



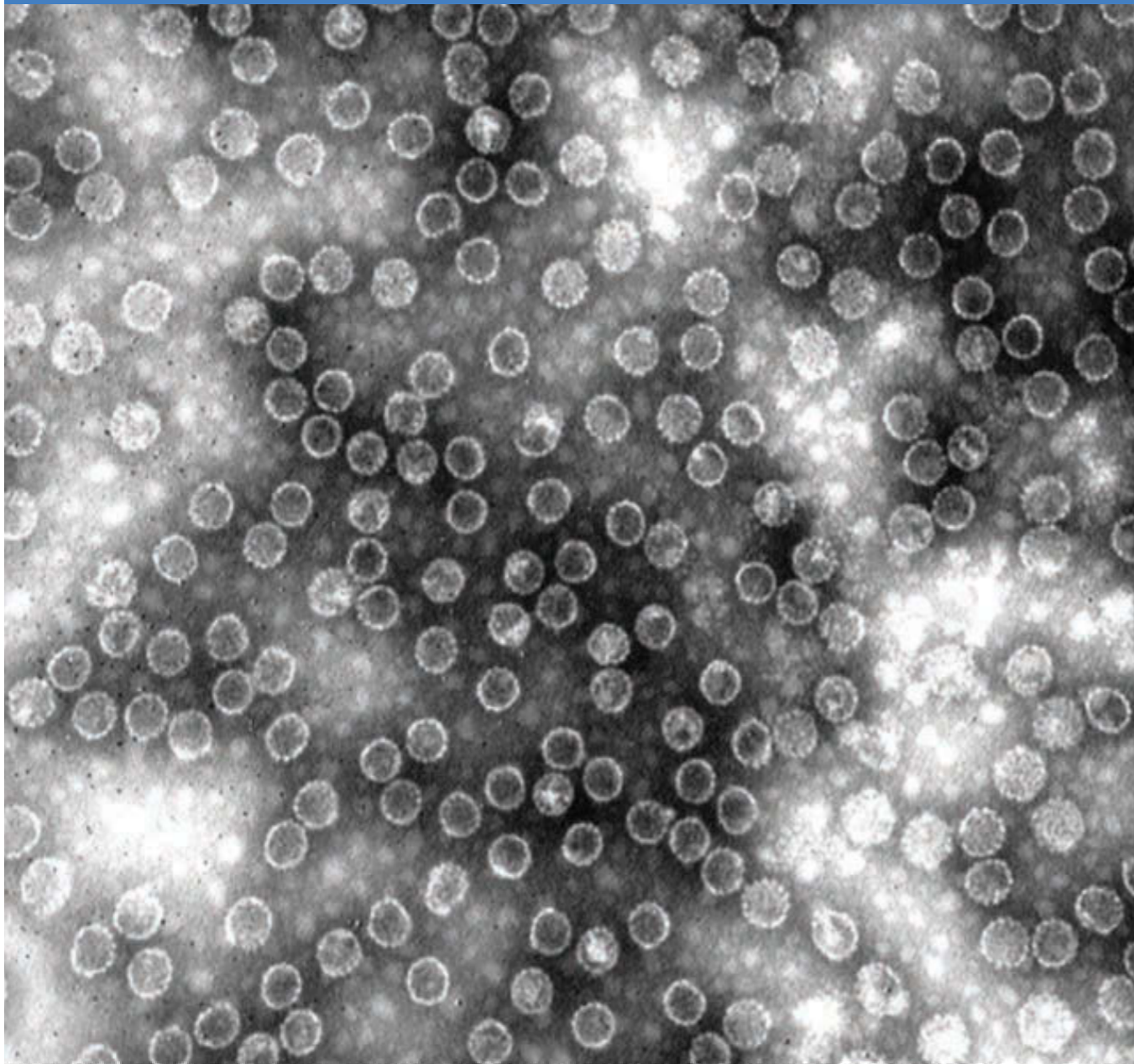
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M.Reza Sadeghi

Molecular Biology and Clinical Occurrence of Emerging Human Polyomaviruses

## Molecular Biology and Clinical Occurrence of Emerging Human Polyomaviruses

Mohammadreza Sadeghi



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Department of Virology  
Haartman Institute  
University of Helsinki  
Finland

# **Molecular Biology and Clinical Occurrence of Emerging Human Polyomaviruses**

**Mohammadreza Sadeghi**

ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in Lecture Hall 1, Haartman Institute, Haartmaninkatu 3, on September 12<sup>th</sup>, 2014, at 12 noon.

Helsinki 2014

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**To my family, Neda and Pärham**

*especially*

**to the memory of my mother**

To be joyous is an art,  
should other hearts beat  
with the drum of happiness  
inspired by us,  
life would be the unique  
scene of art.

You sing your song and depart,  
the scene is eternal.

Greet the song that stays  
in the people's memory to infinity.

Jaleh Esfahani (1921-2007)

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## ABSTRACT

Human polyomaviruses (HPyVs) are known to cause benign initial infection at an early age. They have a high prevalence in the population with frequent incidences of reactivation, and pathologic consequences in those who are elderly or immunosuppressed. Thus far 12 HPyV species have been known. The first two HPyVs, JC virus (JCPyV) and BK virus (BKPyV) were described in 1971. They are associated with specific diseases, progressive multifocal leukoencephalopathy (PML) and polyomavirus-associated nephropathy (PVAN), respectively. Since 2007, 10 additional HPyVs have been identified by molecular genetic techniques. Study of the role of these new viruses in human disease is a new challenge in the HPyV area. The Karolinska Institute (KIPyV) and Washington University (WUPyV) viruses were discovered in respiratory secretions of patients with unidentified causes of pneumonia. Other examples of newly found HPyVs are the Merkel cell polyomavirus (MCPyV) in Merkel cell carcinoma (MCC), and trichodysplasia spinulosa-associated polyomavirus (TSPyV) in *Trichodysplasia spinulosa* (TS), both skin diseases in immunocompromised patients. The potential pathogenicity of the remaining HPyVs awaits assessment. Seroprevalence studies indicate that HPyVs infect 30 to 90% of the general population and are transmitted apparently independently of one another. Thus far, although their modes of transmission have yet to be resolved, HPyVs are frequently detectable at different body sites and in bodily fluids of healthy immunocompetent individuals, including the skin, hair follicles, saliva, urine, feces, and respiratory secretions, and they can also be found in the environment.

To investigate their tropism, persistence site, reactivation, transmission route, and contribution to disease, we have developed for KI, WU, MC, and TS polyomaviruses comprehensive diagnostic methods. We studied the occurrence of their DNAs and antibodies to these viruses from birth to death.

In our study, using recombinant fusion protein antigens, IgG antibodies were detectable for KIPyV in 55% and for WUPyV in 69%. Rapidly increasing and high IgG seroprevalences showed that KIPyV and WUPyV are acquired early in childhood and supported the notion that these polyomaviruses are widespread. Our results not only suggested the significance of protein conformation in immunoreactivity of VP1, the major capsid protein, but also pointed to the antigenic importance of the minor proteins VP2 and VP3.

Among aging individuals, by employment of recombinant virus-like particles (VLPs) as antigens in ELISA, MCPyV and TSPyV IgG seroprevalences were 59.6% and 67.3%. Among 462 pregnant women, MCPyV IgG seroprevalence was 46% and in constitutionally healthy individuals, TSPyV IgG seroprevalence among children was 39% and among adults 70%.

In addition, our DNA PCR studies of respiratory specimens indicated exposure to KIPyV and WUPyV, as well as to MCPyV. We observed MCPyV and TSPyV DNAs particularly often in tonsillitis or hypertrophic tonsillar tissues, unlike for KIPyV or WUPyV. MCPyV and TSPyV DNA in the tonsillar biopsies suggested lifelong persistence in lymphoid tissue or mucosa. MCPyV DNA occurred in tonsils more frequently in adults than in children. By contrast, WUPyV DNA was found preferentially in children. MCPyV occurred also in nasal swabs and NPAs, at a frequency similar to that of KIPyV and WUPyV. The tonsil may be an initial site of WUPyV infection and a site of MCPyV persistence. On the other hand, TSPyV PCR positivity of tonsillar samples of individuals with long-term immunity provided evidence of TSPyV persistence in tonsils and suggests lymphoid tissue as a latency site also for this emerging human pathogen.

Our results indicated that MCPyV DNA, unlike TSPyV DNA, occurs in low copy numbers in serum in a notable proportion of aging individuals. Whether the enhanced viral replication in our elderly participants is a reflection of waning immune surveillance and is correlated with increased MCC risk deserves further exploration.

Furthermore, to investigate the frequency of fetal infections by these new viruses, we sought the KIPyV, WUPyV, and MCPyV DNAs by PCR, from 535 fetal autopsy samples (heart, liver, placenta) from intrauterine fetal deaths (IUFDs), miscarriages, or induced abortions. Examining by PCR 535 fetal autopsy samples and the corresponding pregnant women by serology, we obtained data to rule out vertical transmission of the new polyomaviruses KI, WU, and MC. Our data suggest that none of the three often cause miscarriages or IUFDs, nor are they transmitted to fetuses.

By means of new molecular methods several emerging polyomaviruses have been discovered. Although it is still too early to reach a conclusion on this point, it seems apparent that these novel viruses follow the pattern established for the JC and BK polyomaviruses: a mild initial infection at an early age, high prevalence in the general population, lymphoid tissue as a latency site, and pathologic consequences among the immunosuppressed and/or the elderly.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their Roman numerals. The copyright holders have given their permission to reprint the publications herein.

**I.\*Kantola K, Sadeghi M, Lahtinen A, Koskenvuo M, Aaltonen LM, Möttönen M, Rahiala J, Saarinen-Pihkala U, Riikonen P, Jartti T, Ruuskanen O, Söderlund-Venermo M, Hedman K.** Merkel cell polyomavirus DNA in tumor-free tonsillar tissues and upper respiratory tract samples: implications for respiratory transmission and latency. *J Clin Virol* 2009;45,292-5.

