Seroprevalence, Molecular Epidemiology and Immunological Aspects of Human Herpesvirus 8 in Ireland

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except where duly acknowledged in the text. Full and informed consent was obtained from human subjects.

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Summary

Human herpesvirus 8 (HHV-8) seroprevalence and molecular epidemiology vary both geographically and between sub-populations. Recent work has highlighted the importance of proinflammatory cytokines in the pathogenesis of HHV-8 infection. The purpose of this study was to determine the seroprevalence of HHV-8 in an Irish population, including specific risk groups, to examine the molecular epidemiology of HHV-8 in an Irish population by characterising four regions of the HHV-8 genome, K1, T0.7 (K12), open reading frame (ORF)75 and K15, and to perform cytokine analysis over time in a population with known human herpesvirus 8-related disease and thereby to investigate the role of proinflammatory cytokines in the diagnosis and monitoring of HHV-8-related disease. Cytokine levels were also compared between HHV-8 subtypes to investigate whether a relationship exists between subtype and immunological response.

A cross-sectional seroprevalence study of 200 blood donors and 200 Genitourinary Medicine (GUM) and Infectious Diseases (ID) clinic patients was performed. All 200 blood donor samples were negative for HHV-8 IgG antibodies. 21% of GUM and ID patients were positive for HHV-8 IgG antibodies. 100 of these patients were men who have sex with men (MSM), 35% of whom were HHV-8 seropositive (46% of human immunodeficiency virus (HIV) positive MSM and 24% of HIV negative MSM). Of 100 heterosexual patients, only 7% were HHV-8 seropositive.

HHV-8 sequencing data were obtained for 23 specimens from 19 patients with HHV-8-related diseases. K1 subtypes A, B, C and F were present in 37%, 11%, 47% and 5%, respectively. For T0.7, subtypes A/C, J, B, R and Q were present in 58%, 17%, 8%, 8% and 8%, respectively. For ORF75, subtypes A, B, C and D were present in 58%, 8%, 25% and 8%, respectively. For K15, 69% had the P allele and 31% had the M allele.

The cytokine analysis study included 30 patients who had HHV-8 DNA detected in plasma. Plasma levels of interferon (IFN)-γ, interleukin (IL)-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and tumour necrosis factor (TNF)-α over time were assessed. Results were obtained for 76 specimens from 28 patients. Cytokine levels were compared between K1 subtypes. A statistically significant difference between subtypes was identified for IFN-γ, IL-1β, IL-6, IL-8, IL-13 and TNF-α, with higher levels for subtype F compared to the other subtypes. IL-1β and TNF-α levels were significantly higher in patients with the K15 M allele compared to the K15 P allele. Cycle threshold (Ct) values for HHV-8 PCR were significantly higher following treatment and a negative correlation between Ct value and IL-10, IFN-γ, IL-4, IL-6, IL-13 and TNF-α levels was identified.

This is the first study of HHV-8 seroprevalence and molecular epidemiology performed in Ireland. The absence of seropositivity in 200 Irish blood donors suggests that Ireland has a low population HHV-8 seroprevalence. The proportion of HHV-8 seropositivity in the MSM population was significantly higher than in the heterosexual population and most marked in HIV positive MSM. A broad variety of HHV-8 subtypes are represented in patients exhibiting HHV-8-related disease in Ireland. The predominance of C and A K1 subtypes was as expected for a western European population. The 31% prevalence for K15 subtype M was higher than expected for a western European population. This may represent the changing and evolving epidemiology in Ireland due to altered migration patterns. To our knowledge, this is the first study comparing cytokine levels between different subtypes of HHV-8. Further research is warranted to determine whether different subtypes can be linked with differing disease severity and, if so, what role proinflammatory cytokines have to play. The results of this study support previous reports that higher levels of proinflammatory cytokines are associated with higher HHV-8 viral loads and disease flares compared to remission.

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List of Abbreviations

AABB	American Association of Blood Banks
AIDS	Acquired immunodeficiency syndrome
ARE	AU-rich element
ART	Antiretroviral therapy
cART	Combined antiretroviral therapy
CDC	Centers for Disease Control and Prevention
CDK6	Cyclin dependent kinase-6
Ct	Cycle threshold
CMV	*Cytomegalovirus*
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EBV	Epstein–Barr virus
ELISA	Enzyme-linked immunofluorescence assay
GMHS	Gay Men's Health Service
GUM	Genitourinary Medicine
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HLH	Haemophagocytic lymphohistiocytosis
HPV	Human papilloma virus
HSV-1	Herpes simplex virus type 1

HSV-2	Herpes simplex virus type 2
HTLV-1	Human T-lymphotropic virus type 1
IARC	International Agency for Research on Cancer
IBTS	Irish Blood Transfusion Service
IC	Internal control
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive care unit
ID	Infectious diseases
IFA	Immunofluorescence assay
IFN	Interferon
IL	Interleukin
IRF	Interferon response factors
IRIS	Immune reconstitution inflammatory syndrome
IVDU	Intravenous drug user
KICS	KSHV inflammatory cytokine syndrome
KSHV	Kaposi's sarcoma-associated herpesvirus
KS	Kaposi’s sarcoma
LANA	Latency-associated nuclear antigen
MCD	Multicentric Castleman’s disease
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
NF-κB	Nuclear factor-κB
NTC	Non-template control
NVRL	National Virus Reference Laboratory
ORF	Open reading frame

PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PHV	Phocine herpesvirus
TBE	Tris-borate-EDTA
TNF	Tumour necrosis factor
UK	United Kingdom
US	United States
UV	Ultraviolet
v-cyclin	Viral-cyclin
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE-inhibitory protein
vGPCR	Viral G-protein coupled receptor
VIP	Variable ITAM-containing protein
vIRF	Viral interferon response factor
VZV	Varicella zoster virus

Publications

Sadhbh O'Rourke, Niamh O'Flaherty, Dermot Coyne, Almida Lynam, Susan Clarke, Siobhán O'Dea, Sarah Fitzpatrick, Jeff Connell, Brendan Crowley**. Seroprevalence of human herpesvirus 8 in Ireland among blood donors, men who have sex with men, and heterosexual genitourinary medicine and infectious diseases clinic attendees.** *J Med Virol*. 2021 Aug;93(8):5058-5064. doi: 10.1002/jmv.26813. Epub 2021 Mar 9.

Sadhbh O'Rourke, Bairbre Ni Laoi, Susan Clarke, Brendan Crowley. **Molecular epidemiology of human herpesvirus 8 in patients with HHV-8-related diseases in Ireland.** *J Med Virol*. 2024 May;96(5):e29654. doi: 10.1002/jmv.29654.

Chapter 1

Introduction

1.1 Family Orthoherpesviridae

1.1.1 Overview

Based on a recent taxonomic proposal in 2022, the name of the family *Herpesviridae* was changed to *Orthoherpesviridae*. The family *Orthoherpesviridae* contains three subfamilies, seventeen genera and one hundred and eighteen species of virus (Gatherer et al., 2021). Members of the family *Orthoherpesviridae* are large deoxyribonucleic acid (DNA) viruses with hosts including mammals, birds and reptiles. These viruses are highly adapted to their host species, as they are known to have evolved in tandem with their hosts. Invariably, herpesviruses initially cause primary infection in the host species, followed by life-long latent infection, with limited gene expression. Reactivation can occur leading to lytic viral production. Herpesviruses frequently cause mild infection, with severe infection usually observed only in vulnerable individuals, including immunocompromised and elderly individuals, the very young, in pregnancy and in foetal infection.

Table 1.1. Characteristics of members of the family *Orthoherpesviridae*.

Characteristic	Description
Virion	Spherical (150-200nm) particles with condensed DNA core, icosahedral capsid, tegument and a lipid envelope containing glycoproteins
Genome	125-241 kbp of linear dsDNA
Replication	Infection has lytic and latent phases; transcription occurs in the nucleus by a kinetic cascade; DNA replicates by a rolling- circle mechanism to generate concatemers, from which genomes are cleaved and packaged into preformed capsids; virions mature in the cytoplasm
Translation	Occurs from capped, polyadenylated mRNAs, some of which are spliced
Host range	Mammals, birds and reptiles
Taxonomy	Realm *Duplodnaviria*, kingdom *Heunggongvirae*, phylum *Peploviricota*, class *Hervivericetes*, order *Herpesvirales*; 3 subfamilies, >10 genera and >100 species

Adapted from Gatherer et al. (2021)

1.1.2 Structure and Genome

The herpesvirus morphology consists of both symmetric and nonsymmetric components. The herpesvirus virion is spherical and comprises a core, capsid, tegument and envelope (Heming et al., 2017). The genome consists of linear, double stranded DNA, 125–241 kbp in length, containing 70–170 genes (Davison & Scott, 1986; Davison et al., 2003). The capsid is icosahedral and contains 162 capsomers arranged as 150 hexons, 11 pentons and one portal. The tegument has both inner and outer layers. The lipid envelope forms a network of spikes consisting of integral viral glycoproteins (Heming et al., 2017).

There are four classes of herpesvirus genome structure, based on the arrangement of terminal and internal repeat sequences (Gatherer et al., 2021). Class 1 genomes contain a unique sequence with a terminal repeat region at either end. Similarly, Class 2 genomes contain a unique sequence but, unlike Class 1 genomes, the unique sequence is flanked by a variable number of direct repeats at each terminus. Class 3 genomes contain different terminal repeats at either end, which are present in inverted orientation internally also, creating one long unique region and one short unique region. Class 4 genomes also consist of a long and a short unique region, each flanked by large, inverted repeats. They have an additional short terminal direct repeat, which is present in inverse orientation at the junction region. Human herpesvirus 8 (HHV-8) belongs to Class 2.

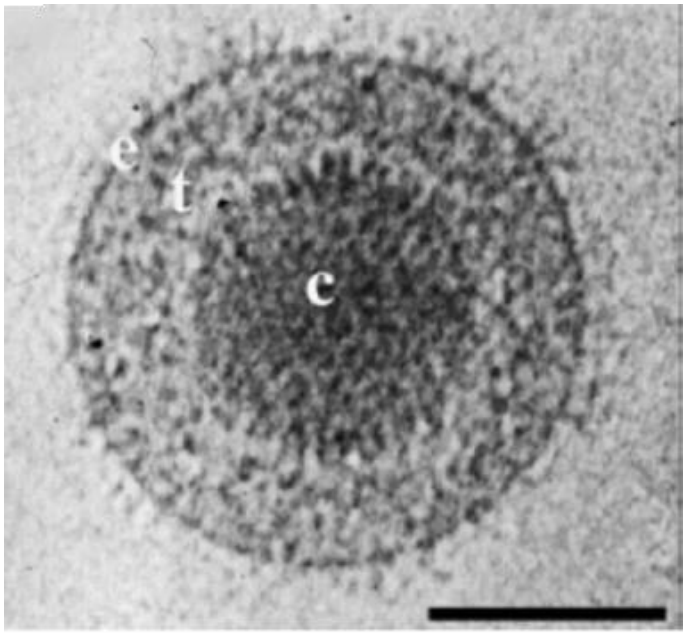

Figure 1.1. *Orthoherpesviridae* virion structure. Cryo-electron microscopy image of a single herpes simplex virus type 1 (HSV-1) virion. The capsid (c) is embedded in the tegument (t) and enclosed by a lipid envelope containing glycoproteins (e). Scale bar, 100 nm. (McGeoch et al., 2006)

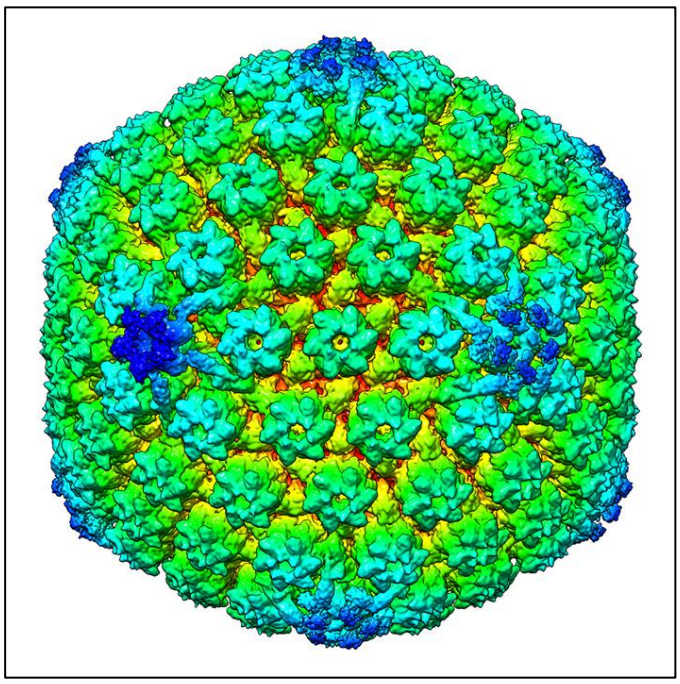

Figure 1.2. *Orthoherpesviridae* capsid structure. Three-dimensional cryo-electron microscopy image reconstruction of a single HSV-1 capsid. (McElwee et al., 2018)

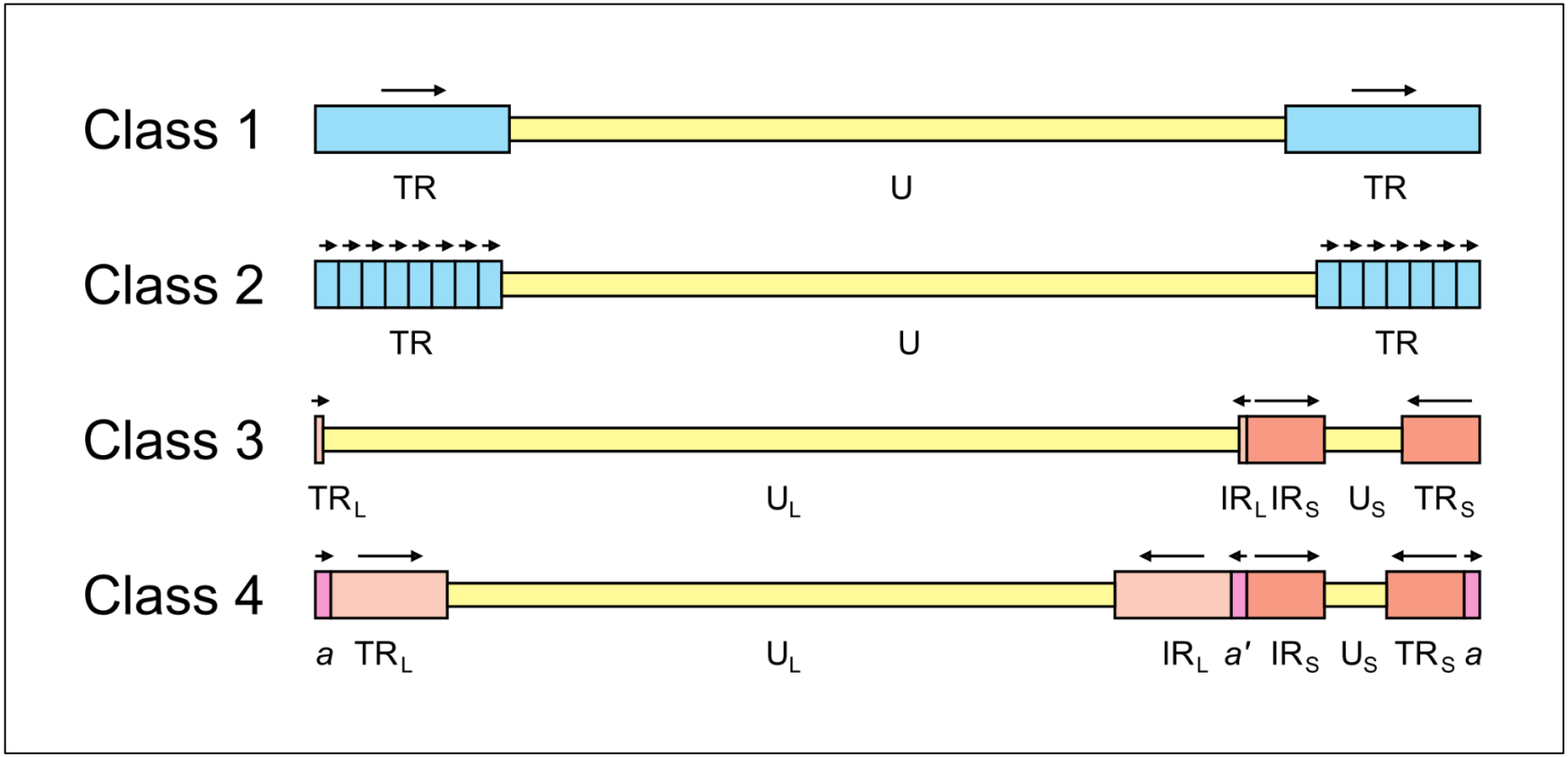

Figure 1.3. Classes of orthoherpesvirus genome structure. (Gatherer et al., 2021)

Abbreviations: U, unique; U_L, long unique; U_S, short unique; TR, terminal repeat); TR_L, terminal long repeat; IR_L, internal long repeat; TR_S, terminal short repeat; IR_S, internal short repeat

Class 4 genomes contain a terminal direct repeat (*a*) that is also present internally in inverse orientation (*a'*)

1.1.3 Classification and Taxonomy

The family *Orthoherpesviridae* consists of three subfamilies, the *Alphaherpesvirinae*, the *Betaherpesvirinae* and the *Gammaherpesvirinae* (Gatherer et al., 2021). The subfamilies were originally defined broadly based on their biological features. Currently, they are demarcated based on genetic content, with each subfamily characterised by the presence of specific genetic properties. This method of categorisation has been generally found to be consistent with the historical method of categorisation, based on biological features. The genera are now similarly defined based on genetic content.

The *Alphaherpesvirinae* classically demonstrate rapid cytolytic growth and are known to establish latent infection in the nervous system (Weidner-Glunde et al., 2020). The *Betaherpesvirinae* affect a limited range of hosts and have a long reproductive cycle, usually associated with the formation of enlarged, cytomegalic cells (Weidner-Glunde et al., 2020). The *Gammaherpesvirinae* establish latent infection in lymphocytes and have been linked with the development of lymphoproliferative diseases (Jha et al., 2016; Weidner-Glunde et al., 2020).

Table 1.2. Herpesvirus classification and taxonomy.

ICTV Designation	
Subfamily Alphaherpesvirinae	
Genus Iltovirus	
Iltovirus cacatuidalpha2 *Iltovirus gallidalpha1*	*Iltovirus psittacidalpha1* *Iltovirus psittacidalpha5*
Genus Mardivirus	
Mardivirus anatidalpha1 *Mardivirus columbidalpha1* *Mardivirus gallidalpha2*	*Mardivirus gallidalpha3* *Mardivirus meleagridalpha1* *Mardivirus spheniscidalpha1*
Genus Scutavirus	
Scutavirus chelonidalpha5	*Scutavirus testudinidalpha3*
Genus Simplexvirus	
Simplexvirus atelinealpha1 *Simplexvirus bovinealpha2* *Simplexvirus cercopithecinealpha2* *Simplexvirus humanalpha1* *Simplexvirus humanalpha2* *Simplexvirus leporidalpha4* *Simplexvirus macacinealpha1* *Simplexvirus macacinealpha2* *Simplexvirus macacinealpha3*	*Simplexvirus macropodidalpha1* *Simplexvirus macropodidalpha2* *Simplexvirus macropodidalpha4* *Simplexvirus panינealpha3* *Simplexvirus papiinealpha2* *Simplexvirus pteropodidalpha1* *Simplexvirus pteropodidalpha2* *Simplexvirus saimiriinealpha1*
Genus Varicellovirus	
Varicellovirus bovinealpha1 *Varicellovirus bovinealpha5* *Varicellovirus bubalinealpha1* *Varicellovirus canidalpha1* *Varicellovirus caprinealpha1* *Varicellovirus cercopithecinealpha9* *Varicellovirus cervidalpha1* *Varicellovirus cervidalpha2* *Varicellovirus cervidalpha3* *Varicellovirus equidalpha1*	*Varicellovirus equidalpha3* *Varicellovirus equidalpha4* *Varicellovirus equidalpha6* *Varicellovirus equidalpha8* *Varicellovirus equidalpha9* *Varicellovirus felidalpha1* *Varicellovirus humanalpha3* *Varicellovirus monodontidalpha1* *Varicellovirus phocidalpha1* *Varicellovirus suidalpha1*
Subfamily Betaherpesvirinae	
Genus Cytomegalovirus	
Cytomegalovirus aotinebeta1 *Cytomegalovirus cebinebeta1* *Cytomegalovirus cercopithecinebeta5* *Cytomegalovirus humanbeta5* *Cytomegalovirus macacinebeta3* *Cytomegalovirus macacinebeta8*	*Cytomegalovirus mandrillinebeta1* *Cytomegalovirus paninebeta2* *Cytomegalovirus papiinebeta3* *Cytomegalovirus papiinebeta4* *Cytomegalovirus saimiriinebeta4*
Genus Muromegalovirus	
Muromegalovirus muridbeta1 *Muromegalovirus muridbeta2*	*Muromegalovirus muridbeta8*
Genus Proboscivirus	

Proboscivirus elephantidbeta1 *Proboscivirus elephantidbeta3*	*Proboscivirus elephantidbeta4* *Proboscivirus elephantidbeta5*
Genus Quwivirus	
Quwivirus caviidbeta2 *Quwivirus miniopteridbeta1*	*Quwivirus tupaiidbeta1*
Genus Roseolovirus	
Roseolovirus humanbeta7 *Roseolovirus humanbeta6a* *Roseolovirus humanbeta6b*	*Roseolovirus macacinebeta9* *Roseolovirus muridbeta3* *Roseolovirus suidbeta2*
Subfamily Gammaherpesvirinae	
Genus Bossavirus	
Bossavirus delphinidgamma1	
Genus Lymphocryptovirus	
Lymphocryptovirus callitrichinegamma3 *Lymphocryptovirus gorillinegamma1* *Lymphocryptovirus humangamma4* *Lymphocryptovirus macacinegamma4* *Lymphocryptovirus macacinegamma10*	*Lymphocryptovirus macacinegamma13* *Lymphocryptovirus paninegamma1* *Lymphocryptovirus papiinegamma1* *Lymphocryptovirus ponginegamma2*
Genus Macavirus	
Macavirus alcelaphinegamma1 *Macavirus alcelaphinegamma2* *Macavirus bovinegamma6* *Macavirus caprinegamma2* *Macavirus hippotraginegamma1*	*Macavirus ovinegamma2* *Macavirus suidgamma3* *Macavirus suidgamma4* *Macavirus suidgamma5*
Genus Manticavirus	
Manticavirus phascolarctidgamma1	*Manticavirus vombatidgamma1*
Genus Patagivirus	
Patagivirus vespertilionidgamma3	
Genus Percavirus	
Percavirus equidgamma2 *Percavirus equidgamma5* *Percavirus felidgamma1* *Percavirus mustelidgamma1*	*Percavirus phocidgamma3* *Percavirus rhinolophidgamma1* *Percavirus vespertilionidgamma1*
Genus Rhadinovirus	
Rhadinovirus atelinegamma2 *Rhadinovirus atelinegamma3* *Rhadinovirus bovinegamma4* *Rhadinovirus colobinegamma1* *Rhadinovirus cricetidgamma2* *Rhadinovirus humangamma8* *Rhadinovirus macacinegamma5*	*Rhadinovirus macacinegamma8* *Rhadinovirus macacinegamma11* *Rhadinovirus macacinegamma12* *Rhadinovirus muridgamma4* *Rhadinovirus muridgamma7* *Rhadinovirus saimiriinegamma2*

The virus names that have been underlined represent the nine human herpesviruses.

Abbreviations: ICTV, International Committee on Taxonomy of Viruses.

1.1.4 Human Herpesviruses

There are currently nine herpesviruses that are known to infect the human species, and these are detailed in Table 1.3. These viruses generally have very high prevalence rates worldwide. Human herpesvirus 8 is an exception to this rule, as it exhibits varying prevalence rates in different populations (*Section 1.3*). The human herpesviruses frequently cause asymptomatic or mild infection but may cause severe and sometimes fatal infection in at risk groups, for example, in immunosuppressed patients, in pregnancy, in the foetus and infancy, and in the elderly. Following primary infection, these viruses establish lifelong latent infection in the host and may cause subsequent reactivation. Latency occurs by differing mechanisms. Generally, the *Alphaherpesvirinae* establish latency in the neuron, the *Betaherpesvirinae* establish latency in the monocytic lineage, and the *Gammaherpesvirinae* establish latency in the lymphocytes (Weidner-Glunde et al., 2020). Epstein–Barr virus (EBV) and HHV-8 have both been associated with human malignancies (Jha et al., 2016; Means et al., 2007; Carlo Parravicini et al., 2000).

Table 1.3. Human herpesviruses.

ICTV Designation	Common Name
Simplexvirus humanalpha1	Herpes simplex virus-1 (HSV-1)
Simplexvirus humanalpha2	Herpes simplex virus-2 (HSV-2)
Varicellovirus humanalpha3	Varicella zoster virus (VZV)
Lymphocryptovirus humangamma4	Epstein–Barr virus (EBV)
Cytomegalovirus humanbeta5	*Cytomegalovirus* (CMV)
Roseolovirus humanbeta6a *Roseolovirus humanbeta6b*	*Roseolovirus*
Roseolovirus humanbeta7	
Rhadinovirus humangamma8	Kaposi's sarcoma-associated herpesvirus (KSHV)

Abbreviations: ICTV, International Committee on Taxonomy of Viruses

1.2 Human herpesvirus 8

1.2.1 History

Human herpesvirus 8, or Kaposi's sarcoma-associated herpesvirus (KSHV) was first described by Chang et al. in 1994, in association with Kaposi's sarcoma (KS). Representational difference analysis, a molecular technique for non-directed genomic searching, was used to identify and characterise unique DNA sequences in KS tissue from patients with acquired immunodeficiency syndrome (AIDS)-KS (Chang et al., 1994; Chang & Moore, 2014). The virus was discovered following the advent of the AIDS epidemic, when epidemiologic studies concluded that AIDS-KS was likely to be caused by a virus (Beral et al., 1990).

As well as being identified as the etiologic agent responsible for Kaposi's sarcoma, in May 1995, Cesarman et al. reported in the New England Journal the identification of KSHV in AIDS-related body-cavity-based lymphomas. These lymphomas would later become known as primary effusion lymphoma (PEL) (Nador et al., 1996). In August 1995, Soulier et al. subsequently reported the presence of the virus in multicentric Castleman's disease (MCD).

1.2.2. Classification

When first identified, in 1994, this new virus was termed Kaposi's sarcoma-associated herpesvirus, due to its association with Kaposi's sarcoma. It belongs to the family *Orthoherpesviridae* and is the most recent human herpesvirus to be identified (Table 1.3). It was subsequently given the formal designation human herpesvirus 8, in keeping with the systematic nomenclature for human herpesviruses (IARC Working Group on

the Evaluation of Carcinogenic Risks to Humans, 2012). It's current formal designation is *Rhadinovirus humangamma8*, following an update by the International Committee on Taxonomy of Viruses (ICTV) in 2022 (Gatherer et al., 2021). HHV-8 is an enveloped gamma-2 herpesvirus (rhadinovirus), with a double-stranded DNA genome, and is the only known human gamma-2 herpesvirus (rhadinovirus). It is related to other rhadinoviruses that primarily infect primates (Ablashi et al., 2002; Edelman, 2005). It's closest relative among the human herpesviruses is Epstein-Barr virus.

1.2.3 Structure and Morphology

HHV-8 is a large, enveloped virus, with a diameter of 140 nm and a morphology similar to that of the other herpesviruses. It has an electron-dense central core and a lipid envelope. The virus has a double-stranded DNA genome, containing a unique region of approximately 140 kb. The capsid is icosahedral, with 162 capsomeres (Wu et al., 2000). The tegument, made up of proteinaceous material, lies between the capsid and the envelope. During lytic infection, the viral genome is linear. Whereas, in latent infection, the genome has a circular conformation (Renne et al., 1996).

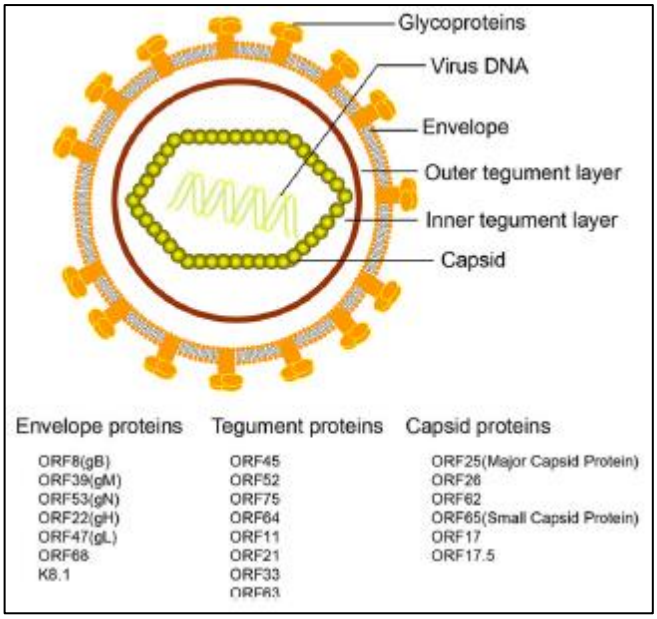

Figure 1.4. Schematic representation of the HHV-8 virion structure. The viral proteins found in the HHV-8 capsid, tegument, and envelope are listed below. (Yan et al., 2019)

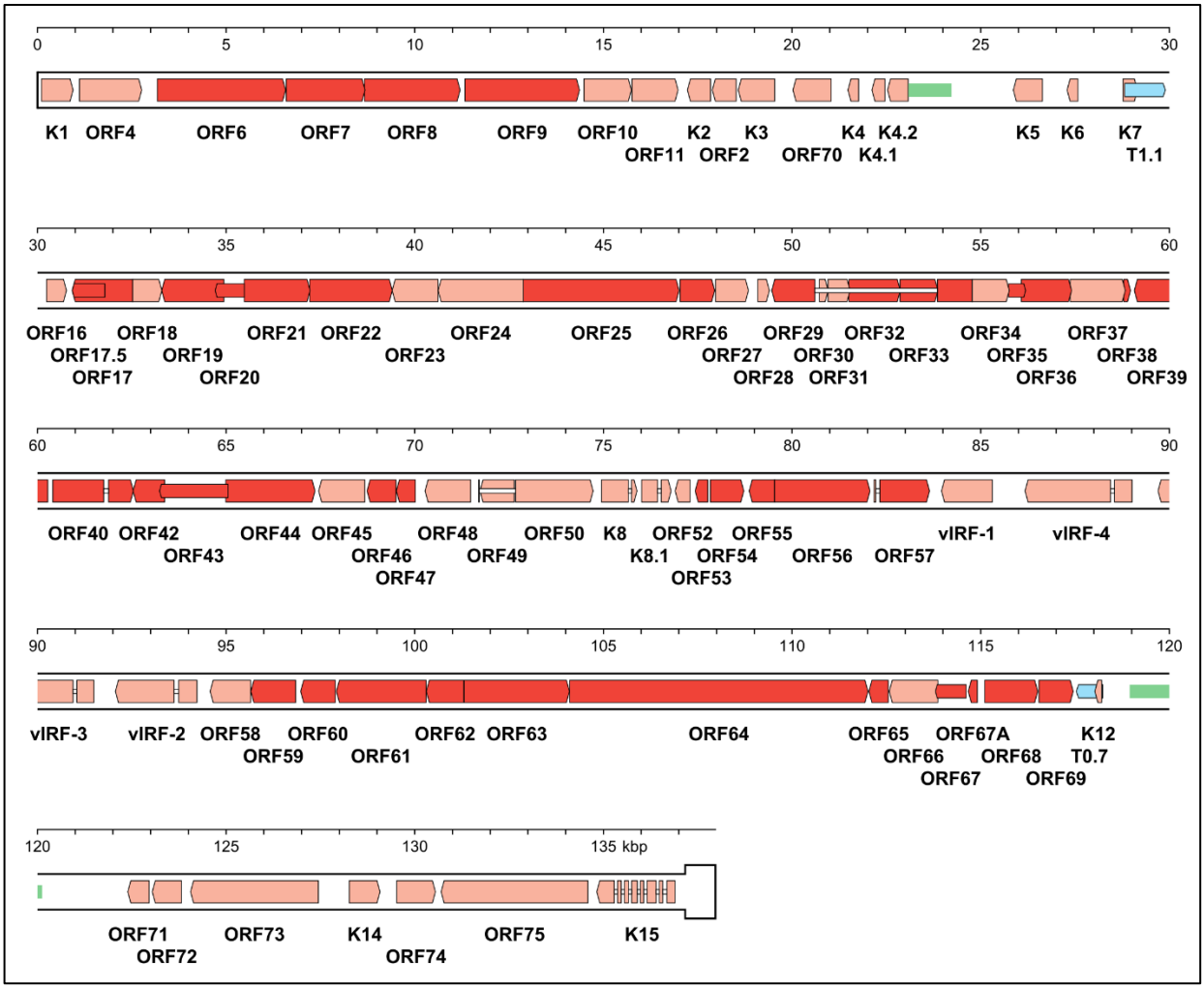

Figure 1.5. Schematic representation of the HHV-8 genome.

HHV-8 strain GK18 (AF148805) (Gatherer et al., 2021)

1.2.4. Life Cycle

Like other herpesviruses, HHV-8 can cause both lytic and latent infection, and latent infection persists for life (Ablashi et al., 2002). In lytic infection, viral progeny that are produced in an infected cell are released as the cell dies. The majority of HHV-8 genes are only expressed during lytic infection and encode the proteins responsible for viral DNA replication (Ray et al., 2012).

Cells that are latently infected are a potential reservoir for amplification of progeny virus, which may be disseminated within the host or to another host (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). The predominant latency reservoir for HHV-8 is CD19+ B-cells (Arvin et al., 2007). During latency, the HHV-8 viral genome only expresses the minimum number of genes required for the purpose of maintaining latency and cell survival (Ray et al., 2012).

Latent infection is predominant in HHV-8-infected tumours, such as Kaposi's sarcoma, and PEL, and lytic infection affects only a small number of infected cells (Ray et al., 2012). Lytic reactivation from cells that have been latently infected is an important step in the pathogenesis of HHV-8-related disease. The immune system plays a critical part in deterring lytic reactivation of HHV-8 in immunocompetent individuals.

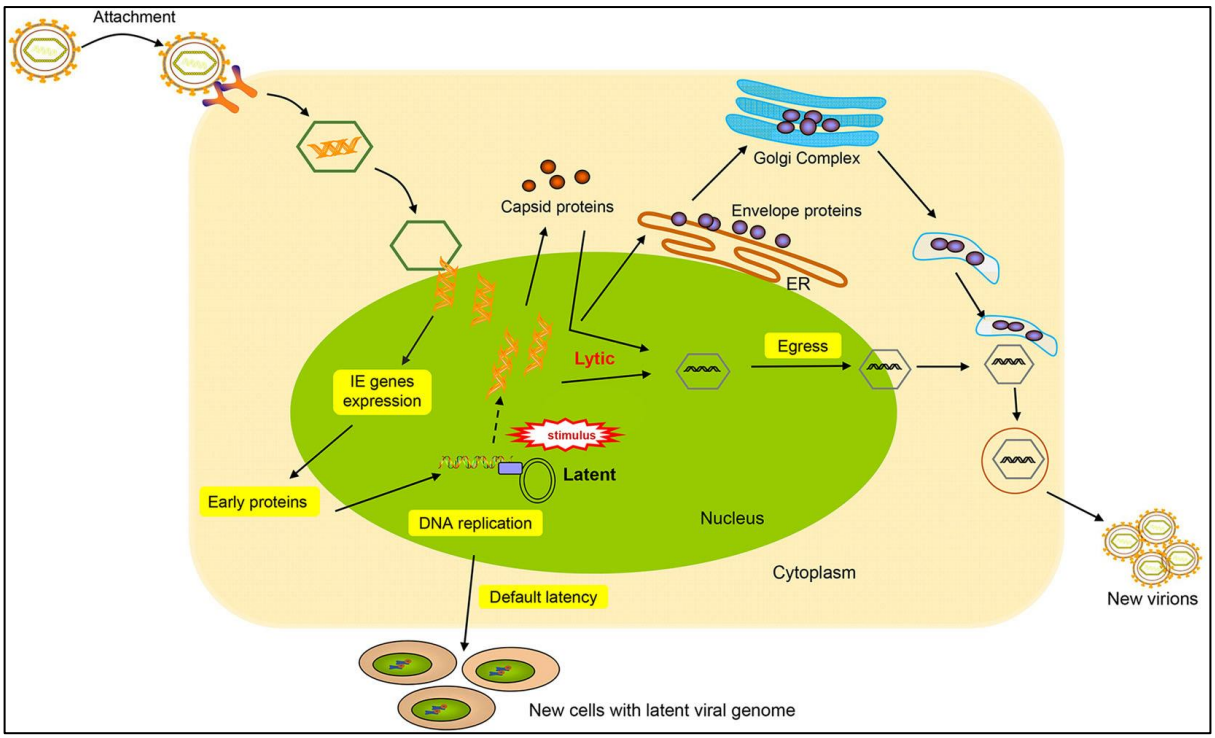

Figure 1.6. Schematic representation of HHV-8 life cycle. (Yan et al., 2019)

1.2.5. Pathogenesis

Infection with HHV-8 rarely leads to disease. The virus predominantly causes latent infection. Genes expressed in both latent and lytic infection of cells have a role in tumorigenesis (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Immunosuppression contributes significantly to the development of HHV-8-related disease, and this is well described. However, although HHV-8 is well described as the etiologic agent underlying Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease, there appear to be other factors at play which are poorly understood. For example, Kaposi's sarcoma occurs more frequently in men than in women, despite similar prevalence of HHV-8 infection, and disease also appears to be more common in certain geographical areas, despite similar seroprevalence rates. The reasons for this remain unclear and warrant further investigation (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012).

HHV-8, along with human papilloma virus (HPV), EBV, human T-lymphotropic virus type 1 (HTLV-1) and hepatitis C virus (HCV), is one of the small number of viruses that appear to drive oncogenesis through neoplastic change in infected cells (Riva et al., 2010). The virus produces a number of proteins that contribute to the inhibition of apoptosis and lead to cell proliferation. For example, the latency-associated nuclear antigen (LANA) affects the function of tumour suppressor proteins, thereby leading to transformation of infected cells and contributing to cell survival (Friborg et al., 1999; Radkov et al., 2000).

Recent work has also highlighted the importance of proinflammatory cytokines in the pathogenesis of HHV-8 infection. Inflammatory symptoms associated with HHV-8-related diseases have been attributed to the presence of HHV-8-encoded viral interleukin-6 and elevated levels of other cytokines, particularly human interleukin-6 and interleukin-10 (Aoki, Tosato, et al., 2001; Brandt et al., 1990; Oksenhendler et al., 2000; Polizzotto et al., 2013). Elevated serum levels of proinflammatory cytokines have been linked to poorer clinical outcomes (Polizzotto et al., 2013).

1.2.6. Viral Proteins

A number of latent and lytic proteins are involved in the pathogenesis of HHV-8-related disease. Several of these proteins have been demonstrated to have transforming properties, while others have been reported to influence cell cycle regulation and cell survival (Table 1.4).

1.2.6.1. Latency Proteins

The latency proteins of note include LANA, viral-cyclin (v-cyclin), viral FLICE-inhibitory protein (vFLIP), the kaposin proteins and viral interferon response factor-3 (vIRF-3) (Yan et al., 2019).

1.2.6.1.1. Latency-associated nuclear antigen (LANA)

LANA is encoded by ORF73. It is the viral protein that is most consistently detected in tumour cells associated with HHV-8 disease (Parravicini et al., 2000). LANA is necessary for viral replication and maintenance of the episomal DNA, and it is required in order to establish viral latency (Ballestas et al., 1999). It suppresses the activity of p53 and Rb, resulting in a pro-survival effect (Friborg et al., 1999; Radkov et al., 2000).

Human interleukin-6 (hIL-6) is induced by LANA, and has been found to correlate with disease severity. There is evidence that hIL-6 may stimulate KS cell growth, and it has been associated with both MCD and PEL (Asou et al., 1998; Miles et al., 1990; Yoshizaki et al., 1989).

1.2.6.1.2. Viral-cyclin (v-cyclin)

v-cyclin is encoded by ORF72, which is immediately adjacent to the gene encoding LANA. Like LANA, v-cyclin is expressed during latency. It is homologous with human cellular cyclin D, and thereby may contribute to cell cycle deregulation. v-cyclin aids in protein phosphorylation by interacting with cyclin dependent kinase-6 (CDK6) (Sherr, 1996). Through this interaction, v-cyclin also plays a role in the maintenance of viral latency (Sarek et al., 2010).

1.2.6.1.3. Viral FLICE-inhibitory protein (vFLIP)

Encoded by ORF71, vFLIP is a homologue of cellular FLICE (caspase-8)-inhibitory protein (FLIP) (Hu et al., 1997). vFLIP interacts with the IκB kinase complex, leading to activation of nuclear factor-κB (NF-κB) signaling (Matta et al., 2003). This activity protects cells against growth factor withdrawal-induced apoptosis (Sun et al., 2003). Through NF-κB, vFLIP modulates MHC-I expression in HHV-8-infected endothelial cells (Lagos et al., 2007). vFLIP activity has been implicated in the development of spindle cell tumours and PEL (Keller et al., 2000; Sakakibara et al., 2009).

1.2.6.1.4. Kaposins

ORF-K12, via alternative reading frames, encodes for the kaposin proteins (Sadler et al., 1999). Kaposin A is a latency protein. It interacts with cytohesin-1, and has been

shown to demonstrate transforming properties in rodent models (Kliche et al., 2001; Muralidhar et al., 1998). Kaposin B binds to and activates the kinase MK2, a target of the p38 pathway. This action inhibits the decay of messenger ribonucleic acids (mRNAs) with AU-rich elements (AREs), including cytokine mRNAs, suggesting that kaposin B may result in the production of proinflammatory cytokines in HHV-8 infected cells (McCormick & Ganem, 2005).

1.2.6.1.5. Viral interferon response factor-3 (vIRF-3)

vIRF-3, encoded by ORF-K10.5 and also known as LANA-2, is one of four viral interferon response factors (IRFs) encoded by HHV-8. These are homologues of cellular IRFs (Baresova et al., 2013). vIRF-3 is a latency protein. Interestingly, vIRF-3 is not detected in Kaposi's sarcoma tissues, but is frequently expressed in PEL and MCD (Rivas et al., 2001). vIRF-3 binds to and inhibits p53 function, and modulates interferon responses (Baresova et al., 2014; Lubyova et al., 2004).

1.2.6.2. Lytic Proteins

The proteins of note expressed during lytic infection include variable ITAM-containing protein (VIP), the viral interferon response factors (vIRFs), viral G-protein coupled receptor (vGPCR) and viral interleukin-6 (vIL-6).

1.2.6.2.1. Variable ITAM-containing protein (VIP)

VIP is encoded by the K1 gene, at the extreme left-hand end of the HHV-8 genome and is expressed during lytic replication. VIP demonstrates anti-apoptotic activity through activation of PI3K/AKT, inhibition of FKHR-mediated apoptosis, and inhibition of Fas-induced apoptosis (Tomlinson & Damania, 2004; Uddin et al., 2005). The protein also

induces the expression of vascular endothelial growth factor (VEGF), promoting angiogenesis (Wang et al., 2004).

1.2.6.2.2. Viral interferon response factors (vIRFs)

vIRF-1, vIRF-2 and vIRF-4 expression occurs during lytic infection, unlike that of vIRF-3, which is a latency protein. The vIRFs contribute to immune evasion by down-regulating MHC-I and MHC-II (Lagos et al., 2007; Schmidt et al., 2011). They also play a role in inhibiting p53 (Rivas et al., 2001). They help to modulate interferon signalling and inhibit activation-induced death signalling (Kirchhoff et al., 2002).

1.2.6.2.3. Viral G-protein coupled receptor (vGPCR)

vGPCR is a lytic protein, encoded by ORF74, and is a homologue of the IL-8 receptor, CXCR1. vGPCR activates multiple anti-apoptotic pathways, including JNK/SAPK, p38 MAPK and NFκB (Bais et al., 1998; Schwarz & Murphy, 2001). Through NFκB activation, vGPCR has the ability to activate a number of proinflammatory factors, including IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α and the AP-1-dependent basic fibroblast growth factor (bFGF) (Schwarz & Murphy, 2001).

1.2.6.2.4. Viral interleukin-6 (vIL-6)

HHV-8 vIL-6 is encoded by ORF-K2 and is a homologue of cellular IL-6. It is upregulated during lytic activation (Chatterjee et al., 2002). vIL-6 induces STAT3 phosphorylation and induces VEGF, promoting angiogenesis (Aoki & Tosato, 1999; Hoischen et al., 2000). It has been shown in mouse models to induce proliferation of PEL cell lines and promote features consistent with MCD (Aoki et al., 1999; Aoki & Tosato, 1999; Suthaus et al., 2012).

Table 1.4. Viral proteins and their role in pathogenesis.

Protein	Role in Pathogenesis
Latency Proteins	
LANA (ORF73)	Necessary for viral replication and maintenance of the episomal DNA. Required to establish latency. Suppresses activity of p53 and Rb resulting in pro-survival effect. Induces hIL-6.
v-cyclin (ORF72)	Contributes to cell cycle dysregulation. Aids in protein phosphorylation through interaction with CDK6. Role in maintenance of viral latency.
vFLIP (ORF71)	Protects cells against growth factor withdrawal-induced apoptosis. Modulates MHC-I expression in infected endothelial cells. Implicated in the development of spindle cell tumours and PEL.
Kaposin A and B (ORF-K12)	Kaposin A has demonstrated transforming properties in rodent models. Kaposin B inhibits the decay of mRNAs with AREs and may result in the production of proinflammatory cytokines in HHV-8 infected cells.
vIRF-3 (ORF-K10.5)	Frequently expressed in PEL and MCD. Inhibits p53 function and modulates interferon responses.
Lytic Proteins	
VIP (ORF-K1)	Anti-apoptotic activity. Induces expression of VEGF, promoting angiogenesis.

vIRF-1 (ORF-K9) **vIRF-2 (ORF-K11)** **vIRF-4 (ORF-K10)**	Contribute to immune evasion by down-regulating MHC-I and MHC-II. Inhibit p53. Modulate interferon signalling, inhibit activation-induced death signalling.
vGPCR (ORF74)	Activates multiple anti-apoptotic pathways. Activates proinflammatory cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α and bFGF.
vIL-6 (ORF-K2)	Induces STAT3 phosphorylation and VEGF, promoting angiogenesis. In mouse models induces proliferation of PEL cell lines and promotes features consistent with MCD.

1.3. Epidemiology of Infection

1.3.1. Seroprevalence

Human herpesviruses are generally ubiquitous organisms. Asymptomatic shedding may occur, and these viruses often persist as latent infections. HHV-8 is similar to other human herpesviruses in this regard.

The epidemiology of HHV-8 is interesting and differs from that of other herpesviruses in that its seroprevalence varies significantly geographically and between different sub-populations (Rohner et al., 2014). Interestingly, the rate of Kaposi's sarcoma does not necessarily correlate with the rate of HHV-8 infection in a given population (Chen et al., 2004).

