical isolates of HCMV (Erice 1999).

The L213I mutation found in the infant's pre and post GCV therapy sample does not confer GCV^R as measured in the baculovirus system. This finding is not entirely

surprising since the mutation was present before the introduction of GCV in the infant. Using antiviral agents to control HHV-6 infections in infants is not well defined. The failure of the infant to respond to GCV therapy may have been caused by the therapeutic dosage or strategy rather than the infecting virus itself. Comparison of the U69 gene from at least 7 different isolates of HHV-6B suggests that amino acid 213 is actually a rare case of a polymorphism in HHV-6 (Manichanh *et al.* 2001). The strains analyzed contained either a leucine or isoleucine residue at amino acid 213.

The function of the HCMV UL97 protein kinase, and the HHV-6 U69 homologue, in the viral lifecycle is unknown. Both proteins have been shown to act as serine/threonine specific protein kinases capable of phosphorylating themselves and other proteins (Ansari & Emery 1999; Michel *et al.* 1998; 1999). However, autophosphorylation has recently been shown not to be required for phosphorylation of exogenous proteins (Baek *et al.* 2002b). It is also known that natural nucleosides are not substrates of the HCMV UL97 protein kinase (Michel *et al.* 1996), suggesting nucleoside kinase activity is not the protein's chief function. Recently, histone H2B (as well as H3 and H1) was identified as a specific target for phosphorylation by the HCMV UL97 protein kinase (Baek *et al.* 2002a). The importance of this interaction in the viral lifecycle remains to be proven. Other reports have shown the function of the viral protein kinase is required for viral replication in tissue culture systems (Prichard *et al.* 1999). It has been suggested the protein kinase plays a role in viral DNA synthesis, viral assembly, and nucleocapsid egress from the nucleus (Wolfe *et al.* 2001). Inhibitors of the protein kinase are effective anti-HCMV agents in tissue culture (Michel *et al.* 1996). These

findings have encouraged a new direction for potential antiviral agents against the viral protein kinase itself (Baek *et al.* 2002a; Marschall *et al.* 2001).

The field of antiviral agents is constantly expanding as more details of viral lifecycles are elucidated. Although currently only a few antiviral agents are recommended for use against the beta-herpesviruses, research has identified several more viral inhibitors which may become the antiviral agents of choice in the future. Fomivirsen (a phosphorothioate oligonucleotide), several benzimidazole riboside compounds (including BDCRB, TCRB, and others), A771726 (the active metabolite of the anti-inflammatory drug leflunomide), synadenol and synguanol (the Z-isomers of 2-hydroxymethylcyclopropylidenemethyl adenine and guanine respectively) have all been shown to inhibit different stages of viral replication (Baldanti *et al.* 2002; Biron *et al.* 2002; Chulay *et al.* 1999; Perry & Balfour, 1999; Waldman *et al.* 1999 a, b;). Resistance in HCMV to some of these compounds has already been observed and is due to UL97 protein kinase mutations (Biron *et al.* 2002). The efficacy of these agents against HHV-6/7 has not been addressed nor has their clinical value against any of the beta-herpesviruses.

HHV-6/7 have been shown to be highly prevalent in immunocompromised hosts. Studies have demonstrated between 28% and 75% (median 48%) of bone marrow recipients and up to 82% (median 32%) of solid organ transplant recipients have evidence of HHV-6 infections or reactivations (reviewed in Clark, 2002). Regardless if HHV-6/7 or HCMV is the specific target of antiviral therapy, these viruses are all being regularly exposed to GCV and other inhibitory agents. Due to this, GCV^R is well documented in HCMV (reviewed in Erice, 1999). GCV^R in HHV-6 is most likely under estimated. The

importance of defining potential GCV^R mutations in the U69 protein kinase is only now being seen with the realization of the effects HHV-6 has in immunocompromised hosts (Clark, 2002; Ljungman, 2002). In order to diagnosis GCV^R isolates of HHV-6, the GCV^R causing mutations first need to be defined and documented in the laboratory, as we show here for the HHV-6 U69 protein kinase. Mutations in the HHV-6 polymerase also need evaluation as they are more severe due to their ability to cause multi-antiviral resistance (as seen in HCMV).

The next step with the mutations we described would be to evaluate how they cause GCV^R. Although this is not required for diagnostic purposes it is important to understand the mechanisms of resistance for future development of antiviral agents. Experiments focusing on the activity of the mutant proteins with respect to how well they phosphorylate GCV should be performed. The beauty of the baculovirus system is the proteins can know be easily purified allowing such experiments to be carried out.