**II.Kantola K, Sadeghi M, Ewald MJ, Weissbrich B, Allander T, Lindau C, Andreasson K, Lahtinen A, Kumar A, Norja P, Jartti T, Lehtinen P, Auvinen E, Ruuskanen O, Söderlund-Venermo M, Hedman K.** Expression and serological characterization of polyomavirus WUPyV and KIPyV structural proteins. *Viral Immunol* 2010;23,385-93.

**III.Sadeghi M, Riipinen A, Väisänen E, Chen T, Kantola K, Surcel HM, Karikoski R, Taskinen H, Söderlund-Venermo M, Hedman K.** Newly discovered KI, WU, and Merkel cell polyomaviruses: no evidence of mother-to-fetus transmission. *Virology* 2010;7,251.

**IV.Sadeghi M, Aronen M, Chen T, Jartti L, Jartti T, Ruuskanen O, Söderlund-Venermo M, Hedman K.** Merkel cell polyomavirus and trichodysplasia spinulosa-associated polyomavirus DNAs and antibodies in blood among the elderly. *BMC Infect Dis* 2012;12,383.

**V.Sadeghi M, Aaltonen LM, Hedman L, Chen T, Söderlund-Venermo M, Hedman K.** Detection of TS polyomavirus DNA in tonsillar tissues of children and adults: Evidence for site of viral latency. *J Clin Virol* 2014;59,55-8.

*\*Equal contribution of first and second authors.*



## ABBREVIATIONS

aa	amino acids
BLAST	Basic Local Alignment Search Tool
BKPyV	BK polyomavirus
BKV	BK virus
bp	base pair
BMT	bone marrow transplant
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
DTS	digital transcriptome subtraction
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
HPV	human papillomavirus
HPyV6	human polyomavirus 6
HPyV7	human polyomavirus 7
HPyV9	human polyomavirus 9
HPyV10	human polyomavirus 10
HPyV12	human polyomavirus 12
HSCT	hematopoietic stem cell transplantation
ICTV	International Committee on Taxonomy of Viruses
JCPyV	JC polyomavirus
JCV	JC virus
KIPyV	KI polyomavirus
LTag	large tumor antigen
LPyV	lymphotropic polyomavirus
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MPyV	mouse polyomavirus
MWPyV	Malawi polyomavirus
MXPyV	Mexico polyomavirus
MDA	multiple displacement amplification
MS	multiple sclerosis
MK	monkey kidney
NP	nasopharyngeal
NPA	nasopharyngeal aspirate
NCCR	non-coding control region
PCR	polymerase chain reaction
PML	progressive multifocal leukoencephalopathy
PyV	polyomavirus
PyVAN	polyomavirus-associated nephropathy
PyVHC	polyomavirus hemorrhagic cystitis
qPCR	quantitative real-time PCR

RT	renal transplant
RDA	representational difference analysis
Rb	retinoblastoma
RCA	rolling circle amplification
sTag	small tumor antigen
SA12	simian agent 12
SDS-page	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SCC	squamous cell carcinoma
STLPyV	St Louis polyomavirus
SV40	simian vacuolating virus 40
TS	trichodysplasia spinulosa
TSPyV	trichodysplasia spinulosa polyomavirus
VP1	viral capsid protein 1
VLP	virus-like particle
WUPyV	WU polyomavirus
WHIM	warts, hypogammaglobulinaemia, infections, myelokathexis

## INTRODUCTION

Polyomaviruses (PyVs) are ubiquitous; they infect many different mammalian species including humans. For about 35 years (1971-2007) the *Polyomaviridae* family was thought to include only two viruses in humans; the BK polyomavirus (BKPyV or BKV) and the JC polyomavirus (JCPyV or JCV). These two human viruses are associated with serious diseases, respectively including polyomavirus-associated nephropathy (PVAN) and progressive multifocal leukoencephalopathy (PML).

Since 2007, with the aid of new molecular techniques, particularly deep sequencing, subtractive hybridization, rolling circle amplification (RCA), degenerate polymerase chain reaction (PCR), pyrosequencing, multiple displacement amplification (MDA), and complementary DNA library screening, the number of known human members of the *Polyomaviridae* family has grown six-fold, by the discovery of 10 new species infecting humans.

In 2007, the first two novel human polyomaviruses (HPyVs), Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV) were identified in children suffering from acute respiratory tract infections. A year later, the carcinogenic Merkel cell polyomavirus (MCPyV) was discovered, and shown to be mutated and chromosomally integrated in Merkel cell carcinoma (MCC).

In 2010, human polyomaviruses 6 (HPyV6) and 7 (HPyV7) were discovered on the forehead skin of healthy volunteers. In that same year, another skin-associated polyomavirus, trichodysplasia spinulosa-associated polyomavirus (TSPyV), was isolated from the face of an immunocompromised patient suffering from trichodysplasia spinulosa (TS). In 2011, human polyomavirus 9 (HPyV9) was amplified from the blood of a renal transplant patient.

HPyV10 and its two isolates Malawi polyomavirus (MWPyV) and Mexico polyomavirus (MXPyV) were identified in 2012, and their whole-genome identity criteria proved them to belong to a single species. MXPyV-like MWPyV was identified in stool samples of children; analysis of condyloma specimens from a patient with warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome demonstrated the presence of HPyV10.

In December 2012, the 11th novel polyomavirus was isolated from stool specimens obtained from both the Gambia and the United States. This was provisionally called St.

Louis polyomavirus (STLPyV); finally in March, 2013, the last novel polyomavirus thus far was initially identified in resected human liver tissue and provisionally called human polyomavirus 12 (HPyV12).

With the discovery of 10 new viruses, the nomenclature of polyomaviruses has continued to evolve. The *Polyomaviridae* Study Group of the International Committee on Taxonomy of Viruses (ICTV) proposed several criteria for polyomavirus classification, including dividing the polyomaviruses into three genera, with *Orthopolyomavirus* and *Wukipolyomavirus* containing all the mammalian species and *Avipolyomavirus* restricted to bird polyomaviruses. In addition, the committee proposed that for a polyomavirus to be designated a unique species within the family; the whole-genome nucleotide sequence must have less than 81% nucleotide identity to the sequence of known polyomavirus species.

During the past four decades, much effort has been focused on previously discovered JCPyV and BKPyV. Basic questions about the viruses themselves and their interactions with the host still remain unanswered. The recent discoveries of new HPyVs certainly raise several fundamental questions. The involvement of these novel HPyVs in a disease and their prevalences remain to be determined. Furthermore, the routes of transmission, latency sites, host cells, and epidemiology of these emerging viruses will need to be determined. We therefore developed for four of the newly found HPyVs (KIPyV, WUPyV, MCPyV, and TSPyV) comprehensive diagnostics, and examined their occurrence from life to death and possible association with other diseases and disorders.

It is impossible to tell how many new HPyVs are yet to be found; thus far 10 new viruses have been reported since 2007. This literature review focuses almost entirely on the 12 HPyVs discovered thus far in two chapters. The first section, however, discusses the biology of polyomaviruses in general including the history, classification and phylogeny, genome and capsid structure, pathogenicity, associated diseases, cancer and clinical aspects. The second section of the literature review deals specifically with the 12 HPyVs and focuses on the discovery methods, molecular and serological occurrence and their clinical significance.

# REVIEW OF THE LITERATURE

## 1. BIOLOGY OF POLYOMAVIRUSES

### 1.1. Brief history, classification, and phylogeny

Polyomaviruses are viruses known since 1953 which have the ability to cause multiple tumors in rodents. These small DNA tumor viruses have been subjects of intensive study since their discovery (Gross, 1953). The derivation of the family name *Polyomaviridae* originates from the observation that the first-discovered member of this family could induce multiple (poly) tumors (oma) in mice (Eddy et al., 1958). The first polyomavirus was the mouse polyomavirus (MPyV), found over 50 years ago. When scientists independently sought to isolate a leukemogenic viral agent by injecting cell-free extracts from leukemic AKR mice into newborn mice, they by chance discovered this polyomavirus (Gross, 1953, Stewart, 1953). MPyV is a ubiquitous and persistent pathogen in wild mice. Its natural transmission most likely occurs through a respiratory route. MPyV is shed from infected carriers via their urine and also occurs in saliva and feces. We do, however, lack understanding of the mechanism by which these lytic viruses maintain life-long persistent infections in their natural host reservoirs (Teunissen et al., 2013).

The taxonomy of polyomaviruses grouped them originally together with the papillomaviruses under the designation of papovaviruses until their separation into two separate and distinct families in 2000 (<http://ICTVonline.org>). The *Papovaviridae* family comprised papillomaviruses, polyomaviruses, and simian virus 40 (SV40). The family name came from three abbreviations: Pa for (rabbit) papillomavirus, Po for (mouse) polyomavirus, and Va for (simian) vacuolating agent 40, later named SV40.

These two first members of the polyomavirus family, MPyV and SV40, were identified as filterable agents that could cause tumors in newborn mice and hamsters (Gross, 1953, Eddy et al., 1958, Sweet and Hilleman, 1960). SV40 was first isolated in 1960 from cultures of Rhesus monkey kidney cells which were being used to produce poliovirus vaccines (Sweet and Hilleman, 1960). Thus far, however, no evidence implicating SV40 as a human pathogen has been convincing (Atkin et al., 2009). Although many people may have been exposed to SV40 by a contaminated polio vaccination, evidence of widespread SV40 infection in the human population, of increased tumor incidence in those receiving contaminated vaccine, or any direct role for SV40 in human cancer remains insufficient

(Poulin and DeCaprio, 2006). Historically, polyomaviruses, especially SV40, have improved our understanding of basic eukaryotic cellular processes, but they need to be studied as human pathogens as well.