1.3.1.1. North America

Early studies among blood donors in the United States (US) found HHV-8 seropositivity rates of 0-1% (Gao et al., 1996; Kedes et al., 1996). The majority of studies, including a number of more recent publications, have reported the HHV-8 seroprevalence to be approximately 5% in the general population (Ablashi et al., 1999; Engels et al., 2007; Pellett et al., 2003; Simpson et al., 1996). Interestingly, higher rates of up to 15-23% have been reported in Texas (Baillargeon et al., 2001; Hudnall et al., 2003; Kedes et al., 1996; Kleinman et al., 2012; Pellett et al., 2003). In 2007, Engels et al. demonstrated significantly higher HHV-8 seroprevalence rates among MSM in the US, when compared to heterosexual individuals, with rates of 8-25% among MSM. HIV-infected individuals in the US have also been noted to have higher seroprevalence rates of HHV-8 (Ablashi et al., 1999; Gao et al., 1996; Martin et al., 1998). One study

reported a relatively higher rate of 10% seropositivity among intravenous drug users (IVDUs) (Atkinson et al., 2003).

1.3.1.2. Northern Europe

HHV-8 seroprevalence in Northern Europe is similar to that of the US, with overall reported seroprevalence rates of approximately 5-6% (Regamey, Cathomas et al., 1998; Whitby et al., 1998). The reported rates among blood donors vary marginally between countries, ranging from 0% in Greece and parts of Spain, to rates of 2-6% in the majority of European countries, including rates of 6.5% in other parts of Spain, 0.7-5% in the United Kingdom, 0.83-1.6% in Hungary, 5% in Switzerland, 2% in France and 3% in Germany (Alcalde et al., 2008; Dakovic Rode et al., 2005; Gambús et al., 2001; Juhász et al., 2001; Politou et al., 2014; Preiser et al., 2001; Regamey, Cathomas et al., 1998; Simpson et al., 1996; Tettmar et al., 2016). Similar to the US, studies performed in European countries among MSM and HIV infected individuals showed higher rates of HHV-8 seropositivity (Gambús et al., 2001; Preiser et al., 2001; Regamey, Cathomas et al., 1998; Simpson et al., 1996). Studies among IVDUs demonstrated varying prevalences, from 3.2% in the United Kingdom (UK) to 12 % in Spain (Gambús et al., 2001; Simpson et al., 1996).

1.3.1.3. Mediterranean

KS was originally described among elderly male patients of Mediterranean descent. As a result, this is an area of particular interest in terms of HHV-8 epidemiology, and HHV-8 seroprevalence is known to be higher in countries of the Mediterranean compared to neighbouring northern European countries. Seroprevalence is approximately 10% in the Mediterranean, approaching 30% in areas of Italy (Whitby et

al., 1998). Sardinia, which has one of the highest incidence rates of KS in the world, was found to have a seroprevalence of 11% in the general population and 39% among relatives of patients with KS (Angeloni et al., 1998).

1.3.1.4. South America

Seroprevalence studies have been performed in a number of South American countries. Seroprevalences among blood donors in Brazil, Chile and Argentina range from 2-4% (Pérez et al., 2004). In Peru, HHV-8 seroprevalence rates are noted to be elevated among the MSM population. 66.5% of HIV infected MSM and 26.7% of HIV negative MSM were HHV-8 seropositive (Guanira et al., 2008). Of note, very high seroprevalence rates have been identified among the indigenous Brazilian Amerindian population (53%) and the Amerindian population of Ecuador (28-38%) (Biggar et al., 2000; Whitby et al., 2004). Among Ecuadorian Amerindians the Huaorani were specifically noted to have a HHV-8 seroprevalence of 100% by LANA immunofluorescence assay (IFA) and LANA enzyme-linked immunofluorescence assay (ELISA) (Whitby et al., 2004).

1.3.1.5. Asia

The incidence of KS is generally relatively low in Asian countries. However, HHV-8 seroprevalence is variable and there are a number of specific populations with higher seroprevalence rates. Seroprevalence studies performed in India, Thailand, Malaysia and Sri Lanka have demonstrated prevalences of approximately 4% (Ablashi et al., 1999). Higher rates have been reported in Taiwan, from 11.7-23% of blood donors tested (Huang et al., 2000; Wang et al., 2002). Seroprevalence is approximately 10% in China (Zhang et al., 2014). However, a significantly higher seroprevalence of 47% has

been reported among the Uygur people of Xinjiang in north-western China (Dilnur et al., 2001). The seroprevalence in Japan is very low, at 0.2% among blood donors, which correlates with a very low incidence of KS. However, KS is more common in Okinawa, particularly in the Miyako Islands, and a higher HHV-8 seroprevalence of 15% has been identified in this region (Awazawa et al., 2017; Fujii et al., 1999). The indigenous population of Papua New Guinea has also been identified as a population of high HHV-8 seroprevalence, with antibodies detected in approximately 25% of the population tested (Rezza et al., 2001).

1.3.1.6. Africa

Sub-Saharan Africa has the highest rate of HHV-8 infection worldwide, with approximately 50% of the population infected in some areas (Butler et al., 2011; Dollard et al., 2010). Equatorial nations such as Uganda, Tanzania, and the Democratic Republic of Congo make up what is known as the "KS Belt", with one of the highest incidence rates of KS in the world (Dollard et al., 2010). HHV-8 seroprevalence rates of 37.5%, 38.7%, 41.9%, 69-82%, 20.3% and 17.6-36.3% have been reported in Zambia, Uganda, Ghana, the Democratic Republic of Congo, Zimbabwe and South Africa, respectively (Ablashi et al., 1999; Dollard et al., 2010; Engels et al., 2000; Sitas et al., 1999). Rates among the San and Bantu populations of Botswana have been reported to be 54.7-90% (Engels et al., 2000; Whitby et al., 2004). Of note, in contrast to findings in North America and Northern European countries, studies performed in sub-Saharan Africa have shown that seroprevalence rates are comparable among HIV positive individuals compared to the general population (Ablashi et al., 1999; Simpson et al., 1996). This demonstrates a different pattern of infection to be evident in endemic settings.

1.3.2. Risk Factors

As well as varying geographically, HHV-8 seroprevalence rates have been found to vary among sub-groups in certain populations and a number of risk groups for HHV-8 infection have been identified. Sub-populations at higher risk of HHV-8 infection compared to the overall population are more apparent in countries where the overall HHV-8 seroprevalence is low, for example in the United States of America. High seroprevalence rates have been specifically described among MSM, HIV-infected individuals, African migrants and IVDUs (Atkinson et al., 2003; Gambús et al., 2001; Rohner et al., 2014; Simpson et al., 1996).

1.3.2.1. Men Who Have Sex with Men

In 1981, the Centers for Disease Control and Prevention (CDC) reported an increased incidence of Kaposi's sarcoma among MSM in the United States (Centers for Disease Control (CDC), 1981). This was the harbinger of the AIDS epidemic in the United States. From the time HHV-8 was first identified as the causative agent in Kaposi's sarcoma, studies have identified higher seroprevalence rates among MSM, particularly among HIV positive MSM (Kedes et al., 1996; Lennette et al., 1996). These findings are most notable in non-endemic countries and many subsequent studies have agreed with these findings, including a meta-analysis by Liu et al. (2018), which demonstrated that, among 15,914 subjects, HIV positive MSM were more likely to be HHV-8 seropositive than those who are HIV negative.

1.3.2.2. Human Immunodeficiency Virus Infection

HIV infection has been associated with higher HHV-8 seroprevalence rates and, in countries where HHV-8 is endemic, HIV infection appears to be a risk factor for HHV-8 infection in childhood (Malope et al., 2007; Minhas et al., 2008).

The evidence for a specific association with HIV has been conflicting, and some studies have shown an association, where others have not (Baeten et al., 2002; Biryahwaho et al., 2010; Klaskala et al., 2005; Nuvor et al., 2001). A systematic review by Rohner et al. (2016) demonstrated an overall increased HHV-8 seroprevalence associated with HIV infection and, in particular, a stronger association in MSM compared to heterosexual adults.

The association between HIV infection and HHV-8 prevalence appears to be most pronounced in regions where HHV-8 is not endemic. In endemic settings, HHV-8 seroprevalence rates are more comparable between HIV infected individuals and those who are not HIV infected (Ablashi et al., 1999; Simpson et al., 1996).

1.3.2.3. African Migrants

In countries with a low HHV-8 prevalence, it has been noted that there are higher seroprevalence rates among African migrants and migrants from other endemic regions (De Tejada et al., 2011). Given the increase in global travel, we may expect a change over time in the global epidemiology of this virus and the diseases it is associated with.

1.3.2.4. Intravenous Drug Use

A number of studies have shown intravenous drug use to be a potential risk factor for HHV-8 infection. Studies performed in the US, UK and Spain have demonstrated this (Atkinson et al., 2003; Gambús et al., 2001; Simpson et al., 1996). The evidence, however, is conflicting, and other studies have suggested that there is no association (Bernstein et al., 2003; Renwick et al., 2002).

1.3.3. Transmission

Several patterns of HHV-8 transmission have been reported, including via saliva, via sexual transmission and via bloodborne transmission, and the mode of transmission may vary depending on the degree of endemicity of the virus (Cannon et al., 2003). Vertical transmission may also occur (Brayfield et al., 2003; Cannon et al., 2003).

In endemic countries, there is evidence that infection frequently occurs in childhood, and that prevalence increases with age (Malope et al., 2007; Whitby et al., 2000). There is also evidence of transmission between mother and child and within families (Dedicoat et al., 2004; Malope et al., 2007; Minhas et al., 2008). These findings suggest that a non-sexual route of transmission is predominant in these settings, with salivary transmission being the likely route (Butler et al., 2011; Dedicoat et al., 2004).

Due to the finding of increased prevalence among MSM in non-endemic settings, it was postulated that HHV-8 infection was sexually transmitted, and several early studies showed sexual activity to be a strong risk factor for HHV-8 infection, as well as demonstrating higher prevalence in MSM and a strong association with HIV infection (Lennette et al., 1996; Martin et al., 1998).

Polymerase chain reaction (PCR)-based studies have been performed to investigate potential transmission routes by aiming to identify the presence of HHV-8 DNA at various sites. HHV-8 has been shown in numerous studies to be present in saliva, and at relatively high levels compared to other sites (Casper et al., 2007; Pauk et al., 2000).

Based on the evidence available, from both epidemiological and virological data, it is likely that the predominant route of transmission of HHV-8 is via saliva, and this is likely to be the case in MSM populations in low prevalence countries, as well as for familial spread in endemic countries (Butler et al., 2011; Dedicoat et al., 2004; Martró et al., 2007; Pauk et al., 2000).

1.3.4. Blood Transfusion

HHV-8 is detectable in peripheral blood and blood-borne transmission is known to be possible.

Hladik et al. (2006) investigated the risk of HHV-8 transmission in an endemic setting in Uganda, providing strong evidence that HHV-8 may be transmitted by blood transfusion. They found that blood units stored for more than four days were less likely to be associated with seroconversion.

Studies in the US have found no evidence of transmission associated with blood transfusion in settings of low HHV-8 seroprevalence, where blood products routinely undergo leukoreduction (Cannon et al., 2009).

With regard to blood transfusion, the AABB (formerly American Association of Blood Banks) have designated HHV-8 a risk category of yellow, described as agents with absent to low scientific/epidemiologic evidence of risk regarding blood safety (Stramer et al., 2009). Although transfusion transmission may occur in the absence of leukoreduction, no clinically significant disease has been documented via transfusion (Cannon et al., 2009; Dollard et al., 2005; Engels et al., 1999; Hladik et al., 2003).

1.3.5. Organ Transplantation

Numerous studies have been undertaken to assess the HHV-8 serostatus of solid organ transplant donors and recipients before and after transplantation (Chiereghin et al., 2017; Muñoz et al., 2002; Parravicini, Olsen et al., 1997; Regamey, Tamm et al., 1998). HHV-8 transmission though organ donation has been well documented, and may lead to transplantation-associated Kaposi's sarcoma (Chiereghin et al., 2017; Muñoz et al., 2002; Parravicini, Olsen et al., 1997; Regamey, Tamm et al., 1998).

Molecular studies have provided further evidence of HHV-8 transmission related to solid organ transplantation (Barozzi et al., 2003; Luppi, Barozzi, Schulz, Setti et al., 2000). Luppi, Barrozi, Shulz, Setti et al., (2000) reported two cases of fulminant disease following renal transplantation with documented seroconversion and viraemia.

As well as occurring due to transmission from donor-derived cells, HHV-8 infection relating to solid organ transplantation can also occur due to reactivation of latent HHV-8 in the recipient (Parravicini, Olsen et al., 1997, 2003).

In addition to an association with disease post solid organ transplantation, there has also been documentation of acute illness characterised by fever, rash and hepatitis due to HHV-8 infection post autologous peripheral blood stem cell transplantation (Luppi, Barozzi, Schulz, Trovato et al., 2000).

1.3.6. Serological Testing

Numerous serological assays have been developed to test for antibodies expressed in latent and lytic phases of HHV-8 infection. However, these assays are known to differ in sensitivity and specificity, and concordance between assays remains only moderate. There is no Food and Drug Administration approved method of testing. Serological assays in use for research purposes include IFAs, incorporating HHV-8 infected cell lines, western blot assays using both latent and lytic antigens and ELISAs using both whole virus lysate and recombinant proteins (Edelman, 2005). A recent study by Chiereghin et al., (2017) provides a thorough comparison of HHV-8 serological tests, with lytic antigen based IFAs showing the best performance and excellent agreement to the reference standard.

1.4. Molecular Epidemiology

1.4.1 Genetic Structure

The HHV-8 genome is composed of double-stranded linear DNA, and was characterised by Russo et al. in 1996, who found the genome to consist of a long 140.5 kb unique coding region, which is flanked by terminal repeats (Russo et al., 1996). Due to the varying length of the terminal repeat region, the overall size of the HHV-8

genome varies, and has been reported to be approximately 165-170 kb in length (Renne et al., 1996; Russo et al., 1996). Overall, the HHV-8 genome is highly conserved, with sequence identity of approximately 99% between viral strains (Sallah et al., 2018). There is greater variability at the 5′ and 3′ ends of the HHV-8 genome and therefore, these regions have been used in order to characterize different strains of the virus (Sallah et al., 2018).

1.4.2. Molecular Classification

Variability within the HHV-8 genome has been particularly noted to occur within the ORF-K1 gene at the left hand side of the HHV-8 genome, and within the ORF-K15 gene at the extreme right hand side of the genome (Poole et al., 1999; Zong et al., 1999). The intervening central segment of the genome represents a constant area (Hayward & Zong, 2007). HHV-8 has been classified, for epidemiological purposes, according to K1 subtyping, K15 subtyping, and according to analysis of variable sequence loci within the central constant region of the genome (Hayward & Zong, 2007).

1.4.2.1. K1

ORF-K1 encodes the variable ITAM-containing protein, a transmembrane protein (Nicholas, 2007; Zong, Arav-Bolger et al., 2007). Amino acid sequence divergence levels have been found to range from 30-60% between isolates from different geographical regions (Liu et al., 2017b). Variability in the ORF-K1 region is most marked in two hypervariable segments known as VR1 and VR2 (Cook et al., 1999; Lacoste, Kadyrova et al., 2000).

A number of classification and nomenclature systems for HHV-8 genotyping were originally employed by different investigators (Table 1.5) (Meng et al., 2001).

HHV-8 can now be classified by sequence analysis of ORF-K1 into six main molecular subtypes, A, B, C, D, E and F (Tornesello et al., 2010). The process of K1 subtyping and the existence of subtypes A-D were described by Zong et al. (1997, 1999) Subtype 'Z' was reported in 1998 by Kasolo et al. in a population of children in Zambia. The sequences that were initially designated subtype Z in this study were subsequently assigned to the A5 genotype (Kasolo et al., 2007). Subtype E was reported by Biggar et al. in 2000 in a population of Brazilian Amerindians. Subtype F was reported by Kajumbula et al. in 2006 in a Ugandan Bantu population. Subtypes A and C are generally more prevalent in Europe and the US, Subtypes A and B are predominant in Africa, subtype D is predominant in the Pacific Islands, subtype E is predominant in Brazil, French Guiana and Ecuador, and subtype F is predominant in the Ugandan Bantu tribe, and has also been sporadically identified in parts of Africa, North and South America and France (Lopes et al., 2021; Tornesello et al., 2010; Zong et al., 1999). Approximate global distributions for each subtype are 47.6% for subtype A, 22.7% for subtype B, 25.6% for subtype C, 1.3% for subtype D, 0.9% for subtype E and 1.8% for subtype F, based on sequences deposited in GenBank from 1997 to 2020 (Lopes et al., 2021). These K1 subtypes can be further divided into subtype variants (Zong et al., 1999). Hayward & Zong (2007) propose the existence of a subgroup G and suggest that all subtypes fall into four main branches; A/C, B, D/E and F/G. Given the level of diversity within the K1 gene and the association of different subtypes with different geographical regions, K1 has been used as a marker to study the epidemiological spread of HHV-8.

Table 1.5. Original classification of HHV-8 K1 genotypes by different investigators

Investigators	First level (highest)	Second level	Third level
Zong et al. (1999)	Subtypes (A-D)	Subgroups A1-A5, C1-C5, D1 and D2	Clades C3' and A1'
Cook et al. (1999)	Subtypes (A-C)	Subgroups A1, A3 and A5, large subgroup A', C' and C"	-
Meng et al. (1999)	Genotypes (I-IV)	Subtypes I-A to I-F	-
Biggar et al. (2000)	Subtype E	-	-

Adapted from Meng et al. (2001)

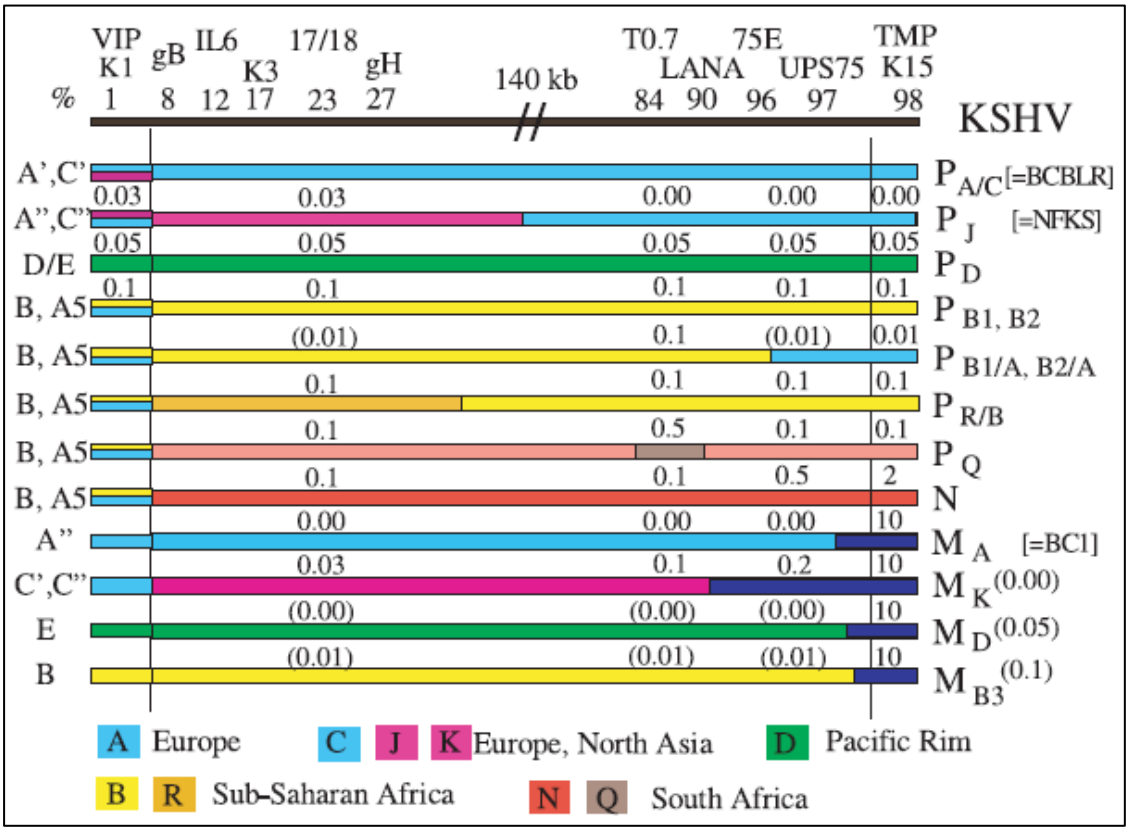

Figure 1.7. The 12 major KSHV genotypes model. (Hayward & Zong, 2007)

Table 1.6. Genotypic distribution of HHV-8 by geographic regions.

K1 Subtype	A	B	C	D	E	F
Africa	48.1%	34.5%	15.9%	0%	0%	1.6%
America (US)	38.5% 66.7%	25.9% 0%	31.1% 33.3%	0% 0%	3.7% 0%	0.7% 0%
Asia	47.1%	0%	41.2%	11.8%	0%	0%
Europe	56.5%	0.9%	38.0%	0%	0%	4.6%
Oceania	60%	0%	20%	20%	0%	0%
Global	47.6%	22.7%	25.6%	1.3%	0.9%	1.8%

Adapted from Lopes et al. (2021)

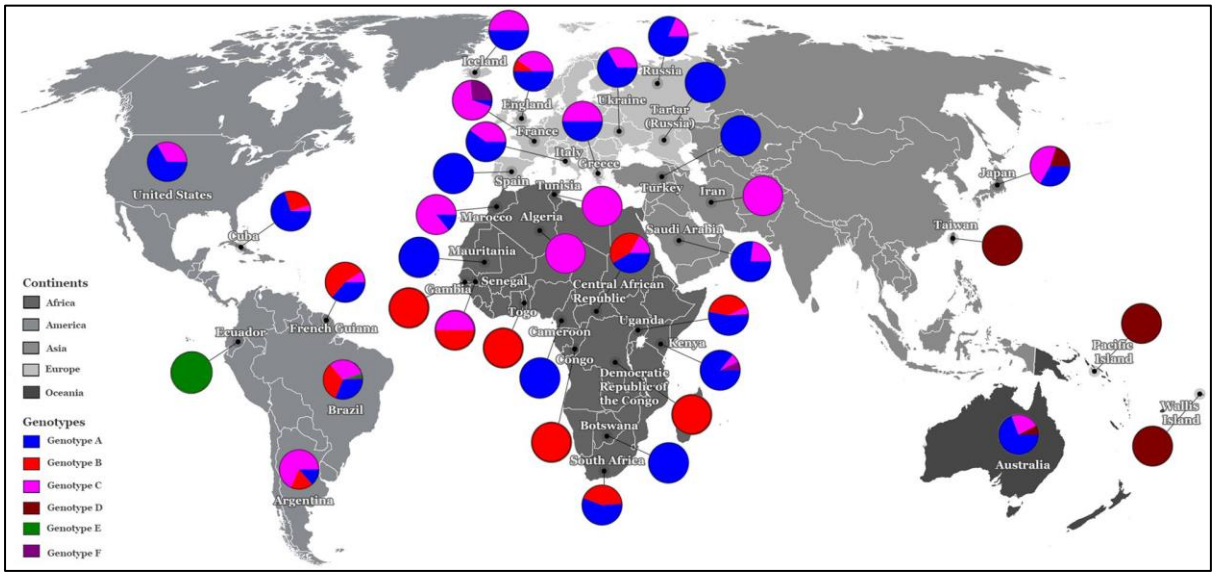

Figure 1.8. The HHV-8 genotypic diversity per country/origin. (Lopes et al., 2021)

1.4.2.2. K15

The virus may also be classified according to the K15 gene, which encodes the TMP protein, or Latency Associated Membrane Protein (LAMP) (Zong, Arav-Bolger et al., 2007). LAMP is a latency membrane protein with twelve transmembrane domains and comprises 500 amino acids (Hayward & Zong, 2007; Nicholas, 2007).

The K15 region of the HHV-8 genome has been examined by numerous investigators since it was originally described by Poole et al. in 1999. Until recently, two distinct P (Predominant or Prototype) and M (Minor) subtypes were thought to exist (Poole et al., 1999). HHV-8 isolates were thought to contain either a P or an M allele. More recently, Alagiozoglou et al. (2000) identified evidence of a new subtype N, through sequencing of the UPS75 locus of South African HHV-8 isolates. UPS75 lies in close proximity to the ORF-K15 gene and further work by Hayward & Zong (2007) has determined a new subset to fall between the P and M K15 patterns, representing a new, third N allele.

The P and M alleles are equally represented among West African patients but the P subtype predominates in Eastern Africa, among French patients and among the A and C K1 subtypes (Lacoste, Judde et al., 2000). Overall, only approximately 20% of HHV-8 isolates belong to subtype M and these have been associated with all three of the major K1 branches, A/C, B and D/E. A much smaller minority belong to subtype N (Hayward & Zong, 2007).

1.4.2.3. Constant Region

The remaining genome, when the variable left hand side and right hand side regions are excluded, is known as the constant region (Hayward & Zong, 2007). A number of areas

of variability have been studied within this constant region, including T0.7, ORF75 and ORF26E.

In 1999, Poole et al. expanded on the initial analysis of the K1 gene to examine six other loci across the HHV-8 genome. This included analysis of areas of the conserved region, notably the ORF26, T0.7 (ORF-K12) and ORF75 gene regions. Analysis of these regions demonstrated overall typical ORF-K1-linked subtype patterns. The ORF75 and T0.7 loci can also be linked with the K15 subtype (Poole et al., 1999). Alagiozoglou et al. (2000) analysed the ORF75 gene of 40 South African HHV-8 isolates and identified a novel (N) subgroup. Further detailed work examining 139 different samples, evaluating these constant region loci and their relation to the K1 and K15 genotypes was published by Zong et al. in 2002, who identified an additional subtype Q in ORF75 and T0.7.

The extended ORF26 gene (ORF26E) was investigated by Zong, Kajumbula et al. in 2007. Segments of ORF26 have been used widely as a target for identifying the presence of HHV-8 by PCR in clinical samples. Zong, Kajumbula et al. (2007) further evaluated an expanded locus of 965 bp to define eight different ORF26E genotypes. Five of these genotypes were determined to be of sub-Saharan African origin, three were of Eurasian origin and one originated in the Pacific Rim. The eight subtypes were designated A/C, J, K/M, D/E, B, Q, R and N.

Overall, Hayward & Zong (2007) have proposed 12 major HHV-8 genotypes based on the overall picture of the HHV-8 genome, through analysis of the K1 gene, K15 gene and the central constant segment.

1.4.3. Whole Genome Sequencing

Until recently, comparison of whole genomes has not been possible on a large scale. This is due to the fact that next generation sequencing data analysis can be difficult in species such as HHV-8 that have long and complex genomes containing direct or inverted repeats, making sequence assembly difficult. The process requires an abundance of DNA and many of the studies to date investigating HHV-8 have not had the availability of cultured or enriched samples required to provide sufficient viral reads for accurate assembly (Strahan et al., 2016).

Prior to 2015 there were only six previously published complete HHV-8 genomes. The majority of these sequences were obtained from individual cases and all samples were from non-endemic Western countries (Olp et al., 2015; Strahan et al., 2016).

Olp et al. (2015) subsequently sequenced 16 additional isolates of Zambian origin. Comparisons of the six Western HHV-8 genomes demonstrated a high level of conservation. This was also the case when the Zambian genomes were compared. However, distinct clustering was demonstrated when the Zambian sequences were compared with those of Western countries (Olp et al., 2015).

In 2018, Sallah et al. sequenced 45 HHV-8 genomes from an asymptomatic endemic Ugandan cohort and compared these sequences to 25 sequences that had been published at the time internationally.

Jary et al. (2020) recently utilised whole genome sequencing to confirm the identification of a new K1 F2 variant discovered among Caucasian MSM in France.

1.5. Spectrum of Disease

1.5.1 Risk Factors for Disease

Kaposi's sarcoma was first described by Moritz Kaposi in 1872, 122 years prior to the identification of HHV-8 by Chang et al. At this time, Kaposi reported an aggressive, incurable and rapidly fatal disease in a number of patients (Breimer, 1994). Subsequent to Kaposi's initial description of the disease, KS was noted to be predominantly an indolent disease in elderly male patients of eastern European and Mediterranean origin. This cohort remain a risk group for the disease known as Classical KS, with increasing age and male sex considered to be risk factors (Brown et al., 2006).

Immunosuppression is the most important risk factor in the development of HHV-8-related disease. An increased incidence of KS among MSM in the United States in the early 1980s heralded the AIDS epidemic, and HIV infection is now known to be a significant risk factor for HHV-8-related disease (Centers for Disease Control (CDC), 1981).

Other forms of immunosuppression are also significant risk factors for HHV-8-related disease, in particular, solid organ transplantation poses a significant risk, and this is well described in the literature (Engels et al., 2011). Inherited immunodeficiencies are another less common risk factor. Immunosuppressive medications such as cyclosporine, azathioprine, glucocorticoids and rituximab, used in the setting of rheumatological disorders, treatment of malignancy and transplantation are potential contributors to the development of HHV-8-related disease (Brambilla et al., 2017; Klepp et al., 1978).

1.5.2. Primary Infection

The majority of HHV-8 primary infections are likely to be asymptomatic. Symptoms such as maculopapular rash, upper respiratory tract symptoms, fever, lymphadenopathy, fatigue, diarrhoea, arthralgia and splenomegaly have been described. Severe primary infection has been reported in severely immunosuppressed patients, for example in renal transplant recipients, characterised by fever, splenomegaly, pancytopenia, or disseminated or rapid-onset KS (Luppi, Barozzi, Schulz, Setti et al., 2000).

1.5.3. Kaposi's Sarcoma

KS typically involves the skin and causes characteristic lesions which are violaceous initially, becoming brown in colour over time. The purple colouration is caused by vascular involvement. KS lesions are characterised by the formation of spindle cells (Hong et al., 2004). KS can be subdivided into four variants. Classical KS affects male patients of Mediterranean and eastern European origin, endemic KS is found in areas of sub-Saharan Africa, epidemic (or HIV-associated) KS is often aggressive and may affect multiple body systems, and iatrogenic KS occurs in the setting of iatrogenic immunosuppression, for example, in solid organ transplantation (Table 1.7).

KS may present differently, depending on the KS variant. Classical KS typically presents with cutaneous lesions on the lower extremities and the disease is generally of an indolent nature (Iscovich et al., 2000). Endemic KS can be more aggressive, often in children, and may demonstrate lymph node involvement (Hengge et al., 2002). It is known to occur in HIV-negative, as well as HIV-positive individuals. Epidemic KS, as mentioned, may demonstrate skin and lymph node involvement and may also disseminate to multiple organs such as the lung, liver, spleen and gastrointestinal tract

(Schwartz, 1996). As well as the lower extremities, skin lesions often affect other parts of the body, such as the face, oral cavity and genitalia (Hengge et al., 2002). Iatrogenic KS also tends to be acute and aggressive, and may be disseminated, particularly in the setting of organ transplantation (Luppi, Barozzi, Schulz, Setti et al., 2000).

Latent HHV-8 infection persists for life, and studies have shown that immunosuppression is associated with an increased risk of developing Kaposi's sarcoma in infected individuals, for example in solid organ transplant recipients and HIV-infected individuals (Mbulaiteye & Engels, 2006; Sullivan et al., 2008). HHV-8 is classified as a Group 1 carcinogenic agent by the International Agency for Research on Cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). The National Cancer Registry of Ireland report a Kaposi's sarcoma estimated incidence of 7 cases per year on average, with an annual age-standardised incidence rate of 0.3 per 100,000 in males and 0.0 per 100,000 in females (*National Cancer Registry (2017) Cancer in Ireland 1994-2015 with Estimates for 2015-2017: Annual Report of the National Cancer Registry. NCR, Cork, Ireland*).

Table 1.7. Classification of Kaposi's Sarcoma.

KS Variant	Population and Clinical Details
Classical KS	Elderly males of Mediterranean and eastern European origin. Indolent presentation. Cutaneous lesions on lower extremities.
Endemic KS	Sub-Saharan Africa. More aggressive presentation, particularly in children. May demonstrate lymph node involvement. Affects HIV-positive and HIV-negative individuals.
Epidemic (HIV-associated) KS	Associated with HIV infection. Worldwide distribution. Often aggressive, may affect multiple body systems.
Iatrogenic KS	Associated with iatrogenic immunosuppression e.g. solid organ transplantation. Often acute and aggressive. May be disseminated.

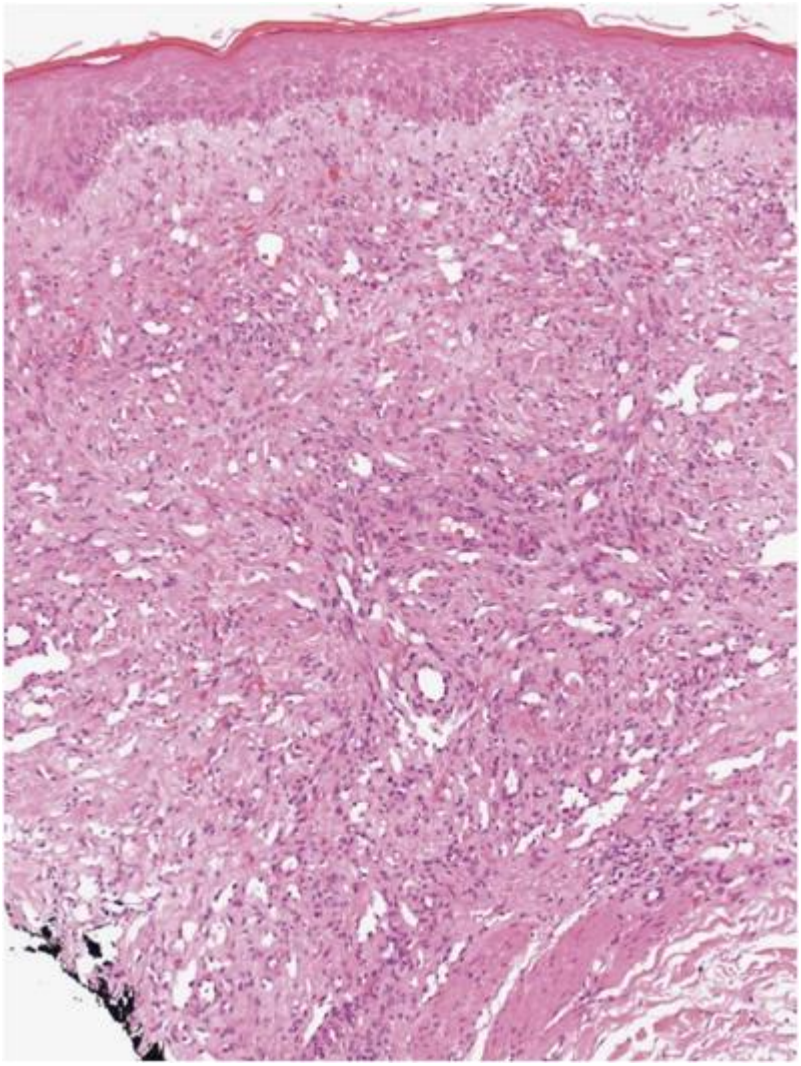

Figure 1.9. Plaque phase of Classic Kaposi Sarcoma. The perivascular spindled cells with extravasated erythrocytes and hemosiderin have coalesced to form a plaque. (Hiatt et al., 2008)

1.5.4. Primary Effusion Lymphoma

Primary effusion lymphoma was first described in 1989 and is an aggressive B-cell lymphoma which may cause pleural, peritoneal and pericardial effusions and predominantly affects AIDS patients, although it may rarely present in HIV negative individuals (Ascoli et al., 2002; Knowles et al., 1989). It has been occasionally documented to occur in the setting of organ transplantation (Jones et al., 1998; Melo et al., 2008). Similarly to KS, PEL more commonly presents in male patients. The PEL cells are infected with HHV-8, often with EBV co-infection, and they tend to remain localised to the site at which they originate (Cesarman et al., 1995; Nador et al., 1996). HHV-8 viral loads tend to be higher in PEL than in Kaposi's sarcoma, although lower than in MCD (Marcelin et al., 2007). PEL is a relatively rare condition, accounting for less than 1-4% of AIDS-related lymphomas (Mbulaiteye et al., 2002; Simonelli et al., 2003). The prognosis with this disease is generally poor (Simonelli et al., 2003).

1.5.5. Multicentric Castleman's Disease

Multicentric Castleman's disease was first recognised in 1956 and is a rare B-cell lymphoproliferative disorder (Castleman et al., 1956). This disorder is characterised by intermittent inflammatory-type symptoms including fever, hepatosplenomegaly and lymphadenopathy. It may be aggressive and has a high mortality rate. HHV-8 is particularly implicated in HIV-associated multicentric Castleman's disease. The vast majority of HIV positive MCD patients are HHV-8 positive, while only approximately 40% of HIV negative MCD cases are associated with HHV-8 (Gessain et al., 1996; Soulier et al., 1995). It is important to distinguish between HHV-8-associated MCD and HHV-8-negative MCD as the clinical features, management and outcomes vary between subtypes of the disease. HHV-8 viral loads in peripheral blood tend to be

relatively high in MCD, and higher than with KS and PEL (Marcelin et al., 2007). The viral load tends to correlate with disease activity and active disease has been associated with higher viral loads compared to when disease is in remission (Oksenhendler et al., 2000). HHV-8 viral loads may therefore be a useful monitoring tool for disease activity during treatment (Marcelin et al., 2007). Increased viral loads have been noted to correlate with elevated levels of IL-6 and IL-10, which may contribute to symptomatology (Oksenhendler et al., 2000). vIL-6 levels are also known to be expressed at high levels in HHV-8-associated MCD and may play a role in the pathogenesis of the disease (Polizzotto et al., 2013). Many patients with HHV-8-associated MCD have concurrent KS. In HIV positive patients, 60-70% will have KS at some time during the course of their illness (Oksenhendler et al., 1996, 2002).

1.5.6. Other Disease associations

There are a number of other conditions that have been linked with HHV-8 infection. However, the evidence in the literature is often conflicting and the relevance of HHV-8 in these conditions remains unproven. Many of the reported associations have not been reproducible in subsequent studies, and Zong, Arav-Bolger et al. (2007) have postulated that PCR contamination or DNA sequencing errors may have been factors in some of the initial reports.

1.5.6.1. Skin disease

HHV-8 has been linked with a number of skin conditions. A high incidence of KS has been noted to occur in patients with pemphigus and HHV-8 has been identified in lesion biopsies from patients with both pemphigus vulgaris and pemphigus foliaceus (Memar et al., 1997). Other studies have found no association (Bezold et al., 2000; Dupin et al.,

1999). KS has also been reported in the setting of bullous pemphigoid (Gaspari et al., 1997). This may have been associated with immunosuppressive treatment.

HHV-8 has been identified by PCR in patients with large plaque parapsoriasis and mycosis fungoides but, again, the evidence is conflicting (Henghold et al., 1997; Kreuter et al., 2008; Trento et al., 2005)

Several case reports have demonstrated the presence of HHV-8 DNA in angiosarcoma tumour tissue, but other studies have contradicted this (Gyulai et al., 1997; Lasota & Miettinen, 1999; McDonagh et al., 1996).

HHV-8 has been identified in a number of other skin conditions and malignancies, including premalignant Bowen's disease, squamous cell carcinoma, actinic keratosis and Paget's disease (Inagi et al., 1996). However, the significance of this finding remains uncertain.

1.5.6.2 Sarcoidosis

HHV-8 has been previously linked with sarcoidosis. Di Alberti et al. (1997) detected HHV-8 DNA in biopsy samples from sarcoidosis patients in higher numbers compared to control specimens. However, this has not been confirmed in subsequent studies.

1.5.6.3. Kikuchi's disease

Kikuchi's disease, also known as histiocytic necrotising lymphadenitis, is a benign condition characterised by fever and cervical lymphadenopathy. Huh et al. (1998) proposed the involvement of HHV-8 in the pathogenesis of this condition following the

detection of HHV-8 in six samples from twenty-six patients with the disease. This has not been corroborated by further research.

1.5.6.4. Multiple myeloma

It has been postulated that HHV-8 may be implicated in the aetiology of multiple myeloma as a number of early studies detected the virus in bone marrow biopsies of patients with the disease (Rettig et al., 1997; Said et al., 1997). This has been supported by serological studies demonstrating high HHV-8 seroprevalence levels among multiple myeloma patients (Gao et al., 1998). Other studies have not supported these findings. For example, serological studies by Mackenzie et al. (1997), Parravicini, Lauri et al. (1997) and Whitby et al. (1997) did not find elevated seroprevalence rates in this patient group. Other researchers have also been unable to detect HHV-8 DNA in bone marrow biopsies or dendritic cell cultures (Bouscary et al., 1998; Mitterer et al., 1998; Olsen et al., 1998). In addition, the significance of some of the earlier PCR-based studies has been questioned by Zong, Arav-Bolger et al. (2007).

1.5.6.5. Haemophagocytic syndrome

There have been several case reports of HHV-8 detection or infection in association with haemophagocytic syndrome (Löw et al., 1998; Pastore et al., 2000). In one case foscarnet was used to treat the patient with good clinical response (Löw et al., 1998).

1.5.6.6. Primary pulmonary hypertension

It has been suggested that there may be an association between HHV-8 and primary pulmonary hypertension. Cool et al. (2003) examined lung-tissue specimens from 16 patients with primary pulmonary hypertension and 14 patients with secondary

pulmonary hypertension using both PCR and immunohistochemistry techniques to look for evidence of HHV-8 infection. Evidence of HHV-8 was present in specimens from 10 cases in the primary pulmonary hypertension group, both on immunohistochemical analysis and using PCR. These findings have not been confirmed in further studies (Laney et al., 2005).

1.5.6.7. Ketosis-prone diabetes mellitus

Sobngwi et al. postulated as to whether HHV-8 could be implicated in the development of ketosis-prone diabetes mellitus. In a cross-sectional study carried out in France of patients of African descent presenting with diabetes, the seroprevalence of HHV-8 was noted to be significantly higher (88%) among patients with ketosis-prone diabetes, compared with 15% among patients with non-ketotic diabetes. HHV-8 DNA was detected in plasma samples that were tested in 46% of the ketotic patients compared to zero in the non-ketotic group (Sobngwi et al., 2008). Additional studies are required to investigate these findings further.

1.5.6.8. Multiple sclerosis

It has been proposed that viral infection may play a role in the pathogenesis of multiple sclerosis and several studies have investigated HHV-8 as a potential contributor (Marashi et al., 2018). Again, the evidence is conflicting (Opsahl & Kennedy, 2006). These studies had small sample sizes and therefore, further work is required in this area.

1.5.7. Diagnosis

Generally, the diagnosis of KS requires clinical and histological assessment. Additional laboratory investigations to demonstrate evidence of HHV-8 infection may be supportive of the diagnosis.

With regard to laboratory investigation, the measurement of HHV-8 viral loads may be performed by PCR of viral DNA either in plasma or in peripheral blood mononuclear cells (PBMCs) (Campbell et al., 2000). Viral load has been shown to correlate with disease activity (Campbell et al., 2000; Engels et al., 2003). However, the clinical utility of viral loads for monitoring KS activity, or as a guide to treatment is limited by the relatively low levels of HHV-8 viraemia (Campbell et al., 2000; Engels et al., 2003). Genome targets that have been used for the molecular detection of HHV-8 in clinical samples include the ORF26 locus originally described by Chang et al. in 1994, ORF72 and ORF65 (Tedeschi et al., 2002).

HHV-8 DNA may be detected in tissue from Kaposi's sarcoma lesions but copy numbers are often low (Chang et al., 1994). Detection of HHV-8 in the peripheral blood may support a diagnosis of KS (Campbell et al., 2000). However, viraemia is not universal, and may be intermittent (Campbell et al., 2000; Engels et al., 2003).

As previously discussed, serological assays are evolving to test for antibodies expressed in latent and lytic phases of infection. However, there is no Food and Drug Administration approved method of testing, and therefore these assays are generally used only for research purposes at present (Rohner et al., 2014).

1.5.8. Management

The first important step in the management of HHV-8-related disease is to correct any underlying immunodeficiency. In the setting of AIDS, effective combined antiretroviral therapy (cART) should be instituted as a priority (Bower et al., 2014; Centers for Disease Control and Prevention (CDC), 2019). Where patients are on immunosuppressive medications or steroids, these should be held or reduced where possible (Trattner et al., 1993). The main aims of treatment are to improve symptoms, prevent progression of the disease and to reduce the disease burden.

There are a variety of treatment methodologies in use for the treatment of KS. These include local therapies to improve the patient's symptoms locally. Examples include intralesional chemotherapy with agents such as vinblastine, local radiation therapy and topical alitretinoin administered as a topical gel (Bodsworth et al., 2001; Donato et al., 2013; Ramírez-Amador et al., 2002). Systemic therapies for more advanced disease include pegylated liposomal doxorubicin, liposomal daunorubicin, paclitaxel, bleomycin, vinblastine, vincristine and etoposide (Bower et al., 2014; Centers for Disease Control and Prevention (CDC), 2019). There may also be a role for interferon-alpha (Susan E. Krown et al., 2002; Shepherd et al., 1998). However, the side effect profile is significant. Pomalidomide is an oral treatment option for more indolent disease or as a second line agent (Polizzotto et al., 2016). Other agents, which are experimental at present, include imatinib, rapamycin and temsirolimus, the monoclonal antibody bevacizumab, directed against VEGF, the angiogenesis inhibitors fumagillin and thalidomide, the molecularly targeted antiangiogenic agent pazopanib, and intralesional injection of human chorionic gonadotropin (Bower et al., 2014; Centers for Disease Control and Prevention (CDC), 2019; Ganjoo & Jacobs, 2010).

For PEL, the current mainstay of treatment, in addition to cART, is combination chemotherapy, usually in the setting of a clinical trial. Treatment regimens in use based on the available evidence include dose-adjusted EPOCH (cyclophosphamide, doxorubicin, etoposide, vincristine, prednisolone) or CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) (Centers for Disease Control and Prevention (CDC), 2019). Rituximab may be incorporated into the treatment regimen in specific cases, where CD20-positive cells are identified (Centers for Disease Control and Prevention (CDC), 2019). Bortezomib has shown activity against PEL in vitro and may be an option for treatment in combination with other agents (Siddiqi & Joyce, 2008). Local radiation therapy may provide symptomatic relief in certain cases. Brentuximab vedotin is currently an investigational agent which has also demonstrated activity against PEL (Bhatt et al., 2013).

In the treatment of HHV-8-associated MCD, choice of treatment depends on the presence of concurrent KS and life-threatening organ failure. For patients without either of these factors, initial treatment is with rituximab and ganciclovir (Centers for Disease Control and Prevention (CDC), 2019). Where there is life-threatening organ failure, patients receive rituximab in combination with pegylated liposomal doxorubicin or etoposide. Where there is concurrent Kaposi's sarcoma, patients are treated with pegylated liposomal doxorubicin in addition to rituximab (Centers for Disease Control and Prevention (CDC), 2019). These patients are also treated with ganciclovir.

In terms of antiviral treatment, ganciclovir, foscarnet, cidofovir and adefovir inhibit HHV-8 lytic replication (Kedes & Ganem, 1997). The majority of HHV-8 infected cells

in KS, PEL and MCD are latently infected and, therefore, these agents have thus far had only a limited role in the treatment of these diseases, for example, the use of ganciclovir in MCD. There has been some suggestion in the literature that antiviral treatment may be beneficial in the setting of acute primary infection, for example post solid organ transplantation (Löw et al., 1998; Luppi et al., 2002). The protease inhibitors, indinavir and nelfinavir, have demonstrated some potential as treatment agents and clinical trials are currently ongoing to further assess this potential (Monini et al., 2009; Sgadari et al., 2003).

Monoclonal antibodies, particularly IL-6 inhibitors, such as siltuximab and tocilizumab are of interest and are currently under investigation as treatment options, particularly in the setting of MCD, where disease flares are known to be associated with elevated IL-6 levels (Ramaswami et al., 2020).

1.5.9. Monitoring

Patients should be monitored closely during and following treatment. Disease may be relapsing and remitting. MCD patients in particular may require multiple additional treatment cycles and should be followed up long term, even following complete response and with ongoing remission.