The HHV-6A and B protein kinases share 94% amino acid identity (De Bolle *et al.* 2002), however autophosphorylation of the HHV-6A protein kinase is less than that of the HHV-6B protein kinase. (Ansari & Emery 1999). Although only subtle differences exist between the proteins, this work should be carried on the HHV-6A (and HHV-7) protein kinase to determine what, if any, differences these mutations have.

Final Conclusions

Diagnostics for HHV-6/7 still need significant improvements and standardization. The diagnosis of HHV-6/7 primary infection in the E.R./clinic using a rapid point-of-care test would be very useful. Although ultimately not fully successful with the initial goals,

our "roseola study" was useful in that a new real-time LightCycler PCR assay was developed which is the first reported method to simultaneously quantify and differentiate HHV-6A, 6B and 7. In addition, important study design/protocol issues came to light which will help in the future development of similar roseola disease profile studies. Lastly, the data collected here suggests whole blood may be considered a potential sample for developing a rapid point-of-care test for HHV-6/7.

With respect to the diagnostics of drug resistant HHV-6, we have successfully characterized HHV-6 protein kinase mutations conferring GCV resistance. Understanding the development of drug resistant HHV-6 strains is important for the use and monitoring of antiviral therapy.

Appendix 1

Table 6: Oligo sequences

Oligo	Sequence 5'→ 3'	Purpose
VZV P1	GTCGTGTTTGATTTTCAAAGTTTATATCC	HHV-6/7 PCR
VZV P2	ATAAACACACAATCCGTATCACCATAAATAACCT	HHV-6/7 PCR
H6 FL	AACATAATCCACCGTGGAACAAAGCATCX	HHV-6 probe
H6 LC	CTCTTCCAAGACACGTTACAGAAGCAGCp	HHV-6 probe
H7 FL	TTACAGGTCCAACATGCACAGTGAGAATGX	HHV-7 probe
H7 LC	CATCTGCATGTAAACCAATTACTGCATTTCp	HHV-7 probe
BG-1	CAACTTCATCCACGTTCACC	HKG[a] primer
BG-2	GAAGAGCCAAGGACAGGTAC	HKG primer
U69 1	TCGGACAATGCGGAACACCA	U69 seq[b]
U69 2	AGTAAACATGACACAAGCGAACAA	U69 seq
GCVK A1	ATGAAACTGTCGATGCC	U69 seq
GCVK A2-B	CAAATTCCGTTTGTATGGATC	U69 seq
GCVK A3	TGGCAATGTATGCGAGGC	U69 seq
GCVK A4	GTTGTTCTTGATCACTTCTTCTGC	U69 seq
GCVK B1-B	CGACCTGAGCAAGGC	U69 seq
GCVK B2	CCGAGAACTCGAGCCATAG	U69seq
GCVK B3	CCTGTCTGGTGGCAAATTTATCG	U69 seq
POL A	TAGACAGGATCAGGTATAGAGG	U38 seq
POL ABIS	CAGGTATAGAGGTTATAAGAG	U38 seq
POL A1	GATGTTAACCGGCGAGGTT	U38 seq

Oligo	Sequence 5'→ 3'	Purpose
POL A2	GACGGCGTGCATATATATG	U38 seq
POL A3	GGGATAGGTTACAAAGGGGC	U38 seq
POL A4	GCATCTTTATGTCTGTCAG	U38 seq
POL A5	TGGAGTTTTCTCACATGACAC	U38 seq
POL A-MOR	CAGACGCATAACTATGAATTA	U38 seq
POL B	CATGTACAAACGACAAAACCT	U38 seq
POL B1	CGCAAGAAGTCTTTCTCACC	U38 seq
POL B2	CTATAACACAGATTATGCGCC	U38 seg
POL B3	GGTAAATCTTATCCATCC	U38 seq
POL B4	GTGTTGGCATTGTACCCATAC	U38 seq
POL B-MOR	CATTACCTCTGCAATAGCAG	U38 seq
IP1-*BamHI*	gatcgatggatccgaataattATGGACAACGGTGTGGA	U69 cloning
IP2-*HindIII*	tgcagtcaagctttccattactataTCACATCTGAAAG	U69 cloning
Baculo F	TTTACTGTTTTCGTAACAGTTTTG	MCS[c] PCR
Baculo R	CAACAACGCACAGAATCTAGC	MCS PCR
U69-L213I	GGTGTTTGATATAGATAAAGTGGCC	Mutagen[d]
U69-M318V	CGATATCTCACCT**G**TGAATATCTTTATAAATC	Mutagen
U69-C448G	GCTAACGAGGCC**G**GTCGATTGAATC	Mutagen
U69-L450S	GGCCTGTCGAT**C**GAATCCTCTTAG	Mutagen
U69-C463Y	CAGGGACGCTT**A**CTGTAAAGTATTGG	Mutagen
BsmI For	GCTTTGTCACGAGTCGCTTTTAG	Mutagen
PmlI For	GTTAGCTGATGCTGTAAGGTTTCTA	Mutagen