Polyomaviruses are ubiquitous, and in addition to MPyV and SV40, other PyVs are detectable in many vertebrate hosts including human beings, other nonhuman primates, mice, cattle, birds, bats, rabbits, and rodents (Krumbholz et al., 2009, Johne et al., 2011). At least 32 different polyomaviruses hitherto known infect both mammalian and avian species (Figure 1) (Feltkamp et al., 2013); among them are 12 PyVs detected in humans (Gardner et al., 1971, Padgett et al., 1971, Allander et al., 2007a, Gaynor et al., 2007, Feng et al., 2008, Schowalter et al., 2010, van der Meijden et al., 2010, Scuda et al., 2011, Buck et al., 2012, Siebrasse et al., 2012b, Yu et al., 2012b, Korup et al., 2013, Lim et al., 2013).

The explosion of discovery of new viruses has led to a steady evolution in the classification of polyomaviruses. First divided into three major clades (genetically-related groups), the family of *Polyomaviridae* included the SV40 clade, the avian clade, and the murine polyomavirus clade (Perez-Losada et al., 2006). In October 2010, the polyomaviridae study group of the ICTV recommended the naming of all polyomaviruses to be standardized (Figure 2) (Johne et al., 2011). Taxonomical revisions meant that the family *Polyomaviridae* now contains two mammalian genera designated *Orthopolyomavirus* and *Wukipolyomavirus* (Figure 2). The *Orthopolyomavirus* genus includes not only BKPyV and JCPyV, but also the recently discovered human Merkel cell polyomavirus (MCPyV) and the trichodysplasia spinulosa-associated polyomavirus (TSPyV). The *Wukipolyomavirus* genus comprises the KI polyomavirus (KIPyV), WU polyomavirus (WUPyV), and Human polyomaviruses 6, 7, and 9 (Johne et al., 2011). Three further polyomaviruses isolated from humans MW polyomavirus, STL polyomavirus, and HPyV12, have not been fully classified or officially thus far accepted; hence, the taxonomy of this family is ongoing. However, the current official ICTV report regarding the family of *Polyomaviridae* still holds only one *Polyomavirus* genus and 13 species, of which 3 are human viruses (<http://ICTVonline.org>).

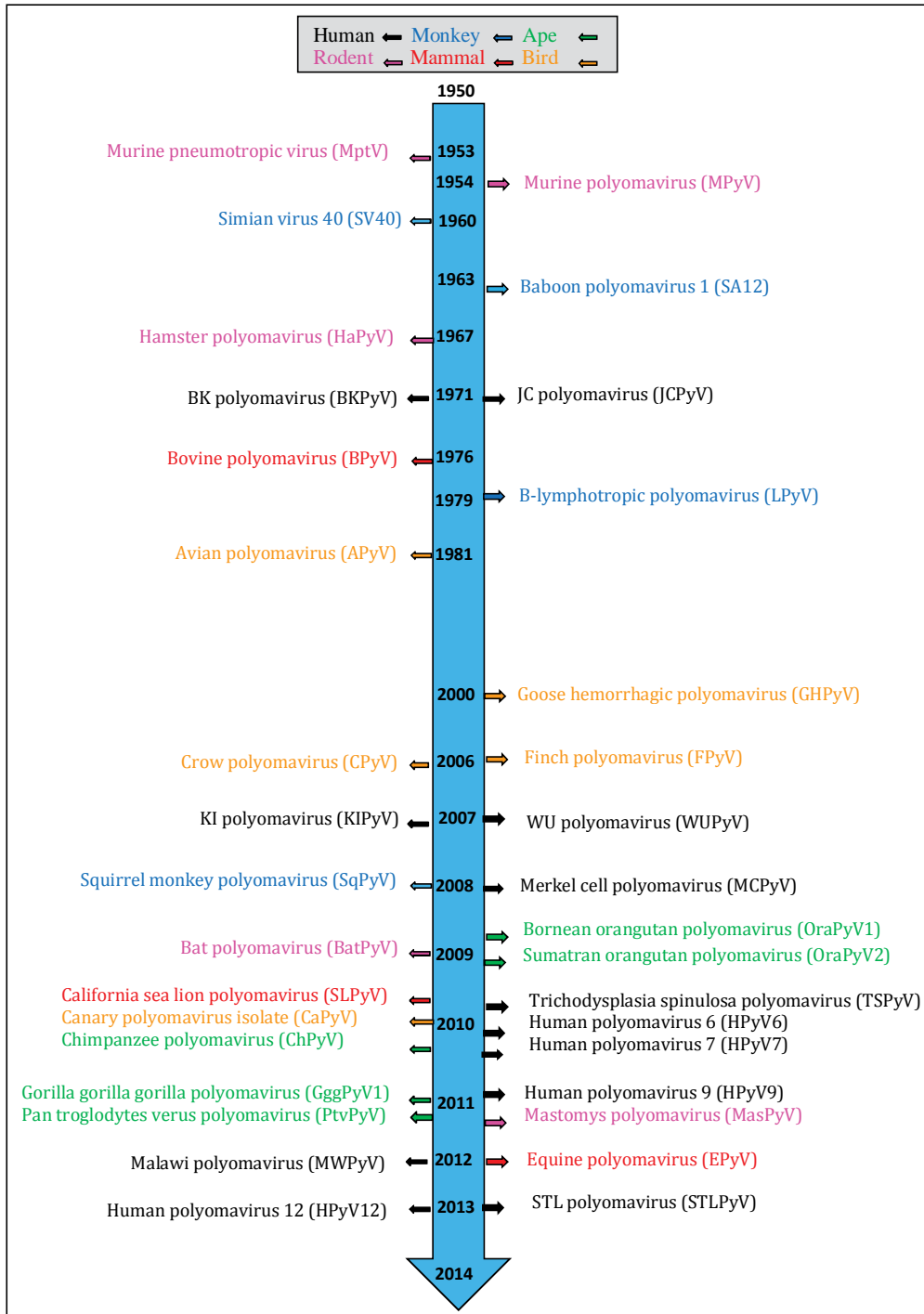


Figure 1. Time-line indicating 32 (putative) polyomavirus species discovered up to June 2014.

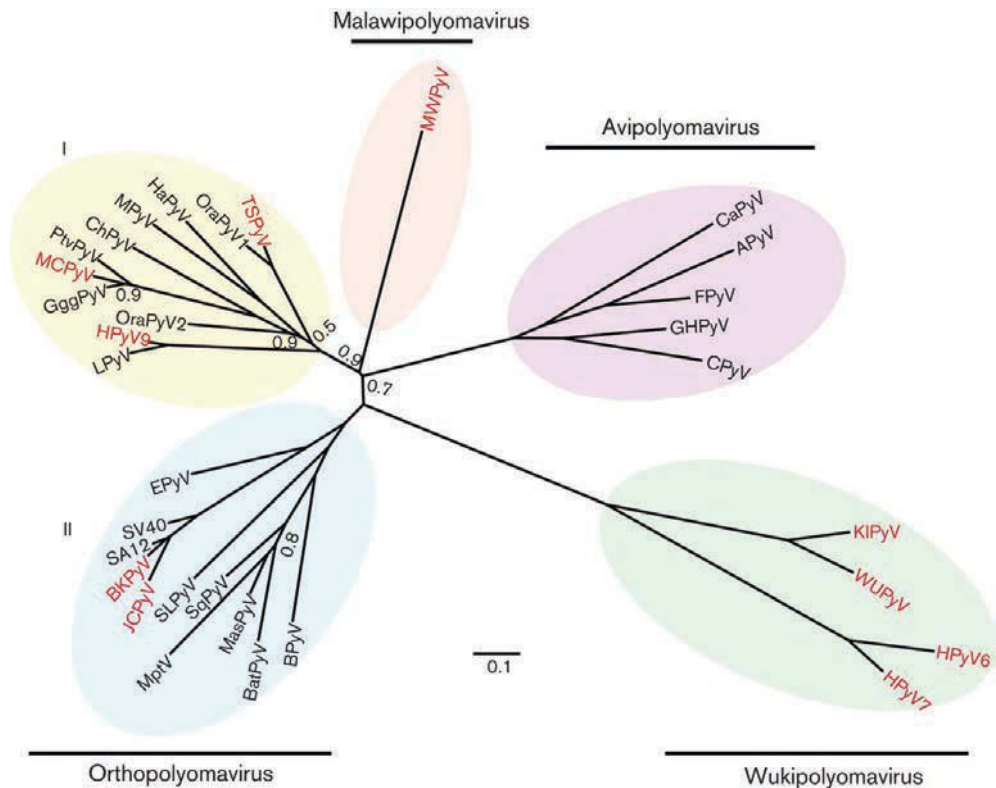


Figure 2. Unrooted phylogenetic tree of 32 (putative) polyomavirus species based on LT, VP1, and VP2 protein comparisons. Reproduced with permission from Feltkamp, M.C., Kazem, S., van der Meijden, E., Lauber, C., Gorbalenya, A.E., 2013. From Stockholm to Malawi: recent developments in studying human polyomaviruses. *The Journal of General Virology* 94, 482-496.

## 1.2. Discovery of human polyomaviruses

Many viruses cannot be cultivated in the laboratory and can be characterized only by molecular methods. Recent advances in molecular methods have revolutionized the discovery of new viruses. These methods have, now also for polyomaviruses, gained increasing use (Figure 3).

The gap between the two initially discovered HPyVs and the first new ones discovered in 2007 is over 35 years (Table 1). Reports on the first two, JCPyV and BKPyV, appeared concurrently in 1971 in *The Lancet* (Gardner et al., 1971, Padgett et al.,



1971). JCPyV was cultured from PML brain tissue in a patient with Hodgkin's lymphoma; BKPyV was isolated from the urine of a nephropathic kidney-transplant patient. These two viruses were first identified by traditional methods of cell culture in 1971. Evolution of methods of nucleic acid amplification and detection bypassed the need for cytopathic changes observed in cell culture in order to detect the presence of a virus. Recent versions of these molecular techniques allow sensitive and high throughput analysis of large numbers of clinical samples, revolutionizing the means of virus discovery. Because nucleic acids isolated from both diseased and healthy persons' tissues can serve as a template for such techniques, the identified viruses are therefore not necessarily pathogenic. Reduction in the content of their hosts' genomic DNA makes possible this and other strategies to enrich the original sample for viral DNA or RNA (Delwart, 2007).