In MCD, HHV-8 viral loads in peripheral blood tend to be relatively high and the viral load tends to correlate with disease activity (Marcelin et al., 2007; Oksenhendler et al., 2000). HHV-8 viral loads may therefore be a useful monitoring tool for disease activity during treatment (Marcelin et al., 2007). It may be of interest to monitor IL-6 and IL-10 levels in MCD as these also fluctuate with disease activity. However, this is not

currently routinely recommended as there is insufficient evidence available (Centers for Disease Control and Prevention (CDC), 2019; Oksenhendler et al., 2000).

1.5.10. Prevention

As HHV-8-associated disease poses a particular risk in the HIV patient population, a number of potential methods for the prevention of HHV-8 transmission have been investigated.

There have been no comprehensive studies examining interventions for the prevention of sexual transmission of HHV-8. As saliva is likely to be the most common route of transmission, providing an intervention is problematic and there are no specific precautions advised for the prevention of transmission in this setting (Centers for Disease Control and Prevention (CDC), 2019).

Studies, including a double blind, placebo-controlled trial, have shown that antiviral therapy, because of its activity against lytic replication, may reduce rates of HHV-8 transmission. Valganciclovir use was shown to significantly reduce oropharyngeal shedding of the virus (Casper et al., 2008). However, the side effect profiles of these agents mean that it is not feasible for them to be used for the prevention of transmission. Interestingly, the use of antiretroviral therapy (ART) has been associated with a reduction in the frequency of HHV-8 detection in the oropharynx, suggesting that transmission should be reduced among HIV positive individuals being treated with ART (Cattamanchi et al., 2011).

Vaccination is the most effective method for the prevention of viral infections and since HIV-infected individuals remain at increased risk of developing HHV-8-related diseases, the development of a vaccine against HHV-8 would be the ideal solution in order to prevent transmission of HHV-8 infection in this patient population. Work in this area is currently ongoing (Chauhan et al., 2019).

1.6. Immunopathogenesis of HHV-8 Disease

1.6.1. Role of Proinflammatory Cytokines in HHV-8-related Disease

Dysregulation of certain inflammatory cytokines has been found to occur during flares of HHV-8-related MCD and other HHV-8-related lymphoproliferative disorders, resolving along with symptoms. Levels of vIL-6, hIL-6 and IL-10 and HHV-8 viral loads have been found to be significantly higher in patients with symptomatic HHV-8-associated MCD and KSHV inflammatory cytokine syndrome (KICS), compared to control patients with KS only (Uldrick et al., 2010). Increased production of HHV-8 vIL-6, human IL-6 and IL-10, perhaps along with other proinflammatory cytokines, is felt to be the cause of the systemic inflammatory symptoms that are manifest in MCD (Uldrick et al., 2010). Increased production of these proinflammatory cytokines may be a contributory factor in the progression of HHV-8-related disease and impaired response to treatment (Uldrick et al., 2010). This has been postulated as a factor in the pathogenesis of these diseases. HHV-8 causes cellular cytokines such as IL-6 and IL-10 to be expressed, and these cytokines are known to promote the development of KS and PEL (Jones et al., 1999; Polizzotto et al., 2013; Qin et al., 2010). Elevated levels of IL-

10 have also been noted in AIDS-MCD patients during the course of the disease (Oksenhendler et al., 2000).

The genome of HHV-8 contains a number of genes that are homologous to cellular genes. Among these is a viral homolog of human IL-6, vIL-6. In patients with HHV-8-associated MCD, affected lymph nodes have been found to contain plasmablasts, infected with HHV-8, that express vIL-6, and these patients have been noted to have elevated levels of vIL-6, hIL-6 and IL-10 systemically (Aoki, Tosato, et al., 2001; Oksenhendler et al., 2000; Polizzotto et al., 2013).

The initial development of KS tumours is characterised by the presence of immune cells, including T cells and monocytes, which promote the production and local infiltration of proinflammatory cytokines, leading to disease progression. Infection with HHV-8 results in high levels of proinflammatory cytokines locally within KS lesions, including IL-1α, IL-1β, and IL-6, and this has been postulated to contribute to initial tumour development (Ensoli & Stürzl, 1998). IFNγ and TNFα have also been implicated in KS development and progression (Ensoli & Stürzl, 1998).

KS patients with systemic inflammatory-type symptoms have been noted in numerous studies to have a higher mortality rate (Krown et al., 1989; Mosam et al., 2012; Nasti et al., 2003). Inflammation driven by HHV-8 is felt to be a major contributor to these symptoms and increased mortality (Polizzotto et al., 2015). These implicated inflammatory cytokines may be a potential target for treatment modalities to alleviate symptomatology. Human IL-6 has particularly been targeted in this manner. For example, a clinical pilot study was recently undertaken by Ramaswami et al. (2020) to

examine the effect of tocilizumab, an anti-IL-6 agent, in the management of symptomatic HHV-8-associated MCD. A response was demonstrated in 5 out of 8 patients (63%) with tocilizumab monotherapy. It was concluded that tocilizumab may have a role in the management of HHV-8-associated MCD and further research in this area is warranted.

1.6.2. KSHV Inflammatory Cytokine Syndrome

KSHV inflammatory cytokine syndrome is a recently described syndrome which is associated with severe multicentric Castleman's-like inflammatory symptoms but without evidence of Castleman's disease clinically. These patients may have KS or PEL. KICS is associated with a poor prognosis and the mortality rate associated with this syndrome is approximately 50% (Prieto-Barrios et al., 2018).

Systemic inflammation associated with HHV-8, while originally found to occur predominantly in the setting of MCD, has more recently been found also to occur in the absence of MCD. A retrospective case series of these patients was described in 2010 by Uldrick et al. This group of patients were determined not to have a diagnosis of MCD, but KSHV was detectable in blood and vIL-6, hIL-6 and IL-10 levels were found to be elevated, similar to those found in active HHV-8-associated MCD. In this group of patients, the levels of these cytokines were found to be significantly elevated when compared with controls with KS only. Based on this study, Uldrick et al., 2010 determined that overproduction of vIL-6 and hIL-6 may be responsible for the inflammatory-type symptoms present in this syndrome. They felt that excessive production of other cytokines, possibly including IL-10 may also contribute. This syndrome has been entitled the KSHV inflammatory cytokine syndrome and the case

definition, as proposed by Polizzotto et al. in 2012, is detailed in Table 1.8. According to Polizzotto et al., at least two clinical manifestations from at least two categories must be present, along with an elevated C-reactive protein (CRP), elevated HHV-8 viral load, and there must be no evidence of HHV-8-associated MCD for the working case definition to be fulfilled.

Table 1.8. KICS case definition, as proposed by Polizzotto et al. (2012, 2015).

1. Clinical Manifestations	
a) Symptoms Fever Fatigue Oedema Cachexia Respiratory symptoms Gastrointestinal disturbance Arthralgia and myalgia Altered mental state Neuropathy with or without pain	**b) Laboratory abnormalities** Anaemia Thrombocytopenia Hypoalbuminaemia Hyponatraemia **c) Radiographic abnormalities** Lymphadenopathy Splenomegaly Hepatomegaly Body cavity effusions
2. Evidence of Systemic Inflammation	
Elevated C-reactive protein (≥3 g/dL)	
3. Evidence of HHV-8 Viral Activity	
Elevated HHV-8 viral load in plasma (≥1000 copies/mL) or peripheral blood mononuclear cells (≥100 copies/10^6 cells)	
4. No Evidence of HHV-8-associated Multicentric Castleman Disease	
Exclusion requires pathologic assessment lymph node, bone marrow or spleen	

It is felt that HHV-8 lytic activation resulting in increased production of vIL-6 and dysregulation of the immune response may lead to the symptoms observed in KICS (Uldrick et al., 2010). Polizzotto et al. (2015) carried out a prospective study of 10 KICS patients, aiming to define the natural history of KICS and its relationship to HHV-8 viraemia and associated cytokines. None of these patients had radiological evidence of substantial lymphadenopathy or splenomegaly that would be consistent with MCD. Patients were commonly found to be critically ill, with the majority requiring inpatient management and 50% requiring intensive care unit (ICU) management. Patients with KICS were found to have significantly elevated IL-6 and IL-10 levels. Of note, the mortality rate in the 10 patients with KICS was 60%.

It is known that patients with HIV, in the absence of HHV-8-related disease, particularly where HIV infection is poorly controlled, may also have elevated levels of certain inflammatory cytokines (Aoki et al., 2001; Breen et al., 1990; Stylianou et al., 1999). Whereas HHV-8-associated-MCD is generally more common in well-controlled HIV infection (Bower et al., 2011; Polizzotto et al., 2015; Uldrick et al., 2010), it is interesting to note that Polizzotto et al. (2015) found their KICS patients to have elevated HIV viral loads and low CD4 counts.

In 2018, Prieto-Barrios et al. described in a case report the association of haemophagocytic lymphohistiocytosis (HLH) with periods of KICS exacerbation. There have been several similar cases reported in the literature also (Fardet et al., 2003).

The management of KICS involves maintaining an appropriate balance between controlling the patient's disease, along with their degree of immunosuppression (Prieto-

Barrios et al., 2018). Several therapeutic approaches have been attempted, including the use of ganciclovir and valganciclovir, aiming to target viral replication, and liposomal doxorubicin, used in the treatment of HHV-8-related disease (Prieto-Barrios et al., 2018). New treatment methods need to be considered and evaluated to address this syndrome and the likelihood that patients' symptoms are due to excessive production of inflammatory cytokines such as vIL-6, IL-6 and IL-10 (Casper et al., 2004; Davis et al., 2007; Uldrick et al., 2010, 2011). Agents such as tocilizumab and sirolimus are under investigation as potential treatment methodologies for MCD in HIV positive individuals and could be future considerations in the management of KICS (Goncalves et al., 2017; Polizzotto et al., 2015).

1.6.3. Kaposi's Sarcoma Immune Reconstitution Syndrome

Immune reconstitution inflammatory syndrome (IRIS) is a disorder resulting in a paradoxical clinical deterioration following commencement of highly active antiretroviral therapy (HAART) in HIV-infected individuals. Pre-existing infections or subclinical infections may trigger systemic or local inflammatory reactions in the setting of a rapid improvement in the individual's immune function and ability to mount an inflammatory response.

IRIS can occur in patients with KS, following commencement of antiretroviral therapy, and can lead to the rapid progression of KS (Bower et al., 2005). The first case report of what would later be known as KS IRIS was reported by Weir & Wansbrough-Jones in 1997. Reported cases have generally demonstrated a notable decrease in HIV viral load along with a rise in $CD4^+$ T-lymphocyte count following initiation of HAART (Leidner & Aboulafia, 2005). Onset of KS flare generally occurs within a 2 month period after

HAART is commenced, and can occur as early as 3 weeks following response to HAART (Leidner & Aboulafia, 2005).

In a case series of 9 cases at the University of Washington and a literature review including an additional 10 cases, 4 deaths associated with pulmonary KS were reported, underlining the significant morbidity and mortality that may be associated with KS IRIS and the importance of clinical follow up during HAART and early treatment of symptomatic KS in this setting (Leidner & Aboulafia, 2005). Interestingly, 21% of cases were not known to have KS prior to their KS IRIS flare. The fatality rate associated with KS IRIS has been reported as up to one third of cases. One study of IRIS associated with AIDS-defining opportunistic infections demonstrated a 100% mortality and long term morbidity rate among patients with visceral KS-IRIS, the highest of any infection included in the study (Achenbach et al., 2012). Regarding management of this syndrome, HAART is usually continued in conjunction with KS treatment. Patients generally appear to respond to treatment with systemic chemotherapy (Leidner & Aboulafia, 2005).

KS-IRIS incidence and mortality have been noted to be higher in limited resource settings. Letang et al. (2013) prospectively studied 417 patients from cohorts in sub-Saharan Africa and the UK and found a significantly higher incidence rate and mortality rate among sub-Saharan African patients compared to those of the UK cohort. The authors attributed these findings to patients from sub-Saharan Africa having more advanced KS disease and limited access to chemotherapeutic agents.

KS IRIS has been reported in treatment-naïve patients, patients who are switched to a new treatment regimen following failure of a previous HAART regimen, and patients who have recommenced treatment following an interruption (Leidner & Aboulafia, 2005).

Of note, it has been reported that patients with IRIS have higher levels of IL-6 than non-IRIS HIV patients and HIV negative individuals (Stone et al., 2001, 2002). In addition, activation of HHV-8 in the setting of IRIS may lead to MCD-like symptoms or KICS in certain patients (Uldrick et al., 2010).

1.7. Aims and Objectives for this Thesis

HHV-8 is a gamma-2 herpesvirus (rhadinovirus) that causes infection worldwide and is endemic in certain populations. It is a large, enveloped virus, with a double-stranded DNA genome, and is the aetiologic agent underlying a number of malignant diseases, contributing to significant morbidity and mortality, particularly in vulnerable and immunosuppressed groups.

Since the discovery of HHV-8, the epidemiology of this virus has been investigated in many countries worldwide. However, little is known about its seroprevalence or molecular epidemiology in the UK and Ireland. No studies in this area have been performed in Ireland to date. Work has been done recently on the role of proinflammatory cytokines in HHV-8 disease but there is the potential for further investigation in this area.

1.7.1 Aim 1: Seroprevalence of HHV-8 in Ireland

To investigate the seroprevalence of HHV-8 among blood donors in Ireland and to compare with HHV-8 seroprevalence among risk groups for HHV-8 infection.

Objectives

1. To assess the seroprevalence of HHV-8 among 200 blood donors in Ireland and 200 patients using an indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp., Denville, NJ, USA).
2. To compare the HHV-8 seroprevalence between blood donors and patient groups.

1.7.2 Aim 2: Molecular Epidemiology of HHV-8 in Ireland

To investigate the molecular epidemiology of HHV-8 in Ireland among patients with known HHV-8-related diseases.

Objectives

1. To use molecular methods to characterise four regions of the HHV-8 genome, K1, including the hypervariable regions VR1 and VR2, T0.7 (K12), ORF75 and K15, using samples from patients with known HHV-8-related diseases at St. James's Hospital.
2. To perform phylogenetic analysis in order to determine the HHV-8 subtypes present among these patients.

1.7.3 Aim 3: Immunological aspects of HHV-8 infection

To perform cytokine analysis over time using paired plasma samples from patients with HHV-8 related diseases and to compare the levels of these cytokines between HHV-8 subtypes and before and after treatment for HHV-8-related disease.

Objectives

1. To determine the levels of 10 specific cytokines in HHV-8 positive patient samples over time using a commercial 10-plex assay, V-PLEX Proinflammatory Panel 1, for IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNF-α (Meso-Scale Discovery, Rockville, MD, US).

2. To compare cytokine levels between HHV-8 subtypes.

3. To compare cytokine levels before and after treatment in patients with HHV-8-related diseases.

Chapter 2

Seroprevalence of Human Herpesvirus 8 in Ireland

2.1 Introduction

The global epidemiology of HHV-8 differs from that of other herpesviruses as it exhibits significant geographical variation in seroprevalence, as well as demonstrating contrasting seroprevalence rates among different sub-populations (Rohner et al., 2014). Sub-Saharan Africa has the highest rate of infection, with approximately 50% of the population seropositive in some areas (Dollard et al., 2010; Etta et al., 2018). Seroprevalence is approximately 10% in China, 10% in the Mediterranean, approaching 30% in areas of Italy, and approximately 5-6% in the United States and northern Europe (Engels et al., 2007; Regamey, Cathomas et al., 1998; Whitby et al., 1998; Zhang et al., 2014). However, the rate of Kaposi's sarcoma does not necessarily correlate with the rate of HHV-8 infection in a given population (Chen et al., 2004). High seroprevalence rates have been specifically ascribed to men who have sex with men (MSM), African migrants and HIV-infected individuals (Rohner et al., 2014) (*Section 1.3.1*).

Transmission of HHV-8 may occur via a number of mechanisms, in particular via salivary transmission, but also through sexual transmission, bloodborne transmission and vertical transmission (Brayfield et al., 2003; Cannon et al., 2003). However, it remains unclear to what degree each of these mechanisms of transmission contributes to the spread of this virus. It may be the case that different mechanisms of transmission predominate depending on the particular population sub-group in question or depending on the seroprevalence of the population (Henke-Gendo & Schulz, 2004) (*Section 1.3.3*).

The majority of HHV-8 infection represents asymptomatic and latent infection. Immunosuppression is a clear contributing factor in the development of HHV-8-related

diseases, such as Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). However, further investigation is required to endeavour to explain why these diseases vary in prevalence in different populations, often despite similar prevalence of HHV-8 infection (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). For example, the worldwide incidence of Kaposi's sarcoma is higher in men than in women, while overall HHV-8 seroprevalence is similar in the two groups (Begré et al., 2016; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012).

Serological testing methodologies for HHV-8 antibodies remain unstandardised and there are a number of different assays used for research purposes currently, including IFAs, western blot assays and ELISAs. This lack of standardisation makes comparison between studies of seroprevalence difficult. It also limits the potential for screening for this virus, for example in blood donor samples and in organ transplantation (Chiereghin et al., 2017; Rohner et al., 2014) (*Section 1.3.6*).

Since the discovery of HHV-8, the epidemiology of this virus has been investigated in many countries, but little is known about its seroprevalence in Ireland.

2.1.1 Aims

The aims of this study were firstly, to investigate the seroprevalence of HHV-8 in an Irish population and, secondly, to examine HHV-8 seroprevalence in specific risk groups for HHV-8 infection. The risk groups for investigation included patients from St. James's Hospital Genitourinary Medicine (GUM) and Infectious Diseases (ID)

clinic and the Gay Men's Health Service (GMHS), subdivided into populations of HIV positive and HIV negative MSM and male and female heterosexual GUM and ID clinic attendees.

2.1.2 Objectives

1. To determine the seroprevalence of HHV-8 among 200 blood donors in Ireland from serum samples using an indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp., Denville, NJ, USA).
2. To determine, using the same method, the seroprevalence among 200 patients from St. James's Hospital GUM and ID clinic and the GMHS, subdivided into populations of HIV positive and HIV negative MSM and male and female heterosexual GUM and ID clinic attendees.
3. For the purposes of test verification, to refer 200 samples to be tested blindly at the CDC Infectious Diseases Laboratories using three different assays, an IFA for detection of IgG antibodies to HHV-8 lytic antigens and two peptide ELISAs based on ORF-K8.1 and ORF65 (Dollard et al., 2010).
4. To compare the HHV-8 seroprevalence between blood donors and patient groups.

2.2 Materials and Methods

2.2.1 Study Design and Setting

We designed a cross-sectional study investigating the seroprevalence of HHV-8 in an Irish population, including various risk groups. The study was carried out from April to November 2019 at the Department of Clinical Microbiology, St. James's Hospital, Dublin, Ireland. The department processes specimens for both the GUM and ID unit at St. James's Hospital and for the GMHS. This study was performed in conjunction with these services and with the Irish Blood Transfusion Service (IBTS).

2.2.2 Study Population

The study population included 400 individuals aged ≥ 18 years, including 200 blood donors from the IBTS and 200 patients from St. James's Hospital GUM and ID clinic and the GMHS, subdivided into populations of HIV positive and HIV negative MSM and male and female heterosexual GUM and ID clinic attendees (Figure 2.1). Written informed consent was obtained from all the study participants.

Serum samples were collected in the IBTS between 6th April 2019 and 14th May 2019. Specimens collected in the IBTS were anonymised prior to transfer to St. James's Hospital and retained donor data included age bracket, gender and region where the specimen was collected.

Serum samples were collected in St. James's Hospital GUM and ID clinic and the GMHS between 27th March 2019 and 3rd July 2019. Each specimen was given a unique

study number and data collected included age, gender, geographical area of origin, HIV status, and whether the patient was MSM or heterosexual.

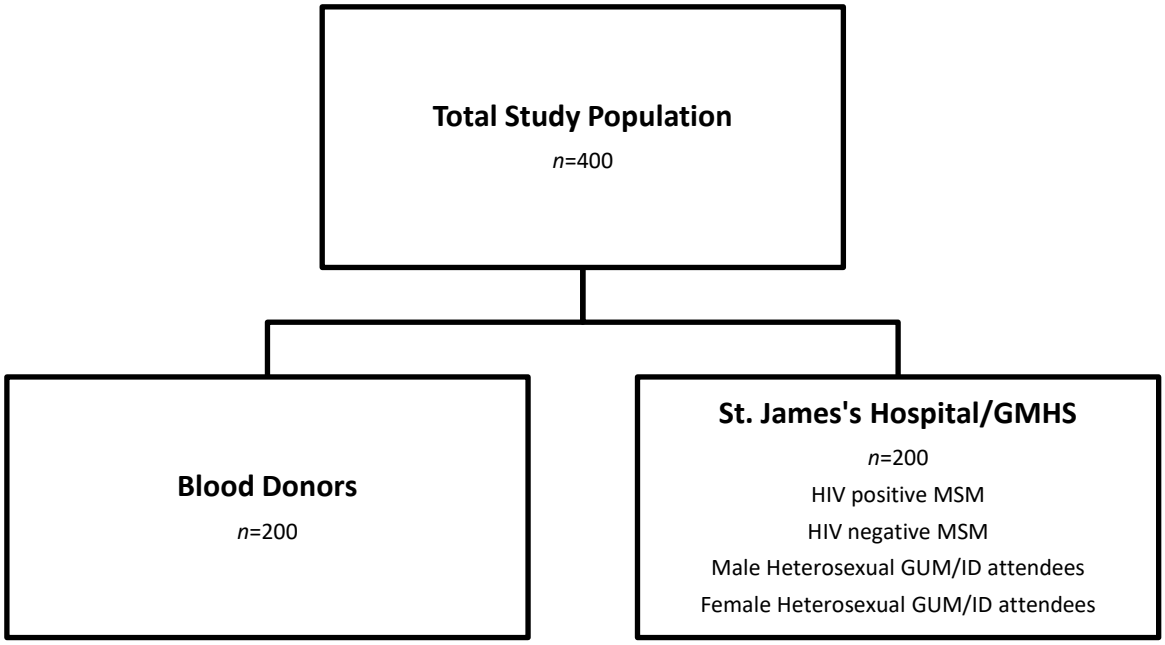

Figure 2.1. Seroprevalence study population and sub-groups for analysis.

HIV, human immunodeficiency virus; MSM, men who have sex with men, GUM, Genitourinary Medicine; ID, Infectious Diseases.

2.2.3 Serological Testing

Four hundred samples were collected. All samples were frozen at -20°C pending testing. All samples collected were tested using a commercial indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp., Denville, NJ, USA), according to the manufacturer's instructions. Testing was performed using facilities at the National Virus Reference Laboratory (NVRL), Dublin. Reactivity was observed under fluorescence microscopy using 20-40X magnification and fluorescence reaction was graded according to the following intensity scale: 4+ (brilliant), 3+ (bright), 2+ (moderate) and 1+ (weak). Samples reactive at a 1:64 dilution were considered positive (Figure 2.2).

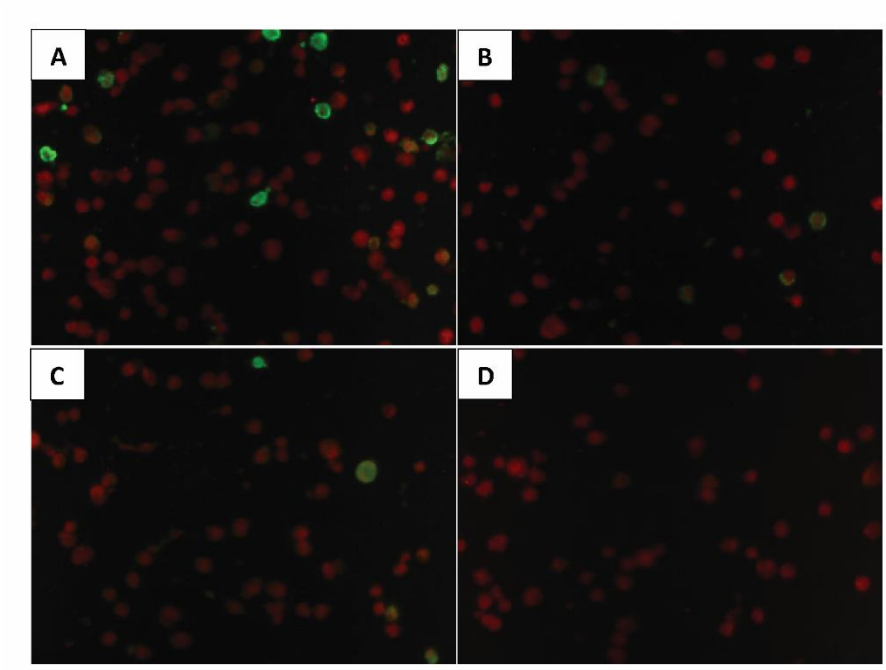

Figure 2.2. Indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp., Denville, NJ, USA).

(A) 3+ fluorescence demonstrated in a positive sample at 1:64 dilution; (B) 1+ fluorescence demonstrated in a positive sample at 1:64 dilution; (C) Scimedx kit positive control; (D) Negative sample at 1:64 dilution.

For the purposes of test verification, 200 samples were also tested blindly at the CDC Infectious Diseases Laboratories using three different assays, an IFA for detection of IgG antibodies to HHV-8 lytic antigens and two peptide ELISAs based on ORF-K8.1 and ORF65 (Dollard et al., 2010).

2.2.4 Statistical Analysis

Statistical analysis was performed using IBM® SPSS® Statistics (version 26). HHV-8 seroprevalence was examined in the total study population and in all sub-groups. Comparisons were performed between sub-groups using the Chi-square test. Fisher's exact test was used where the expected frequency was < 5. A p value of < 0.05 was considered statistically significant.

2.2.5 Ethical Approval

Ethical approval for this study was obtained from Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee and from the Royal College of Physicians of Ireland Research Ethics Committee.

2.3 Results

From 6th April 2019 to 14th May 2019, 200 blood donor serum samples were collected by the IBTS, anonymised and sent to St. James's Hospital for storage at -20°C prior to testing by indirect fluorescence assay for IgG antibodies to HHV-8 (Table 2.1). 57.5% of samples were collected in Leinster, 33% in Munster, 4.5% in Connacht and 4.5% in Ulster. 40% of samples were collected in relatively urban areas and 59.5% in relatively rural areas. The median age of blood donor participants was 44 years (range 19-77 years). 58% of blood donor participants were male and 42% were female. All blood donor samples tested negative or equivocal using indirect fluorescence assay for the detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp., Denville, NJ, USA) and all equivocal results were confirmed to be negative following verification and confirmatory testing in the CDC using the three test protocol described.

From 27th March 2019 to 3rd July 2019, 200 serum samples were collected from St. James's Hospital GUM and ID clinic and the GMHS (Table 2.1). All samples were stored at -20°C prior to testing by indirect fluorescence assay for IgG antibodies to HHV-8. Of these samples, 42 (21%) were confirmed to be positive for HHV-8 antibodies. This was a significantly higher rate of positivity compared with the blood donor population ($p < 0.001$) and the difference remained significant ($p < 0.001$) when both the heterosexual group and the MSM group were compared separately to the blood donor group. Overall, the age group with the highest percentage positivity were the 39-48 year olds category (Table 2.2).

Of these 200 samples, 100 were collected from MSM patients. 35% of MSM samples were positive for HHV-8 antibodies, 46% in HIV positive MSM and 24% in non-HIV positive MSM. The difference in seroprevalence between HIV positive MSM and non-HIV positive MSM was statistically significant ($p = 0.021$). Seven percent of heterosexual patients were positive for HHV-8 antibodies, demonstrating a significantly lower prevalence when compared with the MSM population ($p < 0.001$). Eight percent of heterosexual males and 6% of heterosexual females were found to have antibodies to HHV-8. This difference was not statistically significant. The majority of the 200 GUM and ID clinic participants were Irish ($n = 117$) (59%) and 15% of these patients were observed to have antibodies to HHV-8. This was a lower proportion compared to that of participants from other parts of Europe, South/Central America and Africa, although the numbers in these groups were small (Table 2.3).

Table 2.1. Age and percentage IgG positivity of study population and sub-groups.

Study Population	No. Participants	Median Age (Range)	% IgG Positive
Blood Donors	200	44 (19-77)	0
GMHS/GUM/ID Clinic Attendees	200	29 (19-71)	21
MSM	100	32.5 (19-71)	35
HIV Positive	50	36 (19-57)	46
HIV Negative	50	31 (19-71)	24
Heterosexuals	100	26 (19-60)	7
Male	50	27 (20-60)	8
Female	50	25 (19-53)	6

Abbreviations: GMHS, Gay Men's Health Service; GUM, Genitourinary Medicine; ID, Infectious Diseases; MSM, men who have sex with men; HIV, human immunodeficiency virus.

Table 2.2. Proportion of patients with HHV-8 IgG antibodies stratified according to sub-group and age category.

Age (years)	Proportion (%) of patients with HHV-8 IgG antibodies				
	HIV Positive MSM	HIV Negative MSM	Male Heterosexuals	Female Heterosexuals	All Patients
19-28	3/8 (37.5)	6/19 (31.6)	2/28 (7.1)	2/38 (5.3)	13/93 (14)
29-38	10/23 (43.5)	5/22 (22.7)	2/14 (14.3)	0/7 (0)	17/66 (25.8)
39-48	9/16 (56.3)	1/4 (25)	0/4 (0)	1/4 (25)	11/28 (39.3)
49-58	1/3 (33.3)	0/1 (0)	0/3 (0)	0/1 (0)	1/8 (12.5)
59-68	0/0 (0)	0/3 (0)	0/1 (0)	0/0 (0)	0/4 (0)
69-78	0/0 (0)	0/1 (0)	0/0 (0)	0/0 (0)	0/1 (0)

Abbreviations: HIV, human immunodeficiency virus; MSM, men who have sex with men.

Table 2.3. Geographical area of origin and percentage IgG positivity in GUM/ID patient population.

Geographical area of origin	Proportion (%) of patients with HHV-8 IgG antibodies
Europe *n* = 140	23 (16.43)
(Ireland) *n* = 117	18 (15.38)
(Europe Other) *n* = 23	5 (21.74)
North America *n* = 1	0 (0)
South/Central America *n* = 35	15 (42.86)
Africa *n* = 10	2 (20)
Asia *n* = 10	1(10)
Unknown *n* = 1	0 (0)

Abbreviations: GUM, Genitourinary Medicine, ID, Infectious Diseases.

Two hundred specimens were sent to the CDC for test verification purposes and confirmatory testing using the described methodology. All blood donor samples were negative using the CDC testing methodology, and the overall result concordance demonstrated moderate agreement with a kappa value of 0.404 ($p < 0.001$). However, the level of agreement was reduced due to the number of results reported as equivocal using the commercial kit.

2.4 Discussion

All 200 Irish blood donors tested were HHV-8 seronegative. This was assessed in a population of blood donors with a nationwide representation, both male and female, and over a broad range of age groups. Seroprevalence studies in blood donors in other European countries and in North America with similar participant numbers have demonstrated varying rates, ranging from 0% in Greece and parts of Spain, to rates of 2-6% in the majority of European countries, including rates of 0.7-5% in the United Kingdom (Alcalde et al., 2008; Dakovic Rode et al., 2005; Gambús et al., 2001; Politou et al., 2014; Preiser et al., 2001; Regamey, Cathomas et al., 1998; Simpson et al., 1996; Tettmar et al., 2016). Rates of 24% have been identified among blood donors in parts of southern Italy, an area known to have high rates of KS, and up to 35% in Sicily (Whitby et al., 1998). Rates of 1-5% have been reported in North America, but up to 15-23% in Texas (Baillargeon et al., 2001; Hudnall et al., 2003; Kedes et al., 1996; Kleinman et al., 2012; Pellett et al., 2003). These studies utilised a range of different testing methodologies. This demonstrates that HHV-8 prevalence rates may depend both on the region studied and on the individual assays and testing methodologies utilised. The absence of seropositivity in 200 Irish blood donors may suggest that Ireland has a lower overall population HHV-8 seroprevalence than other European countries. However, 200 is a small sample size for the low seroprevalence. Also, blood donors are a select subset of the population and therefore results cannot be extrapolated to the general population which is a limitation of the study.

As expected, the proportion of individuals identified to have antibodies to HHV-8 in the MSM population was significantly higher than that in the heterosexual population. It

was most marked in the HIV positive MSM population who had a significantly higher HHV-8 seroprevalence when compared with the non-HIV positive MSM group.

From the time HHV-8 was first identified as the causative agent in Kaposi's sarcoma, studies have identified higher seroprevalence rates among MSM, particularly among HIV positive MSM (Kedes et al., 1996; Lennette et al., 1996). It was postulated that HHV-8 infection was sexually transmitted, and studies undertaken by Lennette et al. (1996) and Martin et al. (1998) were among the first to attempt to demonstrate evidence of sexual transmission. Both groups showed sexual activity to be a strong risk factor for HHV-8 infection, as well as demonstrating higher prevalence in MSM and a strong association with HIV infection. These findings are most notable in non-endemic countries and many subsequent studies have agreed with these findings (Engels et al., 2007). A meta-analysis published by Liu et al. in 2018 demonstrated that, among 15,914 subjects, HIV positive MSM were more likely to be HHV-8 seropositive than those who are HIV negative (OR=3.70, 95% CI 2.93-4.67). Similarly, a significant association between sexually transmitted infections (STIs) and HHV-8 was observed (OR=2.40, 95% CI 2.04-2.82), providing further evidence in support of sexual transmission. The evidence for association with HIV specifically has been conflicting, with some studies demonstrating an association and others not (Baeten et al., 2002; Biryahwaho et al., 2010; Klaskala et al., 2005; Nuvor et al., 2001). Rohner et al. (2016) performed a systematic review on this subject and demonstrated an overall increased HHV-8 seroprevalence associated with HIV infection, with a stronger association in MSM compared to heterosexual adults. The current study in an Irish population is consistent with these previous findings in the literature. Although the evidence available suggests that HHV-8 may be sexually transmitted, the exact mode of transmission and

the reasons underlying the propensity for infection in the MSM population and in HIV positive individuals remain unclear. Consistent with the evidence that HHV-8 may be sexually transmitted is the clear association in the literature between recognised STIs and HHV-8 infection (Lennette et al., 1996; Liu et al., 2018; Martin et al., 1998). However, other studies have supported alternative routes of transmission. Pauk et al. (2000) provided evidence in an MSM population of significantly higher detection rates of HHV-8 in oral secretions compared with anal specimens and genital secretions, suggesting that transmission is more likely to occur via saliva rather than genital secretions, and this has been supported by a number of subsequent publications, including a study by Casper et al. (2004) in HIV positive MSM. In the current study, the seroprevalence in the heterosexual GUM and ID clinic group was significantly lower than in the MSM population, and there was no significant difference between the HHV-8 seroprevalence amongst men and women in this group.

There were some limitations to this study. Firstly, the sample size was small both in the blood donor group and in the patient population. However, studies of HHV-8 seroprevalence with similar sample sizes have been performed in other European countries and have provided valuable information on the epidemiology of this infection in Europe. For example, Politou et al. (2014) performed serological testing for HHV-8 IgG antibodies on 179 blood donor samples in Greece and did not identify any positive samples. Regamey, Cathomas et al. (1998) and Rode et al. (2005) performed studies with similar sample sizes in Switzerland and Croatia, respectively, examining the seroprevalence of HHV-8 among blood donors, HIV infected individuals and MSM. Enbom et al. (2000) performed HHV-8 antibody testing of 162 blood donor samples, as well as testing samples from a number of high-risk groups. Similarly, a number of

recent studies in Asia have examined the seroprevalence of HHV-8 in small populations of HIV positive patients and MSM (Liu et al., 2017a; Munawwar et al., 2014; Watanabe et al., 2017). The current study is comparable to these other studies in the literature in terms of sample size and these similar sized studies have provided valuable information regarding the epidemiology of HHV-8.

Secondly, it is not possible to extrapolate blood donor data to the general population in Ireland as blood donors represent a specific subset of the population. Strict donor selection guidelines and deferral criteria lead to the exclusion of many individuals living in Ireland who were born in HHV-8 endemic regions. In addition, MSM (one year exclusion policy) and those with overlapping infectious diseases (HIV, HCV and chronic hepatitis B virus (HBV)) are also temporarily or permanently deferred. The MSM subgroup in this study was recruited from GUM and ID clinics, and therefore may not represent the MSM population as a whole. However, the findings in this group were significant when compared to a similar heterosexual population.

The current study was performed using a lytic indirect fluorescence assay with verification at the CDC, as described. A latent antigen-based assay was not used. Studies have shown that use of latent antigen-based IFAs does not contribute to sensitivity. A recent study by Chiereghin et al. (2017) demonstrates this, with lytic antigen-based IFAs showing the best performance and excellent agreement to the reference standard. This publication provides a thorough comparison of HHV-8 serology tests and concludes that the lytic IFA, when used properly, is the optimal test.

In summary, the first seroprevalence study of HHV-8 in Ireland, indicates low seropositivity in the blood donor population, and suggests an overall low seroprevalence in the Irish population compared with other countries. As expected, a significantly higher rate of seropositivity was identified in the GUM and ID clinic attendee population, in particular among the MSM population and notably the HIV positive MSM population. HHV-8 seropositivity was confined to specific risk groups such as MSM and HIV positive individuals.

Chapter 3

Molecular Epidemiology of Human Herpesvirus 8 in Ireland

3.1 Introduction

The molecular epidemiology of HHV-8 varies significantly between geographical regions. The two areas of the HHV-8 genome that have been used most extensively to classify this virus in to different subtypes are the K1 gene at the left hand side of the HHV-8 genome and the K15 gene at the extreme right hand side of the genome (Poole et al., 1999). Variability in the ORF-K1 region is concentrated within the VR1 and VR2 hypervariable segments (Cook et al., 1999; Lacoste, Kadyrova et al., 2000) (*Sections 1.4.2.1-1.4.2.2*).

Additional areas of study within the constant central region of the HHV-8 genome include T0.7, ORF75 and ORF26. Analysis of these regions demonstrates overall typical ORF-K1-linked subtype patterns (Poole et al., 1999). However, the ORF75 and T0.7 loci can also be linked with the K15 subtype (Poole et al., 1999) (*Section 1.4.2.3*).

HHV-8 is highly genetically variable and has been classified by sequence analysis of ORF-K1 into six main molecular subtypes, A, B, C, D, E, F (Tornesello et al., 2010). Each of these subtypes has been shown to be prevalent in different regions geographically. For example, subtypes A and C predominate in Europe and the US, subtype B is more prevalent in Africa, subtype D and E are more unusual and are associated with specific populations in the Pacific Islands and in Brazil, French Guiana and Ecuador, respectively. Subtype F is also a rare subtype and has been found more sporadically in Africa and Europe and America (Lopes et al., 2021) (*Section 1.4.2.1*).

Regarding the K15 region of the HHV-8 genome, Poole et al. (1999) originally described two distinct P and M subtypes, or alleles. A third subtype, N was identified more recently by Alagiozoglou et al. (2000). K15 subtype P predominates overall globally, with subtype M representing only approximately 20% of HHV-8 sequences internationally and subtype N representing an even smaller proportion. Subtype M tends to be more prevalent in Eastern Europe and Western Africa (Lacoste, Judde et al, 2000) and subtype N has been identified primarily in sequences from South Africa and Uganda (Hayward & Zong, 2007) (*Section 1.4.2.2*).

The molecular epidemiology and the proportions of different subtypes of HHV-8 have been extensively examined and characterised internationally. However, no studies in this area have been performed in Ireland to date. The purpose of this study was to examine the molecular epidemiology according to phylogenetic analysis of HHV-8 in a large tertiary referral centre in Ireland, in the context of data available from other countries.

3.1.1 Aims

The aims of this study were to investigate the molecular epidemiology of HHV-8 in Ireland among patients with known HHV-8-related diseases, firstly by using molecular techniques to characterise four regions of the HHV-8 genome and, secondly, using phylogenetic analysis to examine the HHV-8 subtypes present in this population. Phylogenetic analysis of the samples described will enable us to determine the pattern of HHV-8 subtypes causing disease in susceptible patients in Ireland. It will also allow us to compare the patterns present in Ireland to those in other countries internationally.

3.1.2 Objectives

1. To use nested PCR to characterise four regions of the HHV-8 genome, K1, including the hypervariable regions VR1 and VR2, T0.7 (K12), ORF75 and K15, using samples from patients with known HHV-8-related diseases at St. James's Hospital.
2. To construct six phylogenetic trees, one for each locus, in order to determine the HHV-8 subtypes present among these patients.

3.2 Methods

3.2.1 Study Design and Setting

A retrospective study was designed to investigate the molecular epidemiology of HHV-8 in Ireland. The study was carried out from March 2019 to December 2021 at the Department of Clinical Microbiology, St. James's Hospital, Dublin, Ireland. St. James's Hospital is a large, acute tertiary referral centre incorporating multiple national specialty services, including the National Haematopoietic Stem Cell Transplant Unit and a large Genitourinary Medicine and Infectious Diseases unit.

3.2.2. Study Population

From January 2012 to August 2019 in St. James's Hospital, of all plasma sample requests for diagnostic HHV-8 PCR testing based on clinical suspicion for HHV-8-related disease, HHV-8 DNA was detected in 153 samples. 30 patients consented to the use of their clinical samples for the purposes of this study, providing 88 specimens for use in the study. Written informed consent was obtained from all the study participants. All patients included in the study were living in Ireland and were not referred to the centre from other countries. However, not all participants were born in Ireland. Some participants moved to live in Ireland from other countries and were living in Ireland for varying periods of time before presenting to the study centre.

3.2.3. HHV-8 Real-time PCR Assay

All plasma samples were previously positive on diagnostic HHV-8 PCR testing using real-time PCR and were stored at -80°C. The diagnostic real-time PCR assay currently in use in St. James's Hospital uses real-time PCR technology for the direct qualitative

detection of HHV-8 DNA in clinical samples, using the conserved LANA gene of the HHV-8 genome as the target. It is a singleplex assay that permits testing of HHV-8 in conjunction with a heterologous assay to detect the internal control (IC), Phocine herpesvirus (PHV) in the same microtitre tray. The PHV internal control is introduced into each individual sample to monitor the entire procedure from extraction to detection. KSHV/HHV-8 (KS-1 Strain) Quantitated Viral DNA (Advanced Biotechnologies Inc., Columbia, MD) is used as a positive control with each run.

3.2.3.1 DNA Extraction

Viral DNA was isolated from plasma using the NucliSENS® easyMAG® automated extraction system (Biomérieux, Marcy-L'Étoile, France). This system yields purified viral DNA from plasma or serum samples and efficiently removes contaminants and PCR inhibitors. It also facilitates the introduction of an internal control into each specimen, which serves as an extraction and amplification control for each individual specimen and detects inhibition to PCR.

3.2.3.2 Amplification and Detection

Real-time PCR was carried out in a 96-well microtitre plate (Applied Biosystems, Foster City, California) using the ABI 7500 FAST Sequence Detection System, which combines rapid amplification and real-time detection of amplicons in an automated and closed system. The conserved LANA gene of the HHV-8 genome was used as the target. The primers and probes used in the assay were those previously described by Bourboulia et al. (2004). The forward primer HHV-8 F 5' TTGCCACCCACGCAGTCT 3' and the reverse primer HHV-8 R 5' GGACGCATAGGTGTTGAAGAGTCT 3' generate an 89 bp amplicon. The HHV-8

hydrolysis probe (5' FAM TCTTCTCAAAGGCCACCGCTTTCAAGTC TAMRA 3') is labelled at the 5' end with FAM and at the 3' end with TAMRA. The PHV glycoprotein gB gene is used as the target for the PHV primer-probe set. The forward and reverse primers 5'-ATTTTGGGCGAATCACAGATTGAATC-3' and 5'-GGCGGTTCCAAACGTACCA-3' generate a 94 bp amplicon. The PHV TaqMan™ probe (5'-ATGGTGGCGGACACATA-3') is also labelled at the 5' end with VIC. The nucleotide sequences of the primers and probe used in the real-time PCR assay are described in Table 3.1.

The HHV-8 assay is a singleplex assay and was designed to include a second heterologous amplification assay for PHV as an internal control in the same microtitre tray. The amplification protocol is the same for both gene targets and includes a step at 50°C for 2 minutes followed by 95°C for 10 min and 45 cycles of 15 sec at 95°C (denaturation), and 60°C for 1 min (combines annealing and primer extension). At the end of each cycle, the fluorescent signal of each reaction is measured. Data are acquired during the annealing stage. The measure of the reporter signal (Rn) emitted by each sample is normalised against a passive reference dye (ROX). The fluorescence of a non-template control (NTC) (Rn-) is subtracted from the Rn of each sample to yield a signal, delta Rn (ΔRn). ΔRn is plotted against PCR cycle number to determine the Ct of each individual sample. The higher the starting copy number of the HHV-8 target, the sooner a significant increase in the measure of the reporter signal (Rn) is observed. Following baseline and threshold adjustment samples are considered positive when the amplification plot (ΔRn versus cycle number) demonstrates a sigmoidal curve that rises exponentially and crosses the threshold. Negative samples demonstrate little change in ΔRn and do not cross the threshold.

Table 3.1. Primers and probe used in HHV-8 real-time PCR assay.

Primer/Probe	Sequence
HHV-8-F HHV-8-R HHV-8-Probe (Hydrolysis)	5'**TTGCCACCCACGCAGTCT** 3' 5'**GGACGCATAGGTGTTGAAGAGTCT** 3' 5'**FAM TCTTCTCAAAGGCCACCGCTTTCAAGTC TAMRA** 3'
PHV-F PHV-R PHV-Probe (MGB)	5' **ATTTTGGGCGAATCACAGATTGAATC** 3' 5' **GGCGGTTCCAAACGTACCA** 3' 5' **VIC ATGGTGGCGGACACATA NFQ** 3'

Abbreviations: MGB, minor groove binder DNA probe, FAM, 6-caboxy fluorescein fluorescent dye, VIC™ fluorescent dye, TAMRA, Tetramethyl-6-Carboxyrhodamine

3.2.4 HHV-8 Typing

In order to carry out HHV-8 subtyping, nested end-point PCR was used to characterise four regions of the HHV-8 genome, K1, including the hypervariable regions VR1 and VR2, T0.7 (K12), ORF75 and K15. The nested end-point PCR protocol was performed using specimens that were known to be positive for HHV-8 based on real-time PCR (*Section 3.2.3*).

3.2.4.1 DNA Extraction

Viral DNA was extracted from the study samples using the NucliSENS® easyMAG® (Biomérieux, Marcy-L'Étoile, France) (*Section 3.2.3.1*).

3.2.4.2 HHV-8 DNA Amplification

To perform HHV-8 subtyping, a nested end-point PCR protocol was designed for characterisation of the K1, including the hypervariable regions VR1 and VR2, T0.7 (K12), ORF75 and K15 regions of the HHV-8 genome. Nested PCR primers used in this study are listed in Table 3.2. PCR was performed using DreamTaq Master Mix (ThermoFisher Scientific, Waltham, MA, USA). This is a hot start DNA polymerase, which is supplied as a ready-to-use solution containing DreamTaq DNA Polymerase, optimised DreamTaq Green buffer, $MgCl_2$ and deoxynucleotides (dNTPs). A dye is also incorporated to allow for the direct loading of PCR product on to an agarose gel. All PCR reactions were carried out in a final volume of 25 µl using the same reaction components. Round 1 and Round 2 PCR reactions were performed and the amplified product from Round 1 was used as a template DNA for Round 2. The components of the PCR reaction are described in Table 3.3 and Table 3.4.

Table 3.2. Nested PCR primer pairs used to amplify and sequence HHV-8 genome regions.