Oligo	Sequence 5'→ 3'	Purpose
PmlI Rev	TGGCACAAGTTGAAGATTTCTG	Mutagen
HindIII Rev	CACCAGGGAAACTTCAAGGA	Mutagen

[a]HKG = house keeping gene (Beta globin); [b]seq = sequencing; [c]MCS = multiple cloning site; [d]Mutagen = mutagenesis. In oligos IP1-*Bam HI* and IP2-*HindIII* the under case nucleotides indicate where the primer does not bind the target sequence, the nucleotides in red are the respective restriction enzyme sites. Bolded nucleotides in oligos U69-L213I, U69-M318V, U69-C448G, U69-L450S, and U69-C463Y are the positions were the primers do not match the U69 sequence (the changed bases introduce the desired mutations).

Appendix 2

Table 7: LightCycler PCR program parameters

Parameter 1: Pre-incubation

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	Segment 1
Target Temperature (°C)	95
Incubation Time (mins:sec)	10:00
Temperature Transition Rate (°C/s)	20.0
Acquisition Mode	None

Parameter 2: Amplification

Cycle Program Data	Value		
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target Temperature (°C)	95	47	72
Incubation Time (s)	10	15	20
Temp. Transition Rate (°C/s)	20.0	20.0	2.0
Acquisition Mode	None	Single	None

Parameter 3: Melting Curve Analysis

Cycle Program Data	Value		
Cycles		1	
Analysis Mode		Melting Curve	
Temperature Targets	Segment 1	Segment 2	Segment 3
Target Temperature (°C)	95	56	85
Incubation Time (s)	20	20	0
Temp. Transition Rate (°C/s)	20.0	20.0	0.2
Acquisition Mode	None	None	Continuous

Parameter 4: Cooling

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	Segment 1
Target Temperature (°C)	40
Incubation Time (mins:sec)	0:30
Temperature Transition Rate (°C/s)	20.0
Acquisition Mode	None

Fluorescence Parameters:

When using the probes for HHV-6 (H6 FL and H6 LC Red 640) choose fluorescent channel F2 for detection during a run and fluorescent channel F2/F1 for data analysis after the run has finished.

When using the probes for HHV-7 (H7 FL and H7 LC Red 705) choose fluorescent channel F3 for detection during a run and fluorescent channel F3/F1 for data analysis after the run has finished.

Appendix 3

Figure 11. Sample calculation: Weight of one genome of HHV-6B

Background information:

HHV-6B genome (or molecule) = 162114 bp or 162.114 kb

1 kb of DNA = 6.6×10^5 g/mole

g = [# molecules x molecular weight (g/mole) / 6.02×10^{22} (molecules/mole)]

1 A_{260} unit of dsDNA = 50μg /mL H_2O
For pure DNA: $A_{260} / A_{280} \geq 1.8$ (< 1.8 indicates contamination, with protein for example)

The calculation:

Molecular weight of HHV-6B= 162.114kb x 6.6×10^5 g·mole^{-1}/kb= **1.07×10^8 g/mole**

Therefore the mass of 1 molecule (genome equivalent) of HHV-6B =

g = [1 x 1.07×10^8 g/mole / 6.02×10^{22} (molecules/mole)]