One such large-scale molecular virus-screening system led, in 2007, to the discovery of two new HPyVs: KIPyV and WUPyV. These were named after the institutions in Stockholm, Sweden, and in St. Louis, Missouri, USA, where the identifications took place (Allander et al., 2007a, Gaynor et al., 2007). Both viruses came from children with respiratory tract infections, though whether these viruses are responsible for symptomatic infection is yet unknown (Norja et al., 2007).

MCC is a rare but aggressive human skin cancer usually affecting those who are elderly or immunosuppressed. Studying MCC samples by digital transcriptome subtraction (DTS), detected a fusion transcript between a previously undescribed virus T antigen and a human-receptor tyrosine phosphatase (Feng et al., 2008). Their further work led to the identification and sequence analysis of an about 5 kilobase (kb) genome of a previously unknown MCPyV.

One of the new molecular techniques allowing the discovery of several new polyomaviruses is RCA (Schowalter et al., 2010, van der Meijden et al., 2010, Buck et al., 2012, Siebrasse et al., 2012b). Development and improvement of the RCA technique allowed the isolation from healthy human skin swabs of circular DNA viral genomes of HPyV6 and HPyV7 (Schowalter et al., 2010). RCA also allowed the discovery of the new HPyV associated with *trichodysplasia spinulosa* (TS), TSPyV, and identified it as the possible cause of TS in an immunocompromised patient (van der Meijden et al., 2010). RCA analysis also introduced two almost identical previously unknown polyomaviruses called Malawi polyomavirus (MWPyV) in stool from a 1-year-old healthy child and human HPyV10 from an anal wart of an immunocompromised patient with warts,

hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome (Siebrasse et al., 2012b, Buck et al., 2012).

Other sequence-independent approaches like random PCR, degenerate oligonucleotide-primed PCR, and multiple displacement amplification (MDA) also can detect totally novel HPyVs (Scuda et al., 2011, Yu et al., 2012b, Korup et al., 2013, Lim et al., 2013). Random PCR and an unbiased deep sequencing approach led to the identification, in stool samples from children, of the Mexico polyomavirus (MXPpyV), a closely related variant of the MWPpyV and HPyV10 (Yu et al., 2012b).

Shotgun 454 pyrosequencing of DNA amplified by multiple displacement amplification techniques (MDA) applied to the stool of a healthy 15-month-old child in Malawi led to the identification of HPyV11 or STLPyV. STLPyV also were detected in clinical stool specimens from the USA and the Gambia at up to 1% frequency (Lim et al., 2013).

A generic PCR assay using degenerate and deoxyinosine-substituted primers identified two novel viruses; the HPyV9 from a kidney transplant patient receiving immunosuppressive treatment (Scuda et al., 2011) and the HPyV12 from resected human liver tissue (Korup et al., 2013).

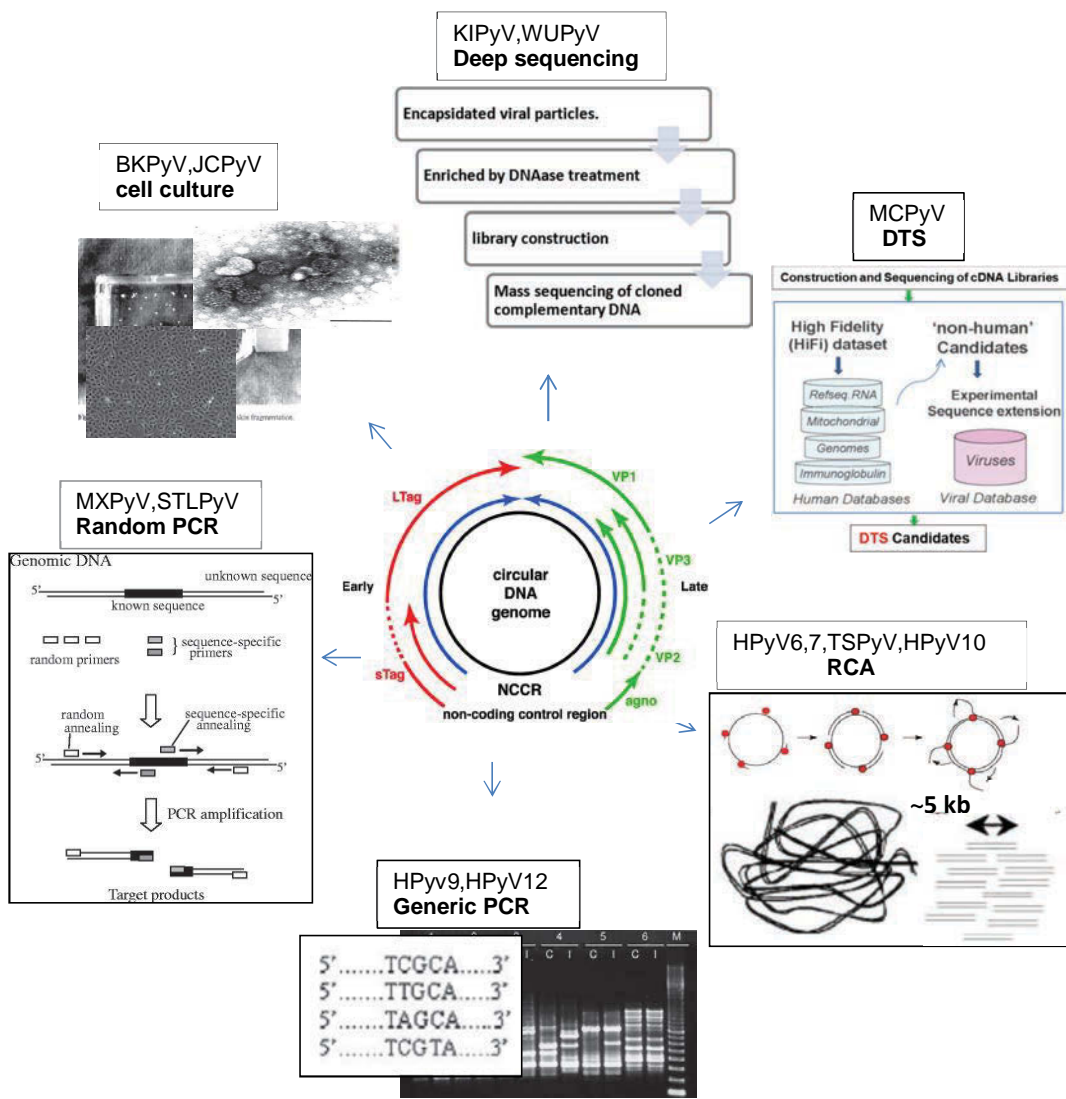


Figure 3. Methods used for discovery of 12 human polyomaviruses.

<b>Nomenclature</b>	<b>Discovery</b>	<b>Method of identification</b>	<b>Prevalence (%)</b>	<b>Disease associations</b>
<b>BK polyomavirus, BKPyV, BKV</b>	(Gardner et al., 1971)	Culture isolation from urine of renal transplant recipient	82-99	PVAN, haemorrhagic cystitis, ureteral stenosis
<b>JC polyomavirus, JCPyV, JCV</b>	(Padgett et al., 1971)	Culture isolation from brain tissue with progressive multifocal Leukoencephalopathy(PML)	30-81	PML
<b>Karolinska Institute polyomavirus, KIPyV, KI</b>	(Allander et al., 2007a)	Deep sequencing of DNAase-treated respiratory fluids	55-90	Not defined
<b>Washington University polyomavirus, WUPyV, WU</b>	(Gaynor et al., 2007)	Deep sequencing of DNAase-treated respiratory fluids	69-98	Not defined
<b>Merkel cell polyomavirus, MCPyV, MCV</b>	(Feng et al., 2008)	Digital transcriptome subtraction of Merkel cell carcinoma tissue	60-81	Merkel cell carcinoma (MCC)
<b>Human polyomavirus 6, HPyV6</b>	(Schowalter et al., 2010)	Rolling-circle amplification of skin and hair samples	69	Not defined
<b>Human polyomavirus 7, HPyV7</b>	(Schowalter et al., 2010)	Rolling-circle amplification of skin and hair samples	35	Not defined
<b>Trichodysplasia spinulosa-associated polyomavirus, TSPyV, TSV</b>	(van der Meijden et al., 2010)	Rolling-circle amplification of trichodysplasia spinulosum lesion in transplant recipient	70-80	Trichodysplasia spinulosum (TS)
<b>Human polyomavirus 9, HPyV9</b>	(Scuda et al., 2011)	Consensus PCR and deep sequencing of blood sample of a renal transplant patient	21-53	Not defined
<b>Malawi or Mexico polyomavirus, MWPyV, MXPpyV, HPyV10</b>	(Yu et al., 2012b, Siebrasse et al., 2012b, Buck et al., 2012)	Rolling-circle amplification, Multiple displacement amplification, random PCR of stool and wart	26-68	Not defined
<b>St Louis polyomavirus, STLPyV, HPyV11</b>	(Lim et al., 2013)	Pyrosequencing and multiple displacement amplification of stool	Not defined	Not defined
<b>Human polyomavirus 12, HPyV12</b>	(Korup et al., 2013)	Generic polyomavirus PCR of liver tissue	12-33	Not defined

*Table 1. Twelve human polyomaviruses thus far discovered.*

### 1.3. Genome and capsid structure of the polyomaviruses

Polyomavirus particles are non-enveloped and approximately 40 to 45 nm in diameter icosahedral particles with 72 capsomers in a skewed ( $T=7$ ) arrangement (Fields et al., 2013). The virion consists of 72 pentamers of the capsid protein VP1 with a single copy of VP2 and VP3 associated with each pentamer (Stehle et al., 1994). The capsids enclose a single molecule of circular double-stranded DNA of fairly uniform size within the family, ranging in size from 4754 (STLPyV) to 5387 (MCPyV) base pair (bp) and able to be divided by transcriptional criteria into three regions (Van Ghelue et al., 2012). In mature particles, the viral DNA is associated with the host-cell histone proteins H2a, H2b, H3, and H4 in a supercoiled, chromatin-like complex. No evidence shows virus particles to contain any carbohydrates or lipids (Fields et al., 2013).