Locus	Primer	Sequence	Amplicon size (nts)	Reference
K1 (hemi-nested)	LGH 2089 K1AG1200AS LGH2089 LGH2088	GTTCTGCCAGGCATAGTC AGGCCATGCTGTAAGTAGCACGGTT GTTCTGCCAGGCATAGTC AATAAGTATCCGACCTCAT	1086 1029	Tozetto-Mendoza et al. (2016)
VR1	LGH 2089 LGH 2500 VR1BF VR1BR	GTTCTGCCAGGCATAGTC GTAACATGCTGACCACAAG CTGGCGGCCCTTGTGTAAAC GACTGTGTTTGATGGCTGTGC	389 340	Tozetto-Mendoza et al. (2016)
VR2	LGH 2507 VR2AR VR2BF VR2BR	CGTCTCGCCTGTCAAATC ACTGGTTGCGTATAGTCTTCC GTATATGTTTTTGGGCGCGTTG CCGTGCACAAATCGTGTAGGG	335 294	Tozetto-Mendoza et al. (2016)
T0.7/K12 **(5')** **(3')**	LGH 2076 LGH 2075 LGH 2076 LGH 2017 LGH 2075 LGH2018	GCTGCAATGTACTGCCATG CTCCAATCCCAATGCATGGA GCTGCAATGTACTGCCATG CATTCAGGACACGAGTTGC CTCCAATCCCAATGCATGGA TGCTTTAATGCGGAGAGG	646 390 470	Poole et al. (1999) Zong et al. (2002) Zong et al. (2002)
ORF75	KS 1000 KS 1034 LGH 2000 LGH 2034	CGGTTCGGTGGCATACAGGC CTGACTACAGAGGGTGTCCCCG GGAAACAGGGTGCTGTG CATGGCCTACGACGTCAC	895 804	Kakoola et al. (2001) Poole et al. (1999)
K15 (P) **K15 (M)**	K15P-OF K15P-OR K15-3C K15-4C K15M-OF K15M-OR LGH 2473 LGH2474	TGCAGGCTTGGTCATGGGTTAC GGGACCACGCTGCAATTAAATG ACGCATACATGTACTGCCAC CTTTGATATTGCCAGTGGTG TGTTGGTTGCAATGCTTAGGTG GCCTTTGCCAGTTGGAGTTTC CATGCAGCGAGCTTGAGA CTTTGAGTACTGTTTGTG	365 285 486 370	Whitby et al. (2004) Kakoola et al. (2001) Whitby et al. (2004) Poole et al. (1999)

Table 3.3. Round 1 nested PCR reaction components.

Component	Volume (µL)	Final Concentration
DreamTaq Mastermix	12.5	2X
F-Primer	0.25	1 µM
R-Primer	0.25	1 µM
PCR grade H_2O	7	-
cDNA	5	-
Total Volume	**25**	**-**

Table 3.4. Round 2 nested PCR reaction components.

Component	Volume (µL)	Final Concentration
DreamTaq Mastermix	12.5	2X
F-Primer	0.25	1 µM
R-Primer	0.25	1 µM
PCR grade H_2O	10	-
Amplified DNA from Round 1	2	-
Total Volume	**25**	**-**

The PCR reactions were carried out using the Veriti® Thermal Cycler (Applied Biosystems, Foster City, California). The cycling conditions of all nested PCR reactions were similar, differing only with respect to annealing temperatures. First and second round PCR cycling conditions consisted of an initial hot start at 94°C for 5 minutes, followed by 45 cycles at 94°C for 25 seconds (denaturation), 35 seconds at the appropriate annealing temperature, 72°C for 65 seconds (extension), followed by a final elongation step at 72°C for 10 minutes. Annealing temperatures for the outer and inner primers were 59°C and 59°C respectively for K1, 55°C and 59°C for VR1, 57°C and 55°C for VR2, 58°C and 57°C for T0.7 5', 58°C and 55°C for T0.5 3', 60°C and 57°C for ORF75, 57°C and 55°C for K15 P, and 54°C and 57°C for K15 M. Careful measures were taken to prevent PCR contamination. Separate rooms were used for DNA extraction, reagent preparation and PCR amplification. All equipment was UV-irradiated. A non-template control was analysed with each PCR reaction.

3.2.4.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using 1.5% (W/V) agarose gel at 90V for 90 minutes. Gels were prepared using 1.26 g Agarose Type II (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany), 84 mL 1x Tris-borate-EDTA (TBE) buffer (National Diagnostics, Atlanta, Georgia) and 4 µL Gel Red™ nucleic acid stain (Biotium, Fermont, USA). 4 µL of amplified PCR product was loaded onto the gel prior to electrophoresis and a 100 bp ladder (Promega, Madison, Wisconsin) was used as a molecular weight marker to estimate band size. Gels were visualised using ultraviolet (UV) light in a Gel Doc XR system (Bio-Rad, Hercules, CA).

3.2.4.4 PCR product purification

PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Briefly, the PCR product (approximately 21 µL) was added to five times the volume of PB binding buffer. This mixture was applied to the silica column and washed with PE wash buffer and eluted into a final volume of 30 µL of room temperature nuclease free water.

3.2.4.5 Estimation of DNA concentration

The concentration of DNA in each purified sample was estimated using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). 2 µL of purified PCR product was analysed and the DNA concentration in ng/µL was calculated.

3.2.4.6 DNA Sequencing

Purified PCR products were diluted to a concentration of 10 ng/µL and referred to a commercial genomics company (Source BioScience Ltd., Nottingham, UK) for Sanger sequencing using the second round primers shown in Table 3.2, diluted to a concentration of 3.2 ng/µL. Results were received as sequence trace files.

3.2.4.7. DNA Sequencing analysis

The sequence chromatograms were analysed, and consensus sequences were obtained using Lasergene® SeqMan software (DNASTAR, Madison, WI). The consensus sequences were aligned with HHV-8 reference strains using Lasergene® MegAlign software (DNASTAR, Madison, WI). Reference strains used in this study are detailed in Table 3.5.

Table 3.5. Reference strains used in this study.

Locus	Name	GenBank reference	Nucleotide position	Origin	Reference
K1	K1-18/Hec	AF178787-C3	1 to 855	NK	Lacoste et al., 2000
	D14-1	DQ394049-C3	1 to 855	Morocco	Duprez et al., 2006
	D10-1	DQ394044-C3	1 to 855	Morocco	Duprez et al., 2006
	BC2	AF133042-C3	1 to 1005	USA	Zong et al., 1999
	78/48	AF201851-C4	1 to 855	Russia	Lacoste et al., 2000
	I7	DQ394060-C3	1 to 855	Morocco	Duprez et al., 2006
	D4-2	DQ394038-C5	1 to 855	Morocco	Duprez et al., 2006
	Icam1	AF130274-C3	1 to 855	NK	Cook et al., 1999
	J21	AF278843-C	1 to 866	Japan	Meng et al., 2001
	UKma8	AF130304-C7	1 to 855	NK	Cook et al., 1999
	BR66_C7_K1	KT215124-C7	1 to 855	Brazil	Tozetto-Mendoza et al., 2016
	D13	DQ394048-C2	1 to 855	Morocco	Duprez et al., 2006
	GK17	AF130267-C	1 to 855	NK	Cook et al., 1999
	D19	DQ394055-C1	1 to 855	Morocco	Duprez et al., 2006
	ASM72	AF133041-C1	1 to 999	USA	Zong et al., 1999
	I4	DQ394058-C2	1 to 855	Morocco	Duprez et al., 2006
	I10	DQ394064-C5	1 to 831	Morocco	Duprez et al., 2006
	K1-25/Cas	AF178794-A	1 to 870	NK	Lacoste et al., 2000
	Ema7	AF130305-A2	1 to 870	NK	Cook et al., 1999
	US-216	FJ884626-A1	1 to 933	USA	Tornesello et al., 2010
	BCBL-B	AF133039-A4	1 to 1008	USA	Zong et al., 1999
	K1-40/Bc1	AF178807-A2	1 to 870	NK	Lacoste et al., 2000
	K1-17/Fuj	AF178786-A2	1 to 870	NK	Lacoste et al., 2000
	K1-32/Bcb	AF178799-A3	1 to 870	NK	Lacoste et al., 2000
	Ife5 K1	AF130284-A5	1 to 821	NK	Cook et al., 1999
	K1-58/Sar	AF178823-A5	1 to 867	NK	Lacoste et al., 2000
	Ife1	AF130282-A5	1 to 824	NK	Cook et al., 1999
	K1-43/Berr	AF178810-F	1 to 870	NK	Lacoste et al., 2000
	J26	AF278846-D1	1 to 882	Japan	Meng et al., 2001
	ZKS3	AF133044-D2	1 to 1020	Pacific	Zong et al., 1999
	Hua1	AY329027-E	1 to 844	Ecuador	Whitby et al., 2004
	Hua2	AY329028-E2	1 to 844	Ecuador	Whitby et al., 2004
	Tupi-1	AF220292-E1	1 to 847	Brazil	Biggar et al., 2000
	Tupi-2	AF220293-E2	1 to 847	Brazil	Biggar et al., 2000
	K1-22/Yan	AF178791-B1	1 to 870	NK	Lacoste et al., 2000
	UKma24	AF130301-B1	1 to 870	NK	Cook et al., 1999
	K1-34/E40	AF178801-B1	1 to 870	NK	Lacoste et al., 2000
	K1-27/Ban	AF178796-B1	1 to 870	NK	Lacoste et al., 2000
	K1-12/Mou	AF178783-B1	1 to 869	NK	Lacoste et al., 2000
	431KAP	AF133040-B4	1 to 1020	Congo	Zong et al., 1999
	K1-11/Tho	AF178782-B1	1 to 870	NK	Lacoste et al., 2000
	Ug52	AF130290-B	1 to 856	NK	Cook et al., 1999
	Ugd26	AY042940-B2	1 to 855	Uganda	Kakoola et al., 2001
	K1-23/Kok	AF178792-B2	1 to 870	NK	Lacoste et al., 2000
	K1-37/E44	AF178804-B2	1 to 870	NK	Lacoste et al., 2000
	K1-60/Ced	AF178825-B3	1 to 870	NK	Lacoste et al., 2000
T0.7/K12	AKS2	MH996486-A/C	1 to 648	USA	Zong et al., 2018
	ZKS5	MH996635-A/C	1 to 648	NZ	Zong et al., 2018
	AKS1	MH996485-A/C	1 to 648	USA	Zong et al., 2018
	ZKS1	MH996632-A/C	1 to 648	NZ	Zong et al., 2018
	BC2	MH996492-A/C	1 to 648	USA	Zong et al., 2018
	SKS2	MH996610-A/C	1 to 648	S. Arabia	Zong et al., 2018
	BCBLR	MH996496-A/C	1 to 648	USA	Zong et al., 2018
	OKS16	MH996579-A/C	1 to 648	USA	Zong et al., 2018

	BCP1	MH996498-A/C	1 to 648	UK	Zong et al., 2018
	RGKS5	MH996583-A/C	1 to 648	Italy	Zong et al., 2018
	BKS10	MH996501-A/C	1 to 648	USA	Zong et al., 2018
	OKS14	MH996578-A/C	1 to 648	USA	Zong et al., 2018
	RGKS4	MH996582-A/C	1 to 648	Italy	Zong et al., 2018
	JSC1	MH996566-A/C	1 to 648	USA	Zong et al., 2018
	BKS20	MH996510-A/C	1 to 648	USA	Zong et al., 2018
	C282	MH996513-A/C	1 to 648	USA	Zong et al., 2018
	BKS3	MH996499-A/C	1 to 648	USA	Zong et al., 2018
	BKS11	MH996502-A/C	1 to 648	USA	Zong et al., 2018
	BKS12	MH996503-J	1 to 648	USA	Zong et al., 2018
	OKS18	MH996581-F	1 to 648	USA	Zong et al., 2018
	HKS22	MH996537-F	1 to 648	Uganda	Zong et al., 2018
	MKS2	MH996571-J	1 to 648	Sweden	Zong et al., 2018
	SKS7	MH996613-J	1 to 648	S. Arabia	Zong et al., 2018
	OKS13	MH996577-J	1 to 648	USA	Zong et al., 2018
	BCBL1	MH996497-J	1 to 648	USA	Zong et al., 2018
	BKS14	MH996505-J	1 to 648	USA	Zong et al., 2018
	SKS3	MH996611-J	1 to 648	S. Arabia	Zong et al., 2018
	BKS16	MH996507-J	1 to 648	USA	Zong et al., 2018
	BKS18	MH996508-J	1 to 648	USA	Zong et al., 2018
	BKS22	MH996512-J	1 to 648	USA	Zong et al., 2018
	BKS19	MH996509-J	1 to 648	USA	Zong et al., 2018
	EKS1/2	MH996514-J	1 to 648	USA	Zong et al., 2018
	BC1	MH996491-J	1 to 648	USA	Zong et al., 2018
	SKS1	MH996609-F	1 to 648	S. Arabia	Zong et al., 2018
	SAPB5	MH996606-B4	1 to 648	S. Africa	Zong et al., 2018
	SAPB3	MH996605-B4	1 to 648	S. Africa	Zong et al., 2018
	SAKS32	MH996599-B4	1 to 648	S. Africa	Zong et al., 2018
	OKS8	MH996575-B3	1 to 648	USA	Zong et al., 2018
	OKS7	MH996574-B3	1 to 648	USA	Zong et al., 2018
	JKS15	MH996564-B3	1 to 648	USA	Zong et al., 2018
	RKS4	MH996587-B2	1 to 648	Zambia	Zong et al., 2018
	RKS3	MH996586-B2	1 to 648	Zambia	Zong et al., 2018
	HKS6	MH996525-B2	1 to 648	Uganda	Zong et al., 2018
	OKS4	MH996573-B1	1 to 648	Tanzania	Zong et al., 2018
	SAPB8	MH996607-B	1 to 648	S. Africa	Zong et al., 2018
	SALN6	MH996603-N	1 to 648	S. Africa	Zong et al., 2018
	SAKS23	MH996590-N	1 to 648	S. Africa	Zong et al., 2018
	Hua2	AY329020-E	1 to 564	Ecuador	Whitby et al., 2004
	Hua5	AY329021-E	1 to 564	Ecuador	Whitby et al., 2004
	Hua3	AY329019-E	1 to 564	Ecuador	Whitby et al., 2004
	ZKS4	MH996634-D2	1 to 648	NZ	Zong et al., 2018
	ZKS3	MH996633-D2	1 to 648	NZ	Zong et al., 2018
	TKS10	MH996622-D1	1 to 648	Taiwan	Zong et al., 2018
	OKS3	MH996572-R1	1 to 648	Tanzania	Zong et al., 2018
	RKS2	MH996585-R1	1 to 648	Zambia	Zong et al., 2018
	RKS5	MH996588-R1	1 to 648	Zambia	Zong et al., 2018
	431K	MH996484-R1	1 to 648	Congo	Zong et al., 2018
	HKS3	MH996523-R2	1 to 648	Uganda	Zong et al., 2018
	SAPB9	MH996608-R2	1 to 648	S. Africa	Zong et al., 2018
	KKS2/4	MH996568-M	1 to 648	S. Korea	Zong et al., 2018
	HKS35	MH996544-M	1 to 648	Uganda	Zong et al., 2018
	KKS6	MH996570-M	1 to 648	S. Korea	Zong et al., 2018
	KKS5	MH996569-M	1 to 648	S. Korea	Zong et al., 2018
	KKS1	MH996567-M	1 to 648	S. Korea	Zong et al., 2018
	TKS11	MH996623-M	1 to 648	Taiwan	Zong et al., 2018
	TKS9	MH996621-M	1 to 648	Taiwan	Zong et al., 2018
	BKS21	MH996511-M	1 to 648	USA	Zong et al., 2018
	TKS7	MH996620-M	1 to 648	Taiwan	Zong et al., 2018

	TKS5	MH996619-M	1 to 648	Taiwan	Zong et al., 2018
	TKS2	MH996617-M	1 to 648	Taiwan	Zong et al., 2018
	TKS1	MH996616-M	1 to 648	Taiwan	Zong et al., 2018
	SKS9	MH996615-M	1 to 648	S. Arabia	Zong et al., 2018
	BKS13	MH996504-M	1 to 648	USA	Zong et al., 2018
	ASM70/80	MH996490-M	1 to 648	USA	Zong et al., 2018
	TKS3	MH996618-M	1 to 648	Taiwan	Zong et al., 2018
	San1	AY329022-Q	1 to 551	Botswana	Whitby et al., 2004
	FTKS25	MH996518-Q	1 to 636	S. Africa	Zong et al., 2018
	SAPB1	MH996604-Q	1 to 636	S. Africa	Zong et al., 2018
	SAKS35	MH996602-Q	1 to 636	S. Africa	Zong et al., 2018
ORF75	KS82ZA	AF243819-A	1 to 803	S. Africa	Alagiozoglou, 2000
	PP158ZA	AF243828-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVST1	EF109641-A	1 to 890	Uganda	Zong et al., 1997
	KSHVEKS1	EF109636-A	1 to 890	USA	Zong et al., 1997
	KSHVBCBLR	EF109622-A	1 to 890	USA	Zong et al., 1997
	KS12ZA	AF243814-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVC282	EF109625-A	1 to 890	USA	Zong et al., 1997
	RS901ZA	AF243829-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KS18ZA	AF243800-A	1 to 803	S. Africa	Alagiozoglou, 2000
	RS904ZA	AF243836-A	1 to 803	S. Africa	Alagiozoglou, 2000
	TS652ZA	AF243801-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVAKS4	EF109624-A	1 to 890	USA	Zong et al., 1997
	KS80ZA	AF243818-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KS12ZA	AF243814-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KS23ZA	AF243815-A	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVST2	EF109662-B	1 to 890	Uganda	Zong et al., 1997
	KS65ZA	AF243797-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS85ZA	AF243821-B	1 to 803	S. Africa	Alagiozoglou, 2000
	MEN43ZA	AF243826-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS49ZA	AF243809-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS88ZA	AF243834-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS64ZA	AF243811-B	1 to 803	S. Africa	Alagiozoglou, 2000
	LN7ZA	AF243824-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS83ZA	AF243820-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS81ZA	AF243832-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS31ZA	AF243806-B	1 to 803	S. Africa	Alagiozoglou, 2000
	TS522ZA	AF243807-B	1 to 803	S. Africa	Alagiozoglou, 2000
	TS799ZA	AF243798-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS35ZA	AF243808-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS37ZA	AF243799-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS13ZA	AF243805-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVSAPB1	EF109704-Q	1 to 890	S. Africa	Zong et al., 1997
	KS76ZA	AF243816-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KS61ZA	AF243810-B	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVTKS2	EF109726-M	1 to 890	Taiwan	Zong et al., 1997
	KSHVTKS1	EF109725-M	1 to 890	Taiwan	Zong et al., 1997
	KSHVTKS6	EF109727-M	1 to 890	Taiwan	Zong et al., 1997
	KSHVTKS11	EF109723-M	1 to 890	Taiwan	Zong et al., 1997
	KSHVHBL6/BC1	EF109716-M	1 to 890	USA	Zong et al., 1997
	KSHVSKS3	EF109721-M	1 to 890	S. Arabia	Zong et al., 1997
	KSHVOKS13	EF109724-M	1 to 890	USA	Zong et al., 1997
	KSHVBKS18	EF109717-M	1 to 890	USA	Zong et al., 1997
	KSHVBKS21	EF109718-M	1 to 890	USA	Zong et al., 1997
	KSHVBKS13	EF109720-M	1 to 890	USA	Zong et al., 1997
	DS814ZA	AF243803-C	1 to 803	S. Africa	Alagiozoglou, 2000
	KS70ZA	AF243831-C	1 to 803	S. Africa	Alagiozoglou, 2000
	KS91ZA	AF243823-C	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVZKS3	EF109714-D	1 to 890	NZ	Zong et al., 1997
	KSHVTKS10	EF109713-D	1 to 890	Taiwan	Zong et al., 1997

	LN2ZA	AF243835-N	1 to 803	S. Africa	Alagiozoglou, 2000
	DS895ZA	AF243813-N	1 to 803	S. Africa	Alagiozoglou, 2000
	MEN21ZA	AF243825-N	1 to 803	S. Africa	Alagiozoglou, 2000
	LN4ZA	AF243837-N	1 to 803	S. Africa	Alagiozoglou, 2000
	RS820ZA	AF243804-N	1 to 803	S. Africa	Alagiozoglou, 2000
	KS78ZA	AF243817-N	1 to 803	S. Africa	Alagiozoglou, 2000
	KS89ZA	AF243822-N	1 to 803	S. Africa	Alagiozoglou, 2000
	KS33ZA	AF243830-N	1 to 803	S. Africa	Alagiozoglou, 2000
	KSHVSALN7	EF109732-N	1 to 890	S. Africa	Zong et al., 1997
	KSHVSALN6	EF109731-N	1 to 890	S. Africa	Zong et al., 1997
K15	ZM004	KT271453-P	134255 to 136330	Zambia	Olp et al., 2015
	ZM091	KT271455-P	133941 to 136026	Zambia	Olp et al., 2015
	ZM102	KT271457-P	133886 to 135971	Zambia	Olp et al., 2015
	ZM106	KT271458-P	134061 to 136146	Zambia	Olp et al., 2015
	ZM114	KT271460-P	134193 to 136278	Zambia	Olp et al., 2015
	ZM116	KT271461-P	134042 to 136117	Zambia	Olp et al., 2015
	ZM117	KT271462-P	134323 to 136408	Zambia	Olp et al., 2015
	ZM118	KT271463-P	135011 to 137086	Zambia	Olp et al., 2015
	ZM121	KT271464-P	134599 to 136684	Zambia	Olp et al., 2015
	431K	MH613917-P	1 to 2086	Congo	Poole et al., 1999
	BCBL-R	MH613922-P	1 to 2075	USA	Poole et al., 1999
	JSC-1	GQ994935-P	135001 to 137076	NK	Brulois et al., 2012
	GK18	AF148805-P	134824 to 136899	Greece	Glenn et al., 1999
	ZM130	KT271468-P	134030 to 136115	Zambia	Olp et al., 2015
	ZM124	KT271466-P	134417 to 136492	Zambia	Olp et al., 2015
	ZM123	KT271465-P	133901 to 135976	Zambia	Olp et al., 2015
	ZM108	KT271459-P	133816 to 135901	Zambia	Olp et al., 2015
	ZM027	KT271454-P	134282 to 136367	Zambia	Olp et al., 2015
	ZM095	KT271456-N	134611 to 136714	Zambia	Olp et al., 2015
	SAKS23	MH613959-N	1 to 2106	S. Africa	Poole et al., 1999
	ZM128	KT271467-N	134570 to 136673	Zambia	Olp et al., 2015
	ZKS4	MH613958-P	1 to 2113	NZ	Poole et al., 1999
	HKS49	MH613964-M	1 to 2101	Uganda	Poole et al., 1999
	TKS13	MH613968-M	1 to 2102	Taiwan	Poole et al., 1999
	VG1	MH613969-M	1 to 2101	Haiti	Poole et al., 1999
	Japan1	LC200589-M	134178 to 136276	Japan	Awazawa et al., 2017
	Miyako1	LC200586-M	134321 to 136422	Japan	Awazawa et al., 2017
	Miyako2	LC200587-M	134408 to 136509	Japan	Awazawa et al., 2017
	Miyako3	LC200588-M	134125 to 136226	Japan	Awazawa et al., 2017
	HBL6	MH613962-M	1 to 2102	USA	Poole et al., 1999

Abbreviations: NK, not known, USA, United States of America, NZ, New Zealand.

3.2.4.8 Phylogenetic analysis

In total six phylogenetic trees were constructed; K1 (1029 nts), VR1 (340 nts), VR2 (294 nts), T0.7 (390 nts), ORF75 (804 nts) and K15 (P, 285 nts; M, 370 nts). Phylogenetic reconstructions of the nucleotide alignments, employing maximum likelihood, were carried out using PAUP*4.0a165 software (Swofford, 2002). Multiple sequence alignments were performed using Clustal X (Thompson et al., 1997). The 'best fit' models of evolution for the aligned datasets were determined using jModeltest version 2.1.10 (Posada, 2008) by comparing the likelihood of the different parameters for all models of substitution. The parameters of each model were then estimated using a heuristic search strategy and selecting those with the highest likelihood. Bootstrap resampling was carried out using PAUP* with 1,000 replicates performed to examine the robustness of the trees produced. Details of the models of evolution chosen for each dataset and the out-group used are outlined below.

3.2.4.8.1 K1

Phylogenetic analysis of the complete K1 gene was carried out on five sequences from this study and 46 reference strains, representing subtypes A, B, C, D, E and F. A subtype A1 strain was used as the out-group for analysis (GenBank Accession Number AF133038). The model of evolution chosen by hLRT using modeltest was TrN.

3.2.4.8.2 VR1

Phylogenetic analysis of the VR1 region of the K1 gene was carried out on 16 sequences from this study and the same 46 reference strains. An E1 subtype strain was used as the out-group (GenBank Accession Number AF220292). The model of evolution chosen by hLRT using modeltest was JC+G.

3.2.4.8.3 VR2

Phylogenetic analysis of the VR2 region of the K1 gene was carried out on 17 sequences from this study and the same 46 reference strains. SJH063VR2 was used as the out-group. The model of evolution chosen by hLRT using modeltest was JC+G.

3.2.4.8.4 T0.7/K12

Phylogenetic analysis of the T0.7/K12 region was carried out on 12 sequences from this study and comparison was made to 79 reference strains representing subtypes A/C, B, D, E, F, J, M, N, Q and R. A subtype R2 strain was used as the out-group for analysis (GenBank Accession Number MH996523). The model of evolution chosen by hLRT using modeltest was K81.

3.2.4.8.5 ORF75

Phylogenetic analysis of ORF75 was carried out on 12 sequences from this study and comparison was made to 59 reference strains representing types A, B, C, D, M, N and Q. A subtype M strain was used as the out-group for analysis (GenBank Accession Number EF109725). The model of evolution chosen by hLRT using modeltest was TrN.

3.2.4.8.6 K15

Phylogenetic analysis of the K15 gene was carried out on 13 sequences from this study and 30 reference strains, representing the P, M and N alleles. A P subtype strain, SJH050K15 was used as the out-group for analysis. The model of evolution chosen by hLRT using modeltest was TrNef+G.

3.2.5 Ethical Approval

Ethical approval for this study was obtained from Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee.

3.3 Results

Eighty-eight HHV-8-positive (*n* = 88) specimens from 30 patients were available for use in the study. Sequencing data were obtained for 23 specimens from 19 patients.

3.3.1 Phylogenetic analysis

Six phylogenetic trees were constructed for the K1, VR1, VR2, T0.7, ORF75 and K15 regions of the HHV-8 genome. Each tree was constructed using the sequences obtained for each region from this study, along with a selection of reference strains taken from GenBank to represent the appropriate subtypes for each region. According to the resultant phylogenetic trees, the HHV-8 sequences were classified into subtypes and subgroups. The subtypes that were determined for each patient are detailed in Table 3.6. The nomenclature used is that proposed by Hayward & Zong, (2007).

Table 3.6. HHV-8 subtypes identified in patients with HHV-8 related disease in Ireland.

Patient	Specimen	K1	VR1	VR2	T0.7/K12	ORF75	K15
1	SJH098	-	A	-	-	-	M
3	SJH113	-	A	A	J	C	-
5	SJH014	-	-	A	-	-	P
6	SJH063	-	-	A	-	-	-
10	SJH062	-	A	A	A/C	A	P
11	SJH068	-	-	-	Q	D	M
	SJH081	-	F	F	-	-	-
13	SJH136	-	A	A	A/C	-	-
14	SJH105	-	-	C	-	C	-
16	SJH101	-	C	C	-	A	-
18	SJH137	-	B	B	B	B	M
19	SJH131		C	C	A/C	A	-
	SJH133	C	-	-	-	-	P
20	SJH024	-	B	-	R	A	P
	SJH025	-	-	B	-	-	-
21	SJH053	-	A	A	-	A	P
23	SJH109	C	C	C	A/C	-	P
24	SJH050	C	C	C	A/C	A	P
27	SJH082	-	-	-	A/C	-	-
	SJH083	C	C	C	-	-	P
28	SJH139	-	C	-	-	-	-
29	SJH092	-	C	C	A/C	A	P
30	SJH089	C	C	C	J	C	M

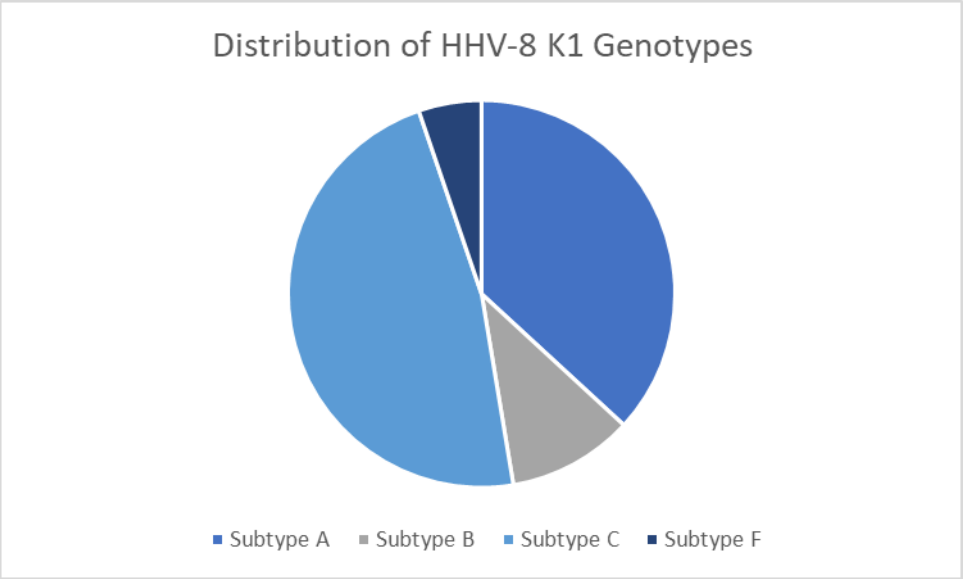

Figure 3.1. Distribution of HHV-8 K1 genotypes.

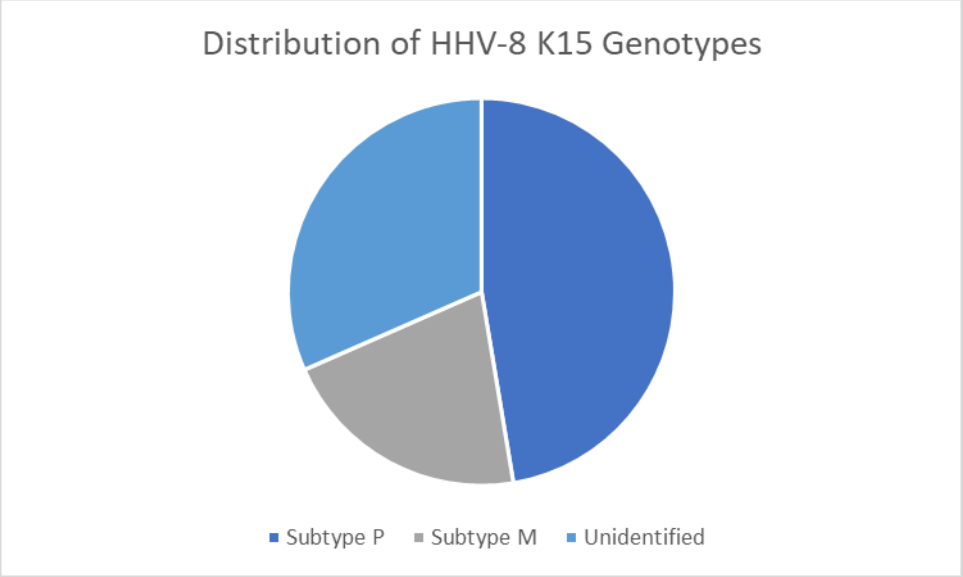

Figure 3.2 Distribution of HHV-8 K15 genotypes.

3.3.1.1 K1 analysis

Using nested PCR, complete or partial ORF-K1 sequences were successfully obtained for 19 patients out of 30 (63%). Complete K1 sequences were obtained for five patients, VR1 sequences were obtained for 16 patients and VR2 sequences were obtained for 17 patients. Not all PCR attempts were successful, largely due to low viral loads in the remaining samples. For the samples where nested PCR was unsuccessful, the diagnostic real time PCR Ct values were all greater than 34. This was also the reason it was not possible to obtain the larger K1 sequence for a greater number of samples. Where it was possible to sequence the complete K1 gene, the Ct values for these specimens were all below 32, using real time PCR. Phylogenetic analyses of the K1, VR1 and VR2 sequences, along with 46 previously published sequences, are shown in Figure 3.3, Figure 3.4 and Figure 3.5, respectively. Of the St. James's Hospital patient K1 sequences, seven (37%) clustered with subtype A, two (11%) with subtype B, nine (47%) with subtype C and one (5%) with subtype F (Figures 3.3-3.5). There were no subtype D or E sequences identified. The predominance of C and A subtypes is as expected for a western European population. Although there were two B subtypes identified, there were no sequences that clustered with the A5 K1 subtype. Interestingly, all five specimens where the complete K1 gene was successfully amplified were identified as belonging to subtype C.

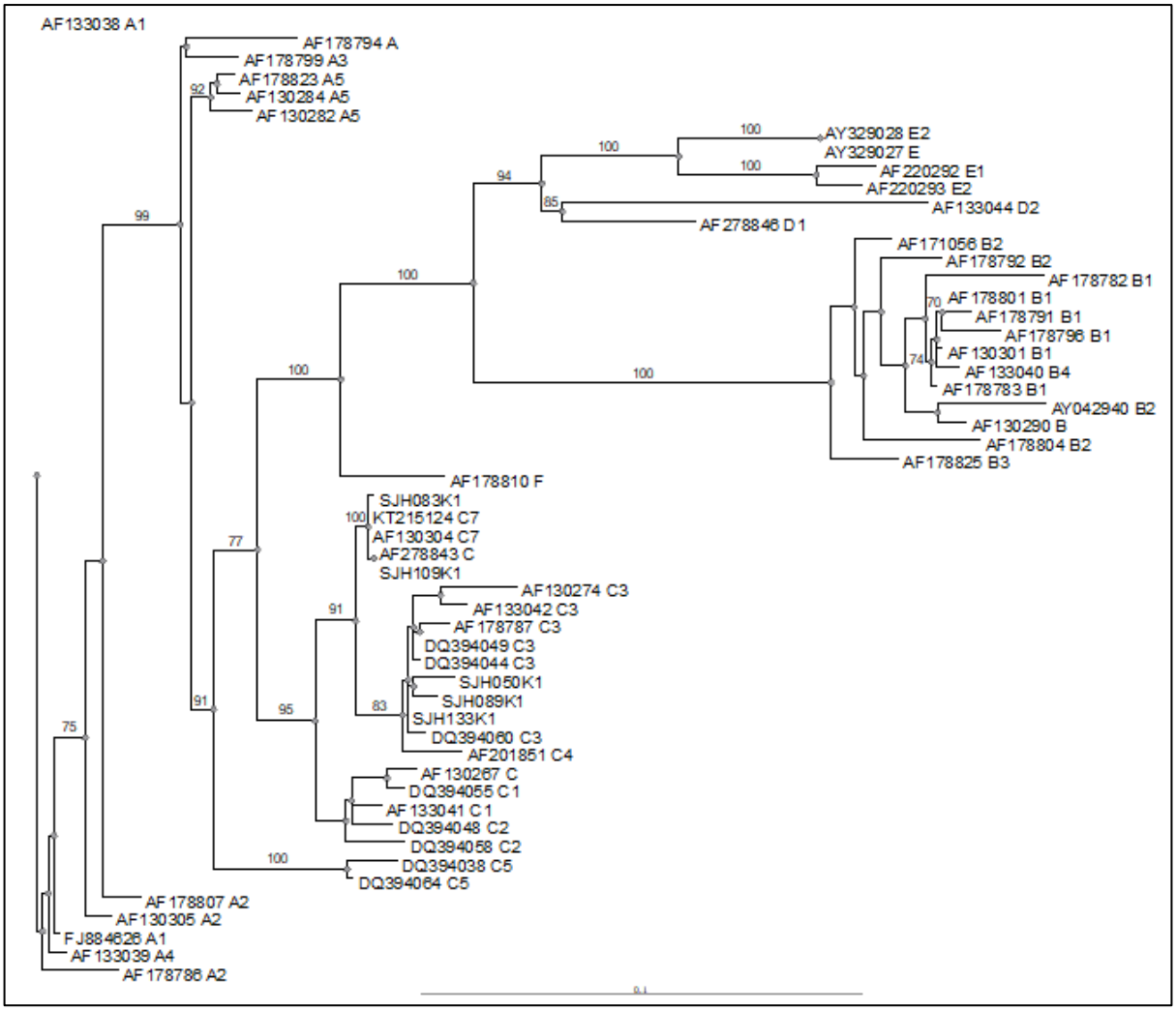

Figure 3.3. A maximum likelihood tree based on the K1 gene (1029 nts) of 5 St. James's Hospital HHV-8-positive specimens, using AF133038 as an out-group.

Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing subtypes A to F, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH133K1: PP129309; SJH109K1: PP129310; SJH050K1: PP129311; SJH083K1: PP129312; SJH089K1: PP129313.

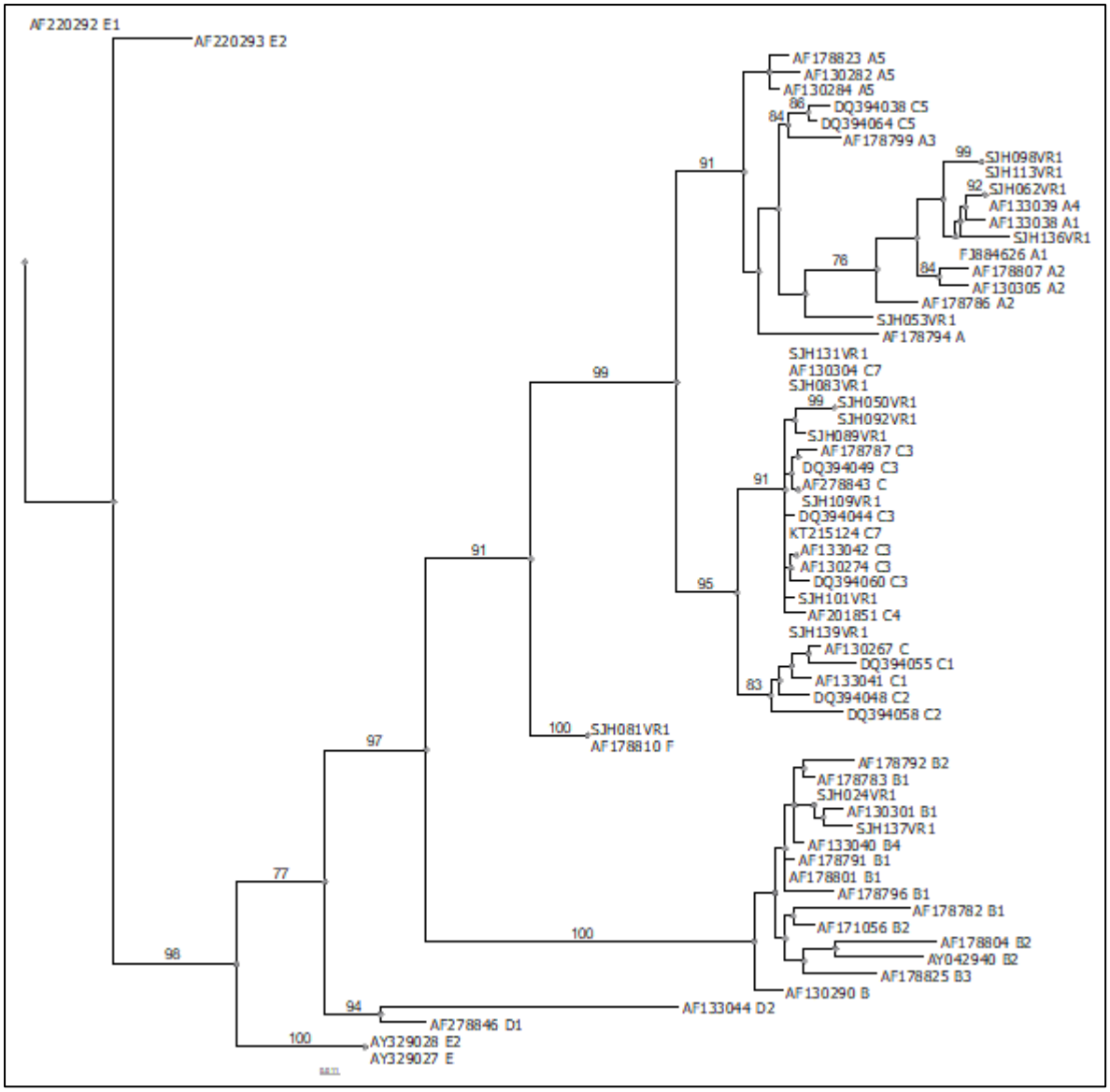

Figure 3.4. A maximum likelihood tree based on the VR1 hypervariable region of the K1 gene (340 nts) for 16 St. James's Hospital HHV-8-positive specimens, using AF220292 as an out-group.

Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing subtypes A to F, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH053VR1: PP129331; SJH062VR1: PP129332; SJH101VR1: PP129333; SJH109VR1: PP129334; SJH113VR1: PP129335; SJH092VR1: PP129336;

SJH024VR1: PP129337; SJH050VR1: PP129338; SJH083VR1: PP129339; SJH131VR1: PP129340; SJH136VR1: PP129341; SJH137VR1: PP129342; SJH139VR1: PP129343; SJH089VR1: PP129344; SJH098VR1: PP129345; SJH081VR1: PP227227.

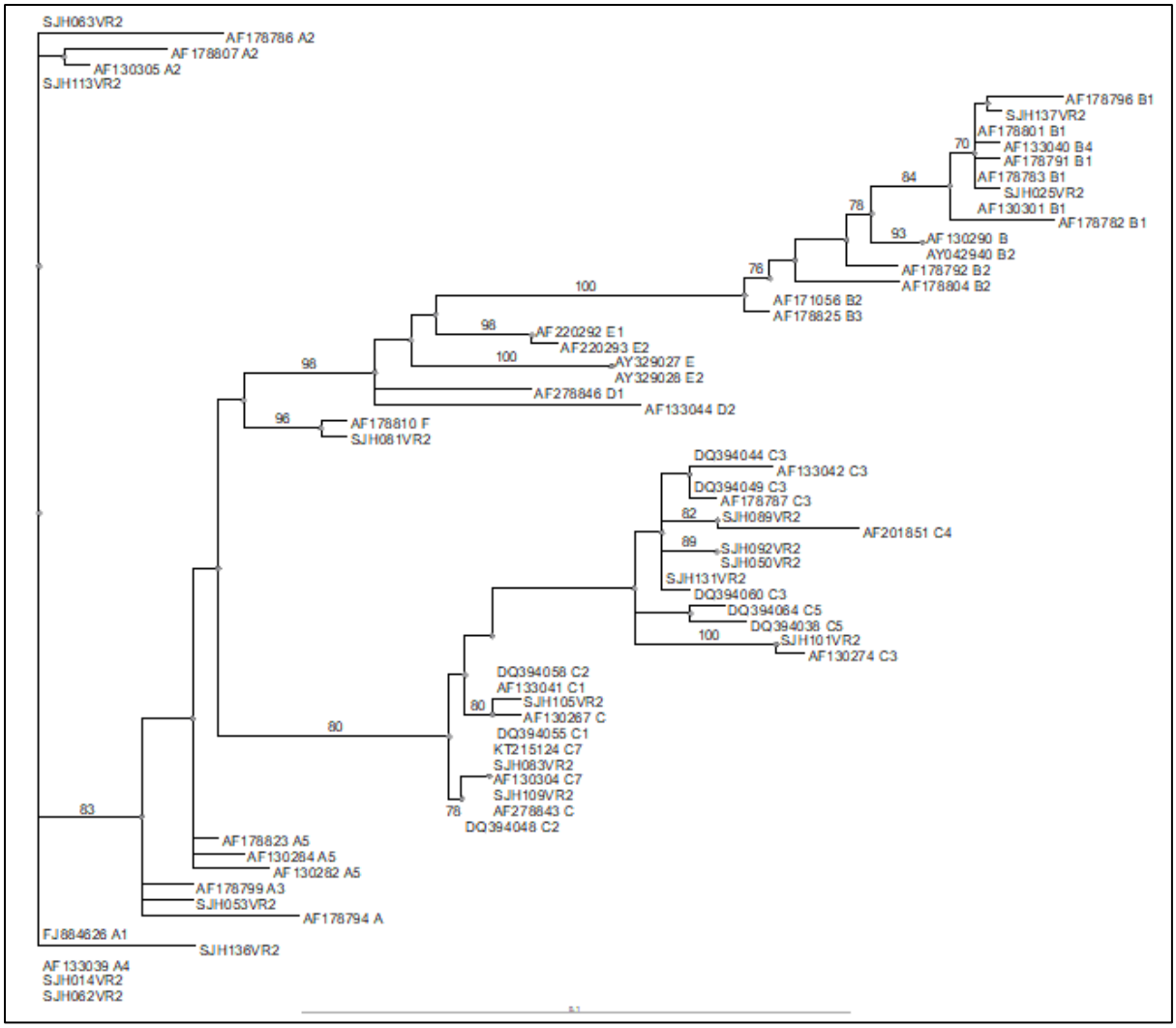

Figure 3.5. A maximum likelihood tree based on the VR2 hypervariable region of the K1 gene (294 nts) for 17 St. James's Hospital HHV-8-positive specimens, using SJH063VR2 as an out-group.

Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing subtypes A to F, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH014VR2: PP129314; SJH053VR2: PP129315; SJH062VR2: PP129316; SJH101VR2: PP129317; SJH113VR2: PP129318; SJH109VR2: PP129319; SJH092VR2: PP129320; SJH050VR2: PP129321; SJH063VR2: PP129322;

SJH081VR2: PP129323; SJH083VR2: PP129324; SJH131VR2: PP129325; SJH136VR2: PP129326; SJH137VR2: PP129327; SJH025VR2: PP129328; SJH105VR2: PP129329; SJH089VR2: PP129330.

3.3.1.2 T0.7/K12 analysis

T0.7 sequences were obtained for 12 patients, seven (58%) clustered with subtype A/C, two (17%) clustered with subtype J, one (8%) clustered with subtype B, one (8%) clustered with subtype R and one (8%) clustered with subtype Q. There were no sequences identified belonging to subtypes D, E, F, N or M. Phylogenetic analysis of these sequences, as well as 79 previously published sequences, is shown in Figure 3.6. As expected, the majority of sequences clustered with subtype A/C, consistent with previous literature demonstrating a predominance of subtype A/C in T0.7 among KS patients in Europe (Tornesello et al., 2010; Zong, Kajumbula et al., 2007). The evidence to date has shown T0.7 subtypes A/C, J and K/M to be associated with HHV-8 genomes originating in Eurasia, D subtypes are noted to originate in aboriginal individuals from the Pacific Rim and subtypes B, F/G, R, Q and N are found in sub-Saharan Africa (Hayward & Zong, 2007).