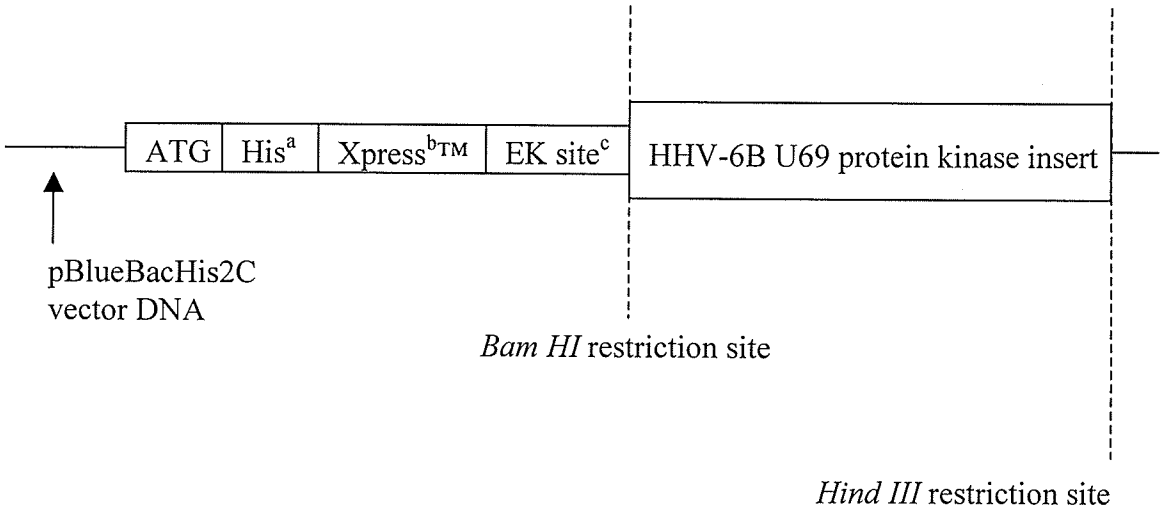
= **1.78×10^{-15} g / molecule**

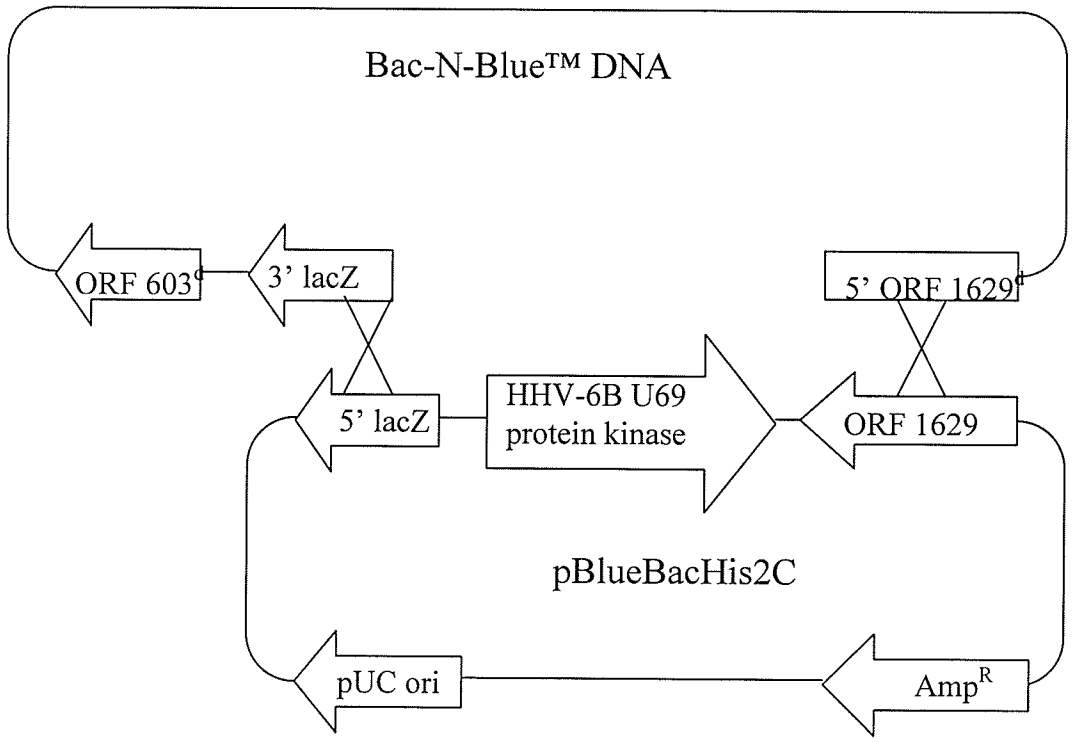
1 g = 10^{15} fg

Therefore the mass of one genome equivalent of HHV-6B = **1.78 fg**

Appendix 4

Figure 12. Overview of HHV-6B U69 protein kinase cloning and recombination





[a]His = 6x His Tag. [b]Xpress = Xpress™ epitope. [c]EK site = enterokinase cleavage site.
[d]ORF 603 and 1629 = open reading frames of the baculovirus system.

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