All polyomaviruses have a similar genomic organization; the genome is almost evenly divided into an early and a late region encoded on opposite strands (Figure 4). Between these two regions is a non-coding control region (NCCR). This region contains the origin of replication, the TATA box, the T-antigen-binding sites, the cellular transcription factor-binding sites, and a bidirectional promoter. It also contains the promoter elements that control transcription of both the early and late transcripts. NCCR is thought to be the main determinant of the viral tropism. Importantly in JCPyV, modifications in the NCCR region are associated with an increase in viral transcription and replication in patients with PML (Tan et al., 2010, Reid et al., 2011).

The early region, transcribed soon after the initial infection of the host cell, encodes at least two proteins: the large tumor antigen (LTag) and the small T antigen (sTag); these share the amino-terminal 75–80 amino acids (aa). This shared part is encoded by exon 1 of the LTag gene. The LTag is a non-structural, multifunctional protein that regulates the switch from early to late viral protein transcription and the replication of the viral genome. This protein interacts with a number of cellular proteins (such as retinoblastoma protein and p53), and its role in cancerogenesis is being investigated (Gjoerup and Chang, 2010). The sTag and other T antigen variants are produced by cellular splicing of the LTag RNA; these proteins perform multiple functions.

Late transcripts generally encode three structural proteins (VP1, VP2, and VP3), as well as additional proteins in some of the polyomavirus species (Fields et al., 2013). Of the three structural proteins, VP1 makes up more than 70% of the total protein content of virus particles and is also called the major structural protein. Five VP1 molecules surround

either a VP2 or VP3 molecule to form stable assembly units, or capsomers; 72 capsomers link together in icosahedral symmetry to form the capsid of each virion. The VP2 and VP3 molecules may be necessary to ensure specific encapsidation of the replicated polyomavirus genome. All of these proteins originate from the same mRNA transcript, but they are produced upon differential splicing and internal translation (Good et al., 1988). VP4, which also originates from the late transcript, thus far has been detectable only in SV40, where it functions as a viroporin, promoting the release of the virus from the cell (Giorda et al., 2012). The human polyomaviruses JCPyV and BKPyV, the monkey polyomaviruses SV40, SV40-Ri257, baboon polyomavirus 1 (isolate SA12), chimpanzee polyomavirus, squirrel monkey polyomavirus and bovine polyomavirus also encode the agnoprotein in the leader region of the late transcript (Gerits and Moens, 2012). No other polyomaviruses are, however, known to encode this agnoprotein. Murine and hamster polyomaviruses encode a middle T antigen which functions as a transforming protein (Cheng et al., 2009). MCPyV encodes a protein phylogenetically related to this middle T antigen, one called ALTO (Carter et al., 2013). Alternative splicing of the STLPyV early region yields, in addition to the expected LTag and sTag, a unique form of T antigen, which is called 229T (Lim et al., 2013).

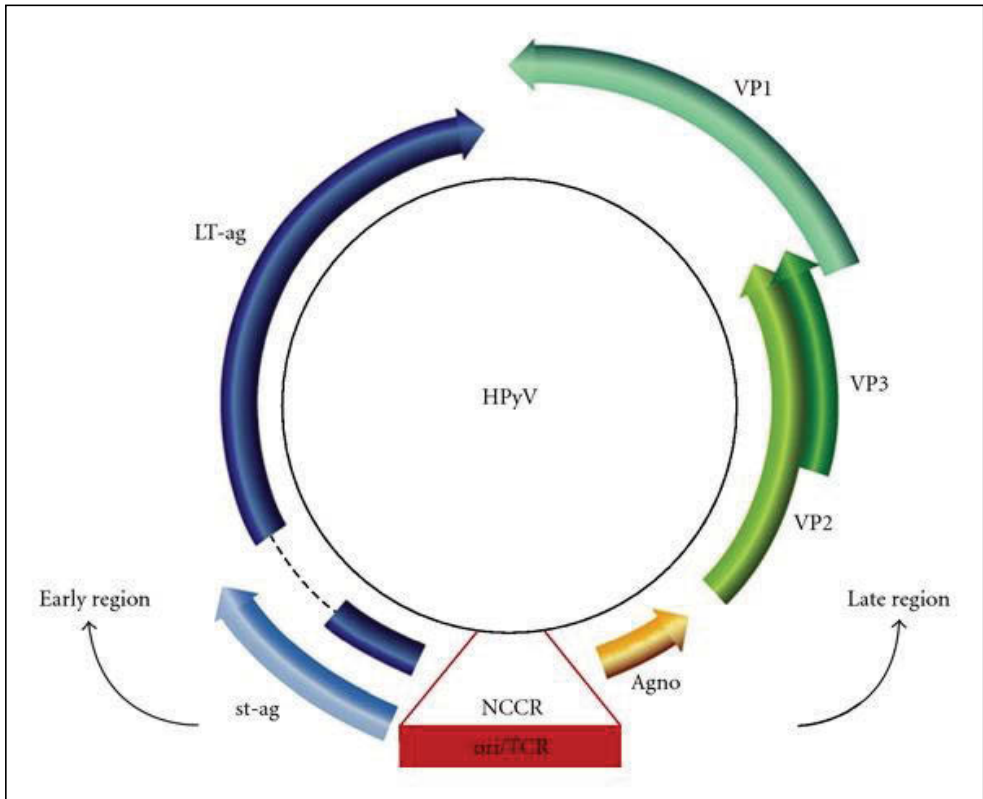


Figure 4 .The prototypical circular double-stranded DNA genome has three main regions: a non-coding control region, an early region, and a late region. Agnoprotein (Agno) is encoded by a late transcript from JC polyomavirus and BK polyomavirus, but has yet to be confirmed in the new human polyomaviruses. Reproduced with permission from Moens, U., Ludvigsen, M., Van Ghelue, M., 2011. Human polyomaviruses in skin diseases. Pathology research international 2011, 123491.

#### **1.4. Human polyomavirus pathogenicity, associated diseases, and clinical aspects**

Among the 12 polyomavirus species identified in humans thus far, only four have been consistently linked to human pathologies (Table 1) (White et al., 2013). BKPyV to PyV-associated nephropathy (PyVAN) and PyV-associated hemorrhagic cystitis (PyVHC), JCPyV to PML and rarely PyVAN, MCPyV to MCC, and TSPyV to *trichodysplasia spinulosa* (TS) of the skin (Dalianis and Hirsch, 2013).

The host cells and tissues that support infection by the new HPyVs *in vivo* are unknown, and their identification is among the most essential task in research. Some of the new HPyVs may transmit horizontally by direct contact, or transmit by aerosol or fecal–oral routes (Babakir-Mina et al., 2011, Csoma et al., 2012, Babakir-Mina et al., 2013, Coursaget et al., 2013, Dalianis and Hirsch, 2013, DeCaprio and Garcea, 2013, Ehlers and Wieland, 2013, Feltkamp et al., 2013). Infection site, the original site of the polyomavirus's identification, and the distant organs in which the virus is detectable may, however, be distinct and be hard to relate to any pathophysiology (Dalianis et al., 2009).

Although several of the new HPyVs have been isolated from nasopharyngeal or stool samples, correlations between infection and respiratory symptoms or diarrheal illness remain circumstantial (Bialasiewicz et al., 2007a, Bialasiewicz et al., 2007b, Wattier et al., 2008, Babakir-Mina et al., 2009b, Bialasiewicz et al., 2009a, Bialasiewicz et al., 2009b, Babakir-Mina et al., 2011). The data remain confounded by the presence of numerous viral agents in the samples studied, such that no single entity has been singled out as the causative agent.

Because the clinical symptoms of primary infection with BKPyV and JCPyV still lack conclusive identification, it is perhaps not surprising that the new HPyVs in this context have not been associated with any clinical symptoms, either (Chen et al., 2011a, Chen et al., 2011b). Attempts to link the new polyomaviruses to clinical disease have focused mostly on immunosuppressed or older individuals, in whom immunity is already waning. In particular, opportunistic diseases in immunocompromised individuals have been studied, but with no specific correlations with HPyVs yet identified (Tables 2-10).