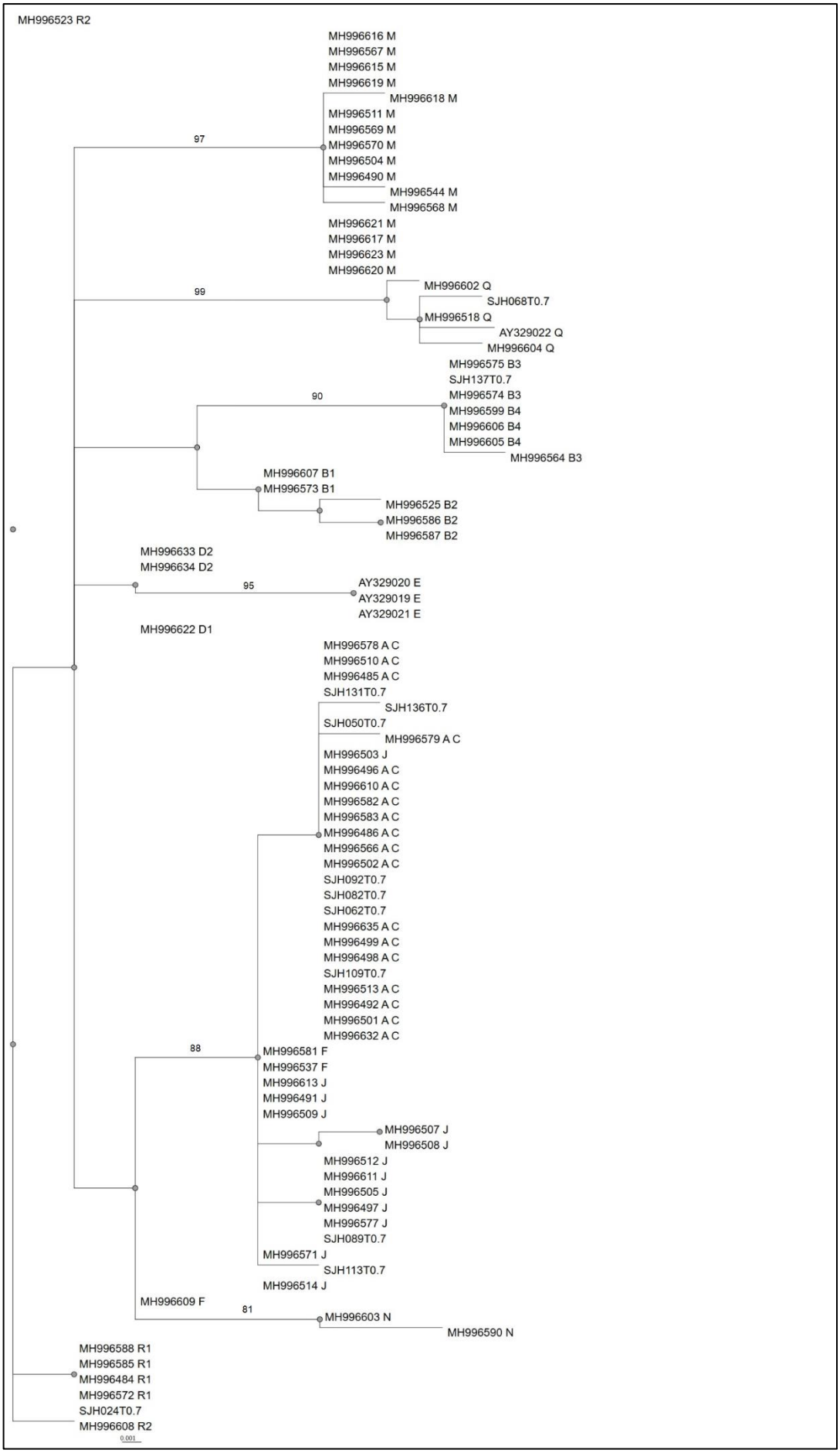
MH996523 R2
MH996616 M
MH996567 M
MH996615 M
MH996619 M
MH996618 M
MH996511 M
MH996569 M
97
MH996570 M
MH996504 M
MH996490 M
MH996544 M
MH996568 M
MH996621 M
MH996617 M
MH996623 M
MH996620 M
MH996602 Q
99
SJH068T0.7
MH996518 Q
AY329022 Q
MH996604 Q
MH996575 B3
SJH137T0.7
90
MH996574 B3
MH996599 B4
MH996606 B4
MH996605 B4
MH996564 B3
MH996607 B1
MH996573 B1
MH996525 B2
MH996586 B2
MH996587 B2
MH996633 D2
MH996634 D2
95
AY329020 E
AY329019 E
AY329021 E
MH996622 D1
MH996578 A C
MH996510 A C
MH996485 A C
SJH131T0.7
SJH136T0.7
SJH050T0.7
MH996579 A C
MH996503 J
MH996496 A C
MH996610 A C
MH996582 A C
MH996583 A C
MH996486 A C
MH996566 A C
MH996502 A C
SJH092T0.7
SJH082T0.7
SJH062T0.7
MH996635 A C
MH996499 A C
MH996498 A C
SJH109T0.7
MH996513 A C
MH996492 A C
MH996501 A C
MH996632 A C
88
MH996581 F
MH996537 F
MH996613 J
MH996491 J
MH996509 J
MH996507 J
MH996508 J
MH996512 J
MH996611 J
MH996505 J
MH996497 J
MH996577 J
SJH089T0.7
MH996571 J
SJH113T0.7
MH996514 J
MH996609 F
81
MH996603 N
MH996590 N
MH996588 R1
MH996585 R1
MH996484 R1
MH996572 R1
SJH024T0.7
MH996608 R2
0.001

Figure 3.6. A maximum likelihood tree based on the T0.7/K12 gene (390 nts) for 12 St. James's Hospital HHV-8-positive specimens, using MH996523 as an out-group. Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing types A/C, B, D, E, F, J, M, N, Q and R, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH024T0.7: PP196517; SJH068T0.7: PP196518; SJH113T0.7: PP196519; SJH136T0.7: PP196520; SJH050T0.7: PP227228; SJH062T0.7: PP227229; SJH089T0.7: PP227230; SJH092T0.7: PP227231; SJH109T0.7: PP227232; SJH131T0.7: PP227233; SJH082T0.7: PP227234; SJH137T0.7: PP227235.

3.3.1.3 ORF75 analysis

ORF75 sequences were obtained for 12 patients, seven (58%) clustered with subtype A, one (8%) clustered with subtype B, three (25%) clustered with subtype C and one (8%) clustered with subtype D. There were no sequences belonging to subtypes M or N identified. Phylogenetic analysis of these sequences, as well as 59 previously published sequences, is shown in Figure 3.7.

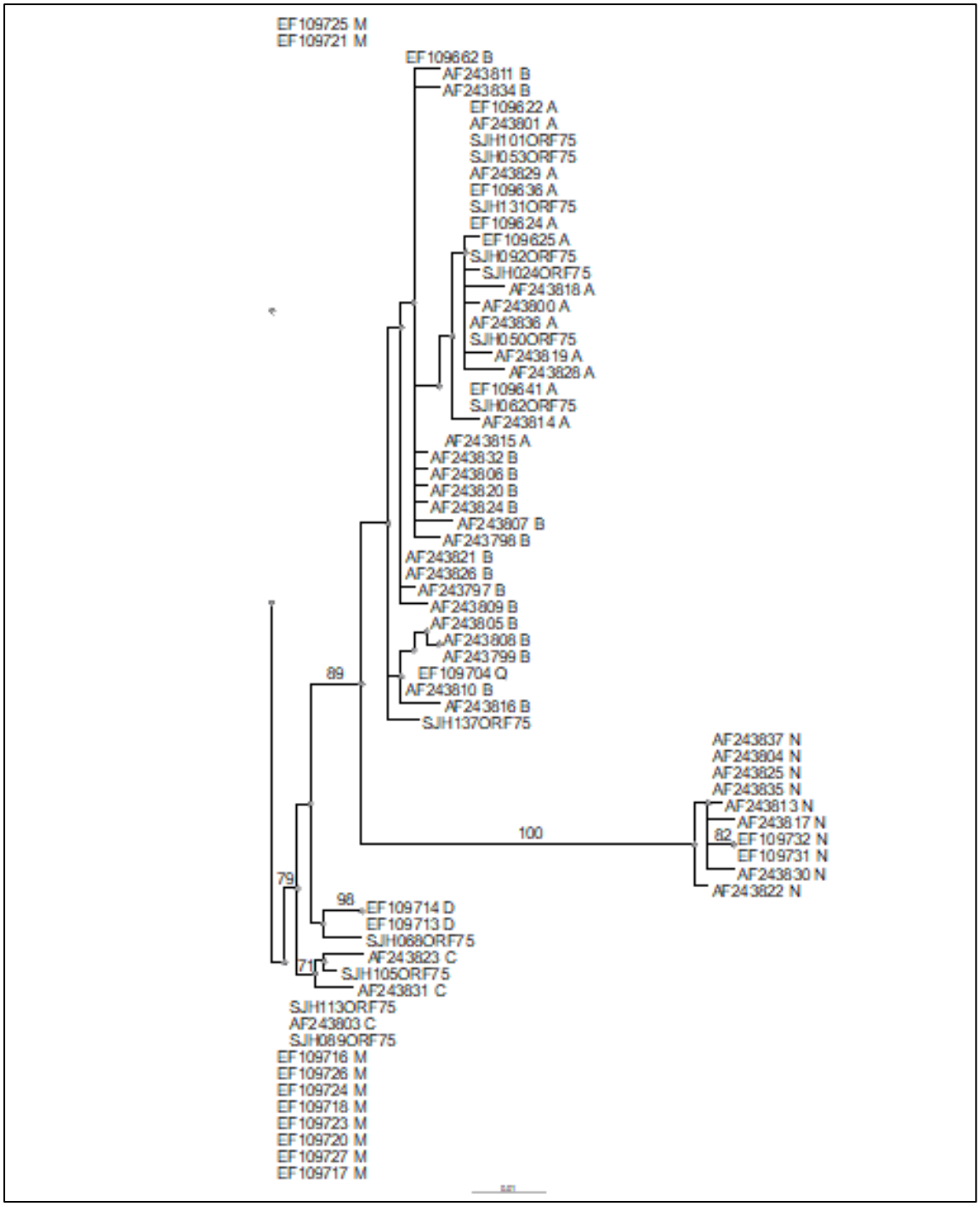

Figure 3.7. A maximum likelihood tree based on the ORF75 (804 nts) for 12 St. James's Hospital HHV-8-positive specimens, using EF109725 as an out-group.

Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing types A, B, C, D, M, N and Q, indicated by their GenBank accession numbers. GenBank new sequence

accession numbers: SJH050ORF75: PP227236; SJH024ORF75: PP227237; SJH062ORF75: PP227238; SJH068ORF75: PP227239; SJH089ORF75: PP227240; SJH092ORF75: PP227241; SJH101ORF75: PP227242; SJH105ORF75: PP227243; SJH113ORF75: PP227244; SJH131ORF75: PP227245; SJH137ORF75: PP227246; SJH053ORF75: PP227247.

3.3.1.4 K15 analysis

K15 sequences were determined for 13 of the St. James's Hospital patients, nine (69%) of HHV-8 isolates had the P allele, and four (31%) patients had the M allele. These results are demonstrated in Table 3.6 and Figure 3.8.

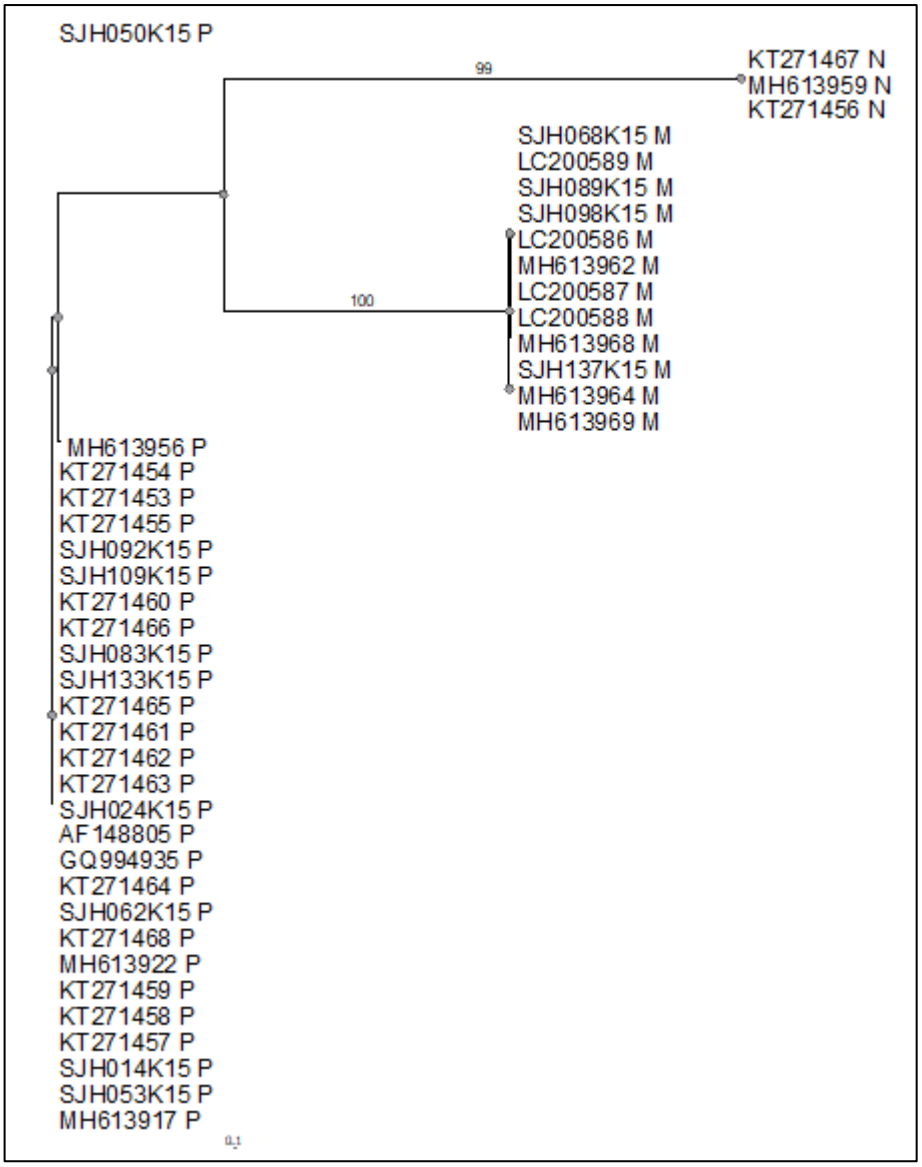

Figure 3.8. A maximum likelihood tree based on the K15 gene (P, 285 nts; M, 370 nts) for 13 St. James's Hospital HHV-8-positive specimens, using SJH050K15 P as an out-group.

Bootstrap analysis was carried out using 1000 replicates of the dataset and the values are written as a percentile beside the appropriate branch. Bootstrap values <70% are not shown. Comparison was made to reference strains representing subtypes M, N and P, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH014K15: PP227248; SJH050K15: PP227249; SJH053K15: PP227250; SJH062K15: PP227251; SJH083K15: PP227252; SJH092K15: PP227253; SJH109K15:

PP227254; SJH133K15: PP227255; SJH024K15: PP227256; SJH098K15: PP227257; SJH068K15: PP227258; SJH137K15: PP227259; SJH089K15: PP227260.

3.4 Discussion

This is the first study detailing the molecular epidemiology of HHV-8 in the Republic of Ireland. Sequencing was successfully performed using specimens from 19 patients with HHV-8-related disease, to characterise four regions of the HHV-8 genome, K1, T0.7, ORF75 and K15, by phylogenetic analysis. The data generated by this study demonstrate that a broad variety of HHV-8 subtypes are represented in the patient population in Ireland exhibiting HHV-8-related disease. Of note, although 30 patients were consented for this study, HHV-8 sequences were successfully obtained for only 19 of these patients, largely due to low viral loads in the remaining samples. This highlights the limitations of using plasma samples for HHV-8 subtyping, compared to biopsy samples from KS lesions, for example.

The current study provides some interesting observations regarding genetic diversity in HHV-8 in Ireland, a low prevalence country. The findings demonstrate significant heterogeneity of HHV-8 subtypes based on the four genome regions examined. There was one patient with an A subtype in K1 and an M allele in K15, and one patient with a C subtype in K1 and an M allele in K15. Two patients were identified to have divergent sequences across the different loci of the HHV-8 genome. One patient with a K1 gene that clustered with the B subtype had a subtype R in K12, a subtype A in ORF75 and a subtype P in K15. One patient was identified with a K1 gene that clustered with the F subtype. This patient had divergent sequences at the other loci, with a subtype Q K12, subtype D ORF75 and a subtype M K15. These findings may represent recombination, which has been demonstrated elsewhere in the literature, and has been shown to occur over time.

The earlier literature suggested that recombination was a relatively slow process in HHV-8. However, a number of more recent studies have suggested that the presence of recombinants may be more common than previously understood. Evidence of recombinants between subtypes has been demonstrated and recombination has been shown to occur at multiple different genetic loci (Kakoola et al., 2001). Poole et al. (1999) originally demonstrated evidence for recombination in approximately 20-30% of HHV-8 strains and this was further demonstrated by Kakoola et al. (2001) who identified recombination in seven out of nine Ugandan strains studied. These findings have been supported by further studies, including the use of whole-genome sequencing on fluid samples in patients with HHV-8-related diseases (Cornejo Castro et al., 2020; Olp et al., 2015; Sallah et al., 2018). Sallah et al. (2018) performed a comprehensive recombination analysis, providing evidence of recombination throughout the HHV-8 genome, identifying recombination in eight genes.

It is also possible that the finding of divergent sequences in these two cases may represent dual infection by different subtypes of HHV-8. This has been shown to be possible, and Cornejo Castro et al. (2020) recently reported a case of an African-American patient with primary effusion lymphoma and multicentric Castleman's disease, where the presence of two different HHV-8 genomes was confirmed in pleural fluid, saliva and peripheral blood mononuclear cell specimens obtained from the same patient. However, overall, the evidence to date would suggest that dual infection by different subtypes of HHV-8 is a rare occurrence and, until recently, the finding of multiple HHV-8 variants in a single host has been thought more likely to be due to "quasispecies formation" rather than multiple episodes of infection (Hayward & Zong, 2007; Kajumbula et al., 2006; Zong, Arav-Bolger et al., 2007; Whitby et al., 2004;

Zong et al., 2002; Leao et al., 2013). The finding of multiple HHV-8 strains appears to be more common in the setting of severe immunosuppression and it is interesting to note that, despite the presence of multiple HHV-8 strains in the blood of a single host, resulting KS lesions generally tend to be caused by a single HHV-8 strain (Leao et al., 2013). Stebbing et al. (2001, 2003) demonstrated that K1 does not change over time in an individual patient and does not change in different tumour sites within an individual, specifically, multiple tumours in a single patient tend to demonstrate identical K1 sequences. Further analysis, for example using whole genome sequencing, would be required to definitively assess whether the divergent sequences from the two cases in the current study represent recombination or dual infection.

Subtype F in K1 has been reported rarely in the literature, in individual cases and small clusters (Jary et al., 2020; Kajumbula et al., 2006; Tornesello et al., 2010; Tozetto-Mendoza et al., 2016). Lacoste, Judde et al. (2000) identified a single patient from France with a K1 gene belonging to this subtype and Marshall et al. (2007) identified an identical K1 sequence in a US citizen who was born in France, a patient with AIDS-KS. Kajumbula et al. (2006) described the presence of the K1 F subtype in an African population from Uganda. Tozetto-Mendoza et al. (2016) and Tornesello et al. (2010) also identified individual patients with subtype F K1 genes from Brazil and Kenya, respectively. Jary et al. (2020) recently proposed a novel F2 variant, which was described in 5 Caucasian MSM living in Paris. On further analysis of the sequence belonging to the K1 F subtype in the current study, it was found to cluster more closely with sequences belonging to the F2 subtype proposed by Jary et al. (2020), as opposed to the F1 subtype identified by Kajumbula et al. (2006) in Uganda. Of note, in the study by Jary et al. (2020), four out of five patients with the F2 subtype were

immunocompromised and presented with severe HHV-8-related disease. Further work is warranted to investigate whether specific HHV-8 subtypes may be associated with severe disease (Jary et al., 2020).

In the literature to date, isolates containing the K15 M allele are in the minority, with approximately 20% of all HHV-8 genomes characterised worldwide belonging to the K15 M subtype, so it is interesting that 31% of the sequences obtained in the current study were belonging to this group (Hayward & Zong, 2007). This may represent the changing and evolving epidemiology in Ireland resulting from changing patterns of migration. The four specimens in the current study containing the M allele (SJH098, SJH068, SJH137, SJH089) were each associated with a different K1 subtype, subtype A, subtype F, subtype B and subtype C, respectively. This suggests that the specimens are likely to be unrelated epidemiologically within this patient group. Nine specimens contained the P allele, and the majority of these were associated with an A or C K1 subtype, which is consistent with the international literature (Lacoste, Judde et al., 2000). The exception is specimen SJH024, which belonged to K1 subtype B and contained the K15 P allele.

A limitation of this study was that it was not possible to identify sequences belonging to the K15 N subtype, as it was not possible to obtain the relevant primers to perform nested PCR for this subtype. Where it was not possible to determine the K15 subtype, it is conceivable that some of these HHV-8-positive specimens may have belonged to the N subtype. However, as there were no sequences identified belonging to subtype N in ORF75, this is unlikely to be the case. Designing primers in order to identify the K15 N

subtype was, therefore, not felt to be warranted in a western European population such as that of the current study.

Early studies on the molecular epidemiology of HHV-8 demonstrated clear geographical patterns with respect to HHV-8 subtypes. For example, in K1, B and A5 subtypes were shown to predominate in sub-Saharan Africa, subtype D among native populations of South Asia, Australia and the Pacific, with subtypes A and C predominating in the majority of European and Asian countries (Cook et al., 1999; Kasolo et al., 1998; Lacoste, Judde et al., 2000; Meng et al., 1999; Poole et al., 1999; Zong et al., 2002). These patterns are felt to be due to the original migration patterns of modern humans (Zong et al., 2002). While there was evidence at this time of some degree of geographical dissemination, for the most part, subtypes tended to conform to these patterns. It is interesting to note, therefore, in the current study, the diverse findings of different K1 subtypes in Ireland, as well as their linkage to different T0.7, ORF75 and ORF-K15 subtypes, which may reflect global migration and population changes.

In K15, the P subtype has been shown to predominate in regions other than Central and West Africa, while M and P subtypes have been present in equal numbers in samples from Central and West Africa (Lacoste, Judde et al., 2000). It is unclear whether the P and M K15 alleles are linked to the corresponding K1 subtype, with conflicting findings reported in different studies (Lacoste, Judde et al., 2000; Poole et al., 1999). Lacoste, Judde et al. (2000) found the significant majority of C and A K1 subtypes to be linked with a P K15 subtype, while B and A5 subtypes showed the P and M alleles to be equally represented. This is in contrast to the findings of Poole et al (1999) who failed

to demonstrate a significant link between K1 and K15 subtypes. Lacoste, Judde et al. (2000) raised the possibility of a functional difference between the P and M subtypes, potentially conferring higher transmissibility to the P subtype, evidenced by the overall predominance of the P subtype in studies performed worldwide. The relatively high rate of M alleles identified in the current study is in contrast to the findings in a number of European studies, for example in France and Greece, where the P allele was found to predominate (Lacoste, Judde et al., 2000; Tornesello et al., 2010). However, the findings in this study are more in keeping with rates reported in European countries such as Russia and Scandinavia (Kadyrova et al., 2003; Lacoste, Judde et al., 2000). This may be due to population movements into Ireland from Eastern Europe and Western Africa, regions known to have higher rates of K15 M alleles (Lacoste, Judde et al., 2000; Lacoste, Kadyrova et al., 2000). Studies by Zong et al. (2002) and Zhang et al. (2001) both identified relatively high proportions of K15 M alleles among populations in several states of the USA, specifically Maryland, Tennessee and Texas. This finding has been attributed to the diverse populations in these states from varying ethnic backgrounds.

The HHV-8 subtypes identified in this study reflect the current population in Ireland. The diversity of subtypes and the presence of subtypes such as B and F in K1 and subtype M in K15, which are unusual to find in a western European setting, most likely reflect the changing population in Ireland, with increasing inward migration due to Ireland's economic development (Tornesello et al., 2010).

Despite evidence initially supporting HHV-8 subtype linkage to the geographic origin of the host individual among immigrant populations, inter-ethnic transmission is known

to occur, as evidenced by multiple previous studies, including a report by Nascimento et al. on the Brazilian population (Kourí et al., 2012; Nascimento et al., 2005; Pérez & Tous, 2017; Zhang et al., 2001). The variety of subtypes identified in the current study using phylogenetic analysis is likely to represent individuals who have been infected with HHV-8 in their native countries, as well as local inter-ethnic transmission in Ireland.

Of note, the sequences obtained represent a relatively small sample of patients attending a single specialised centre, incorporating a large Genitourinary Medicine and Infectious Diseases unit, and therefore may not be an accurate representation of HHV-8 subtypes circulating in Ireland as a whole. This is a limitation of the study and it would be an interesting avenue for future research to include patients from other referral centres nationally.

In conclusion, this study has classified HHV-8 sequences from 19 patients with HHV-8-related disease into their respective subtypes using phylogenetic analysis. K1 subtype C has been found to be the most prevalent subtype (47%), followed by subtype A (37%). This is consistent with similar European populations previously studied (Hayward & Zong, 2007; Tornesello et al., 2010). One K1 sequence clustered with the rare subtype F. Despite the presence of a significant Brazilian population in Ireland, there were no subtype D or E sequences identified. K15 subtype P was found to be more prevalent than subtype M. However, the prevalence of 31% for subtype M was higher than expected for a western European population (Lacoste, Judde et al., 2000; Tornesello et al., 2010).

Studies such as this contribute greatly to our understanding of this virus and its epidemiology, providing additional insight into the geographical distribution of HHV-8 subtypes in different parts of the world. It is interesting to note the existence of a high level of genetic variability in a very low prevalence country such as Ireland, providing valuable additional information on the present-day global epidemiology of this virus. This study is the first of its type in Ireland and will inform future molecular studies of HHV-8.

Chapter 4

Immunological Aspects of Human Herpesvirus 8 in Ireland

4.1 Introduction

In addition to the role of HHV-8 infection, it is clear that the immune system has a significant role to play in the development and severity of HHV-8-related diseases, and chronic inflammation is a notable feature in HHV-8-associated diseases. Proinflammatory cytokines have been demonstrated to play an important part in the pathogenesis of HHV-8-related diseases. Initial infection with HHV-8 in monocytes promotes production of cytokines such as IL-1α, IL-1β, and IL-6 (Host et al., 2017). Early KS lesions are characterised by an inflammatory cell infiltrate, which produces inflammatory cytokines that further promote tumour progression (Ensoli & Stürzl, 1998) (*Section 1.6.1*).

Elevated levels of HHV-8-encoded viral interleukin-6 (vIL-6), human IL-6 and IL-10 have been demonstrated in serum samples from patients with HHV-8-associated diseases, in the presence of inflammatory symptoms and higher levels of these cytokines have been associated with adverse clinical outcomes (Aoki, Tosato, et al., 2001; Brandt et al., 1990; Oksenhendler et al., 2000; Polizzotto et al., 2013). KSHV inflammatory cytokine syndrome (KICS) is a disorder, recently described by Uldrick et al. (2010) that is characterised by elevated levels of vIL-6 and human IL-6, and has been associated with high mortality rates (*Section 1.6.2*).

Internationally, work has been done recently on the role of proinflammatory cytokines in HHV-8 disease but there is the potential for further investigation in this area. The purpose of this study was to perform cytokine analysis over time in a population with known human herpesvirus 8-related disease and thereby to investigate the role of

proinflammatory cytokines in the diagnosis and monitoring of HHV-8-related disease. As a rule, KS lesions involve elevated levels of inflammatory cytokines and it has been postulated that the presence of these cytokines may contribute to the pathogenesis of this disease. Yu et al. (1999) suggested from their work that IL-1β and other inflammatory cytokines have a potential direct effect on HHV-8 and, by modulating the expression of certain HHV-8 genes, may contribute to the pathogenesis of KS.

It is important to understand the role that the immune system and proinflammatory cytokines have to play in the pathogenesis and severity of HHV-8 disease in order to develop optimal treatment strategies for these diseases. Research is currently being undertaken into the use of agents for treatment that target these cytokines, for example the use of tocilizumab, an IL-6 receptor blocking antibody in the treatment of MCD (Alomari & Totonchy, 2020).

The current study is being undertaken to examine the immunological aspects of diseases related to this virus. Cytokine analysis over time using paired patient samples will allow us to determine the role of proinflammatory cytokines in the diagnosis and monitoring of response to treatment of HHV-8-related disease to inform international knowledge in this area.

4.1.1 Aims

The aims of this study were firstly, to perform cytokine analysis over time using paired plasma samples from patients with HHV-8 related diseases by measuring the levels of 10 specific cytokines, IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNF-α. A second aim was to compare the levels of these cytokines between HHV-8

subtypes, specifically K1 and K15 subtypes. Thirdly, the study aimed to compare the levels of these cytokines before and after treatment for HHV-8-related disease. Finally, a fourth aim of this study was to compare HHV-8 Ct values with the cytokine levels for each sample and before and after treatment.

4.1.2 Objectives

1. To determine the levels of 10 specific cytokines in HHV-8 positive patient samples over time using a commercial 10-plex assay, V-PLEX Proinflammatory Panel 1, for IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNF-α (Meso-Scale Discovery, Rockville, MD, US).
2. To compare the levels of these cytokines between HHV-8 K1 subtypes and K15 subtypes, using molecular data previously obtained (*Section 3.3.1*).
3. To compare the levels of each cytokine before and after treatment for HHV-8-related disease.
4. To compare Ct values for HHV-8 PCR before and after treatment and to compare Ct values with cytokine levels before and after treatment.

4.2 Methods

4.2.1 Study Design and Setting

A retrospective study was designed to investigate the presence of proinflammatory cytokines in HHV-8 related disease. The study was carried out from March 2019 to December 2021 at the Department of Clinical Microbiology, St. James's Hospital, Dublin, Ireland. St. James's Hospital is a large, acute tertiary referral centre incorporating multiple national specialty services, including the National Haematopoietic Stem Cell Transplant Unit and a large Genitourinary Medicine and Infectious Diseases unit.

4.2.2 Study Population

From January 2012 to August 2019 in St. James's Hospital, of all plasma sample requests for diagnostic HHV-8 PCR testing based on clinical suspicion for HHV-8-related disease, 153 samples tested positive by PCR for HHV-8 DNA. 30 patients consented to the use of their clinical samples for the purposes of this study, providing 85 specimens for use in the study. Written informed consent was obtained from all the study participants.

4.2.3 Cytokine Analysis

Plasma levels of proinflammatory cytokines in the HHV-8 positive patient samples over time were assessed using a commercial 10-plex assay, V-PLEX Proinflammatory Panel 1, for IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8 and TNF-α (Meso-Scale Discovery, Rockville, MD, US) with the SECTOR® Imager, according to the manufacturer's instructions. The panel consists of sandwich immunoassays, using electrochemiluminescence to provide a quantitative result for each cytokine. All assays

were performed in duplicate. The V-PLEX Proinflammatory Panel 1 kit has undergone extensive validation and a certificate of analysis is provided with each kit detailing specifications for sensitivity, specificity, accuracy and precision. The detection ranges were IFN-γ 0.37–1280 pg/mL, IL-1β 0.05–554 pg/mL, IL-2 0.09-1440 pg/mL, IL-4 0.02–218 pg/mL, IL-6 0.06–742 pg/mL, IL-8 0.07–545 pg/mL, IL-10 0.04–378 pg/mL, IL-12p70 0.11-549 pg/mL, IL-13 0.24-529 pg/mL, TNFα 0.04–349 pg/mL.

4.2.3.1 Selected Cytokines

The proinflammatory cytokines selected for study are detailed below.

4.2.3.1.1 Human interferon gamma (IFN-γ)

IFN-γ is a proinflammatory cytokine and is the only type II interferon. It exists as a non-covalently linked homodimer (Chesler & Reiss, 2002). IFN-γ is produced by natural killer cells, natural killer T cells, CD4+ and CD8+ lymphocytes, and is an activator of macrophages (Schroder et al., 2004). IFN- γ reactivity has been demonstrated during primary HHV-8 infection, with detectable HHV-8 DNA in PBMCs and increases in serum HHV-8 antibody levels (Wang et al., 2001).

4.2.3.1.2 Human interleukin-1 beta (IL-1β)

IL-1β is a proinflammatory cytokine that is produced by activated macrophages. IL-1β is an important mediator of the inflammatory response and, in conjunction with IFN-γ, IL-6, and TNF-α, contributes to the production of prostaglandins, leading to fever induction. IL-1β has been identified at high levels in KS lesions and it has been suggested that IL-1β may contribute to the pathogenesis of KS by modulating the expression of certain HHV-8 genes (Yu et al., 1999).

4.2.3.1.3 Human interleukin-2 (IL-2)

IL-2 contributes to immune system regulation by promoting T-cell proliferation. IL-2 increases the cell killing activity of both natural killer cells and cytotoxic T cells (Liao et al., 2011). Recombinant forms of IL-2 have been trialled in the treatment of a number of cancers and viral infections such as hepatitis B, hepatitis C and HIV. However, it has been shown that therapy involving IL-2 may induce HHV-8 replication (Malnati et al., 2002).

4.2.3.1.4 Human interleukin-4 (IL-4)

IL-4 is produced by T helper type 2 (Th2) cells, mast cells, eosinophils and basophils. It participates in activation of B cells and T cells, and aids the differentiation of B cells into plasma cells (Gadani et al., 2012). It has been shown in vitro that HHV-8 replication in B cells requires pre-activation of the cells with IL-4 (Rappocciolo et al., 2008).

4.2.3.1.5 Human interleukin-6 (IL-6)

IL-6 acts as a proinflammatory cytokine and is secreted mainly by T-cells and macrophages. It is involved in numerous biological processes including inflammation, aging, cell growth, apoptosis, and bone remodelling. IL-6 contributes to the acute phase response and promotes fever induction. A proportion of the inflammatory symptoms associated with KICS and other HHV-8-related diseases have been attributed to the presence of IL-6 (Oksenhendler et al., 2000; Polizzotto et al., 2013).

4.2.3.1.6 Human interleukin-8 (IL-8)

IL-8 is a chemokine that is produced by macrophages, epithelial cells and endothelial cells in response to inflammation. IL-8 induces chemotaxis in neutrophils and other granulocytes, enabling travel to infection sites, and subsequently stimulates phagocytosis at the site. IL-8 is a significant inducer of angiogenesis. It has been postulated that IL-8 production in the setting of HHV-8 may have a role in the pathogenesis of disease associated with this virus (Sun et al., 2006).

4.2.3.1.7 Human interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine. It is produced by monocytes, T-cells, B-cells and mast cells. IL-10 inhibits production of several proinflammatory cytokines, such as IFN-γ, IL-1β, IL-12 and TNF-α, resulting in downregulation of Th1 responses. It also promotes B-cell survival and antibody production. Like IL-6, IL-10 has been reported in a number of studies to be potentially implicated in the pathogenesis of HHV-8-related diseases (Oksenhendler et al., 2000; Polizzotto et al., 2015).

4.2.3.1.8 Human interleukin-12p70 (IL-12p70)

IL-12p70 is made up of IL-12p35 and IL-12p40, two disulfide-linked subunits, resulting in a heterodimer. Production is predominantly by macrophages and T lymphocytes. It is frequently the first cytokine released in the presence of antigen, and is therefore of upmost importance in activating the immune response (Zhang & Wang, 2008).

4.2.3.1.9 Human interleukin-13 (IL-13)

IL-13 is secreted by a number of different immune cells, including Th2 cells, CD4+ T cells, natural killer T cells, mast cells, basophils, eosinophils and nuocytes (Rael & Lockey, 2011). IL-13 contributes to IgE regulation and mediates allergic inflammatory responses (Rael & Lockey, 2011). It is also involved in the regulation of B-cell proliferation, macrophage activation and immunoglobulin production. Elevated IL-13 levels have been noted to occur in effusions of PEL (Ramaswami et al., 2018, 2021).

4.2.3.1.10 Human tumour necrosis factor alpha (TNF-α)

TNF-α is primarily secreted by macrophages and has a primary role in the regulation of immune cells. It acts as a pyrogen, inducing fever and responding to sepsis through the activation of IL-1 and IL-6. It has a role in apoptosis and can inhibit tumorigenesis. HHV-8 is thought to stimulate TNF-α production and TNF-α levels have been correlated with HHV-8 infection and disease progression. Elevated levels of TNF-α have also been observed within KS lesions (Aboulafia et al., 1989; Murakami-Mori et al., 1999; Oxholm et al., 1989).

4.2.3.2 Principle of the Assay

The Proinflammatory Panel 1 (human) kit uses a plate with specific spots that are pre-coated with capture antibodies. The assay protocol entails the addition of a solution containing detection antibodies conjugated with electrochemiluminescent labels to the sample in the plate and incubating them together. Immobilised capture antibodies on the working electrode surface bind to analytes within the sample. Detection antibodies are then recruited by the bound analytes, completing the sandwich immunoassay. A buffer is added that facilitates electrochemiluminescence and the plate is loaded onto the

SECTOR® Imager. A voltage applied to the plate electrodes causes light to be emitted from the captured labels. The SECTOR® Imager measures the intensity of light emitted and provides a quantitative measure of each analyte in the sample.

4.2.3.3 Reagent Preparation

All reagents were brought to room temperature and calibrator dilutions were prepared as per the manufacturer's instructions. Briefly, a lyophilised calibrator blend was reconstituted with 1,000 µL of diluent, this was then inverted three times and equilibrated for 15 minutes at room temperature, then briefly vortexed. A series of 4-fold dilutions were performed to prepare seven calibrator solutions and a zero calibrator was prepared. Each dilution was again briefly vortexed. A combined detection antibody solution was prepared by diluting each 50X detection antibody 50-fold. PBS + 0.05% Tween-20 was used as a wash buffer. Read Buffer T was prepared by diluting 4X Read Buffer T 2-fold with deionised water.

4.2.3.4 Sample Collection and Handling

All plasma samples had previously been analysed by diagnostic HHV-8 PCR testing using real-time PCR and were stored at -80°C prior to testing. After thawing, samples were centrifuged at 2,000g for 3 minutes prior to sample preparation. All samples were diluted 2-fold before adding them to the plate.

4.2.3.5 Assay Protocol

The following protocol was utilised in the performance of the assay.

4.2.3.5.1 Wash and Add Sample

Each plate was washed three times with 150 µL/well of wash buffer. 50 µL of the prepared sample or calibrator were added per well. The plate was sealed with an adhesive plate seal and incubated at room temperature with shaking for 2 hours.

4.2.3.5.2 Wash and Add Detection Antibody Solution

Each plate was washed three times with 150 µL/well of wash buffer. 25 µL of detection antibody solution were added to each well. The plate was sealed with an adhesive plate seal and incubated at room temperature with shaking for 2 hours.

4.2.3.5.3 Wash and Read

Each plate was washed three times with 150 µL/well of wash buffer. 150 µL of 2X Read Buffer T were added to each well and the plate was analysed on the SECTOR® Imager.

4.2.4 Statistical Analysis

Statistical analysis was performed using IBM® SPSS® Statistics (version 26). The cytokine analysis data were not normally distributed and are represented by median, interquartile range and minimum and maximum values. Comparisons between K1 subtypes were made using the Kruskal-Wallis test. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Comparisons between K15 subtypes were made using the Mann-Whitney U Test. Spearman's rank correlation was employed to assess for correlation between cytokine levels and Ct value. The Wilcoxon signed-rank test was used to compare data before and after treatment.

4.2.5 Ethical Approval

Ethical approval for this study was obtained from Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee.

4.3 Results

From January 2012 to August 2019 in St. James's Hospital, 153 samples tested positive for HHV-8 DNA using real-time PCR. 85 specimens from 30 patients were available for use in the cytokine analysis study. Results were obtained successfully for 76 specimens from 28 patients. The sample sizes were insufficient for two patients. It was possible to perform cytokine analysis over time using multiple specimens for 17 patients and 11 patients had analysis performed on a single specimen. For the 17 patients where multiple specimens were analysed, the time frames over which the specimens were analysed ranged from 1 month to 57 months.

4.3.1 Cytokine analysis

Measurements were obtained for 10 cytokines on each patient sample, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α. The results are summarised in Table 4.1.

Table 4.1. Summary of cytokine measurements in plasma from patients PCR positive for HHV-8 (*n*=28).

Cytokine	Median (IQR) (pg/mL)
IFN-γ	27.13 (78.58)
IL-1β	0.22 (0.62)
IL-2	0.64 (0.82)
IL-4	0.02 (0.02)
IL-6	3.20 (7.83)
IL-8	7.11 (8.66)
IL-10	5.53 (45.14)
IL-12p70	0.20 (0.20)
IL-13	0.40 (0.74)
TNF-α	9.14 (7.68)

4.3.1.1 IFN-γ analysis

The median value for IFN-γ was 27.13 pg/mL (range 1.75-1578.79). The mean was 126.7 pg/mL. The results for each patient are demonstrated in Figure 4.1.

4.3.1.2 IL-1β analysis

The median value for IL-1β was 0.22 pg/mL (range 0.0001-5.69). The mean was 0.73 pg/mL. The results for each patient are demonstrated in Figure 4.2.

4.3.1.3 IL-2 analysis

The median value for IL-2 was 0.64 pg/mL (range 0.07-157.1). The mean was 5.90 pg/mL. The results for each patient are demonstrated in Figure 4.3.

4.3.1.4 IL-4 analysis

The median value for IL-4 was 0.02 pg/mL (range 0.0007- 0.25). The mean was 0.03 pg/mL. The results for each patient are demonstrated in Figure 4.4.

4.3.1.5 IL-6 analysis

The median value for IL-6 was 3.20 pg/mL (range 0.13- 61.29). The mean was 7.04 pg/mL. The results for each patient are demonstrated in Figure 4.5.

4.3.1.6 IL-8 analysis

The median value for IL-8 was 7.11 pg/mL (range 1.56-1054.48). The mean was 23.14 pg/mL. The results for each patient are demonstrated in Figure 4.6.

4.3.1.7 IL-10 analysis

The median value for IL-10 was 5.53 pg/mL (range 0.19- 2343.55). The mean was 185.44 pg/mL. The results for each patient are demonstrated in Figure 4.7.

4.3.1.8 IL-12p70 analysis

The median value for IL-12p70 was 0.20 pg/mL (range 0.01-2.37). The mean was 0.26 pg/mL. The results for each patient are demonstrated in Figure 4.8.

4.3.1.9 IL-13 analysis

The median value for IL-13 was 0.40 pg/mL (range 0.02-4.09). The mean was 0.66 pg/mL. The results for each patient are demonstrated in Figure 4.9.

4.3.1.10 TNF-α analysis

The median value for TNF-α was 9.14 pg/mL (range 2.65-31.49). The mean was 10.37 pg/mL. The results for each patient are demonstrated in Figure 4.10.

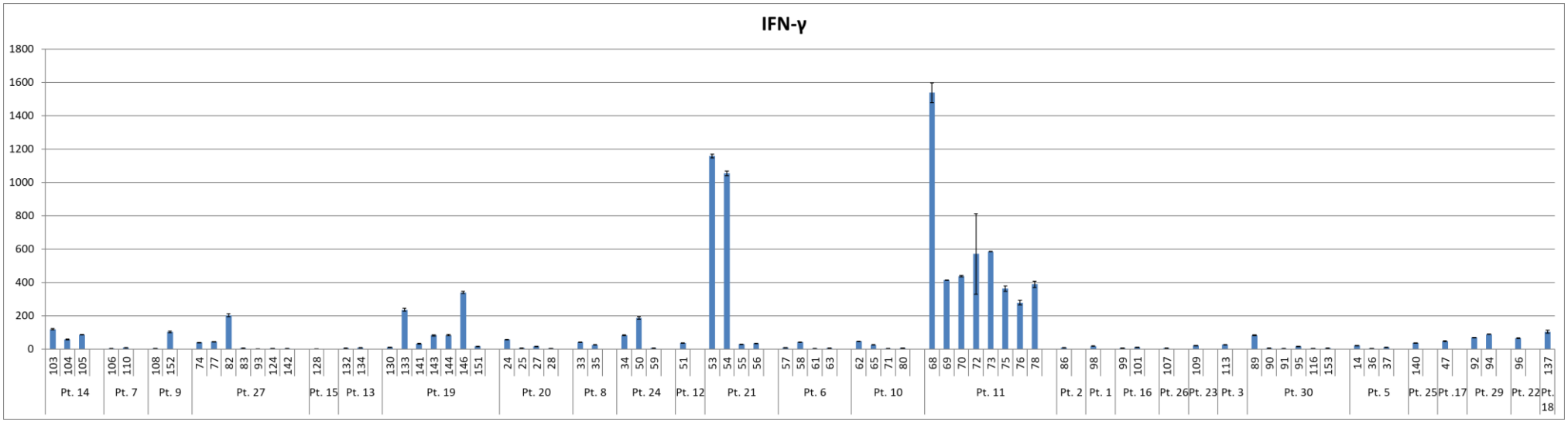

Figure 4.1. Cytokine analysis results for IFN-γ.

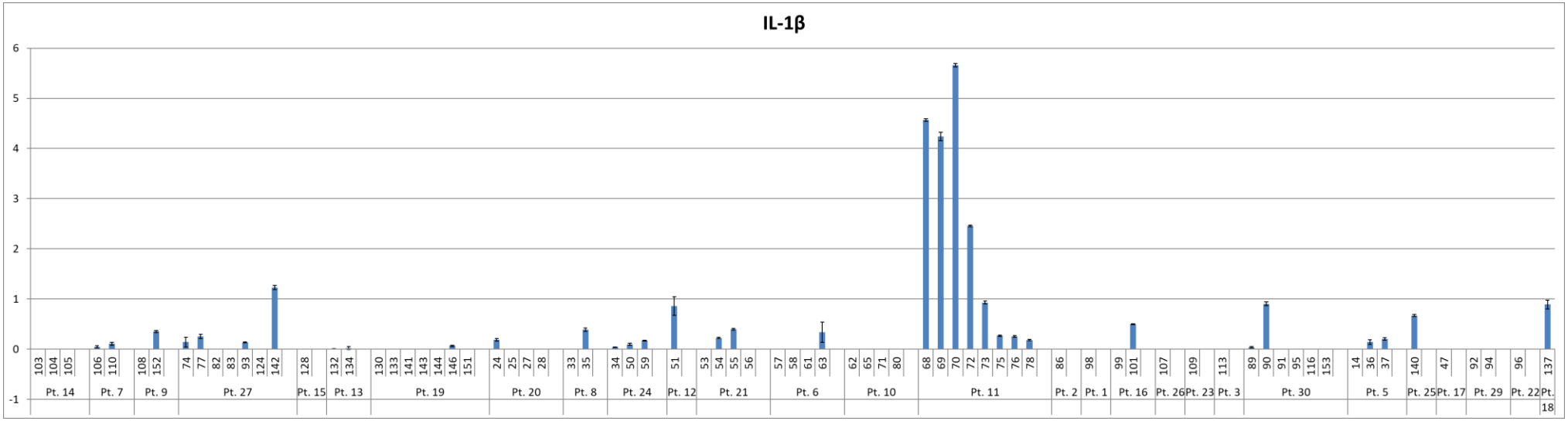

Figure 4.2. Cytokine analysis results for IL-1β.

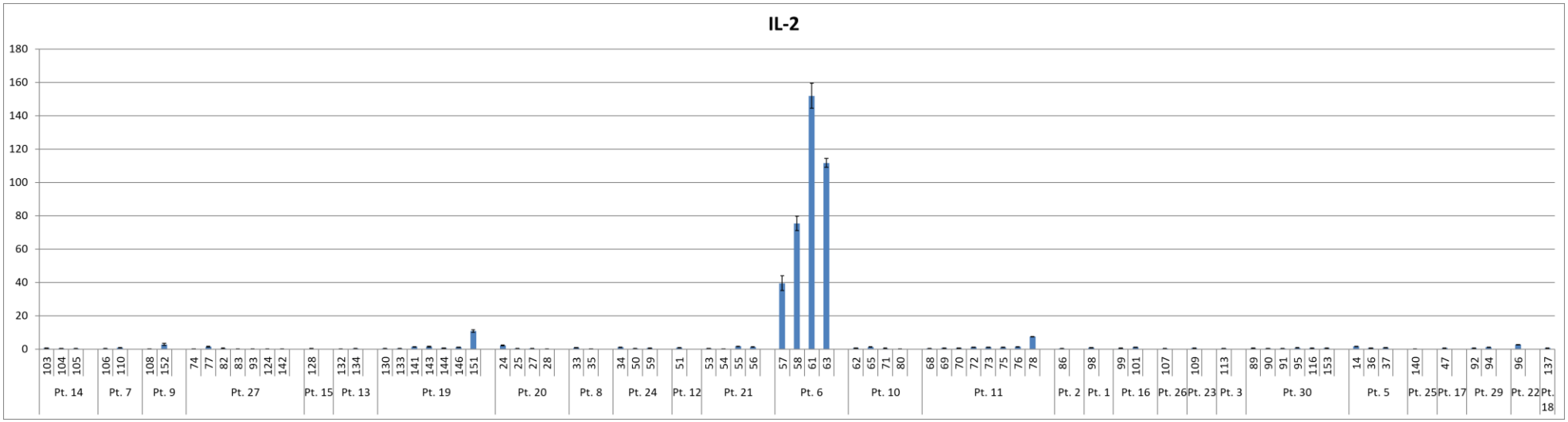

Figure 4.3. Cytokine analysis results for IL-2.

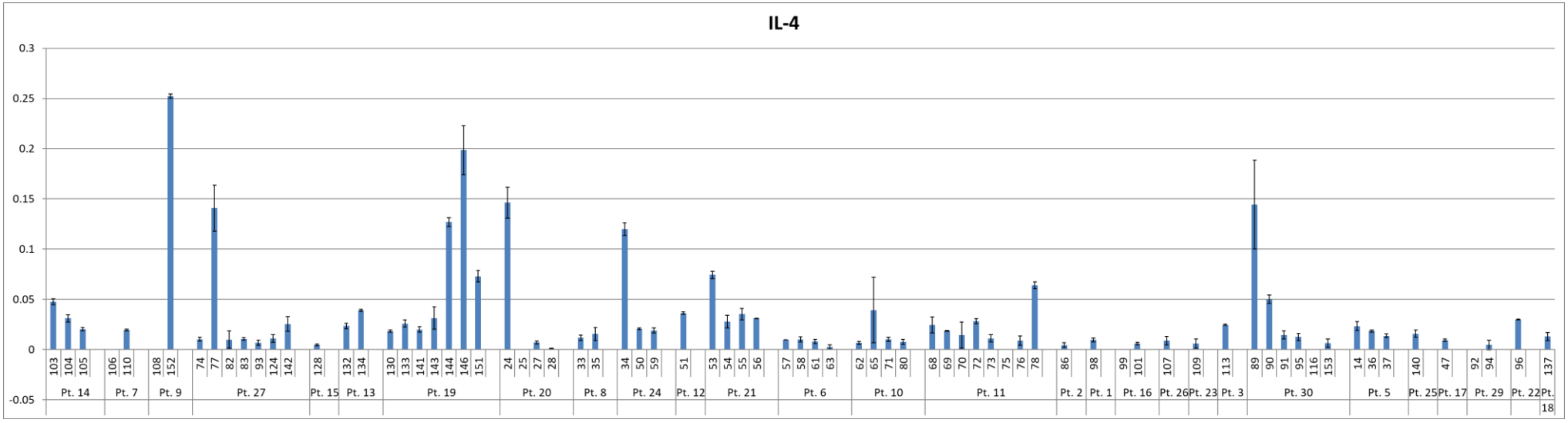

Figure 4.4. Cytokine analysis results for IL-4.

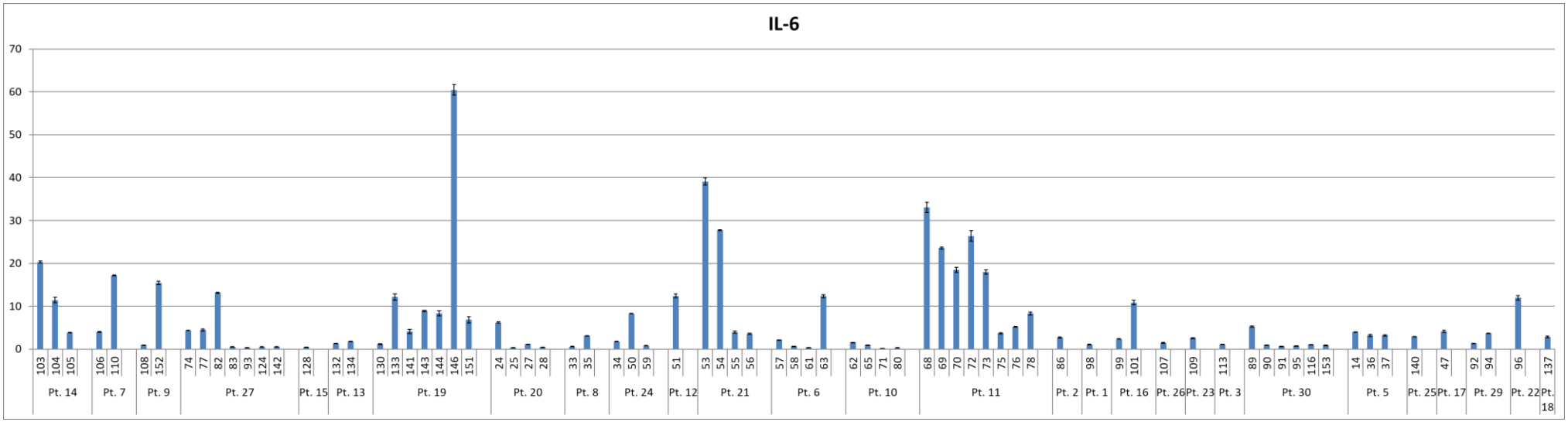

Figure 4.5. Cytokine analysis results for IL-6.

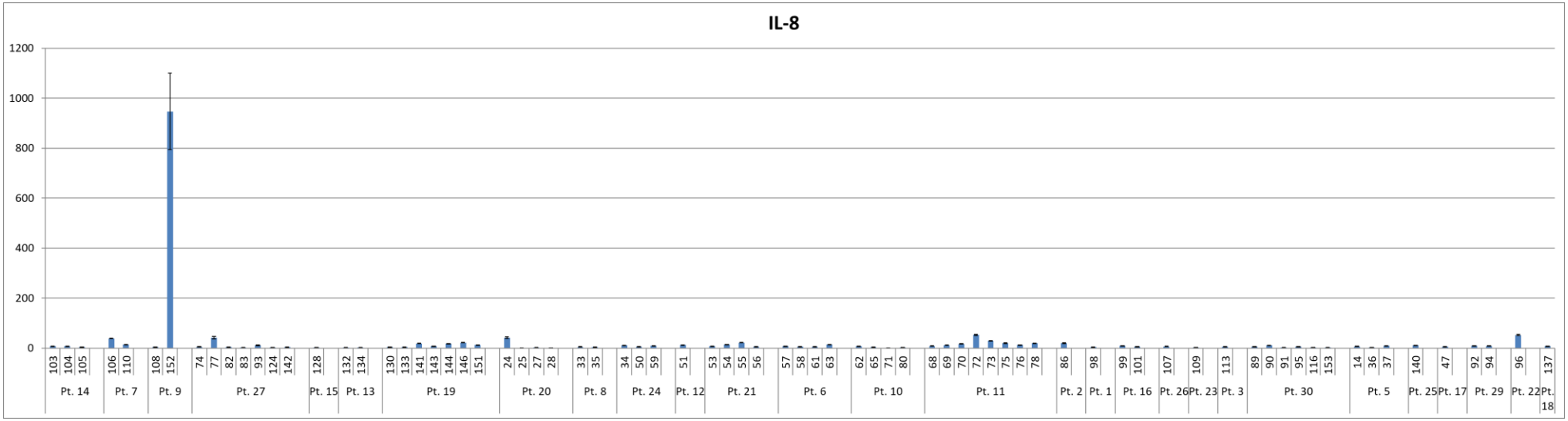

Figure 4.6. Cytokine analysis results for IL-8.

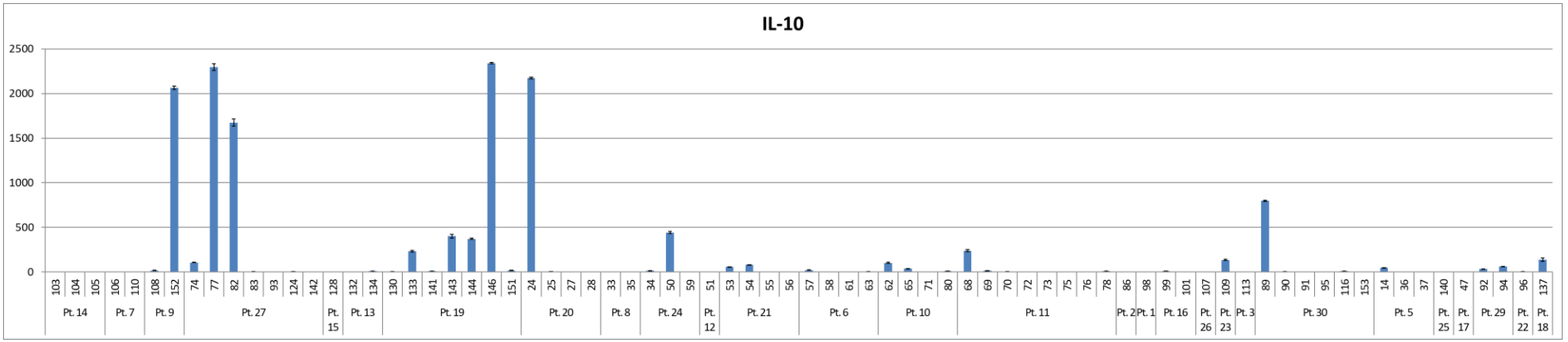

Figure 4.7. Cytokine analysis results for IL-10.

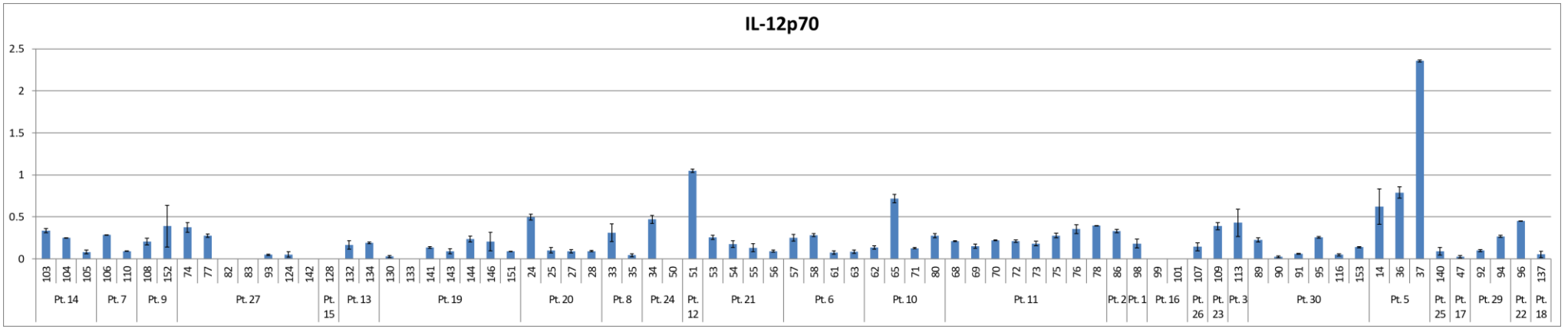

Figure 4.8. Cytokine analysis results for IL-12p70.

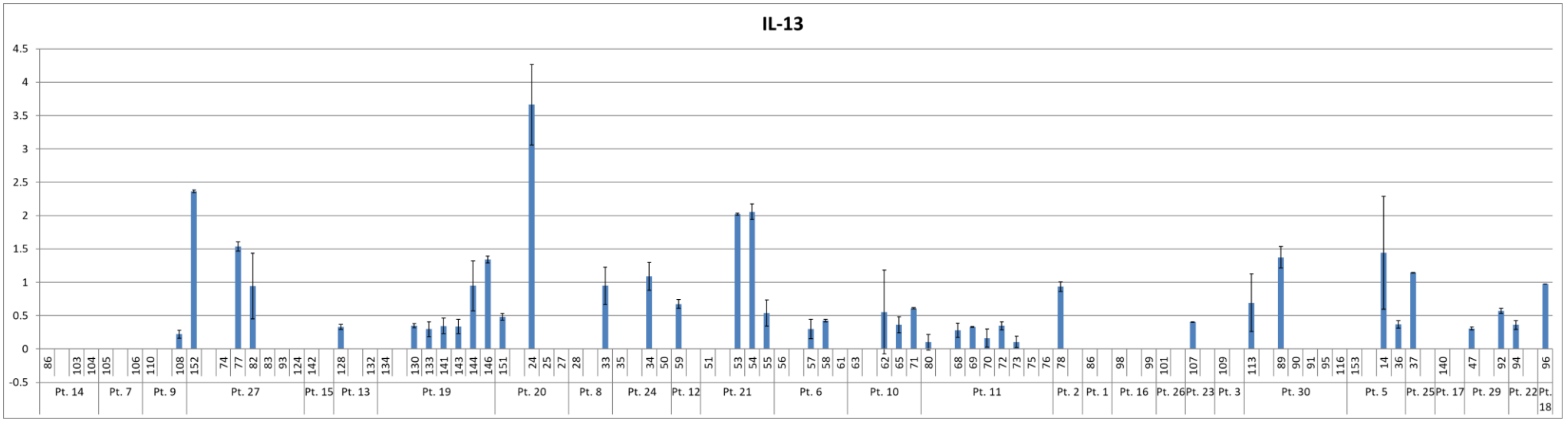

Figure 4.9. Cytokine analysis results for IL-13.

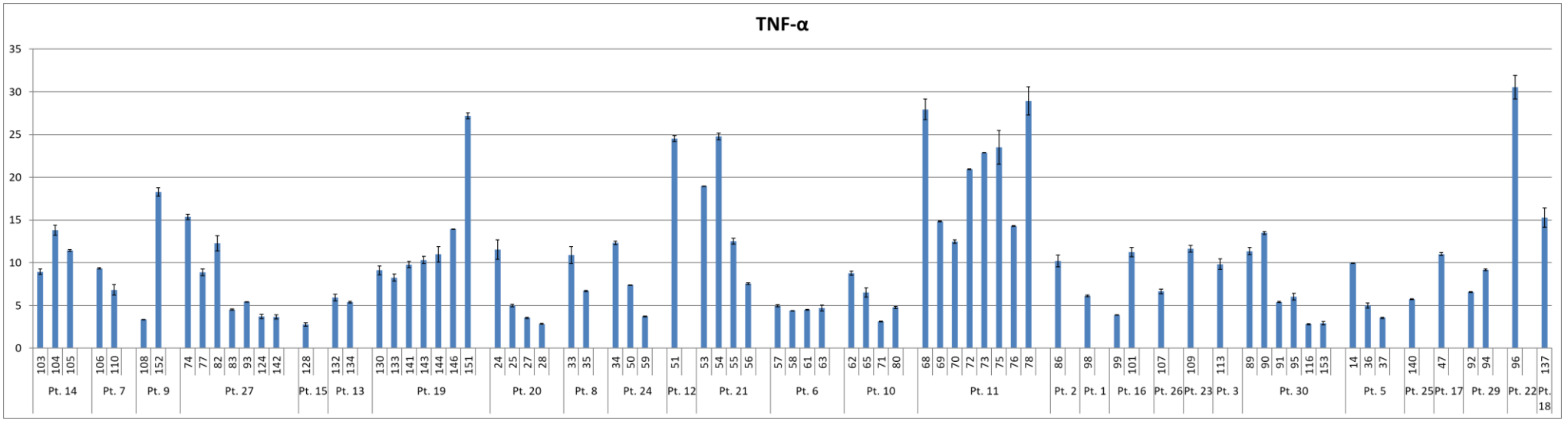

Figure 4.10. Cytokine analysis results for TNF-α.

4.3.2 Cytokine analysis by molecular subtype

Cytokine levels were compared for specimens of different HHV-8 molecular subtypes. Comparisons were performed for K1 subtype and K15 subtype.

4.3.2.1 Cytokine analysis by K1 subtype

Comparisons between K1 subtypes were made using the Kruskal-Wallis test. A statistically significant difference between subtypes was identified for IFN-γ, IL-1β, IL-6, IL-8, IL-13 and TNF-α (Table 4.2). Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Adjusted *p* values are presented. For IFN-γ, this post hoc analysis revealed statistically significant differences between subtypes A and F ($p < 0.0005$), subtypes B and F ($p = 0.018$) and subtypes C and F ($p = 0.001$). For IL-1β, there was a statistically significant difference between subtypes C and F ($p = 0.048$). For IL-6, there were statistically significant differences between subtypes B and F ($p = 0.020$), subtypes A and F ($p = 0.013$) and subtypes C and F ($p = 0.030$). For IL-8, there were statistically significant differences between subtypes B and F ($p = 0.012$), subtypes A and F ($p = 0.005$) and subtypes C and F ($p = 0.013$). For IL-13, there was a statistically significant difference between subtypes F and B ($p = 0.046$). For TNF-α, there were statistically significant differences between subtypes B and F ($p = 0.013$), subtypes A and F ($p = <0.0005$) and subtypes C and F ($p = 0.003$).

Table 4.2. Comparisons between K1 subtypes.

Cytokine	Subtype A (Median, Range)	Subtype B (Median, Range)	Subtype C (Median, Range)	Subtype F (Median, Range)	*p* value
IFN-γ	17.10 (3.57-1157.98)	16.21 (4.11-103.88)	27.14 (2.29-339.61)	425.24 (278.25-1537.12)	<0.0005
IL-1β	0.20 (0.01-0.40)	0.54 (0.18-0.89)	0.14 (0.04-1.23)	1.69 (0.18-5.66)	0.031
IL-2	0.84 (0.22-151.90)	0.50 (0.20-2.13)	0.58 (0.08-10.75)	1.03 (0.36-7.49)	0.250
IL-4	0.02 (0.003-0.07)	0.01 (0.001-0.15)	0.02 (0.005-0.20)	0.02 (0.009-0.06)	0.790
IL-6	1.94 (0.15-39.07)	1.16 (0.37-6.21)	3.07 (0.34-60.45)	18.22 (3.69-33.05)	0.007
IL-8	6.22 (1.71-21.59)	2.32 (1.58-42.23)	6.0 (2.69-41.79)	17.95 (8.63-53.0)	0.003
IL-10	4.38 (0.26-101.41)	7.66 (0.19-2172.56)	10.54 (0.2-2339.96)	3.72 (0.5-238.90)	0.478
IL-12p70	0.22 (0.07-2.36)	0.09 (0.05-0.50)	0.16 (0.03-0.47)	0.22 (0.15-0.39)	0.138
IL-13	0.55 (0.10-2.06)	2.32 (0.97-3.66)	0.62 (0.30-1.53)	0.31 (0.11-0.93)	0.031
TNF-α	5.65 (3.14-24.80)	4.98 (2.84-15.29)	9.03 (2.81-27.19)	21.91 (12.49-28.93)	0.001

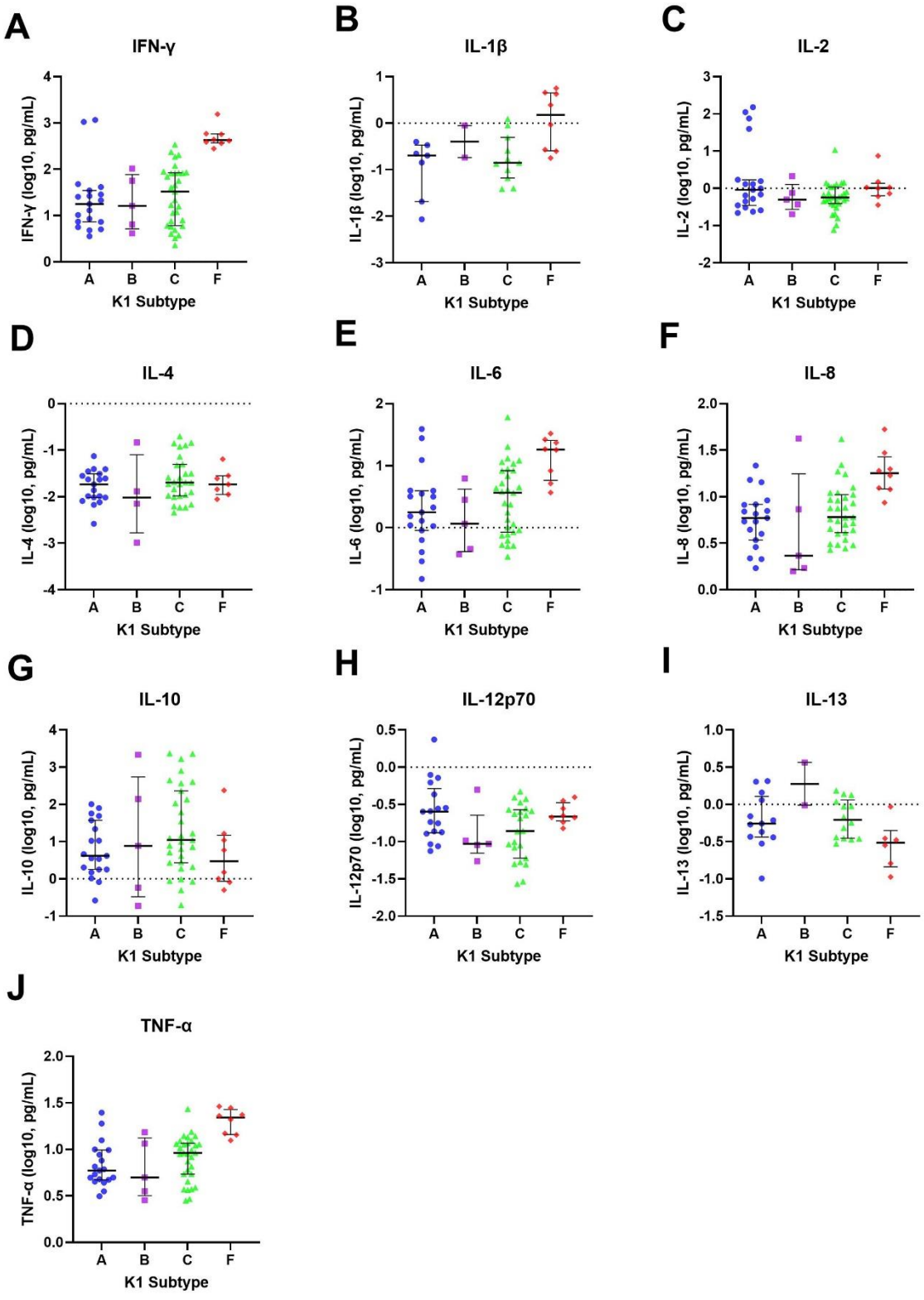

Figure 4.11. Comparison of cytokine levels for each HHV-8 K1 molecular subtype in patients with HHV-8 related diseases.

Midlines represent median values and error bars represent interquartile ranges.

4.3.2.2 Cytokine analysis by K15 subtype

Comparisons between K15 subtypes were made using the Mann-Whitney U test. A statistically significant difference between subtypes P and M was identified for IL-1β ($p = 0.009$) and TNF-α ($p = 0.038$) (Table 4.3).

Table 4.3. Comparisons between K15 subtypes.

Cytokine	Subtype P (Median, Range)	Subtype M (Median, Range)	*p* value
IFN-γ	28.49 (2.29-1157.98)	191.07 (3.73-1537.12)	0.062
IL-1β	0.17 (0.04-1.23)	0.90 (0.04-5.66)	0.009
IL-2	0.58 (0.08-10.75)	0.75 (0.36-7.49)	0.301
IL-4	0.02 (0.001-0.20)	0.01 (0.01-0.15)	0.762
IL-6	3.19 (0.15-60.45)	4.43 (0.62-33.05)	0.310
IL-8	6.92 (1.58-42.23)	9.80 (2.69-53.0)	0.180
IL-10	16.64 (0.19-2339.96)	3.52 (0.49-797.15)	0.059
IL-12p70	0.18 (0.029-2.36)	0.2 (0.03-0.39)	0.652
IL-13	0.59 (0.10-3.66)	0.34 (0.11-1.38)	0.104
TNF-α	8.78 (2.84-27.19)	13.90 (2.81-28.93)	0.038

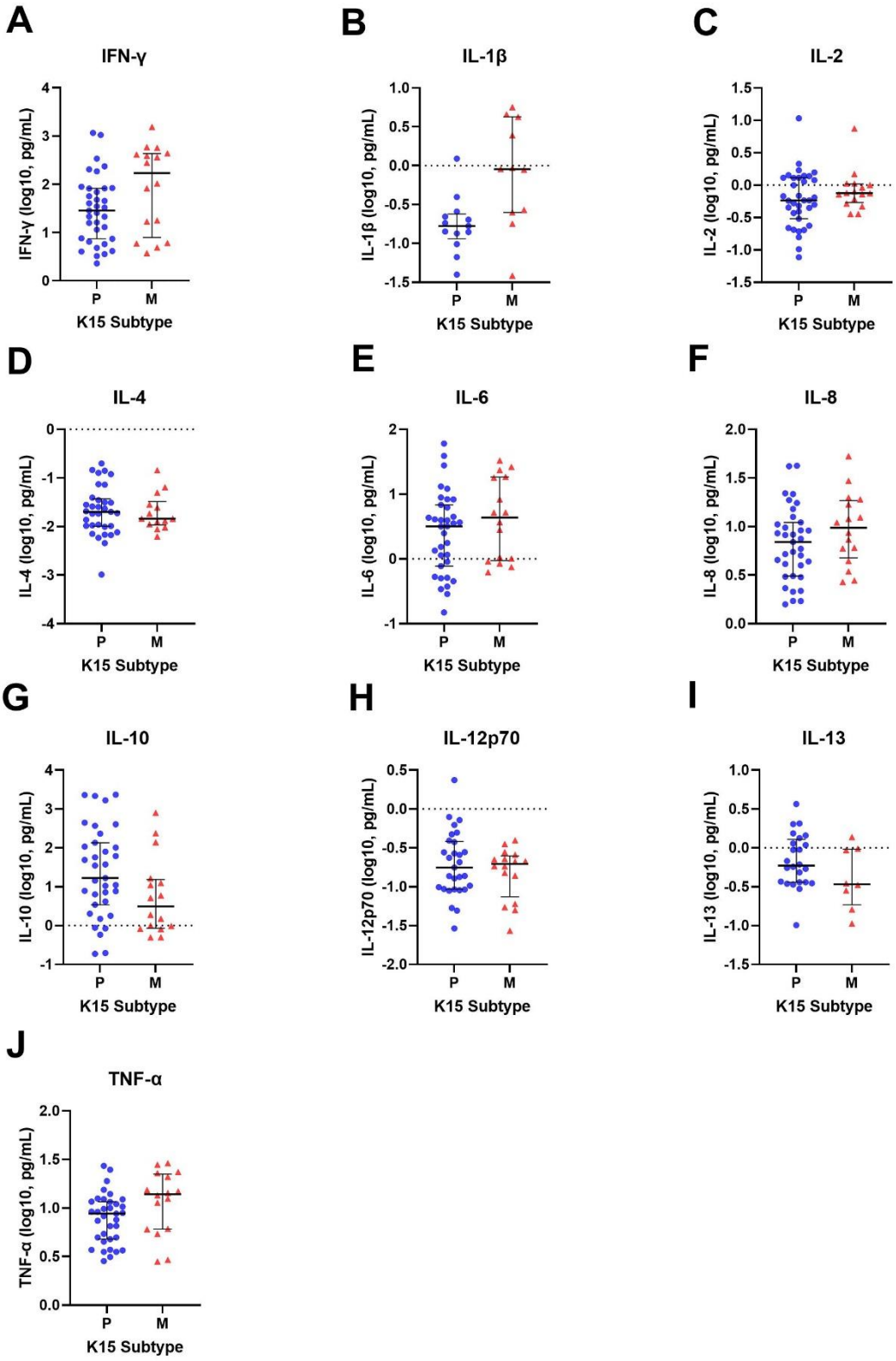

Figure 4.12. Comparison of cytokine levels for each HHV-8 K15 molecular subtype in patients with HHV-8 related diseases. Midlines represent median values and error bars represent interquartile ranges.

4.3.3 Correlation of cytokine levels with Ct value

Spearman's rank correlation was used to assess the relationship between Ct value and each cytokine level. There was a statistically significant, strong negative correlation between Ct value and IL-10 level (r_s = -0.850, $p < 0.0005$). There was a statistically significant, moderate negative correlation between Ct value and IFN-γ level (r_s = -0.403, p = 0.001), and a weaker correlation between Ct value and IL-4 (r_s = -0.311, p = 0.017), IL-6 (r_s = -0.284, p = 0.023), IL-13 (r_s = -0.326, p = 0.049) and TNF-α (r_s = -0.312, p = 0.012) levels. There was no statistically significant correlation between Ct value and IL-1β, IL-2, IL-8 or IL-p70 levels. Ct values were also compared between K1 and K15 subtypes, using the Kruskal-Wallis test and Mann-Whitney U test, respectively and there was no significant difference in Ct values between the different K1 or K15 subtypes.

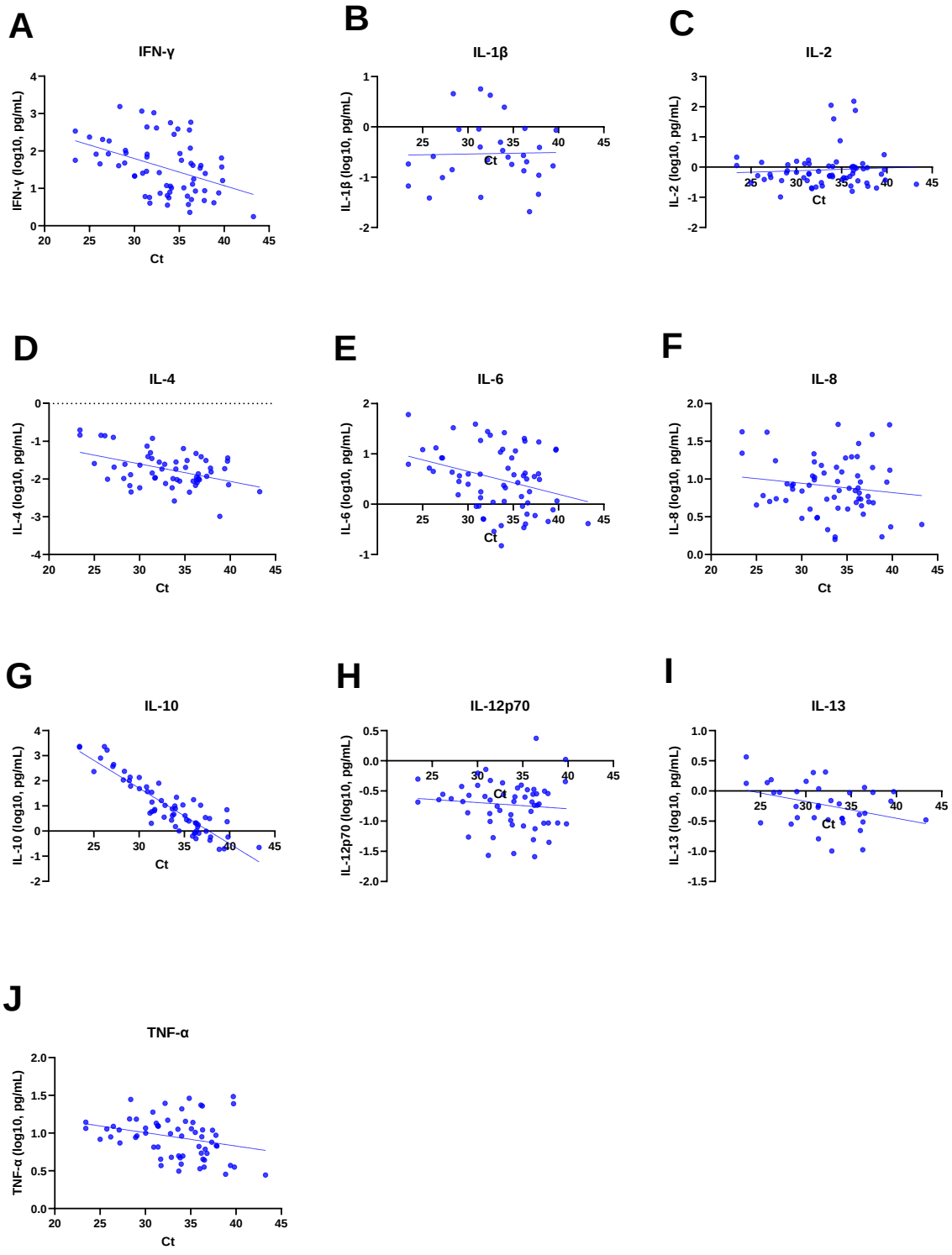

Figure 4.13. Correlation of cytokine levels with Ct value for HHV-8 PCR in patients with HHV-8 related diseases.

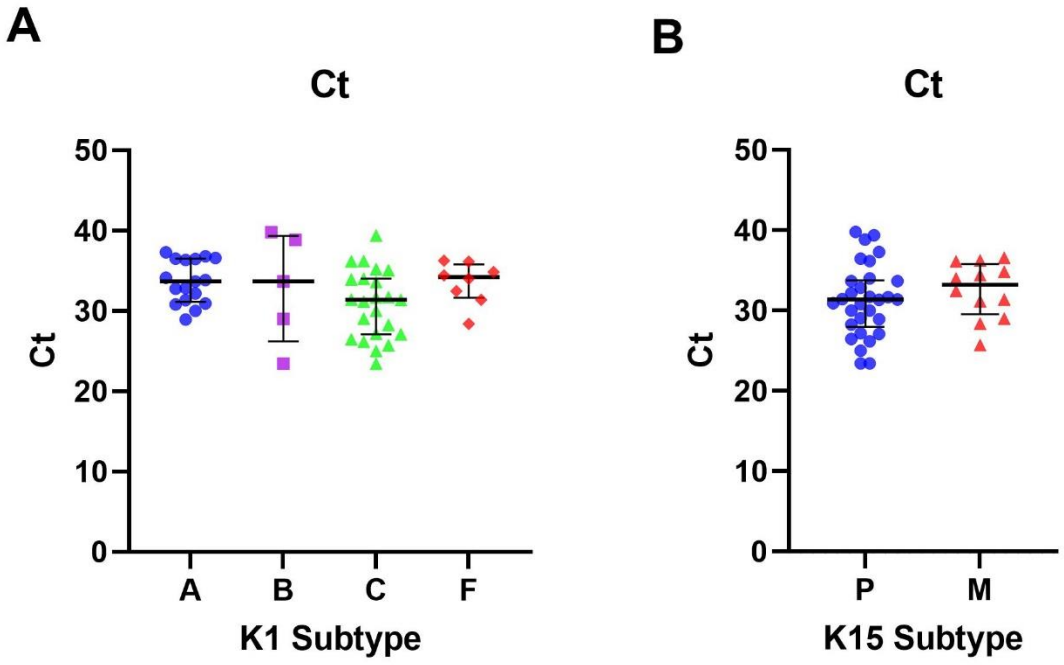

Figure 4.14. Comparison of Ct values for each HHV-8 K1 and K15 molecular subtype in patients with HHV-8 related diseases. Midlines represent median values and error bars represent interquartile ranges.

Patient	Specimen	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNF-α	Ct Value	K1 Subtype	K15 Subtype
1	98	17.67931217	N/A	0.914689272	0.009587297	1.060780505	4.422090761	0.817616136	0.183396862	N/A	6.118576657	36.6	C	M
2	86	10.37264472	N/A	0.43382875	0.004493255	2.676119706	19.72382132	2.500153692	0.331165775	N/A	10.22927063	35.5		
3	113	26.36738788	N/A	0.306793226	0.024607418	1.09515461	5.405933143	3.576222487	0.430095939	0.69252537	9.842886669	32.77	A	
4	84	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
5	14	21.31229766	N/A	1.569451602	0.023408807	3.978411616	6.924190148	48.95038819	0.622931956	1.440450945	9.962777782	30.04	A	P
5	36	4.803723202	0.142595924	0.686356815	0.018421186	3.192978353	2.173967021	1.780945818	0.78987684	0.368076899	4.990327785		A	P
5	37	12.88518887	0.202083296	0.994994304	0.013738016	3.167073184	9.128741365	1.484955241	2.356069382	1.142511536	3.546418304	36.48	A	P
6	57	10.2774319	N/A	39.51305764	0.009652748	2.113838777	7.031655015	21.95476843	0.252508949	0.297452632	4.965608448	34.13	A	
6	58	41.3007869	N/A	75.28740826	0.010249451	0.632405446	5.416858742	1.786628838	0.283904463	0.427590044	4.383843779	36.52	A	
6	61	5.031347606	N/A	151.9029046	0.008108676	0.402924367	6.534415394	1.029545048	0.074677662	N/A	4.507737298	36.35	A	
6	63	5.586666183	0.338751211	111.6563264	0.002613444	12.34309913	14.309063	4.624583753	0.086749483	N/A	4.698879482	33.84	A	
7	106	4.747713282	0.045694899	0.296598534	N/A	4.004248596	38.99586885	3.146966252	0.286781975	N/A	9.349010801	37.78		
7	110	8.644306083	0.109252341	0.960670534	0.019491234	17.21559222	14.21739466	0.431378529	0.093274622	N/A	6.829527111	37.82		
8	33	40.98623478	N/A	0.882139765	0.011798359	0.593878292	4.981710942	0.983577228	0.313275391	0.948034825	10.89103587	37.41		
8	35	25.09294635	0.391029543	0.230477863	0.015476499	3.101099175	4.842686521	0.577306971	0.044626936	N/A	6.712043136	37.89		
9	108	3.705070849	N/A	0.235929131	N/A	0.910660634	4.891951354	17.68334988	0.20785456	0.221716561	3.371503299	36.02		
9	152	103.2021855	0.352156465	2.80507086	0.252354198	15.39037541	946.3885748	2065.84008	0.39067474	2.363961262	18.27136385			
10	62	47.69058742	N/A	0.644152931	0.006684005	1.537976238	8.179292415	101.4134002	0.13756262	0.553500325	8.775482275	28.92	A	P
10	65	25.49897937	N/A	1.30073485	0.039387596	0.902613829	3.981839138	37.33492811	0.718856214	0.360289061	6.514092908	30.93	A	P
10	71	3.569336728	N/A	0.525676283	0.010249058	0.149084068	1.710692297	4.1373059	0.128201736	0.610743419	3.135866207	33.69	A	P
10	80	7.406863363	N/A	0.236834059	0.00763431	0.288244891	2.13573195	10.56261079	0.276557078	0.101413043	4.774644385	32.87	A	P
11	68	1537.11606	4.565602757	0.357323822	0.024570912	33.04609016	8.626480081	238.9004731	0.210745103	0.282009695	27.93865908	28.38	F	M
11	69	413.1432787	4.241820156	0.727468679	0.018421219	23.55330944	11.99235784	16.33232049	0.151026145	0.331811144	14.83246415	32.46	F	M
11	70	437.3342053	5.66327365	0.608956827	0.01440076	18.46066451	16.85535037	5.944656084	0.222954471	0.161268808	12.49129148	31.39	F	M
11	72	570.0192039	2.455543425	1.054047534	0.028314612	26.3951739	53.00495525	1.498058331	0.212179613	0.348227403	20.93966561	34.02	F	M
11	73	584.3850759	0.930778058	1.053358976	0.011194038	17.98316655	29.61934362	0.497951209	0.182700958	0.106107079	22.87239278	36.28	F	M
11	75	363.3681221	0.269221966	1.003927297	N/A	3.686540553	19.86502431	0.815333343	0.27889429	N/A	23.50540531	36.13	F	M
11	76	278.2510713	0.250877939	1.487125937	0.008782452	5.165822666	12.43922918	1.008246171	0.354753493	N/A	14.2992533	34.42	F	M
11	78	387.9971134	0.178915381	7.48910936	0.064095317	8.286086485	19.03504631	10.9401761	0.394792321	0.934684378	28.93220307	34.84	F	M
11	81	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
12	51	37.30012875	0.856733896	0.822077	0.036197342	12.41840057	13.08259279	2.35928763	1.049404976	N/A	24.52606357	39.73		
13	132	7.260645184	0.008603443	0.257731189	0.023502635	1.292946695	2.874142874	0.261438021	0.165407083	N/A	5.917584492		A	
13	134	8.458374863	0.020641956	0.348448814	0.039002113	1.77488726	3.416627202	10.65169787	0.192058886	N/A	5.378010147	36.8	A	
13	136	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
14	103	119.214131	N/A	0.608964149	0.047547388	20.28879656	7.637519786	2.093065458	0.33617152	N/A	8.936778712	36.23	C	
14	104	56.53783851	N/A	0.444766828	0.030987037	11.43497684	7.538065254	2.822643914	0.250941799	N/A	13.81267526	35.24	C	
14	105	85.51165975	N/A	0.388735446	0.020293628	3.844587449	4.003684481	4.107185552	0.08312379	N/A	11.43246918	35.07	C	
15	128	1.752473352	N/A	0.26932178	0.004627942	0.410018978	2.496311596	0.221598532	N/A	0.331533205	2.783332238	43.25		
16	99	7.887042761	N/A	0.536082406	N/A	2.362701784	9.284623468	9.985742033	N/A	N/A	3.877158171	33.95	C	
16	101	11.89105215	0.495320374	1.081978196	0.005854596	10.81904267	5.743418533	2.698681208	N/A	N/A	11.23763005	33.59	C	
17	47	46.65874364	N/A	0.784272778	0.009415281	4.129203476	5.517616337	2.089197115	0.02577595	0.307096308	11.01634258	36.31		
18	137	103.8795561	0.889008156	0.755582023	0.01299289	2.828570215	7.341332315	139.3516818	0.054582633	0.972973461	15.2943071	29	B	M
19	130	11.41845011	N/A	0.478851786	0.01837034	1.13576127	4.120555537	7.81373488	0.02913104	0.351056208	9.126106241	34	C	P

19	131	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
19	133	234.761067	N/A	0.302256573	0.025810076	12.1264084	4.530159847	231.775717	N/A	0.296387608	8.259532615	25	C	P
19	141	32.77439904	N/A	1.373621282	0.02002603	4.079351294	18.73557037	11.09715	0.136616	0.346199611	9.787630826		C	P
19	143	81.65112548	N/A	1.394289552	0.03138601	8.878115769	7.478825324	402.206124	0.091806939	0.338120926	10.33445078		C	P
19	144	83.66450028	N/A	0.572681896	0.126883547	8.360049196	17.45946583	371.6927473	0.235864094	0.948366264	10.9797296	27.1	C	P
19	146	339.6106815	0.066576269	1.130305159	0.198524607	60.44829971	22.01527809	2339.957403	0.20701834	1.339773084	13.9491657	23.42	C	P
19	151	15.74644995	N/A	10.74944682	0.072965408	6.823837923	12.54879094	16.64030814	0.088734408	0.483222845	27.18884085		C	P
20	24	56.56226409	0.182958055	2.130492511	0.146197964	6.213904935	42.2292779	2172.557348	0.49825336	3.661579681	11.53746012	23.42	B	P
20	25	6.417459154	N/A	0.500832932	N/A	0.374705152	1.580616464	7.657577103	0.103381317	N/A	4.981277278	33.68	B	P
20	26	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
20	27	16.20779704	N/A	0.369720531	0.007039539	1.157093183	2.324933859	0.580758709	0.090070959	N/A	3.546511648	39.82	B	P
20	28	4.110820248	N/A	0.201907593	0.001030864	0.453306996	1.711904901	0.186884905	0.093418289	N/A	2.84359873	38.85	B	P
20	29	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
21	53	1157.97685	N/A	0.444404861	0.07430066	39.07306568	8.261884097	57.01073464	0.256518731	2.020785756	18.9603469	30.82	A	P
21	54	1054.581544	0.221099021	0.218714064	0.027976407	27.72518257	15.16630106	80.16760344	0.17633506	2.056204038	24.79659984	32.18	A	P
21	55	28.48692052	0.396063314	1.695259891	0.03519998	3.951372761	21.58655216	2.020802734	0.133545328	0.537901035	12.51817882	31.36	A	P
21	56	34.67995106	N/A	1.300761262	0.031120894	3.542518976	5.897584199	3.440046806	0.092546924	N/A	7.566523704	37.32	A	P
22	96	65.05929767	N/A	2.580679858	0.029897824	11.93834557	52.34205752	7.043396002	0.451477517	0.972973461	30.51998258	39.7		
23	109	21.50061877	N/A	0.6728231	0.005863806	2.486305592	3.012731535	134.8969537	0.390735746	N/A	11.64143721	30.02	C	P
24	34	82.1947176	0.039800481	1.288355099	0.119955273	1.760719337	10.52924398	14.26585401	0.471153219	1.088353311	12.34029206	31.43	C	P
24	50	185.3151906	0.097967553	0.451600178	0.020694859	8.271233859	5.488272079	442.5398027	N/A	N/A	7.391121063	27.19	C	P
24	59	7.560759822	0.167414602	0.581635113	0.018850122	0.772952003	9.098166216	0.196105131	N/A	0.674817806	3.716568334	39.4	C	P
25	135	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
25	140	37.38308354	0.667704168	0.247190404	0.015739397	2.901084353	10.62037027	0.229614378	0.090063336	N/A	5.730465255			
26	107	6.201960651	N/A	0.488611377	0.008825313	1.415076968	7.0158728	0.628531549	0.144912762	0.403884362	6.651132477	35.91		
27	74	40.25806923	0.140483844	0.102511664	0.010367941	4.325505754	5.194715107	107.5087694	0.375380163	N/A	15.37638944	28.26	C	P
27	77	45.20804971	0.257089221	1.42667717	0.140735962	4.470249212	41.78827474	2295.160788	0.278181222	1.534910011	8.892622799	26.17	C	P
27	79	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
27	82	202.919175	N/A	0.3900106	0.009911668	13.12307375	5.03448238	1673.022973	N/A	0.941012479	12.2915869	26.47	C	P
27	83	5.752906928	N/A	0.205152461	0.010605557	0.512390779	3.043666426	6.367145672	N/A	N/A	4.51604949	31.69	C	P
27	93	2.28818976	0.134096385	0.157319054	0.006803762	0.342311715	11.01291535	0.848298061	0.049235702	N/A	5.423382774	36.18	C	P
27	124	4.022447271	N/A	0.193719515	0.010926075	0.504033686	3.093847528	7.179140155	0.053327506	N/A	3.712773949	31.73	C	P
27	142	3.243895778	1.23033215	0.076779835	0.025453907	0.529743832	4.380271698	0.878247915	N/A	N/A	3.670954407		C	P
28	138	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A			
29	92	69.06700451	N/A	0.580347871	N/A	1.343273954	9.769325936	35.03597905	0.098770453	0.571277616	6.552310456	31.39	C	P
29	94	88.90145247	N/A	1.194416551	0.004567544	3.661759559	8.515125967	61.3209845	0.268542747	0.361007807	9.184405667	29.06	C	P
30	89	82.43501556	0.038469733	0.51863437	0.144193692	5.193085627	6.03904261	797.147696	0.226456122	1.375172118	11.34882689	25.74	C	M
30	90	6.066433673	0.902089237	0.358044816	0.050053966	0.916944707	10.9775811	5.151349364	0.027119797	N/A	13.49328112	31.2	C	M
30	91	4.881242181	N/A	0.469751462	0.014544707	0.620741042	3.444653417	1.886306805	0.05995155	N/A	5.413253392		C	M
30	95	16.77499454	N/A	0.797317338	0.012515184	0.746468829	5.951627071	0.976009519	0.257855504	N/A	6.031467818		C	M
30	116	3.734174243	N/A	0.747105571	N/A	1.012952416	2.784055671	12.44492584	0.04990472	N/A	2.809005216		C	M
30	153	5.902566646	N/A	0.716469449	0.006216488	0.846757283	2.691258724	0.492943397	0.138900991	N/A	2.928557122		C	M

Table 4.4. Cytokine analysis results for 85 specimens from 30 patients included in the study.