JCPyV and BKPyV can cause a lytic infection resulting in tissue destruction. JCPyV infection of oligodendrocytes in the central nervous system (CNS) (that is, PML) and BKPyV infection of renal tubule cells (that is, PVAN) result in loss of these cells, similar to the well-described cytopathic effect (CPE) of SV40. However, except for MCPyV and



TSPyV, the new HPyVs have shown no clear association with any clinical syndrome. Whereas some of the HPyVs (JCPyV, BKPyV, TSPyV, and MCPyV) are associated with disease, the respiratory diseases associated with KI and WU polyomaviruses have been questionable, and no disease association has been established for HPyV6, 7, 9, 10, 12, and STLPyV thus far (Figure 5). On the basis of the sites from whence polyomavirus DNA has been isolated, suspected sites of infection include the nasopharynx and lung for KIPyV and WUPyV, the skin for HPyV6, HPyV7, HPyV9, and MWPyV, and the gastrointestinal tract for MWPyV, STLPyV, and HPyV12 (Allander et al., 2007a, Gaynor et al., 2007, Schowalter et al., 2010, Scuda et al., 2011, Buck et al., 2012, Korup et al., 2013, Lim et al., 2013). HPyV6 and HPyV7 in contrast with HPyV9 seem to be a regular part of the human skin microbiome. Therefore, their association with different skin tumors and some non-tumorous skin lesions has been under investigation in some studies (Table 6, 7, and 9). MWPyV and STLPyV are the first two polyomaviruses to be discovered in human stool samples, and we still are not clear as to whether they have primary gastrointestinal tropisms; many outstanding questions relate to the potential role of HPyV12 and other emerging polyomaviruses in human infections. In addition, one study investigated whether reactivation of the newly discovered KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, and HPyV10 is associated with neurological complications after allogeneic hematopoietic stem cell transplantation (HSCT) and found no HPyV DNA in any of the 32 cerebrospinal fluid (CSF) samples (Gustafsson et al., 2012).

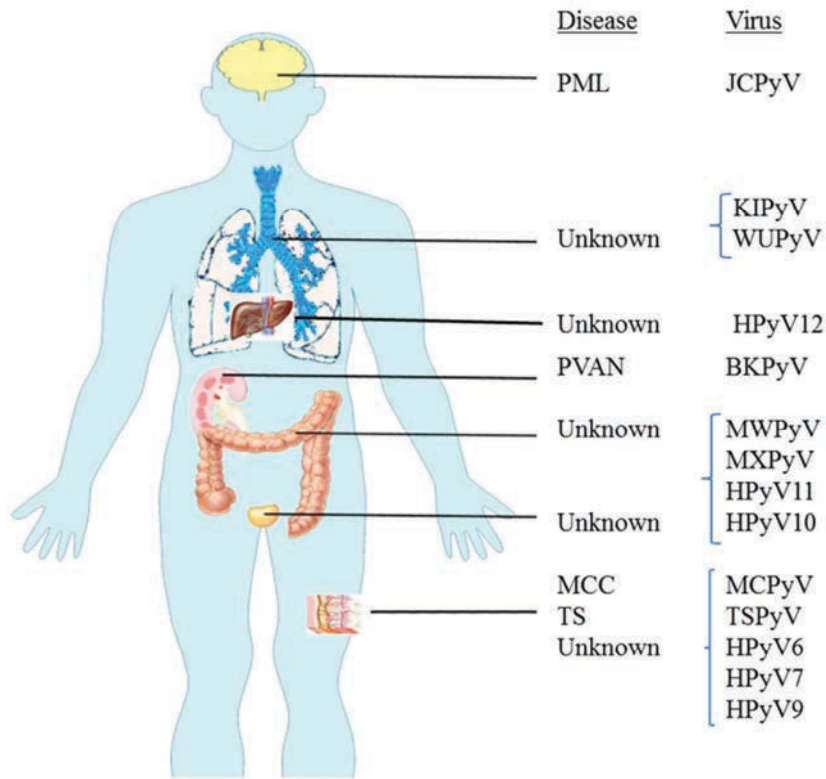


Figure 5. Human polyomaviruses and associated diseases. Reproduced with permission from White, M.K., Gordon, J., Khalili, K., 2013. The rapidly expanding family of human polyomaviruses: recent developments in understanding their life cycle and role in human pathology. *PLoS pathogens* 9, e1003206.

## 1.5. Human polyomavirus cancer association

About a fifth of all human cancers worldwide are caused by infectious agents. In 12% of cancers, seven different viruses have been causally linked to human oncogenesis: Epstein-Barr virus, hepatitis B virus, human papillomavirus (HPV), human T-cell lymphotropic virus, hepatitis C virus, Kaposi's sarcoma herpesvirus, and Merkel cell polyomavirus. Many molecular mechanisms of oncogenesis have been discovered over the decades of study of these viruses (White et al., 2014).

The role of animal polyomaviruses in tumorigenesis has long been known (Gross, 1953, Stewart, 1953, Sweet and Hilleman, 1960). More recently, ten new HPyVs have been discovered, and one of these, MCPyV, was identified in patients with MCC, which is a rare but highly aggressive malignancy (White et al., 2013, White et al., 2014). No evidence exists thus far for any correlation between malignant transformation and the newly discovered HPyV6 and 7, TSPyV, HPyV9, MWPyV, or HPyV11 and 12, but obviously additional investigations are necessary.

A role in malignant transformation for BKPyV and JCPyV was proposed soon after their initial isolation in 1971 (Gardner et al., 1971, Padgett et al., 1971, Walker et al., 1973). In vitro studies supported the oncogenic contribution of their LTag and sTag with the potential to cooperate with other oncogenic alterations. There exist several reports on detection of BKPyV DNA in tumors such as urothelial malignancies and renal tubular malignancies, colorectal tumors, lymphomas, pancreatic cancer, brain tumors, prostate cancer, and a range of sarcomas, and for non-UV light- associated melanomas (Emerson et al., 2008, Giraud et al., 2008, Abend et al., 2009). Interpretation of these reports has been varied and controversial, but based on inadequate evidence in humans and sufficient evidence in experimental animals, a WHO International Agency for Cancer Research Monograph Working Group recently classified BKPyV and JCPyV as possibly carcinogenic to humans (Group 2B), (Bouvard et al., 2012).

Several studies have investigated whether KIPyV or WUPyV is involved in tumor development. Thus far, results for childhood brain tumors and non-UV-exposed melanomas have been negative (Giraud et al., 2009). In one study, KIPyV VP1 DNA was detectable in 9 of 20 lung cancer cases of undefined origin and in both of 2 transplant patients (Babakir-Mina et al., 2009b). In two of the lung-tissue samples, only the C-terminal domain of the early region of KIPyV was successfully identified; analysis of possible integration did not take place. Thus, there is no compelling evidence to date

supporting any role for KIPyV in oncogenesis, although the data may indicate the lung as a site of viral persistence.

The most recent human oncogenic virus to be discovered was MCPyV (Feng et al., 2008). Cell culture and small animal models for MCPyV replication are not yet available, but there is strong mechanistic evidence that it directly contributes to the development of a large proportion of MCCs (Chang and Moore, 2012). First of all, MCPyV sequences were identified in 8 of 10, and integrated in 5 of 8 MCCs as mentioned above, thus immediately linking MCPyV to the pathogenesis of MCC (Feng et al., 2008). This study was followed by others, again detecting MCPyV in around 80% of the MCC samples, revealing that the LTag is frequently truncated before the helicase domain (Duncavage et al., 2009b). In addition, reports have also presented deletions of the VP1 region (Sharp et al., 2009). On the other hand, the role of MCPyV in MCC is further supported by the fact that MCC patients frequently have very high antibody titers to MCPyV as compared to titers of the population in general, indicating that MCPyV infection indeed is associated with MCC (Pastrana et al., 2009). It is therefore possible that cell-mediated immunity (CMI) may be involved in protection against MCPyV-induced malignancy (Kumar et al., 2011a).

Patients with MCC have an increased risk of developing chronic lymphocytic leukemia (CLL) (Koljonen et al., 2009a, Koljonen et al., 2010). An investigation has reported MCPyV sequences in 27.1% of purified malignant cells from CLL samples (Pantulu et al., 2010), yet the causal association remains to be proven. Presence of MCPyV has been sought for numerous other tumors, particularly those of the skin (Table 2). Thus far, evidence is limited that MCPyV plays a role in the oncogenesis of tumors other than MCC.

Diagnosis	No. Samples	No. Positive Lesions/total (%)	Reference
Lung small cell carcinoma	30	2/30 (7.5)	(Andres et al., 2009)
Seborrheic keratosis	12	2/12 (16.7)	(Andres et al., 2010)
Basal cell carcinoma	50	11/50(18.0)	(Imajoh et al., 2013)
Basal cell carcinoma	147	47/147 (32)	(Kassem et al., 2009)
Bowen's disease	23	4/23 (17.4)	(Kassem et al., 2009)
Squamous cell carcinoma	6	1/6 (16.7)	(Wieland et al., 2009)
Squamous cell carcinomas	63	16/63(22.2)	(Imajoh et al., 2013)
Kaposi's sarcoma	49	3/49 (6.1)	(Katano et al., 2009)
Malignant melanoma	12	2/12 (16.7)	(Wieland et al., 2009)
B-cell associated lymphomas	161	5/161 (3.1)	(Shuda et al., 2009)
T-cell associated lymphomas	104	1/104 (1)	(Shuda et al., 2009)
Malignant melanoma	47	10/47(19.1)	(Imajoh et al., 2013)
Langerhans cell sarcoma	7	3/7 (43)	(Murakami et al., 2014)
Lung small cell carcinoma	10	Absent	(Wetzels et al., 2009)
Basal cell carcinoma	11	Absent	(Andres et al., 2010)
Basal cell carcinoma	20	Absent	(Reisinger et al., 2010)
Lentigo malignant melanoma	10	Absent	(Andres et al., 2010)
Squamous cell carcinoma	20	Absent	(Reisinger et al., 2010)
pulmonary neuroendocrine carcinomas	26	Absent	(Busam et al., 2009)
Mesotheliomas	45	Absent	(Bhatia et al., 2010)

Table 2. Presence and absence of MCPyV DNA in some entities.

TSPyV was reported as the eighth HPyV in follicular papules and keratin spines (spicules) of a heart transplant patient with *ts* (van der Meijden et al., 2010). Thereafter, it attempts have been made to find TSPyV in other immunosuppressed patients not suffering from *ts* (Kanitakis et al., 2011, Fischer et al., 2012, Kazem et al., 2012, Siebrasse et al., 2012a, Wieland et al., 2014). There is no evidence for a correlation between TSPyV in other disease or malignant transformation so far, but obviously additional investigations are necessary.