4.3.4 Comparison of cytokine levels and Ct values before and after treatment

Nine patients were identified who had samples sent for HHV-8 PCR before and after treatment with systemic chemotherapy for HHV-8-related diseases. Proinflammatory cytokine levels were compared in these patients before and after treatment using the Wilcoxon signed-rank test. There were no significant differences identified between cytokine levels before and after treatment. Ct values were also compared for the HHV-8 PCR results before and after treatment and were found to be significantly higher following treatment ($p = 0.028$). The median Ct value prior to treatment was 29.21 (range 23.42-31.43) and the median Ct value following treatment was 36.9 (range 34.84-39.4).

Table 4.5. Cytokine analysis results before and after treatment.

Cytokine	Before Treatment (Median, Range)	After Treatment (Median, Range)	*p* value
IFN-γ	56.56 (3.71-1537.12)	15.75 (2.29-388)	0.086
IL-1β	0.14 (0.04-4.57)	0.17 (0.13-0.18)	0.750
IL-2	0.48 (0.1-2.13)	0.99 (0.16-10.75)	0.260
IL-4	0.049 (0.01-0.15)	0.016 (0.001-0.07)	0.161
IL-6	4.33 (0.91-39.07)	3.17 (0.34-15.39)	0.214
IL-8	6.92 (4.12-42.23)	9.13 (1.71-946.39)	0.374
IL-10	57.01 (7.81-2172.56)	1.48 (0.19-2065.84)	0.110
IL-12p70	0.24 (0.03-0.62)	0.18 (0.05-2.36)	0.779
IL-13	0.35 (0.22-1.44)	0.93 (0.48-2.36)	0.625
TNF-α	11.54 (3.37-27.94)	6.03 (2.84-28.93)	0.594

Table 4.6. Cytokine analysis results for 9 patients before and after treatment for HHV-8-related disease.

Patient	Specimen	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNF-α	Ct Value	Before/ After Treatment
5	14	21.31229766	N/A	1.569451602	0.023408807	3.978411616	6.924190148	48.95038819	0.622931956	1.440450945	9.962777782	30.04	Before
5	37	12.88518887	0.202083296	0.994994304	0.013738016	3.167073184	9.128741365	1.484955241	2.356069382	1.142511536	3.546418304	36.48	After
9	108	3.705070849	N/A	0.235929131	N/A	0.910660634	4.891951354	17.68334988	0.20785456	0.221716561	3.371503299	36.02	Before
9	152	103.2021855	0.352156465	2.80507086	0.252354198	15.39037541	946.3885748	2065.84008	0.39067474	2.363961262	18.27136385	N/A	After
11	68	1537.11606	4.565602757	0.357323822	0.024570912	33.04609016	8.626480081	238.9004731	0.210745103	0.282009695	27.93865908	28.38	Before
11	78	387.9971134	0.178915381	7.48910936	0.064095317	8.286086485	19.03504631	10.9401761	0.394792321	0.934684378	28.93220307	34.84	After
19	130	11.41845011	N/A	0.478851786	0.01837034	1.13576127	4.120555537	7.81373488	0.02913104	0.351056208	9.126106241	34	Before
19	151	15.74644995	N/A	10.74944682	0.072965408	6.823837923	12.54879094	16.64030814	0.088734408	0.483222845	27.18884085	N/A	After
20	24	56.56226409	0.182958055	2.130492511	0.146197964	6.213904935	42.2292779	2172.557348	0.49825336	3.661579681	11.53746012	23.42	Before
20	28	4.110820248	N/A	0.201907593	0.001030864	0.453306996	1.711904901	0.186884905	0.093418289	N/A	2.84359873	38.85	After
21	53	1157.97685	N/A	0.444404861	0.07430066	39.07306568	8.261884097	57.01073464	0.256518731	2.020785756	18.9603469	30.82	Before
21	56	34.67995106	N/A	1.300761262	0.031120894	3.542518976	5.897584199	3.440046806	0.092546924	N/A	7.566523704	37.32	After
24	34	82.1947176	0.039800481	1.288355099	0.119955273	1.760719337	10.52924398	14.26585401	0.471153219	1.088353311	12.34029206	31.43	Before
24	59	7.560759822	0.167414602	0.581635113	0.018850122	0.772952003	9.098166216	0.196105131	N/A	0.674817806	3.716568334	39.4	After
27	74	40.25806923	0.140483844	0.102511664	0.010367941	4.325505754	5.194715107	107.5087694	0.375380163	N/A	15.37638944	28.26	Before
27	93	2.28818976	0.134096385	0.157319054	0.006803762	0.342311715	11.01291535	0.848298061	0.049235702	N/A	5.423382774	36.18	After
30	89	82.43501556	0.038469733	0.51863437	0.144193692	5.193085627	6.03904261	797.147696	0.226456122	1.375172118	11.34882689	25.74	Before
30	95	16.77499454	N/A	0.797317338	0.012515184	0.746468829	5.951627071	0.976009519	0.257855504	N/A	6.031467818	N/A	After

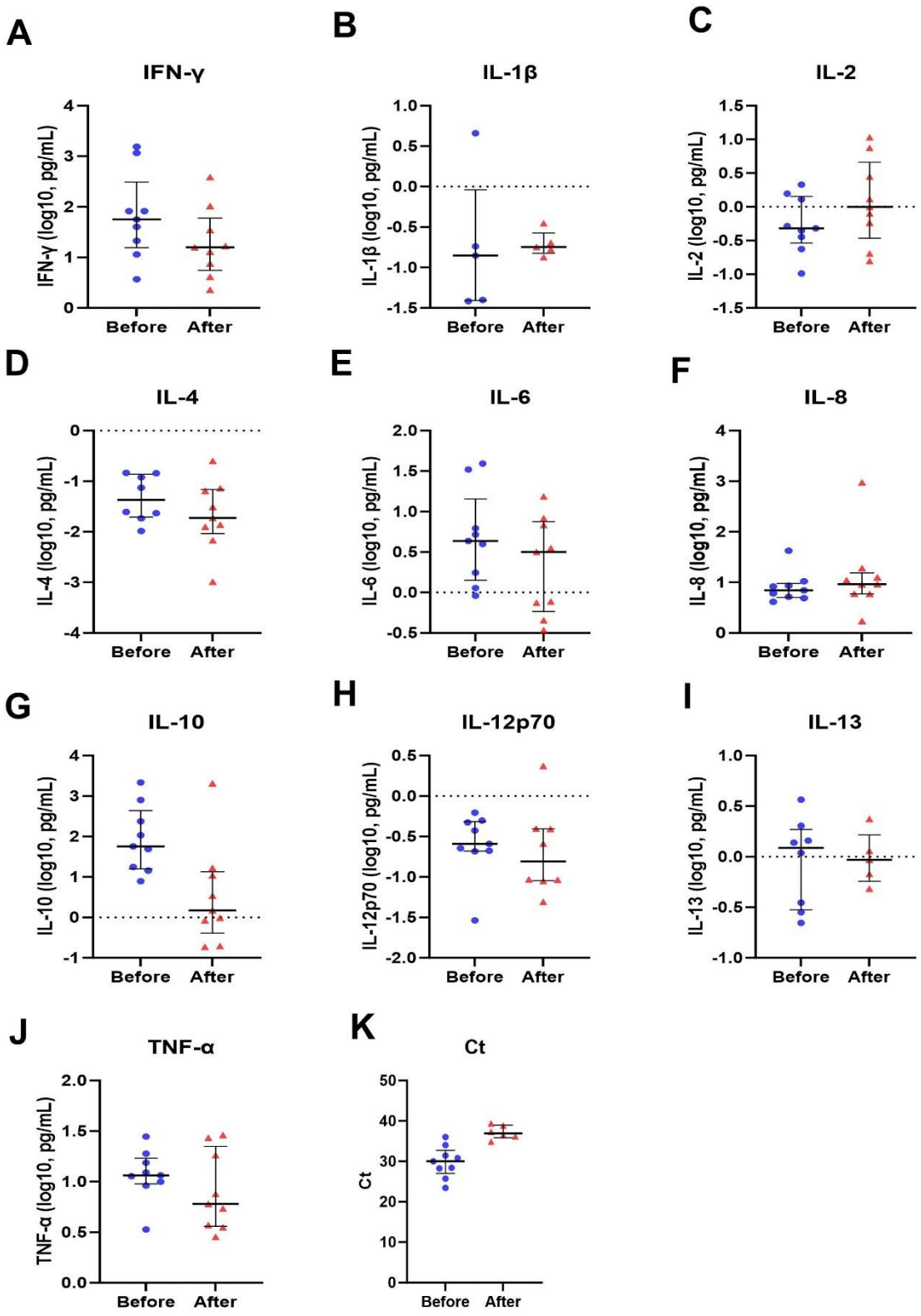

Figure 4.15. Comparison of cytokine levels before and after treatment in patients with HHV-8 related diseases. Midlines represent median values and error bars represent interquartile ranges.

4.4 Discussion

There has been speculation in the literature regarding differing severity of disease based on the subtype of HHV-8 with which the patient is infected (Cordiali-Fei et al., 2015; Isaacs et al., 2016; Jary et al., 2020; Mancuso et al., 2008). To our knowledge, this is the first study comparing cytokine levels between patients with different subtypes of HHV-8.

When cytokine levels were compared between K1 subtypes, a statistically significant difference between subtypes was identified for IFN-γ, IL-1β, IL-6, IL-8, IL-13 and TNF-α. When pairwise comparisons were performed, the difference of note for each cytokine was significantly higher levels for subtype F compared to the other subtypes. A limitation of this study was that all the subtype F samples belonged to the same patient. However, this is an interesting finding as it has been suggested that subtype F, in particular subtype F2 could be associated with increased disease severity and this is an area that warrants further study (Corgiat et al., 2020).

When cytokine levels were compared between K15 subtypes, IL-1β and TNF-α levels were demonstrated to be significantly higher in patients with the K15 M allele compared to the K15 P allele. Of note, there is a significant genetic difference between the K15 P and M alleles, with only 33% amino acid identity between the two, and studies have been performed previously to investigate whether this translates into functional differences between strains of HHV-8 containing the P allele versus the M allele (Poole et al., 1999; Wang et al., 2007). The studies to date have demonstrated there to be qualitatively similar functionality between K15-M and K15-P (Chen et al.,

2018; Wang et al., 2007). It has been postulated as to whether the genetic difference may infer a survival advantage to one type over the other, in particular, because of the higher worldwide prevalence of the P allele, it has been hypothesised that the presence of the P allele may infer a survival advantage. There has been very little research to investigate whether there is a difference in severity between disease due to strains containing the P allele and due to strains containing the M allele. Our findings support the possibility of increased proinflammatory activity associated with the M allele and this raises an area of interest for future research.

It has long been reported that inflammatory cytokines initiate the inflammatory-angiogenic process of KS development and that cytokines such as IFN-γ, TNF-α, IL-1, IL-6 and others are expressed in KS lesions, and are often detectable prior to the detection of HHV-8 (Ensoli & Stürzl, 1998; Host et al., 2017). These cytokines are felt to act synergistically to produce both local and systemic effects, and to promote KS lesion development and progression (Ensoli & Stürzl, 1998). These cytokines are therefore intricately involved in the pathogenesis of HHV-8-related disease.

Oksenhendler et al. found high levels of HHV-8 viral load, IL-6 and IL-10 to correlate with clinical flares compared to clinical remissions in HIV patients with MCD (Oksenhendler et al., 2000). They reported strong correlations between HHV-8 viral load and plasma IL-6 and IL-10 levels and suggested that these cytokines could be involved in the pathogenesis of MCD. Polizzotto et al. (2013) demonstrated that HHV-8 viral load, IL-6, IL-10, TNF- α and IL-1β were each elevated in patients with MCD during initial flares, compared with remission. Patients with KICS are also known to

demonstrate inflammatory symptoms associated with elevated levels of IL-6, IL-10 and HHV-8 viral load (Polizzotto et al., 2015; Uldrick et al., 2010).

In the current study, there was a significant negative correlation between Ct value and IL-10 level identified. There was a moderate negative correlation between Ct value and IFN-γ level and a weaker negative correlation between Ct value and IL-4, IL-6, IL-13 and TNF-α levels. Ct values were also compared for the HHV-8 PCR results before and after treatment and were found to be significantly higher following treatment. These findings support data previously reporting higher levels of proinflammatory cytokines in association with higher HHV-8 viral loads and disease flares compared to remission. (Oksenhendler et al., 2000; Polizzotto et al., 2013). However, interestingly, there were no significant differences identified between cytokine levels measured before and after treatment in patients with HHV-8-related diseases in the current study.

Of note, there were a number of limitations to this study. It was not possible to ascertain at what stage of the infection each specimen was taken and, therefore, the associated impact on proinflammatory cytokine production. The HIV status of the patients was not available, and therefore, it was not possible to compare cytokine production between patients with and without HIV. Due to the retrospective nature of the study, it was not possible to assess or control for other clinical factors that could potentially influence cytokine production, for example concurrent viral infections. Due to the limited number of specimens available for use in this study and the rarity of certain HHV-8 subtypes, for example K1 subtype F, the subanalysis was reduced to small numbers and therefore, it is not certain that the differences identified are necessarily attributable to the presence of different subtypes.

In conclusion, this study compared proinflammatory cytokine levels between both K1 and K15 subtypes of HHV-8, demonstrating significantly higher levels of a number of cytokines in K1 subtype F and K15 subtype M. While the number of patients included in this study was small, these findings present a point of interest for future research. To our knowledge, this is the first study comparing cytokine levels between different subtypes of HHV-8. As expected, Ct values for HHV-8 PCR were found to be significantly higher following treatment. The findings of this study also identified a negative correlation between Ct value and IL-10, IFN-γ, IL-4, IL-6, IL-13 and TNF-α levels, supporting the findings of previous studies in this area.

Chapter 5

General Discussion

5.1 Introduction

This is the first comprehensive study of human herpesvirus 8 in Ireland. It details the seroprevalence and molecular epidemiology of HHV-8 in this country, allowing comparison with the information available internationally on this virus. It also examines the role proinflammatory cytokines play in HHV-8 infection and contributes to the international data on this subject.

5.2. Seroprevalence of HHV-8 among blood donors, MSM, and GUM and ID patients

The population in Ireland has changed significantly over the last 20 years, with increasing inwards migration. It is also a nation of population extremes, with a large urban centre in Dublin but also a notable rural population. All 200 blood donor samples, collected nationwide, that underwent serological testing for HHV-8 antibodies were negative. This study suggests, based on blood donor data, that the seroprevalence of HHV-8 in Ireland, both in urban and rural settings, remains very low despite the country's evolving population. This low rate of seropositivity among blood donors is similar to other countries in Western Europe, and it is interesting to note that these rates have not changed substantially based on studies performed over the last 20 years, despite significant population changes due to migration (Alcalde et al., 2008; Rode et al., 2005; Gambús et al., 2001; Politou et al., 2014; Preiser et al., 2001; Regamey, Cathomas et al., 1998; Simpson et al., 1996; Tettmar et al., 2016).

The results of this study demonstrate significantly higher HHV-8 seroprevalence rates among certain risk groups. The overall GUM and ID patient population was found to have a significantly higher HHV-8 seroprevalence when compared to the blood donor population, with a seroprevalence of 21%. A higher proportion of individuals seropositive for HHV-8 was identified in the MSM population compared to the heterosexual population and the highest seroprevalence of 46% was among HIV positive MSM. This finding was consistent with previous studies reporting higher HHV-8 seroprevalence in MSM, in particular HIV positive MSM populations (Engels et al., 2007; Kedes et al., 1996; Lennette et al., 1996; Liu et al., 2018; Martin et al., 1998; Rohner et al., 2016). This data tentatively supports the inferences made by previous researchers, that there may be an element of sexual transmission involved in the dissemination of this virus (Engels et al., 2007; Lennette et al., 1996; Liu et al., 2018; Martin et al., 1998). However, definitive evidence regarding the exact mode of transmission of HHV-8 remains lacking, and the reasons for higher seroprevalence in MSM and HIV positive populations require clarification. This is an area for potential future research work.

This seroprevalence study was limited by the relatively small sample sizes and the inherent difficulties in extrapolating blood donor data to the general population. There is also a need to standardise serological testing methodologies for HHV-8 antibodies, in order to accurately compare seroprevalence data internationally.

5.3 HHV-8 subtypes and genetic variability

The results of this study demonstrate a high degree of genetic variability among HHV-8 strains circulating in Ireland. Sequencing data were obtained from 23 specimens from 19 patients with HHV-8-related diseases, and the K1, T0.7, ORF75 and K15 regions of the HHV-8 genome were successfully characterised by phylogenetic analysis. K1 subtypes A (37%) and C (47%) were found to predominate, in keeping with the known predominance of subtypes A and C in Europe (*Section 1.4.2.1*). Two patients had sequences belonging to subtype B, usually predominant in Africa, and one had a sequence that clustered with the rare subtype F, which has only previously been reported as individual cases and small clusters in Africa and in an MSM population in Paris, France (Jary et al., 2020; Kajumbula et al., 2006; Tornesello et al., 2010; Tozetto-Mendoza et al., 2016). It has been suggested that subtype F could potentially be associated with more severe disease, but further investigation is required to support this hypothesis (Jary et al., 2020).

Where the K15 gene was successfully sequenced, 69% of patients had the P allele and 31% had the M allele. Globally, K15 subtype M is present in approximately 20% of HHV-8 genomes, so the finding of 31% of sequences containing the M allele in the current study was higher than expected (Hayward & Zong, 2007). The four sequences containing the M allele were associated with different K1 subtypes. It remains unclear whether the P and M K15 alleles are linked to the corresponding K1 subtypes, and the findings from previous studies on this subject have been conflicting (Lacoste, Judde et al., 2000; Poole et al., 1999). Previous studies have also suggested the possibility of a functional difference between the P and M subtypes, as a potential explanation for the

overall predominance of the P subtype globally (Lacoste, Judde et al, 2000). These are both potential areas for future studies on this topic.

Overall, the findings of this study demonstrate significant heterogeneity of HHV-8 subtypes based on the four genome regions examined. The diverse findings of different K1 subtypes in Ireland, as well as their linkage to different right hand side subtypes are in contrast to the clear geographical patterns that were demonstrated based on the early studies of HHV-8 molecular epidemiology. This is likely to reflect the significant inward migration and population changes that have occurred in Ireland over the last 20 years.

5.4. Proinflammatory cytokines in HHV-8-related disease

It is clear from the evidence to date that there is a complex interaction between a number of proinflammatory cytokines and HHV-8 disease. Proinflammatory cytokines have been implicated both in the development and pathogenesis of HHV-8 disease and in the resulting symptomatology and inflammatory process associated with HHV-8 disease (Ensoli & Stürzl, 1998; Host et al., 2017; Oksenhendler et al., 2000; Polizzotto et al., 2013).

This is the first study, to our knowledge, that has compared cytokine levels for different HHV-8 subtypes. Significantly higher levels of IFN-γ, IL-1β, IL-6, IL-8, IL-13 and TNF-α were obtained from K1 subtype F samples compared to the other K1 subtypes. Although the subtype F samples were from a single patient, this is an interesting

finding, as subtype F has been associated with severe cases of HHV-8-related disease, and further study is warranted to determine whether different subtypes can be linked with differing disease severity and, if so, what role proinflammatory cytokines have to play (Jary et al., 2020).

IL-1β and TNF-α levels were significantly higher in patients with the K15 M allele compared to the K15 P allele. This is interesting to note, as studies have been performed previously to investigate whether the genetic difference between K15 alleles corresponds to a functional difference between HHV-8 strains containing the different alleles and it has been hypothesised that the genetic difference between these two alleles could infer a survival advantage to one type over the other (Poole et al., 1999; Wang et al., 2007). To date, no functional difference has been confirmed (Chen et al., 2018; Wang et al., 2007). The findings of the current study suggest that there may be a degree of increased proinflammatory activity associated with the M allele and this raises an area of interest for future research. These findings require confirmation in a larger study.

There were no significant differences between proinflammatory cytokine levels measured before and after treatment in patients with HHV-8-related diseases in this study. However, a significant negative correlation between HHV-8 PCR Ct value and IL-10 level was identified. In addition, there were negative correlations between Ct value and IFN-γ, IL-4, IL-6, IL-13 and TNF-α levels, although these correlations did not reach statistical significance. These results support the findings of previous studies that reported higher levels of proinflammatory cytokines in association with higher

HHV-8 viral loads and disease flares compared to remission (Oksenhendler et al., 2000; Polizzotto et al., 2013).

5.5. Conclusion

This study has examined the seroprevalence, molecular epidemiology and the dynamics of proinflammatory cytokines in HHV-8 disease. Further investigation is warranted to clarify the mode of transmission of HHV-8 and the reasons behind the varying seroprevalence of this virus, both geographically and in risk groups, for example MSM and HIV positive individuals. HHV-8 seroprevalence studies performed in different countries to date have utilised varying methodologies for serological testing. There is a need to standardise serological testing methodologies for HHV-8 antibodies, so that seroprevalence studies such as this can be compared internationally.

Further investigation is also required to determine whether different subtypes of HHV-8 may infer functional differences between strains and whether they may be associated with differing degrees of disease severity. If this proves to be the case, an additional point of interest is the role that proinflammatory cytokines have to play in disease severity.

Treatment options for HHV-8-related disease are still lacking and, in order to optimise therapeutic and disease prevention strategies, it is important to understand the underlying elements determining disease severity, including the role of the immune system and the inflammatory response to infection. Only by understanding these

contributing factors can we determine the optimal treatment strategy for patients suffering from these diseases. The results of this study support the need for future research in this area.

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Appendices

Appendix 1. **HHV-8 genetic sequences described in this study.**

SJH133K1 (GenBank accession PP129309)

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  61 tacatccttg ccaacatcct ggtattgcaa cgatactcgg cttctccgac tgacgcagca
 121 aacattcact gttgtcaccc ttatctgcaa ttttagttgt gtgggacaat ctgggcatcg
 181 acacagcctt tggattacat ggtatccaca acctgtctta caaaccttgt gtggacaacc
 241 atcaaacaca gtcacttgtg gtcagcatgt tactttgtat tgttctacct ctggaaataa
 301 tgttaccgtt tggcatctac caaacggacg aaatgaaacc gtgtcacaaa ctaaatacta
 361 taattttacg ctgatggacc aaacggaggg gtgttatgct tgttctgacg ggctgtcgtc
 421 tcgcctgtca aatcgtctat gtttttcggc gcgttgtgcc aatataactc tagaaactca
 481 tactgtatct gtcagcagta ctacaggctt tagaacattc agtactaata gccatgcaac
 541 cacacatgat gtacttgtaa tgaaagaagc caaatctaca aatctacata ttcaagtgca
 601 ttttcttgta tttatgacac tcgtagctct gataggaacc atgtgtggta tcttaggaac
 661 tattatcttt gcccattgtc aaaaacaaag tgactcaaac aaaacagtgc cacaacaatt
 721 gcgggattat tattccctac acgatttgtg cacgg
```

SJH109K1 (GenBank accession PP129310)

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 121 aacagtcact gttgtcaccc ttatctgcaa ttttagttgt gtgggacaat ctgggcatcg
 181 acacagcctt tggattacat ggtatccaca acctgtctta caaaccttgt gtggacaacc
 241 atcaaacaca gtcacttgtg gtcagcatgt tactttgtat tgttctacct ctggaaataa
 301 tgttaccgtt tggcatctac caaacggacg aaatgaaacc gtgtcacaaa ctaaatacta
 361 taattttacg ctgatggacc aaacggaggg gtgttatgct tgttctgacg ggctgtcgtc
 421 tcgcctgtca aatcgtctat gtttttgggc gcgttgtgcc aatataactc cagaaactca
 481 tactgtatct gtcagcagta ctacaggctt tagaacattc agtactaata gccatgcaac
 541 caaacatgat gtagttgtag tgaaagaagc aaaatttaca aatccacata ttgaagtgcc
 601 ttttcttgta tttatgacac tcgtagctct aataggaacc atgtgtggta tcttaggaac
 661 tattatcttt gcccattgtc aaaaacaaag tgactcaaac aaaacagtgc cacaacaatt
 721 gcgggattat tattccctac acgatttgtg cacgg
```

SJH050K1 (GenBank accession PP129311)

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   1 gtctccaaat ttgtgccctc gagtgatttc aacgccttac acgttgacct gtccgtctaa
  61 tacatccttg ccaacatcct ggtattgcaa cgatactcgg cttttccgac tgacgcagca
 121 aacattgact gttgttaacc ttatctgcaa ttttagttgt gtgggacaat ctgggcatcg
 181 acacagcctt tggattacat ggtatccaca acctgtctta caaaccttgt gtggacaatc
 241 atcaaacaca gtcacttgtg gtcagcatgt tactttgtat tgttctacct ctggaaataa
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 361 taattttacg ctgatggacc aaacggaggg gtgttatgct tgttctgacg ggctgtcgtc
 421 tcgcctgtca aatcgtctat gtttttcggc gcgttgtgcc aatataactc tagaaactca
 481 tactgtatct gtcagcagta ctacaggctt tagaacattc agtactaata gccatgcaac
 541 cacacatgat gtacttgtaa tgaaagaagc caaatctaca aatctaccta ttcaagtgca
 601 ttttttgta tttatgacac tcgtagctct gataggaacc atgtgtggta tcttaggaac
 661 tattatcttt gcccattgtc aaaaacaaag tgactcaaac aaaacagtgc cacaacaatt
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721 gcgggattat tattccctac acgatttgtg cacgg

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121 aacattcact gttgtcaccc ttatctgcaa ttttagttgt gtgggacaat ctgggcatcg
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241 atcaaacaca gtcacttgtg gtcagcatgt tactttgtat tgttctacct ctggaaataa
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361 taattttacg ctgatggacc aaacggaggg gtgttatgct tgttctgacg ggctgtcgtc
421 tcgcctgtca aatcgtctat gtttttgggc gcgttgtgcc aatataactc cagaaactca
481 tactgtatct gtcagcagta ctacaggctt tagaacattc agtactaata gccatgcaac
541 caaacatgat gtagttgtag tgaaagaagc aaaatttaca aatccacata ttgaagtgcc
601 ttttcttgta tttatgacac tcgtagctct aataggaacc atgtgtggta tcttaggaac
661 tattatcttt gcccattgtc aaaaacaaag tgactcaaac aaaacagtgc cacaacaatt
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541 cacacatgat gtacttgtaa tgaaagaagc caaatctaca aatgtatata ttcaagtgca
601 ttttcttgta tttatgacac tcgtagctct gataggaacc atgtgtggta tcttaggaac
661 tattatcttt gcccattgtc aaaaacaaag tgactcaaac aaaacagtgc cacaacaatt
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SJH053VR1 (GenBank accession PP129331)

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121 ttgccaatat cctggtattg caacaatact cggctttggc gactgacgca gccaacagtc
181 actggtgccg ccattgcctg caattttact tgtgtggaac aatctgggca tcgacagagc
241 at

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121 ttgccaatat cctggtattg caacgatact cggcttttcc gactgacgga gagaacactt
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241 at

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121 ttgccaacat cctggtattg caacgatact cggcttctcc gactgacgca gcaaacattc
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241 ct

SJH109VR1 (GenBank accession PP129334)

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SJH053VR2 (GenBank accession PP129315)

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SJH081VR2 (GenBank accession PP129323)

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SJH083VR2 (GenBank accession PP129324)

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SJH101ORF75 (Genbank accession PP227242

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SJH053ORF75 (Genbank accession PP227247)

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SJH062K15 (Genbank accession PP227251)

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61 ccccccctgaa gaagcaggaa cgttaatatt ccacgttttg gtggagccca tgacttatga
121 agagtgctga ttatgccagt gaatcctaca aa

SJH083K15 (Genbank accession PP227252)

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61 ccccccctgaa gaagcaggaa cgttaatatt ccacgttttg gtggagccca tgacttatga
121 agagtgctga ttatgccagt gaatcctaca aa

SJH092K15 (Genbank accession PP227253)

1 ttgccagtgg tgctagtaat tgcaagtagt tcggttgtta aagtttgtgt ggttagcact
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SJH109K15 (Genbank accession PP227254)

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SJH133K15 (Genbank accession PP227255)

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SJH024K15 (Genbank accession PP227256)

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121 agagtgctag tatgccagtg aatcctacaa a

SJH098K15 (Genbank accession PP227257)

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SJH068K15 (Genbank accession PP227258)

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121 aacttaccag taattataga caataaa

SJH137K15 (Genbank accession PP227259)

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121 aacttaccag taattataga caataaa

SJH089K15 (Genbank accession PP227260)

1 actcttaatg ttccaccaag tgatagaatg gctgaaaaac acatacaggg gaattacgtt
61 tttttaaaaa attggaaata ttagatacat aatttttatt taataaaaaa cctttagtaa
121 aacttaccag taattataga caataaa

Appendix 2. **Patient information leaflet for seroprevalence study.**

Patient Information Leaflet

TITLE: A study to investigate the seroprevalence of Human Herpesvirus 8 in Ireland.

BACKGROUND: This study is being undertaken to examine the seroprevalence of the virus Human herpesvirus 8 (HHV-8) in Ireland, in other words, to determine the percentage of people in the Irish population who have been exposed to and developed antibodies to Human herpesvirus 8. Seroprevalence studies have been performed worldwide to help us to better understand infection rates and the specific geographical areas and sub-populations affected by this virus. The rate of infection with HHV-8 is known to vary depending on geographical location. According to studies already performed, the rate of infection has been reported as approximately 5% in Northern Europe and the USA and up to 50% in parts of Africa. The rate of HHV-8 infection in Ireland is unknown as it has never been studied before. In otherwise healthy individuals, infection with HHV-8 does not usually cause any problems or lead to disease and HHV-8 infection does not usually cause any symptoms or require any treatment. Infection with HHV-8 may, in some cases, lead to certain specific conditions in patients who are severely immunosuppressed, for example, patients who have undergone organ transplantation or patients with HIV infection.

WHY?: The reason for this study is to investigate the percentage of people in Ireland who have previously been infected with HHV-8 virus and enable us to compare with the prevalence of this virus internationally.

WHAT WILL HAPPEN?: The study will be explained to you by a member of the medical staff. You will be asked to sign a consent form which shows that you understand the project and what will happen. There will be no need for any additional blood samples to be taken – testing for HHV-8 can be performed on one of your routine blood samples that is already being taken. Your sample will be given a study number and will be tested in the laboratory for antibodies to HHV-8.

If you are found to have antibodies to HHV-8, indicating previous infection with the virus, you will be informed. However, as discussed above, this virus does not usually cause symptoms or require treatment except in very specific circumstances. You will also be informed if you have a negative result for HHV-8 antibodies.

BENEFITS: Your participation will help us to understand how many people in Ireland are positive for HHV-8 and how this number compares to other countries.

RISKS: There will be no need for any intervention or for any additional bloods to be taken for this study as testing can be performed on one of your blood samples that is being requested for testing already by your doctor. Taking your blood samples may be a little bit uncomfortable and occasionally there may be some bruising. There is a very small risk of developing a skin infection at the site where the blood is taken.

ST. JAMES'S HOSPITAL

CONFIDENTIALITY AND DATA PROTECTION: This study is being funded by the St. James's Hospital Department of Clinical Microbiology and St. James's Hospital is the data controller. Your right to confidentiality is very important to us and your consent is required for the collection and processing of your data. After your blood sample has been processed for your usual blood tests, as requested by your doctor, your sample will be given a unique study number, which will be recorded with your date of birth, surname initial and hospital number, kept in the laboratory on a secure hospital server, which will link you to the study number. The data for this study will be processed by the study personnel detailed below and only they will have access to this. There will be no third party access to the study data. At no time will your full name, telephone number or address be recorded. All data will be destroyed 1 year following final completion of the study. The St. James's Hospital Data Liaison Officer, foi@stjames.ie may be contacted for any data related queries.

VOLUNTARY PARTICIPATION: Participation in this study is completely optional and you are under no pressure to be involved. Your participation or non-participation in the study will not affect the care you receive. If you change your mind and don't want your blood sample to be used as part of this study, that is completely within your rights and we will respect your decision.

PERMISSION: The Ethics Committee of the hospital has approved this study. The anonymised data that you provide will be collated with other participants' and may be published as an entire study or submitted as part of a postgraduate university project.

WHAT IF I THINK OF A QUESTION LATER?: You can email one of the study personnel at saorourke@stjames.ie.

Dr. Sadhbh O'Rourke
Virology Registrar
St. James's Hospital

Dr. Brendan Crowley
Consultant Virologist
St. James's Hospital

Appendix 3. Consent form for seroprevalence study.

Patient Consent Form

TITLE: A study to investigate the seroprevalence of Human Herpesvirus 8 (HHV-8) in Ireland.

CONSENT: This form is to state that I agree with the following statements and I am happy to participate in the study.

(Please tick the box where appropriate)

	Yes	No
I am aged 18 years or older.		
I am not pregnant or breastfeeding.		
I agree to have my blood processed for testing for Human Herpesvirus 8 as explained in the patient information leaflet.		
I have received and understand the patient information leaflet.		
Any questions that I have at this stage have been answered.		
I understand and agree to have my personal information processed in accordance with the details provided in the patient information leaflet.		
I understand that I am free to withdraw from the study at any time without giving a reason and without this affecting my future medical care.		

Date: / /

Name (PRINTED):

Signature:

Consent obtained by (PRINTED):

Signature:

Appendix 4. **Patient information leaflet for molecular epidemiology and immunological studies.**

ST. JAMES'S HOSPITAL

Patient Information Leaflet

TITLE: A study to investigate the Molecular Epidemiology and Immunological Aspects of Human Herpesvirus 8 in Ireland.

BACKGROUND: This study is being undertaken to learn more about the virus Human herpesvirus 8 (HHV-8) in Ireland. The aim is to examine the particular subtypes of this virus that are present in Ireland and also the types of immune responses this virus causes. In the majority of patients, infection with HHV-8 does not usually cause any problems or lead to disease and HHV-8 infection does not usually cause any symptoms or require treatment. However, HHV-8 infection may rarely lead to certain specific conditions. When patients do develop these specific conditions, they are often associated with significant inflammatory symptoms associated with the immune response to HHV-8. Studies have been performed worldwide to investigate the different subtypes of this virus present in different countries but there have been no such studies performed in Ireland to date and therefore, little is known about this virus in Ireland. Examining the types of virus present in Ireland will give us a better understanding of the virus in this country and help us to compare the patterns evident in this country with those found internationally. Studies have also been performed internationally looking at markers of the immune response to this virus but no similar studies have been performed in Ireland and by performing this study we hope to learn more and contribute to the knowledge and understanding of this virus. It is important to emphasise that there will be no clinical implications associated with this study.

WHY?: The reason for this study is to examine the subtypes of Human herpesvirus 8 that are present in Ireland and also the types of inflammatory proteins that are present in HHV-8 infection.

WHAT WILL HAPPEN?: If you are happy that you understand the project and consent for us to include you, then there is nothing further that you need to do. Testing can be performed on blood samples that have already been taken. Your sample will be given a study number and will be tested for the presence of HHV-8. If HHV-8 is found to be present, then the subtype of the virus will be determined and your sample will also be tested for certain inflammatory proteins to see if they are present. These tests will be performed on samples that have already been taken, for research purposes, with a view to advancing our knowledge about this virus and how to improve patient management in the future, and the results of these tests will not have any clinical impact.

BENEFITS: Your participation will help us to understand the types of HHV-8 virus present in Ireland and the types of inflammatory proteins that are present in HHV-8 infection. This will help us to know more about this virus and knowing more may help us in the care of patients with diseases related to this virus in the future. It is important to note that the results of the study will have no clinical implications.

RISKS: There will be no need for any intervention or for any additional bloods to be taken for this study as testing can be performed on blood samples that have been taken already. Therefore, there are no risks involved in this study.

CONFIDENTIALITY AND DATA PROTECTION: This study is being funded by the St. James's Hospital Department of Clinical Microbiology and St. James's Hospital is the data controller. Your right to confidentiality is very important to us and your consent is required for the collection and processing of your data. Your sample will be given a unique study number, which will be recorded with your date of birth, surname initial and hospital number, kept in the laboratory on a secure hospital server, which will link you to the study number. The data for this study will be processed by the study personnel detailed below and only they will have access to this. There will be no third party access to the study data. At no time will your full name or address be recorded. All data will be destroyed 1 year following final completion of the study. The St. James's Hospital Data Liaison Officer, foi@stjames.ie may be contacted for any data related queries.

VOLUNTARY PARTICIPATION: Participation in this study is completely optional and you are under no pressure to be involved. Your participation or non-participation in the study will not affect the future care you receive. If you change your mind and don't want your blood sample to be used as part of this study, that is completely within your rights and we will respect your decision.

PERMISSION: The Ethics Committee of the hospital has approved this study. The anonymised results of this study will be collated with other participants' and may be published as an entire study or submitted as part of a postgraduate university project.

WHAT IF I THINK OF A QUESTION LATER?: You can email one of the study personnel at saorourke@stjames.ie.

Dr. Sadhbh O'Rourke
Virology Registrar
St. James's Hospital

Dr. Brendan Crowley
Consultant Virologist
St. James's Hospital

Appendix 5. **Consent form for molecular epidemiology and immunological studies.**

Patient Consent Form

TITLE: A study to investigate the Molecular Epidemiology and Immunological Aspects of Human Herpesvirus 8 in Ireland.

CONSENT: This form is to state that I agree with the following statements and I am happy to participate in the study.

(Please tick the box where appropriate)

	Yes	No
I am aged 18 years or older.		
I agree to have my laboratory samples processed for testing for Human Herpesvirus 8 and subtyping for research purposes as explained in the patient information leaflet.		
I agree to have my laboratory samples processed for inflammatory proteins as explained in the patient information leaflet.		
I have received and understand the patient information leaflet.		
Any questions that I have at this stage have been answered.		
I understand and agree to have my personal information processed in accordance with the details provided in the patient information leaflet.		
I understand that I am free to withdraw from the study at any time without giving a reason and without this affecting my future medical care.		

Date: / /

Name (PRINTED):

Signature:

Consent obtained by (PRINTED):

Signature:

Appendix 6. **Seroprevalence of human herpesvirus 8 in Ireland among blood donors, men who have sex with men, and heterosexual genitourinary medicine and infectious diseases clinic attendees.** ***Journal of Medical Virology*** **(2021).**

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RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Seroprevalence of human herpesvirus 8 in Ireland among blood donors, men who have sex with men, and heterosexual genitourinary medicine and infectious diseases clinic attendees

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Abstract

Human herpesvirus 8 (HHV-8) seroprevalence varies geographically and between subpopulations. High seroprevalence rates have been ascribed to men who have sex with men (MSM), African migrants, and HIV-infected individuals. The objective of this study was to determine the seroprevalence of HHV-8 in an Irish population, including specific risk groups. A cross-sectional study of 200 blood donors and 200 genitourinary medicine (GUM) and infectious diseases (ID) clinic patients was performed, with testing for Immunoglobulin G (IgG) antibodies to HHV-8 lytic antigens using a commercial indirect fluorescence assay (Scimedx Corp.). Verification was performed at the Centers for Disease Control and Prevention (CDC). All 200 blood donor samples were negative for HHV-8 IgG antibodies. 21% of GUM and ID patients were positive for HHV-8 IgG antibodies. One hundred of these patients were MSM, 35% of whom were HHV-8 seropositive (46% of HIV-positive MSM and 24% of HIV-negative MSM). Of 100 heterosexual patients, only 7% were HHV-8 seropositive. The absence of seropositivity in 200 Irish blood donors may suggest that Ireland has a low overall population HHV-8 seroprevalence. The proportion of HHV-8 seropositivity in the MSM population was significantly higher than in the heterosexual population and most marked in HIV-positive MSM.

KEYWORDS
blood donors, HHV-8, HIV, MSM, seroprevalence

1 | INTRODUCTION

Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma (KS)-associated herpesvirus, was first described by Chang et al. in 1994[1], in association with KS. Representational difference analysis was used to identify and characterize unique DNA sequences in KS tissue from patients with AIDS-associated KS.[1] As well as being identified as an etiologic agent responsible for KS, HHV-8 has subsequently been associated with primary effusion lymphoma and multicentric Castleman's disease.[2,3] HHV-8 is an enveloped γ-2 herpesvirus (rhadinovirus), with a double-stranded DNA genome. It can cause both latent and lytic infection. Latent infection persists for life, and studies have shown that immunosuppression is associated with an increased risk of developing KS, for example, in solid organ

transplant recipients and HIV-infected individuals.[4,5] HHV-8 is classified as a Group 1 carcinogenic agent by the International Agency for Research on Cancer.[6] The National Cancer Registry of Ireland reports an average KS incidence of 7 cases per year, with an annual age-standardized incidence of 0.3 per 100,000 in males and 0.0 per 100,000 in females.[7]

The global epidemiology of HHV-8 differs from that of other herpesviruses in that its seroprevalence varies significantly geographically and between different subpopulations.[8] Sub-Saharan Africa has the highest rate of infection, with approximately 50% of the population seropositive in some areas.[9,10] Seroprevalence is approximately 10% in China, 10% in the Mediterranean, approaching 30% in areas of Italy, and approximately 5%–6% in the United States and northern Europe.[11–14] However, the rate of KS does not necessarily correlate with the rate of HHV-8 infection in a given population.[15] High seroprevalence rates have been specifically ascribed to men who have sex with men (MSM), African migrants, and HIV-infected individuals.[8]

Several patterns of HHV-8 transmission have been reported, including via saliva, sexual transmission, and bloodborne transmission. Transmission may also occur from mother to infant.[16,17] With regard to blood transfusion, the American Association of Blood Banks (AABBs) have designated HHV-8 a risk category of yellow, that is, as agents with absent to low scientific/epidemiologic evidence of risk regarding blood safety.[18] Although transfusion transmission may occur in the absence of leukoreduction, no clinically significant disease has been definitively documented via transfusion.[19–23]

Infection with HHV-8 itself rarely leads to disease, and the virus predominantly causes latent infection, but genes expressed in both latent and lytic infection of cells can have a role in tumorigenesis and immunosuppression contributes significantly to the development of HHV-8-related disease.[6] Although HHV-8 is well described as the etiologic agent underlying KS, primary effusion lymphoma, and multicentric Castleman's disease, there appear to be other poorly understood factors at play. For example, KS occurs more frequently in men than in women, despite similar prevalences of HHV-8 infection, and disease also appears to be more common in certain geographical areas, despite similar seroprevalence rates. The reasons for this warrant further investigation.[6]

Serological assays are evolving to test for antibodies expressed in latent and lytic phases of HHV-8 infection. However, these assays are known to differ in sensitivity and specificity and there is no Food and Drug Administration approved method of testing. This limits the potential for screening for this virus, for example, in blood donor samples.[8] Serological assays in use for research purposes include immunofluorescence assays, incorporating HHV-8-infected cell lines, western blot assays using both latent and lytic antigens, and enzyme-linked immunosorbent assays (ELISA) using both whole virus lysate and recombinant proteins.

Since the discovery of HHV-8, the epidemiology of this virus has been investigated in many countries, but little is known about its seroprevalence in Ireland. The objective of this study was to determine the seroprevalence of HHV-8 in an Irish population and examine the seroprevalence in specific risk groups.

2 | MATERIALS AND METHODS

2.1 | Study design and setting

We designed a cross-sectional study investigating the seroprevalence of HHV-8 in an Irish population, including various risk groups. The study was carried out from April to November 2019 at the Department of Clinical Microbiology, St. James's Hospital, Dublin, Ireland. The department processes specimens for both the genitourinary medicine (GUM) and infectious diseases (ID) unit at St. James's Hospital and for the Gay Men's Health Service (GMHS). This study was performed in conjunction with these services and with the Irish Blood Transfusion Service (IBTS).

2.2 | Study population

The study population included 400 individuals aged ≥18 years, including 200 blood donors from the IBTS and 200 patients from St. James's Hospital GUM and ID clinic and the GMHS, subdivided into populations of HIV-positive and HIV-negative MSM and male and female heterosexual GUM and ID clinic attendees (Figure 1). Written informed consent was obtained from all the study participants.

Serum samples were collected in the IBTS between April 6, 2019 and May 14, 2019. Specimens collected in the IBTS were anonymized before transfer to St. James's Hospital and retained donor data included age bracket, gender, and region where the specimen was collected.

Serum samples were collected in St. James's Hospital GUM and ID clinic and the GMHS between March 27, 2019 and July 03, 2019. Each specimen was given a unique study number and data collected included age, gender, geographical area of origin, HIV status, and whether the patient was MSM or heterosexual.

2.3 | Serological testing

Four hundred samples were collected. All samples were frozen at −20°C pending testing. All samples collected were tested using a commercial indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp.), according to the manufacturer's instructions. Testing was performed using facilities at the National Virus Reference Laboratory, Dublin. Reactivity was observed under fluorescence microscopy using ×20–40 magnification and fluorescence reaction was graded according to the following intensity scale: 4 + (brilliant), 3 + (bright), 2 + (moderate), and 1 + (weak). Samples reactive at a 1:64 dilution were considered positive (Figure 2).

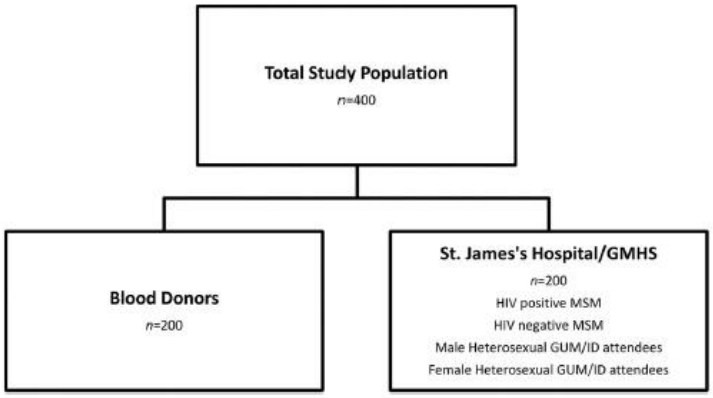

FIGURE 1 Seroprevalence study population and subgroups for analysis. HIV, human immunodeficiency virus; GUM, genitourinary medicine; ID, infectious diseases; MSM, men who have sex with men,

For the purposes of test verification, 200 samples were also tested blindly at the Centers for Disease Control and Prevention (CDC) Infectious Diseases Laboratories using three different assays, an immunofluorescence assay for detection of IgG antibodies to HHV-8 lytic antigens and two peptide ELISAs based on the open reading frame (ORF) K8.1 and ORF 65.[9]

2.4 | Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics (version 26). HHV-8 seroprevalence was examined in the total study population and in all subgroups. Comparisons were performed between subgroups using the χ^2 test. Fisher's exact test was used where the expected frequency was <5. A $p < .05$ was considered statistically significant.

2.5 | Ethical approval

Ethical approval for this study was obtained from Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee and from the Royal College of Physicians of Ireland Research Ethics Committee.