In addition, by use of a bead-based multiplex assay, the presence of HPyVs: BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6, 7, 9, TSPyV, and MWPyV as well as the primate PyVs, SV40, and lymphotropic polyomavirus (LPyV) have been analyzed in 37 mucosal melanomas; none of the samples analyzed were positive for any of the examined viruses (Ramqvist et al., 2014).

## **2. TWELVE SPECIES OF HUMAN POLYOMAVIRUSES**

### **2.1. John Cunningham polyomavirus (JCPyV or JCV)**

JC virus, as the first of 12 polyomavirus (PyV) members identified in humans thus far, was identified in 1964 by electron microscopy (EM), when the neuroscientists Zu Rhein and Sam Chou were investigating formalin-fixed brain tissue sections from patients with PML (Zurhein and Chou, 1965). Numerous non-enveloped viral particles of icosahedral symmetry were visible in the nuclei of oligodendrocytes, either dispersed or arranged in filamentous or crystal-like arrays. Six years later, the virus was isolated by cell culture from post-mortem PML tissue from the brain of a Hodgkin's lymphoma patient with the initials J.C. This allowed further studies on the biology and pathogenesis of JCPyV (Padgett et al., 1971, Padgett and Walker, 1973, Padgett et al., 1976, Padgett et al., 1977a, Padgett et al., 1977b).

JC virus is implicated as a causal agent in a rare but often fatal infection known as PML (Tyler, 2008). JCPyV, a highly human-specific virus, has only one major serotype (Hirsch et al., 2013). It infects approximately half of the general population, but its transmission route is still unresolved (Hirsch et al., 2013). This virus persists in the reno-urinary tract, and viral replication is detectable as asymptomatic viruria in about one-third of all infected immunocompetent individuals. The key JCPyV diseases are associated with immunological failure at parenchymal sites of the body like the CNS and the kidney, but possibly also at other sites, including the colon. Moreover, kidney transplant patients may have PyVAN, as well as other JCPyV-associated pathologies including possibly some forms of cancer (Ferenczy et al., 2012). Thus, JCPyV still remains challenging. Its biology and the clinical implications of JCPyV infection, replication, and disease warrant study.

#### **JCPyV genome and structure**

Taxonomically, JCPyV is proposed to be a member of the *Orthopolyomavirus genus* (Figure 2) (Johne et al., 2011). Characterization of the JCPyV species reveals that only one major VP1 serotype exists, but at least seven major genotypes. These genotypes, initially distinguished by their restriction-site pattern and by sequences from the intergenic region located between the distal early viral gene region and the late viral gene region, were preferentially detected in different geographic areas of the world (Yogo et al., 1990,

Sugimoto et al., 1997). Initially, eight major genotypes were distinguished, based on coding region polymorphisms from 100 full-length JCPyV sequences (Cubitt et al., 2001).

JCPyV virions, like all polyomaviruses, are nonenveloped particles of approximately 42 nm in diameter of  $T = 7$  icosahedral symmetry and hence are morphologically indistinguishable from other PyV particles. The capsid consists of 360 VP1 molecules spontaneously assembled from 72 VP1 pentamers and mediates host cell receptor binding for uptake. Inside the VP1 shell are found the minor capsid proteins, the myristoylated VP2, and the VP3; these probably play a role not only in packaging, but also in uncoating and delivery of the JCPyV genome to the host cell nucleus (Shishido-Hara et al., 2004, Gasparovic et al., 2006, Neu et al., 2009).

The genome size is approximately 5130 bp, although single isolates can differ in length, due to alteration in their noncoding regions. The JCPyV genome is wrapped around histones in a nucleosomal core-like organization (Sugimoto et al., 2002). The NCCR lies between the early and late coding regions. The early coding region spans 2.4 kb and encodes five proteins: the LTag, the sTag, and three different splice variants (Hirsch et al., 2013). The late viral gene region of ~2300 bp contains the ORFs of the capsid proteins VP1, VP2, and VP3, which are also derived by splicing of one major transcript, but going in the other direction from the NCCR. In addition, the JCPyV late viral gene region encodes a small leader protein starting directly adjacent to the NCCR; this is called an agnoprotein, and it has, as thus far determined, a counterpart in several polyomaviruses including BKPyV and SV40 but not in all known PyVs (Suzuki et al., 2010).

### **JCPyV epidemiological aspects**

JC virus is one of the most prevalent viruses worldwide (Knowles et al., 2003, Tan and Koralnik, 2010); aerosol inhalation and ingestion of contaminated water or food may be major modes of its human transmission (Cinque et al., 2009). JCPyV infection is widespread in the general population, and several seroprevalence studies of human populations have detected JCPyV-specific antibodies in 30 to 70% of healthy individuals (Stolt et al., 2003, Kean et al., 2009, Ferenczy et al., 2012). Many reports show that most of their study populations appear to be infected, either transiently or latently, with JCPyV (Hirsch et al., 2013). Based on different detection methods, the epidemiology of JCPyV varies somewhat from study to study, with 10 to 30% of adults excreting JCPyV in the urine. PCR detection of viral DNA allows for accurate and sensitive detection of

individuals with actively replicating virus. Nevertheless, its transmission route is not yet well defined, although the presence of JCPyV DNA in B cells and stromal cells of the tonsils indicates that tonsils may serve as the initial site of viral infection (Monaco et al., 1998). The virus may enter the upper respiratory tract via close interpersonal contact or via fomites, and then presumably spread by a hematogenous route from the primary site of infection to secondary sites such as the kidneys, bone marrow, lymphoid tissues, and the brain to establish focal areas of infection or remain persistent (Figure 6) (Chapagain and Nerurkar, 2010, Aksamit, 2012). Potential alternative modes of transmission include by urino-oral and transplacental routes, via blood transfusion, in semen and by organ transplantation (Jiang et al., 2009).

The archetypal virus is also isolated from sewage samples from various geographic areas, suggesting possible transmission by contaminated food, water, or fomites (White and Khalili, 2011). Transplacental transmission is unlikely (Boldorini et al., 2008), but transmission from parent to child can occur (Zheng et al., 2004, Boldorini et al., 2011). JCPyV infection in immunocompetent hosts is usually subclinical and remains localized in the kidney. It is unclear whether JCPyV reactivation causes viral spread to the CNS or if a latent infection in the CNS becomes locally reactivated. However, JCPyV is permissive in B-lymphocytes of the bone marrow and peripheral blood (Dorries et al., 2003), suggesting that viral spread after primary replication may occur via a hematogenous route. In the CNS, JCPyV infects glial cells, including astrocytes, and myelin-producing cells known as oligodendrocytes (Zurhein and Chou, 1965). Studies suggest that the virus can remain latent in the brains of healthy people (Tan et al., 2010). In cases of immunosuppression, JCPyV can become reactivated, leading to enhanced viral replication and a lytic infection of the CNS causing cytolytic destruction of oligodendroglia, resulting in the fatal disease PML (Khalili and White, 2006). The exact mechanism of oligodendroglia cell death in PML is unknown (Khalili and White, 2006).



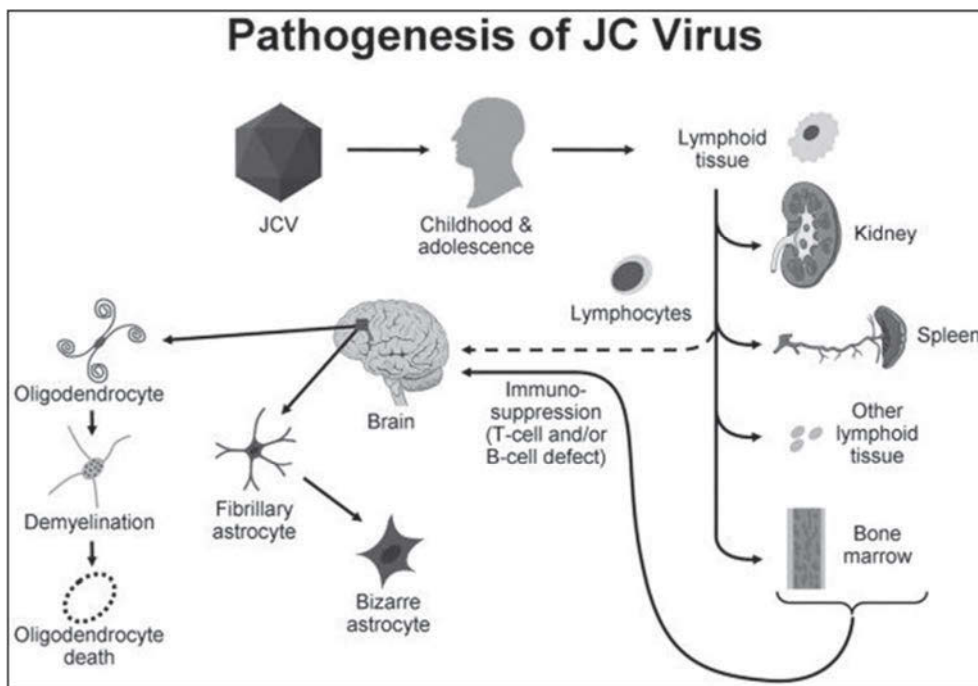


Figure 6. Pathogenesis of JC virus infection causing progressive multifocal leukoencephalopathy. Reproduced with permission from Aksamit, A.J., Jr., 2012. Progressive multifocal leukoencephalopathy. *Continuum* 18, 1374-1391.

### JCPyV disease associations

After asymptomatic primary infection with JCPyV, which occurs in childhood, in addition to the kidney, bone marrow, lymphoid tissues, and tonsils (Tan and Koralnik, 2010, White and Khalili, 2011), may remain latent in the brains of healthy people (Tan and Koralnik, 2010). Asymptomatic reactivation of latent JC virus is common in individuals who are immunocompetent, and the virus can be detected in the urine of about 30% of individuals who are healthy (Tyler, 2010)

Attributed to JC virus are also some new neurological disorders including granule cell neuropathy, encephalopathy, and meningitis (Tan and Koralnik, 2010). Discovery of new neurological syndromes that result from neuronal infection with JC virus, and the presence of this virus in the gray matter are under debate (Tan and Koralnik, 2010). It has been suggested that the JC virus is associated with cognitive decline, dementia, strokes, and brain- and some other tumors (Katona, 2009, Rollison, 2010).