3 | RESULTS

From April 6, 2019 to May 14, 2019, 200 blood donor serum samples were collected by the IBTS, anonymized and sent to St. James's Hospital for storage at −20°C before testing by indirect fluorescence assay for IgG antibodies to HHV-8 (Table 1). 57.5% of samples were collected in Leinster, 33% in Munster, 4.5% in Connacht, and 4.5% in Ulster. 40% of samples were collected in relatively urban areas and

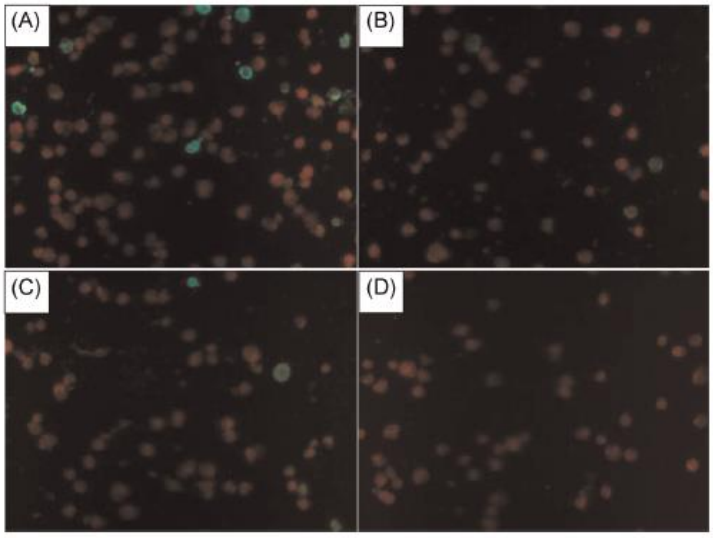

FIGURE 2 Indirect fluorescence assay for the qualitative detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp.). (A) 3+ fluorescence demonstrated in a positive sample at 1:64 dilution; (B) 1+ fluorescence demonstrated in a positive sample at 1:64 dilution; (C) Scimedx kit positive control; (D) negative sample at 1:64 dilution. HHV-8, Human herpesvirus 8; IgG, Immunoglobulin G

TABLE 1 Age and percentage IgG positivity of study population and subgroups

Study population	No. of participants	Median age (range)	% IgG-positive
Blood donors	200	44 (19–77)	0
GMHS/GUM/ID clinic attendees	200	29 (19–71)	21
MSM	100	32.5 (19–71)	35
HIV-positive	50	36 (19–57)	46
HIV-negative	50	31 (19–71)	24
Heterosexuals	100	26 (19–60)	7
Male	50	27 (20–60)	8
Female	50	25 (19–53)	6

Abbreviations: GMHS, Gay Men's Health Service; GUM, genitourinary medicine; HIV, human immunodeficiency virus; ID, infectious diseases; IgG, immunoglobulin G; MSM, men who have sex with men.

TABLE 3 Geographical area of origin and percentage IgG positivity in GUM/ID patient population

Geographical area of origin	Proportion (%) of patients with HHV-8 IgG antibodies
Europe, $n = 140$	23 (16.43)
Ireland, $n = 117$	18 (15.38)
Europe other, $n = 23$	5 (21.74)
North America, $n = 1$	0 (0)
South/Central America, $n = 35$	15 (42.86)
Africa, $n = 10$	2 (20)
Asia, $n = 10$	1(10)
Unknown, $n = 1$	0 (0)

Abbreviations: GUM, genitourinary medicine; ID, infectious diseases.

59.5% in relatively rural areas. The median age of blood donor participants was 44 years (range: 19–77 years). 58% of blood donor participants were males and 42% were females. All blood donor samples tested negative or equivocal using indirect fluorescence assay for the detection of IgG antibodies to HHV-8 lytic antigens (Scimedx Corp.) and all equivocal results were confirmed to be negative following verification and confirmatory testing in the CDC using the three test protocol described.

From March 27, 2019 to July 3, 2019, 200 serum samples were collected from St. James's Hospital GUM and ID clinic and the GMHS (Table I). All samples were stored at −20°C before testing by indirect fluorescence assay for IgG antibodies to HHV-8. Of these samples, 42 (21%) were confirmed to be positive for HHV-8 antibodies. This was a significantly higher rate of positivity compared with the blood donor population ($p < .001$) and the difference remained significant ($p < .001$) when both the heterosexual group and the MSM group were compared separately with the blood donor group. Overall, the age-group with the highest percentage positivity was the 39–48-year-olds category (Table 2).

Of these 200 samples, 100 were collected from MSM patients. 35% of MSM samples were positive for HHV-8 antibodies, 46% in HIV-positive MSM, and 24% in non-HIV-positive MSM. The difference in seroprevalence between HIV-positive MSM and non-HIV-positive MSM was statistically significant ($p = .021$). Seven percent of heterosexual patients were positive for HHV-8 antibodies, demonstrating a significantly lower prevalence when compared with the MSM population ($p < .001$). Eight percent of heterosexual males and 6% of heterosexual females were found to have antibodies to HHV-8. This difference was not statistically significant. The majority of the 200 GUM and ID clinic participants were Irish ($n = 117$) (59%) and 15% of these patients were observed to have antibodies to HHV-8. This was a lower proportion compared with that of participants from other parts of Europe, South/Central America, and Africa, although the numbers in these groups were small (Table 3).

Two hundred specimens were sent to the CDC for test verification purposes and confirmatory testing using the described methodology. All blood donor samples were negative using the CDC testing methodology, and the overall result concordance demonstrated moderate agreement with a κ value of 0.404 ($p < .001$). However, the level of agreement was reduced due to the number of results reported as equivocal using the commercial kit.

TABLE 2 Proportion of patients with HHV-8 IgG antibodies stratified according to subgroup and age category

Age-group (years)	Proportion (%) of patients with HHV-8 IgG antibodies				
	HIV-positive MSM	HIV-negative MSM	Male heterosexuals	Female heterosexuals	All patients
19–28	3/8 (37.5)	6/19 (31.6)	2/28 (7.1)	2/38 (5.3)	13/93 (14)
29–38	10/23 (43.5)	5/22 (22.7)	2/14 (14.3)	0/7 (0)	17/66 (25.8)
39–48	9/16 (56.3)	1/4 (25)	0/4 (0)	1/4 (25)	11/28 (39.3)
49–58	1/3 (33.3)	0/1 (0)	0/3 (0)	0/1 (0)	1/8 (12.5)
59–68	0/0 (0)	0/3 (0)	0/1 (0)	0/0 (0)	0/4 (0)
69–78	0/0 (0)	0/1 (0)	0/0 (0)	0/0 (0)	0/1 (0)

Abbreviations: HIV, human immunodeficiency virus; MSM, men who have sex with men.

4 | DISCUSSION

All 200 Irish blood donors tested were HHV-8 seronegative. This was assessed in a population of blood donors with a nationwide representation, both males and females, and over a broad range of age groups. Seroprevalence studies in blood donors in other European countries and in North America with similar participant numbers have demonstrated varying rates, ranging from 0% in Greece and parts of Spain to rates of 2%–6% in the majority of European countries, including rates of 0.7%–5% in the United Kingdom.[14,24–30] Rates of 24% have been identified among blood donors in parts of southern Italy, an area known to have high rates of KS, and up to 35% in Sicily.[12] Rates of 1%–5% have been reported in North America, but up to 15%–23% in Texas.[31–35] These studies utilized a range of different testing methodologies. This demonstrates that HHV-8 prevalence rates may depend both on the region studied and on the individual assays and testing methodologies utilized. The absence of seropositivity in 200 Irish blood donors may suggest that Ireland has a lower overall population HHV-8 seroprevalence than other European countries. However, 200 is a small sample size for the low seroprevalence. Also, blood donors are a select subset of the population and therefore results cannot be extrapolated to the general population which is a limitation of the study.

As expected, the proportion of individuals identified to have antibodies to HHV-8 in the MSM population was significantly higher than that in the heterosexual population. It was most marked in the HIV-positive MSM population who had a significantly higher HHV-8 seroprevalence when compared with the non-HIV-positive MSM group.

From the time HHV-8 was first identified as the causative agent in KS, studies have identified higher seroprevalence rates among MSM, particularly among HIV-positive MSM.[31,36] It was postulated that HHV-8 infection was sexually transmitted, and studies undertaken by Lennette et al. and Martin et al. were among the first to attempt to demonstrate evidence of sexual transmission. Both groups showed sexual activity to be a strong risk factor for HHV-8 infection, as well as demonstrating higher prevalence in MSM and a strong association with HIV infection.[36,37] These findings are most notable in nonendemic countries and many subsequent studies have agreed with these findings.[13] A meta-analysis published by Liu et al. in 2018 demonstrated that, among 15,914 subjects, HIV-positive MSM were more likely to be HHV-8 seropositive than those who are HIV-negative (OR = 3.70; 95% confidence interval [CI]: 2.93–4.67). Similarly, a significant association between sexually transmitted infections (STIs) and HHV-8 was observed (OR = 2.40, 95% CI 2.04–2.82), providing further evidence in support of sexual transmission.[38] The evidence for association with HIV specifically has been conflicting, with some studies demonstrating an association and others not.[39–42] Rohner et al.[43] performed a systematic review on this subject and demonstrated an overall increased HHV-8 seroprevalence associated with HIV infection, with a stronger association in MSM compared with heterosexual adults. This study in an Irish population is consistent with these previous findings in the literature. Although the evidence available suggests that HHV-8 may be sexually transmitted, the exact mode of transmission and the reasons underlying the propensity for infection in the MSM population and in HIV-positive individuals remain unclear. Consistent with the evidence that HHV-8 may be sexually transmitted is the clear association in the literature between recognized STIs and HHV-8 infection.[36–38] However, other studies have supported alternative routes of transmission. Pauk et al.[44] provided evidence in an MSM population of significantly higher detection rates of HHV-8 in oral secretions compared with anal specimens and genital secretions, suggesting that transmission is more likely to occur via saliva rather than genital secretions, and this has been supported by a number of subsequent publications, including a study by Casper et al.[45] in HIV-positive MSM. In this study, the seroprevalence in the heterosexual GUM and ID clinic group was significantly lower than in the MSM population, and there was no significant difference between the HHV-8 seroprevalence among men and women in this group.

There were some limitations to this study. First, the sample size was small both in the blood donor group and in the patient population. However, studies of HHV-8 seroprevalence with similar sample sizes have been performed in other European countries and have provided valuable information on the epidemiology of this infection in Europe. For example, Politou et al.[24] performed serological testing for HHV-8 IgG antibodies on 179 blood donor samples in Greece and did not identify any positive samples. Regamey et al.[14] and Rode et al.[27] performed studies with similar sample sizes in Switzerland and Croatia, respectively, examining the seroprevalence of HHV-8 among blood donors, HIV-infected individuals, and MSM. Enbom et al.[46] performed HHV-8 antibody testing of 162 blood donor samples, as well as testing samples from a number of high-risk groups. Similarly, a number of recent studies in Asia have examined the seroprevalence of HHV-8 in small populations of HIV-positive patients and MSM.[47–49] This study is comparable to these other studies in the literature in terms of sample size and these similar-sized studies have provided valuable information regarding the epidemiology of HHV-8.

Second, it is not possible to extrapolate blood donor data to the general population in Ireland as blood donors represent a specific subset of the population. Strict donor selection guidelines and deferral criteria lead to the exclusion of many individuals living in Ireland who were born in HHV-8 endemic regions. In addition, MSM (1-year exclusion policy) and those with overlapping infectious diseases (HIV, hepatitis C virus, and chronic hepatitis B virus) are also temporarily or permanently deferred. The MSM subgroup in this study was recruited from GUM and ID clinics and therefore may not represent the MSM population as a whole. However, the findings in this group were significant when compared with a similar heterosexual population.

This study was performed using a lytic indirect fluorescence assay with verification at the CDC as described. A latent antigen-based assay was not used. Studies have shown that the use of latent antigen-based immunofluorescence assays does not contribute to sensitivity. A recent study by Chiereghin et al.[50] demonstrates this,

with lytic antigen-based immunofluorescence assays showing the best performance and excellent agreement to the reference standard. This publication provides a thorough comparison of HHV-8 serology tests and concludes that the lytic immunofluorescence assay, when used properly, is the optimal test.

In summary, the first seroprevalence study of HHV-8 in Ireland indicates low seropositivity in the blood donor population and suggests an overall low seroprevalence in the Irish population compared with other countries. As expected, a significantly higher rate of seropositivity was identified in the GUM and ID clinic attendee population, in particular among the MSM population and notably the HIV-positive MSM population. HHV-8 seropositivity was confined to specific risk groups such as MSM and HIV-positive individuals.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Study concept and design: Brendan Crowley, Sadhbh O'Rourke. *Methodology*: Brendan Crowley, Sadhbh O'Rourke, Niamh O'Flaherty, Dermot Coyne, Almida Lynam, Susan Clarke, Siobhán O'Dea, Sarah Fitzpatrick, Jeff Connell. *Recruitment*: Sadhbh O'Rourke, Niamh O'Flaherty, Dermot Coyne, Almida Lynam, Susan Clarke. *Laboratory analysis*: Sadhbh O'Rourke, Sarah Fitzpatrick, Jeff Connell. *Data analysis and drafting of article*: Sadhbh O'Rourke. *Critical revision of article*: Brendan Crowley, Niamh O'Flaherty. *Study oversight*: Brendan Crowley. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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RESEARCH ARTICLE

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Molecular epidemiology of human herpesvirus 8 in patients with HHV-8-related diseases in Ireland

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Abstract

Human Herpesvirus 8 (HHV-8) has been classified by sequence analysis of open reading frame (ORF) K1, ORF K15, and variable sequence loci within the central constant region. The purpose of this study was to examine the molecular epidemiology of HHV-8 in an Irish population. This retrospective study included 30 patients who had HHV-8 DNA detected in plasma. Nested end-point PCR was used to characterise four regions of the HHV-8 genome, K1, T0.7 (K12), ORF 75, and K15. Sequencing data were obtained for 23 specimens from 19 patients. Phylogenetic analysis of ORF K1 demonstrated that subtypes A, B, C and F were present in 37%, 11%, 47% and 5%, respectively. For T0.7 and ORF 75, sequencing data were obtained for 12 patients. For T0.7, subtypes A/C, J, B, R and Q were present in 58%, 17%, 8%, 8%, and 8%, respectively. For ORF 75, subtypes A, B, C and D were present in 58%, 8%, 25%, and 8%, respectively. K15 sequences were determined for 13 patients. 69% had the P allele and 31% had the M allele. The data generated by this study demonstrate that a broad variety of HHV-8 subtypes are represented in patients exhibiting HHV-8-related disease in Ireland, a low prevalence country. The predominance of C and A K1 subtypes was as expected for a Western European population. The 31% prevalence for K15 subtype M was higher than expected for a Western European population. This may represent the changing and evolving epidemiology in Ireland due to altered migration patterns.

KEYWORDS
HHV-8, Kaposi's sarcoma, molecular, phylogenetic

1 | INTRODUCTION

Chang et al. first associated Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus (KSHV) with Kaposi's sarcoma (KS) in 1994, when representational difference analysis was used to identify and characterise unique DNA sequences in KS tissue from patients with AIDS-KS.[1] In addition, Cesarman et al. and Soulier et al. subsequently associated HHV-8 with primary effusion lymphoma and multicentric Castleman's disease.[2,3] These diseases are typically associated with immunosuppression, and individuals such as solid organ transplant recipients and HIV-infected individuals are known to be at increased risk for their development.[4,5]

Institution at which the work was performed: St. James's Hospital, James's Street, Dublin 8, Ireland.

Genetic variability has been noted to occur within the open reading frame (ORF) K1 gene at the left hand side of the HHV-8 genome, and within the ORF K15 gene at the extreme right hand side of the genome.[6,7] The intervening central segment of the genome represents a constant area.[8] HHV-8 has been classified, for epidemiological purposes, according to K1 subtyping, K15 subtyping, and according to analysis of variable sequence loci within the central constant region of the genome.[8] Variability in the ORF K1 region is most marked in two hypervariable segments known as VR1 and VR2.[9,10]

Since the discovery of HHV-8, the molecular epidemiology of this virus has been investigated in many countries worldwide. HHV-8 is highly genetically variable and has been classified by sequence analysis of ORF K1 into six main molecular subtypes, A, B, C, D, E, F.[11] The process of K1 subtyping and the existence of subtypes A-D were originally described by Zong et al.[6,12] Subtype 'Z' was reported in 1998 by Kasolo et al. in a population of children in Zambia.[13] The sequences that were initially designated subtype Z in this study were subsequently assigned to the A5 subtype.[14] Subtype E was reported by Biggar et al. in 2000 in a population of Brazilian Amerindians.[15] Subtype F was reported by Kajumbula in 2006 in a Ugandan Bantu population.[16] Subtypes A and C are generally more prevalent in Europe and the US, subtype B is predominant in Africa, subtype D is associated with the Pacific Islands and subtype E is present in Brazil, French Guiana and Ecuador. These K1 subtypes can be further divided into subtype variants.[6]

The K15 region of the HHV-8 genome has been examined by numerous investigators since it was originally described by Poole et al. in 1999.[7] Until recently, two distinct P (Predominant or Prototype) and M (Minor) subtypes were thought to exist.[7] HHV-8 isolates were thought to contain either a P or an M allele. More recently, Alagiozoglou et al. identified evidence of a third subtype N, through sequencing of the UPS75 locus of South African HHV-8 isolates.[17] UPS75 lies in close proximity to the ORF K15 gene and further work by Hayward et al. has determined a new subset to fall between the P and M K15 patterns, representing a new, third N allele.[8]

The P and M alleles are equally represented among West African patients but the P subtype predominates in Eastern Africa, among French patients and among the A and C K1 subtypes.[18] Overall, only approximately 20% of HHV-8 isolates belong to subtype M and these have been associated with all three of the major K1 branches, A/C, B and D/E, and a much smaller minority to subtype N.[8]

Additional areas of study within the constant region include T0.7, ORF 75 and ORF 26. Analysis of these regions demonstrate overall typical ORF K1-linked subtype patterns.[7] However, the ORF 75 and T0.7 loci can also be linked with the K15 subtype.[7]

Since the discovery of HHV-8, this virus has been investigated in many countries worldwide. However, no studies in this area have been performed in Ireland to date. The purpose of this study was to examine the molecular epidemiology according to phylogenetic analysis of HHV-8 in a large tertiary referral centre in Ireland, in the context of data available from other countries.

2 | MATERIALS AND METHODS

2.1 | Study design and setting

A retrospective study was designed to investigate the molecular epidemiology of HHV-8 in Ireland. The study was carried out from March 2019 to December 2021 at the Department of Clinical Microbiology, St. James's Hospital, Dublin, Ireland. St. James's Hospital is a large, acute tertiary referral centre incorporating multiple national specialty services, including the National Haematopoietic Stem Cell Transplant Unit and a large Genitourinary Medicine and Infectious Diseases unit.

2.2 | Study population

From January 2012 to August 2019 in St. James's Hospital, of all plasma sample requests for diagnostic HHV-8 PCR testing based on clinical suspicion for HHV-8-related disease, HHV-8 DNA was detected in 153 samples. 30 patients consented to the use of their clinical samples for the purposes of this study, providing 88 specimens for use in the study. Written informed consent was obtained from all the study participants. All patients included in the study were living in Ireland and were not referred to the centre from other countries. However, not all participants were born in Ireland. Some participants moved to live in Ireland from other countries and were living in Ireland for varying periods of time before presenting to the study centre.

2.3 | DNA extraction and amplification

All plasma samples were previously positive on diagnostic HHV-8 PCR testing using real-time PCR and were stored at −80°C. Viral DNA was isolated from plasma using the NucliSENS® easyMAG® automated extraction system (Biomérieux). Nested end-point PCR was used to characterise four regions of the HHV-8 genome, K1, including the hypervariable regions VR1 and VR2, T0.7 (K12), ORF 75, and K15. The nested end-point PCR protocol was performed using specimens that were known to be positive for HHV-8 based on real-time PCR. Nested PCR primers used in this study are listed in Table 1. PCR was performed using DreamTaq Master Mix (Thermo-Fisher Scientific). All PCR reactions were carried out in a final volume of 25 µL, using 5 µL of DNA extract, 12.5 µL X2 DreamTaq Master Mix, and 1 µM of both forward and reverse primers.

The PCR reactions were carried out using the Veriti® Thermal Cycler (Applied Biosystems). The cycling conditions of all nested PCR reactions were similar, differing only with respect to annealing temperatures. First and second round PCR cycling conditions consisted of an initial hot start at 94°C for 5 min, followed by 45 cycles at 94°C for 25 s (denaturation), 35 s at the appropriate annealing temperature, 72°C for 65 s (extension), followed by a final elongation step at 72°C for 10 min. Annealing temperatures for the outer and inner primers were 59°C and 59°C respectively for K1,

TABLE 1 Nested PCR primer pairs used to amplify and sequence HHV-8 genome regions.

Locus	Primer	Sequence	Amplicon size (nts)	Reference
K1 (hemi-nested)	LGH 2089	GTTCTGCCAGGCATAGTC	1086	Tozetto-Mendoza et al.[27]
	K1AG1200AS	AGGCCATGCTGTAAGTAGCACGGTT		
	LGH2089	GTTCTGCCAGGCATAGTC	1029	
	LGH2088	AATAAGTATCCGACCTCAT		
VR1	LGH 2089	GTTCTGCCAGGCATAGTC	389	Tozetto-Mendoza et al.[27]
	LGH 2500	GTAACATGCTGACCACAAG		
	VR1BF	CTGGCGGCCCTTGTGTAAAC	340	
	VR1BR	GACTGTGTTTGATGGCTGTGC		
VR2	LGH 2507	CGTCTCGCCTGTCAAATC	335	Tozetto-Mendoza et al.[27]
	VR2AR	ACTGGTTGCGTATAGTCTTCC		
	VR2BF	GTATATGTTTTTGGGCGCGTTG	294	
	VR2BR	CCGTGCACAAATCGTGTAGGG		
T0.7/K12	LGH 2076	GCTGCAATGTACTGCCATG	646	Poole et al.[7]; Zong et al.[31]
	LGH 2075	CTCCAATCCCAATGCATGGA		
(5′)	LGH 2076	GCTGCAATGTACTGCCATG	390	
	LGH 2017	CATTCAGGACACGAGTTGC		
(3′)	LGH 2075	CTCCAATCCCAATGCATGGA	470	
	LGH2018	TGCTTTAATGCGGAGAGG		
ORF 75	KS 1000	CGGTTCGGTGGCATACAGGC	895	Kakoola et al.[23]; Poole et al.[7]
	KS 1034	CTGACTACAGAGGGTGTCCCCG	804	
	LGH 2000	GGAAACAGGGTGCTGTG		
	LGH 2034	CATGGCCTACGACGTCAC		
K15 (P)	K15P-OF	TGCAGGCTTGGTCATGGGTTAC	365	Whitby et al.[37]; Kakoola et al.[23]; Poole et al.[7]
	K15P-OR	GGGACCACGCTGCAATTAAATG		
	K15-3C	ACGCATACATGTACTGCCAC	285	
	K15-4C	CTTTGATATTGCCAGTGGTG		
K15 (M)	K15M-OF	TGTTGGTTGCAATGCTTAGGTG	486	
	K15M-OR	GCCTTTGCCAGTTGGAGTTTC		
	LGH 2473	CATGCAGCGAGCTTGAGA	370	
	LGH2474	CTTTGAGTACTGTTTGTG		

55°C and 59°C for VR1, 57°C and 55°C for VR2, 58°C and 57°C for T0.7 5′, 58°C and 55°C for T0.5 3′, 60°C and 57°C for ORF 75, 57°C and 55°C for K15 P, and 54°C and 57°C for K15 M. Careful measures were taken to prevent PCR contamination. Separate rooms were used for DNA extraction, reagent preparation and PCR amplification. All equipment was UV-irradiated. A nontemplate control was analysed with each PCR reaction. Agarose gel electrophoresis was performed using 1.5% (W/V) agarose gel containing Gel Red™ nucleic acid stain (Biotium) at 90 V for 90 min. Gels were visualised using ultraviolet (UV) light in a Gel Doc XR system (Bio-Rad). PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH), according to the manufacturer's instructions.

2.4 | DNA sequencing and phylogenetic analysis

Amplicons were diluted to 10 ng/μL and referred to a commercial genomics company (Source BioScience Ltd.) for Sanger sequencing using the second round primers shown in Table 1, diluted to a concentration of 3.2 ng/μL. The sequence chromatograms were analysed, and consensus sequences were obtained using Lasergene® SeqMan software (DNASTAR). The consensus sequences were aligned with HHV-8 reference strains using Lasergene® MegAlign software (DNASTAR). Reference strains used in this study are detailed in Supporting Information: Table 1. In total six phylogenetic trees were constructed; K1 (1029 nts), VR1 (340 nts), VR2 (294 nts), T0.7 (390 nts), ORF 75 (804 nts) and K15 (P, 285 nts; M, 370 nts). Phylogenetic reconstructions of the nucleotide alignments, employing maximum likelihood, were carried out using PAUP*4.0a165 software.[19] Multiple sequence alignments were performed using Clustal X.[20] The "best fit" models of evolution for the aligned datasets were determined using jModeltest version 2.1.10 by comparing the likelihood of the different parameters for all models of substitution.[21] The parameters of each model were then estimated using a heuristic search strategy and selecting those with the highest likelihood. Bootstrap resampling was carried out using PAUP* with 1000 replicates performed to examine the robustness of the trees produced.

3 | RESULTS

Eighty-eight HHV-8-positive ($n = 88$) specimens from 30 patients were available for use in the study. Sequencing data were obtained for 23 specimens from 19 patients. Six phylogenetic trees were constructed for the K1, VR1, VR2, T0.7, ORF 75, and K15 regions of

TABLE 2 HHV-8 subtypes identified in patients with HHV-8 related disease in Ireland.

Patient	Specimen	K1	VR1	VR2	T0.7/ K12	ORF 75	K15
1	SJH098	-	A	-	-	-	M
3	SJH113	-	A	A	J	C	-
5	SJH014	-	-	A	-	-	P
6	SJH063	-	-	A	-	-	-
10	SJH062	-	A	A	A/C	A	P
11	SJH068	-	-	-	Q	D	M
	SJH081	-	F	F	-	-	-
13	SJH136	-	A	A	A/C	-	-
14	SJH105	-	-	C	-	C	-
16	SJH101	-	C	C	-	A	-
18	SJH137	-	B	B	B	B	M
19	SJH131		C	C	A/C	A	-
	SJH133	C	-	-	-	-	P
20	SJH024	-	B	-	R	A	P
	SJH025	-	-	B	-	-	-
21	SJH053	-	A	A	-	A	P
23	SJH109	C	C	C	A/C	-	P
24	SJH050	C	C	C	A/C	A	P
27	SJH082	-	-	-	A/C	-	-
	SJH083	C	C	C	-	-	P
28	SJH139	-	C	-	-	-	-
29	SJH092	-	C	C	A/C	A	P
30	SJH089	C	C	C	J	C	M

the HHV-8 genome. Each tree was constructed using the sequences obtained for each region from this study, along with a selection of reference strains taken from GenBank to represent the appropriate subtypes for each region. According to the resultant phylogenetic trees, the HHV-8 sequences were classified into subtypes and subgroups. The subtypes that were determined for each patient are detailed in Table 2. The nomenclature used is that proposed by Hayward and Zong.[8]

Using nested PCR, complete or partial ORF K1 sequences were successfully obtained for 19 patients out of 30 (63%). Complete K1 sequences were obtained for five patients, VR1 sequences were obtained for 16 patients and VR2 sequences were obtained for 17 patients. Not all PCR attempts were successful, largely due to low viral loads in the remaining samples. For the samples where nested PCR was unsuccessful, the diagnostic real time PCR Ct values were all greater than 34. This was also the reason it was not possible to obtain the larger K1 sequence for a greater number of samples. Where it was possible to sequence the complete K1 gene, the Ct values for these specimens were all below 32, using real time PCR. Phylogenetic analyses of the K1, VR1 and VR2 sequences, along with 46 previously published sequences, are shown in Supporting Information: Figure 1, Figure 1 and Figure 2, respectively. Of the St. James's Hospital patient K1 sequences, seven (37%) clustered with subtype A, two (11%) with subtype B, nine (47%) with subtype C and one (5%) with subtype F (Figures 1–2). There were no subtype D or E sequences identified. The predominance of C and A subtypes is as expected for a Western European population. Although there were two B subtypes identified, there were no sequences that clustered with the A5 K1 subtype. Interestingly, all five specimens where the complete K1 gene was successfully amplified were identified as belonging to subtype C.

T0.7 sequences were obtained for 12 patients, seven (58%) clustered with subtype A/C, two (17%) clustered with subtype J, one (8%) clustered with subtype B, one (8%) clustered with subtype R and one (8%) clustered with subtype Q. There were no sequences identified belonging to subtypes D, E, F, N, or M. Phylogenetic analysis of these sequences, as well as 79 previously published sequences, is shown in Supporting Information: Figure 2. As expected, the majority of sequences clustered with subtype A/C, consistent with previous literature demonstrating a predominance of subtype A/C in T0.7 among KS patients in Europe.[11,22] The evidence to date has shown T0.7 subtypes A/C, J and K/M to be associated with HHV-8 genomes originating in Eurasia, D subtypes are noted to originate in aboriginal individuals from the Pacific Rim and subtypes B, F/G, R, Q, and N are found in sub-Saharan Africa.[8]

ORF 75 sequences were obtained for 12 patients, seven (58%) clustered with subtype A, one (8%) clustered with subtype B, three (25%) clustered with subtype C and one (8%) clustered with subtype D. There were no sequences belonging to subtypes M or N identified. Phylogenetic analysis of these sequences, as well as 59 previously published sequences, is shown in Supporting Information: Figure 3.

K15 sequences were determined for 13 of the St. James's Hospital patients, nine (69%) patients had the P allele, and four (31%) patients had the M allele. These results are demonstrated in Table 2 and Supporting Information: Figure 4.

4 | DISCUSSION

This is the first study detailing the molecular epidemiology of HHV-8 in the Republic of Ireland. Sequencing was successfully performed using specimens from 19 patients with HHV-8-related disease, to characterise four regions of the HHV-8 genome, K1, T0.7, ORF 75, and K15, by phylogenetic analysis. The data generated by this study demonstrate that a broad variety of HHV-8 subtypes are represented in the patient population in Ireland exhibiting HHV-8-related disease. Of note, although 30 patients were consented for this study, HHV-8 sequences were successfully obtained for only 19 of these patients, largely due to low viral loads in the remaining samples. This highlights the limitations of using plasma samples for HHV-8 subtyping, compared to biopsy samples from KS lesions, for example.

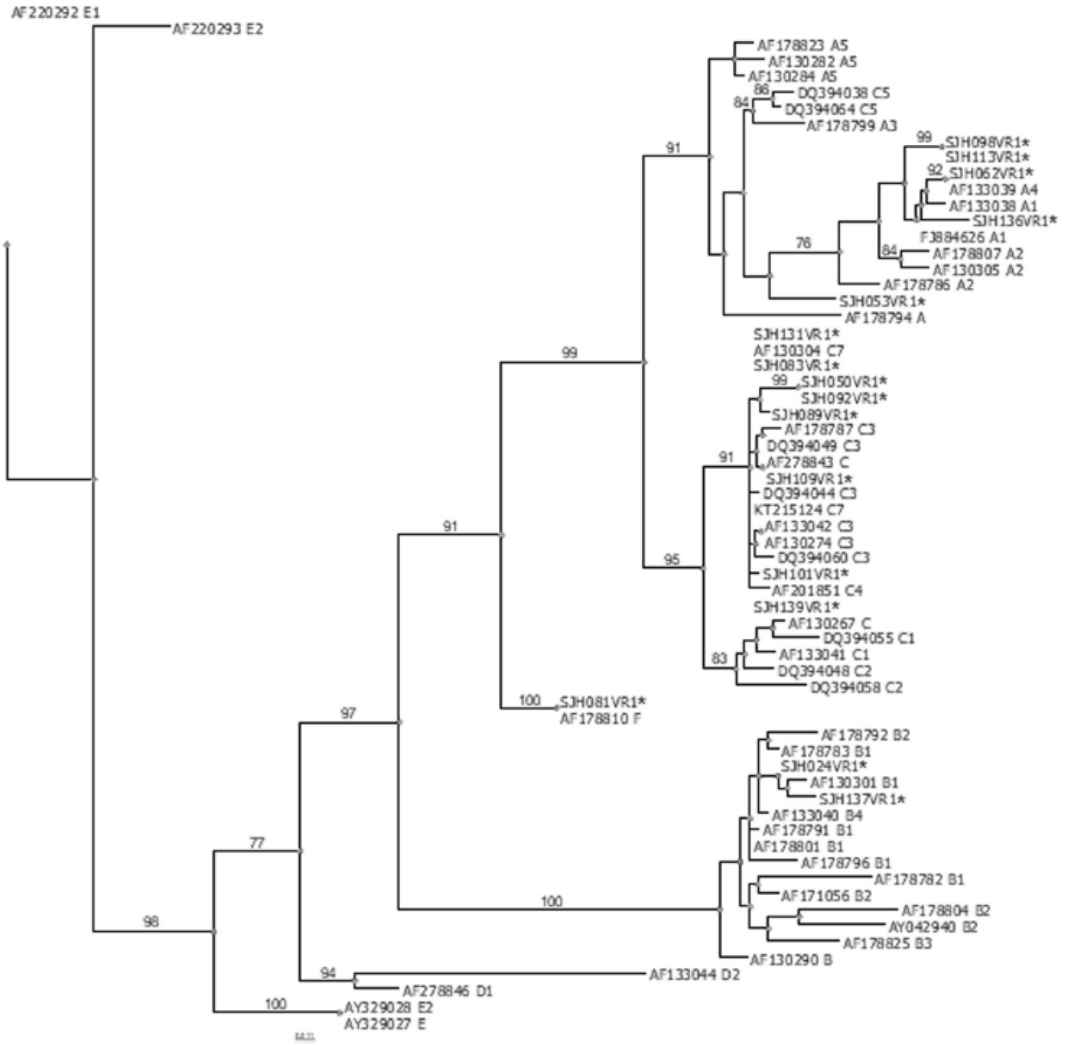

FIGURE 1 A maximum likelihood tree based on the VR1 hypervariable region of the K1 gene (340 nts) for 16 St. James's Hospital HHV-8-positive specimens, using AF220292 as an out-group. Bootstrap analysis was carried out using 1000 replicates of the data set and the values are written as a percentile beside the appropriate branch. Bootstrap values < 70% are not shown. Comparison was made to reference strains representing subtypes A to F, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH053VR1: PP129331; SJH062VR1: PP129332; SJH101VR1: PP129333; SJH109VR1: PP129334; SJH113VR1: PP129335; SJH092VR1: PP129336; SJH024VR1: PP129337; SJH050VR1: PP129338; SJH083VR1: PP129339; SJH131VR1: PP129340; SJH136VR1: PP129341; SJH137VR1: PP129342; SJH139VR1: PP129343; SJH089VR1: PP129344; SJH098VR1: PP129345; SJH081VR1: PP227227. SJH sequences are highlighted with a * symbol.

The current study provides some interesting observations regarding genetic diversity in HHV-8 in Ireland, a low prevalence country. The findings demonstrate significant heterogeneity of HHV-8 subtypes based on the four genome regions examined. There was one patient with an A subtype in K1 and an M allele in K15, and one patient with a C subtype in K1 and an M allele in K15. Two patients were identified to have divergent sequences across the different loci of the HHV-8 genome, possibly representing recombination. One patient with a K1 gene that clustered with the B subtype had a subtype R in K12, a subtype A in ORF 75 and a subtype P in K15. One patient was identified with a K1 gene that clustered with the F subtype. This patient had divergent sequences at the other loci, with a subtype Q in K12, subtype D in ORF 75 and a subtype M in K15.

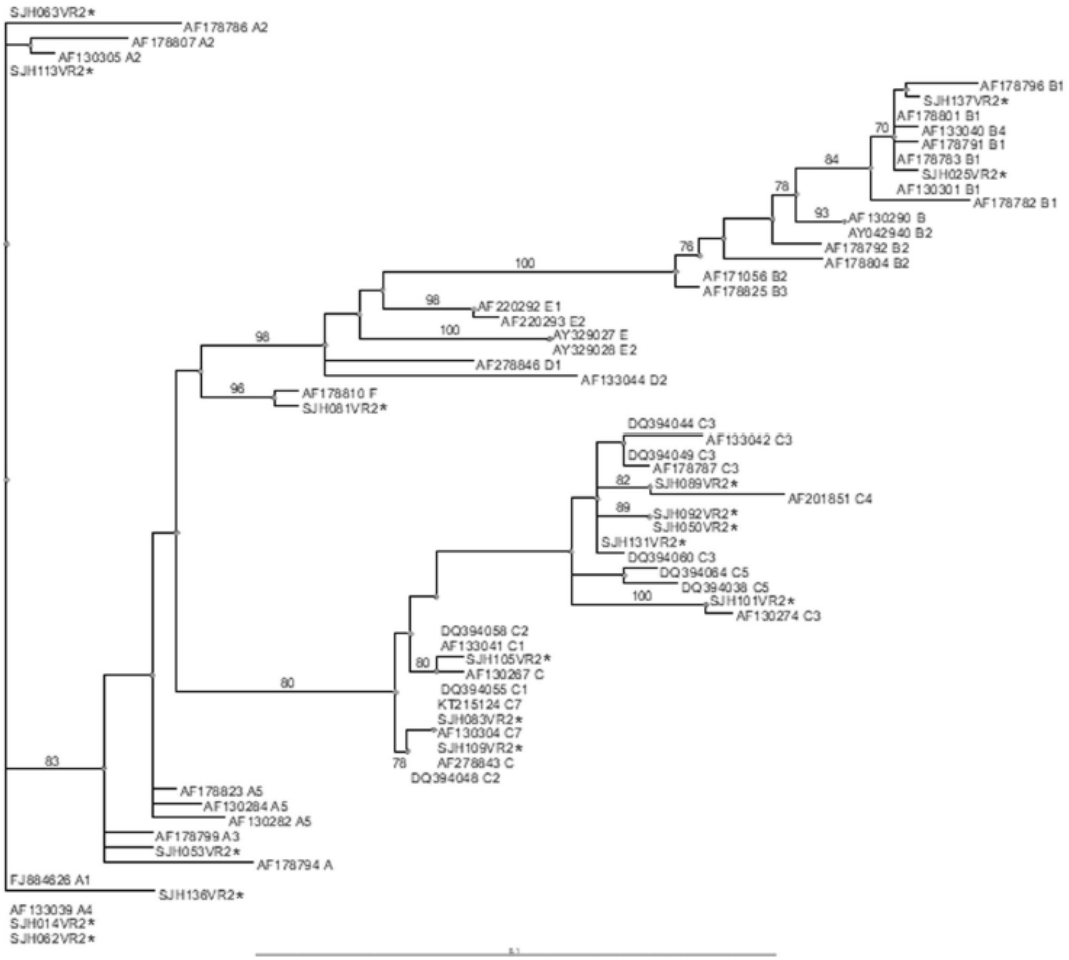

FIGURE 2 A maximum likelihood tree based on the VR2 hypervariable region of the K1 gene (294 nts) for 17 St. James's Hospital HHV-8-positive specimens, using SJH063VR2 as an out-group. Bootstrap analysis was carried out using 1000 replicates of the data set and the values are written as a percentile beside the appropriate branch. Bootstrap values < 70% are not shown. Comparison was made to reference strains representing subtypes A to F, indicated by their GenBank accession numbers. GenBank new sequence accession numbers: SJH014VR2: PP129314; SJH053VR2: PP129315; SJH062VR2: PP129316; SJH101VR2: PP129317; SJH113VR2: PP129318; SJH109VR2: PP129319; SJH092VR2: PP129320; SJH050VR2: PP129321; SJH063VR2: PP129322; SJH081VR2: PP129323; SJH083VR2: PP129324; SJH131VR2: PP129325; SJH136VR2: PP129326; SJH137VR2: PP129327; SJH025VR2: PP129328; SJH105VR2: PP129329; SJH089VR2: PP129330. SJH sequences are highlighted with a * symbol.

The earlier literature suggested that recombination was a relatively slow process in HHV-8. However, a number of more recent studies have suggested that the presence of recombinants may be more common than previously understood. Evidence of recombinants between subtypes has been demonstrated and recombination has been shown to occur at multiple different genetic loci.[23] Poole et al. originally demonstrated evidence for recombination in approximately 20–30% of HHV-8 strains and this was further demonstrated by Kakoola et al. who identified recombination in seven out of nine Ugandan strains studied.[7,23] These findings have been supported by further studies, including the use of whole-genome sequencing on fluid samples in patients with HHV-8-related diseases.[24–26] Sallah et al. performed a comprehensive recombination analysis, providing evidence of recombination throughout the HHV-8 genome, identifying recombination in eight genes.[25]

Subtype F in K1 has been reported rarely in the literature, in individual cases and small clusters.[11,16,27,28] Lacoste et al. identified a single patient from France with a K1 gene belonging to this subtype and Marshall et al. identified an identical K1 sequence in a US citizen

who was born in France, a patient with AIDS-KS.[18,29] Kajumbula et al. described the presence of the K1 F subtype in an African population from Uganda.[16] Tozetto-Mendoza et al. and Tornesello et al. also identified individual patients with subtype F K1 genes from Brazil and Kenya, respectively.[11,27] Jary et al. recently proposed a novel F2 variant, which was described in 5 Caucasian MSM living in Paris.[28] On further analysis of the sequence belonging to the K1 F subtype in the current study, it was found to cluster more closely with sequences belonging to the F2 subtype proposed by Jary et al., as opposed to the F1 subtype identified by Kajumbula et al. in Uganda. Of note, in the study by Jary et al., four out of five patients with the F2 subtype were immunocompromised and presented with severe HHV-8-related disease. Further work is warranted to investigate whether specific HHV-8 subtypes may be associated with severe disease.[28]

In the literature to date, isolates containing the K15 M allele are in the minority, with approximately 20% of all HHV-8 genomes characterised worldwide belonging to the K15 M subtype, so it is interesting that 31% of the sequences obtained in the current study were belonging to this group.[8] This may represent the changing and evolving epidemiology in Ireland resulting from changing patterns of migration. The four specimens in the current study containing the M allele (SJH098, SJH068, SJH137, SJH089) were each associated with a different K1 subtype, subtype A, subtype F, subtype B and subtype C, respectively. This suggests that the specimens are likely to be unrelated epidemiologically within this patient group. Nine specimens contained the P allele, and the majority of these were associated with an A or C K1 subtype, which is consistent with the international literature.[18] The exception is specimen SJH024, which belonged to K1 subtype B and contained the K15 P allele.

A limitation of this study was that it was not possible to identify sequences belonging to the K15 N subtype, as it was not possible to obtain the relevant primers to perform nested PCR for this subtype. Where it was not possible to determine the K15 subtype, it is conceivable that some of these HHV-8-positive specimens may have belonged to the N subtype. However, as there were no sequences identified belonging to subtype N in ORF 75, this is unlikely to be the case. Designing primers to identify the K15 N subtype was, therefore, not felt to be warranted in a Western European population such as that of the current study.

Early studies on the molecular epidemiology of HHV-8 demonstrated clear geographical patterns with respect to HHV-8 subtypes. For example, in K1, B and A5 subtypes were shown to predominate in sub-Saharan Africa, subtype D among native populations of South Asia, Australia and the Pacific, with subtypes A and C predominating in the majority of European and Asian countries.[7,9,13,18,30,31] These patterns are felt to be due to the original migration patterns of modern humans.[31] While there was evidence at this time of some degree of geographical dissemination, for the most part, subtypes tended to conform to these patterns. It is interesting to note, therefore, in the current study, the diverse findings of different K1 subtypes in Ireland, as well as their linkage to different T0.7, ORF 75 and ORF K15 subtypes, which may reflect global migration and population changes.

In K15, the P subtype has been shown to predominate in regions other than Central and West Africa, while M and P subtypes have been present in equal numbers in samples from Central and West Africa.[18] It is unclear whether the P and M K15 alleles are linked to the corresponding K1 subtype, with conflicting findings reported in different studies.[7,18] Lacoste et al. found the significant majority of C and A K1 subtypes to be linked with a P K15 subtype, while B and A5 subtypes showed the P and M alleles to be equally represented.[18] This is in contrast to the findings of Poole et al. who failed to demonstrate a significant link between K1 and K15 subtypes.[7] Lacoste et al. raised the possibility of a functional difference between the P and M subtypes, potentially conferring higher transmissibility to the P subtype, evidenced by the overall predominance of the P subtype in studies performed worldwide.[18] The relatively high rate of M alleles identified in the current study is in contrast to the findings in a number of European studies, for example in France and Greece, where the P allele was found to predominate.[11,18] However, the findings in this study are more in keeping with rates reported in European countries such as Russia and Scandinavia.[18,32] This may be due to population movements into Ireland from Eastern Europe and Western Africa, regions known to have higher rates of K15 M alleles.[10,18] Studies by Zong et al. and Zang et al. both identified relatively high proportions of K15 M alleles among populations in several states of the USA, specifically Maryland, Tennessee and Texas.[31,33] This finding has been attributed to the diverse populations in these states from varying ethnic backgrounds.

The HHV-8 subtypes identified in this study reflect the current population in Ireland. The diversity of subtypes and the presence of subtypes such as B and F in K1 and subtype M in K15, which are unusual to find in a Western European setting, most likely reflect the changing population in Ireland, with increasing inward migration due to Ireland's economic development.[11]

Despite evidence initially supporting HHV-8 subtype linkage to the geographic origin of the host individual among immigrant populations, interethnic transmission is known to occur, as evidenced by multiple previous studies, including a report by Nascimento et al. on the Brazilian population.[33–36] The variety of subtypes identified in the current study using phylogenetic analysis is likely to represent individuals who have been infected with HHV-8 in their native countries, as well as local interethnic transmission in Ireland.

Of note, the sequences obtained represent a relatively small sample of patients attending a single specialised centre, incorporating a large Genitourinary Medicine and Infectious Diseases unit, and therefore may not be an accurate representation of HHV-8 subtypes circulating in Ireland as a whole. This is a limitation of the study and it would be an interesting avenue for future research to include patients from other referral centres nationally.

In conclusion, this study has classified HHV-8 sequences from 19 patients with HHV-8-related disease into their respective subtypes using phylogenetic analysis. K1 subtype C has been found to be the most prevalent subtype (47%), followed by subtype A (37%). This is consistent with similar European populations previously studied.[8,11] One K1 sequence clustered with the rare subtype F. Despite the

presence of a significant Brazilian population in Ireland, there were no subtype D or E sequences identified. K15 subtype P was found to be more prevalent than subtype M. However, the prevalence of 31% for subtype M was higher than expected for a Western European population.[11,18]

Studies such as this contribute greatly to our understanding of this virus and its epidemiology, providing additional insight into the geographical distribution of HHV-8 subtypes in different parts of the world. It is interesting to note the existence of a high level of genetic variability in a very low prevalence country such as Ireland, providing valuable additional information on the present-day global epidemiology of this virus. This study is the first of its type in Ireland and will inform future molecular studies of HHV-8.

AUTHOR CONTRIBUTIONS

Study concept and design: Brendan Crowley, Bairbre Ni Laoi, Sadhbh O'Rourke. *Methodology*: Brendan Crowley, Bairbre Ni Laoi, Sadhbh O'Rourke, Susan Clarke. *Recruitment*: Sadhbh O'Rourke, Susan Clarke. *Laboratory analysis*: Sadhbh O'Rourke, Bairbre Ni Laoi. *Data analysis and drafting of article*: Sadhbh O'Rourke, Bairbre Ni Laoi. *Critical revision of article*: Brendan Crowley. *Study oversight*: Brendan Crowley. All authors read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval for this study was obtained from Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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