## **PML and MS**

PML was originally described in 1958 as a demyelinating disease of the CNS (Astrom et al., 1958), and later, viral particles were detected by EM in the brains of patients with PML (Silverman and Rubinstein, 1965, Zurhein and Chou, 1965). JC virus from the brain tissue of a patient with Hodgkin's lymphoma who developed PML was described in Lancet article in 1971 (Padgett et al., 1971). PML results from lytic JC virus infection of glial cells in immunosuppressed patients (Tan and Koralnik, 2010). It was first described as a complication of immune suppression 50 years ago and emerged as a major complication of HIV infection in the 1980s (Focosi et al., 2010). PML has recently become topical because it is a side-effect of some newly developed immunomodulatory drugs for autoimmune diseases, including natalizumab for multiple sclerosis and Crohn's disease, rituximab for systemic lupus erythematosus, and efalizumab for psoriasis (Focosi et al., 2010, Tan and Koralnik, 2010, White and Khalili, 2011). PML has had a dramatic effect on the use of these drugs. Efalizumab was withdrawn from the market in April 2009, and natalizumab was temporarily withdrawn in February 2005 after three confirmed cases of PML (Tyler, 2010).

Multiple sclerosis (MS) is another primarily inflammatory disorder of the brain and spinal cord in which focal lymphocytic infiltration leads to damage of myelin and axons. MS and PML are both diseases resulting in demyelinated brain lesions; they are distinguished by morphologically distinct bizarre astrocytes, inclusion bodies within the nuclei of oligodendrocytes, and also by the lack of the inflammatory infiltrates which are characteristic of PML (Khalili et al., 2007). Because these diseases are so similar, researchers have studied the association of MS with JCPyV reactivation and PML. Reports of JCPyV DNA and viremia in patients with MS conflict: several report a lack of evidence for JCPyV infection in MS patients (Buckle et al., 1992, Bogdanovic et al., 1998), while others have found JCPyV DNA sequences in CSF of some MS patients but not in healthy controls (Ferrante et al., 1998, Koralnik et al., 1999).

## **2.2. Thea Brennan-Krohn polyomavirus (BKPyV or BKV)**

BKPyV was one of the first two HPyVs identified. It was isolated from both urine and ureteral epithelial cells of a Sudanese renal transplant (RT) recipient presenting with renal failure and ureteral stenosis (Gardner et al., 1971). A urine sample from a patient who underwent kidney transplantation 3.5 months earlier contained many inclusion-bearing epithelial cells. Dr. Anne Field examined the sample by EM and found a large number of viral particles that were similar to the common wart virus (Knowles, 2006). Post-inoculation of urine to cell culture, a CPE occurred in the secondary Rhesus monkey kidney and African monkey kidney (Vero) cell culture; the cell culture supernatant contained viral particles. The virus was called BK after the initials of the patient, and the original isolate was called the Gardner strain (Gardner et al., 1971).

BK virus is a very common polyomavirus globally, similar to the JC virus. BKPyV infects most people subclinically during childhood and establishes a lifelong infection in the reno-urinary tract. In most immunocompetent individuals, infection is completely asymptomatic, but in frequent episodes of viral reactivation, virus is shed into the urine. In immunocompromised patients, reactivation is followed by high-level viral replication, and this can lead to severe disease: 1 to 10% of kidney-transplant patients develop PyVAN, and 5 to 15% of allogenic HSCT patients develop polyomavirus-associated haemorrhagic cystitis (PyVHC) (Rinaldo et al., 2013).

### **BKPyV genome and structure**

Comparative analysis of the BKPyV genome shows its considerable homology to JCPyV (Frisque et al., 1984), and more distantly to other human PyVs. This is evident by their phylogenetic tree (Figure 2).

The non-enveloped, icosahedral virion of BKPyV is about 45 nm in diameter (Takemoto and Mullarkey, 1973). Its capsid is composed of 72 capsomers, each consisting of five copies of the major structural protein VP1 and one internal minor structural protein, either VP2 or VP3, linking the viral genome to the capsid (Li et al., 2003). The BKPyV genome is a circular, double-stranded DNA molecule of 5135 bp (Dunlop strain) packed around the cellular histones H2A, H2B, H3, and H4, thereby forming a minichromosome (Hirsch, 2005). The late coding region of BKPyV codes the viral capsid proteins (VP1, VP2, and VP3) as well as the agnoprotein (Abend et al., 2009).

The two forms of the virus, the archetype virus and rearranged variants, are based on the DNA sequence structure of the NCCR. The archetype virus can often be isolated by transient reactivation events from the urine both of healthy individuals and from diseased patients (Doerries, 2006). In contrast, rearranged variants most often occur in the serum of patients with BKPyV-associated diseases.

There are four sub-types of BKPyV (defined by antigenic variations of some viral capsid proteins) distributed according to the various populations (age, HIV co-infection, etc.); genotype 1 is the most frequent (Ahsan and Shah, 2006).

### **BKPyV epidemiological aspects**

Its high prevalence of infection, low morbidity, asymptomatic reactivation, and host specificity suggest the co-evolutionary adaptation of BKPyV to its human host (Hirsch and Steiger, 2003). Primary infection typically occurs during early childhood, after the waning of maternal antibodies. Before the child reaches the age of 10 years, the seroprevalence increases to 91%, and the overall rate of seropositivity for BKPyV is 81% (Knowles et al., 2003). The natural route of BKPyV transmission has not been resolved and may be respiratory or oral. BKPyV, like JCPyV, is fairly resistant to environmental inactivation, and both are detectable in human sewage. Latency is established in renal tubular epithelial and urothelial cells. Reactivation and low-level replication with asymptomatic viruria occurs in 5% of healthy individuals (Hirsch and Steiger, 2003). The prevalence may increase with pregnancy, older age, or immune dysfunction to rates of > 60% (Rinaldo et al., 2013). In addition, the level of replication may increase to a BKPyV DNA load from  $<10^5$  to  $>10^7$  copies/mL of urine. However, that BKPyV disease is rare suggests that additional factors are required.

Additionally, BKPyV viruria can be detected in up to 25% of pregnant women (Jin et al., 1993). Moreover, BKPyV is a significant human pathogen that causes disease in immunocompromised individuals. BKPyV is able to reactivate in renal transplant- and bone marrow-transplant (BMT) patients in whom a lytic infection results, respectively, in polyomavirus-associated nephropathy (PyVAN) and hemorrhagic cystitis (HC). Rarely, BKPyV reactivation and disease are observable in other immunocompromised conditions such as systemic lupus erythematosus, in addition to solid organ-transplant recipients, and in patients with HIV/AIDS (Jiang et al., 2009).

### **BKPyV disease associations**

High prevalence, latent infection, and asymptomatic reactivation of BKPyV complicate our understanding and evaluation of its pathogenic role. Various disease processes have been linked to BKPyV infection, but the two most important diseases are PyVAN affecting kidney transplant patients and PyVHC affecting allogenic HSCT patients. In immunocompromised individuals, other associated conditions include ureteric stenosis, encephalitis, meningoencephalitis, pneumonia, and vasculopathy. Although BKPyV has been associated with cancer development, especially in the bladder, definitive evidence of its role in human malignancy is lacking.

### **PyVAN and PyVHC diseases**

PyVAN, the most frequent BKPyV-associated disease occurring after renal transplantation, is a form of acute interstitial nephritis (Nickeleit and Mihatsch, 2006). BKPyV viruria and viremia due to reactivation appear in up to 80% of renal transplant patients; 10% of such patients progress to PyVAN, resulting in allograft loss 90% of the time (Hirsch et al., 2002, Bressollette-Bodin et al., 2005, Egli et al., 2007, Huang et al., 2010). Lytic infection in kidney epithelial cells as a result of BKPyV leads to PyVAN, which is characterized by necrosis of proximal tubules and denudation of the basement membrane (Nickeleit et al., 2003). Recently, PyVAN incidence has been rising because of new and more potent immunosuppressive regimens (Nickeleit et al., 2000, Mengel et al., 2003); this finding indicates a relationship between BKPyV reactivation and disruption of the immune system.

Among allogenic HSCT patients, 5 to 15% are affected by PyVHC, a late-onset hemorrhagic cystitis usually starting about 50 days post-transplantation. The pathogenesis of PyVHC is not fully understood, but is suggested to result from the following sequence of events (Azzi et al., 1999). First the bladder mucosa is subclinically damaged by the toxic metabolite of cyclophosphamide, a drug used as a conditioning protocol prior to stem-cell transplant. Next, during the aplastic phase, immunologically uncontrolled high-level BKPyV replication causes denudation of the damaged bladder mucosa. Finally, upon engraftment of the allogeneic stem cell graft inflammation occurs. Though PyVHC usually affects allogenic HSCT patients, some case reports describe PyVHC also in other immunocompromised patients (Hirsch and Steiger, 2003, Kinnaird and Anstead, 2010).

### **2.3. Karolinska Institute polyomavirus (KIPyV)**

In 2007 the Karolinska Institute polyomavirus (KIPyV) was identified in Stockholm, Sweden (Allander et al., 2007a). It was discovered in respiratory-tract samples from individuals with acute respiratory tract infections, but by totally new methods. The Allander group developed a system for large-scale molecular virus screening of clinical samples based on host DNA depletion, random PCR amplification, large-scale sequencing, and bioinformatics (Allander et al., 2005, Allander et al., 2007a). In this technology for virus screening, the nasopharyngeal aspirates were randomly selected, centrifuged, and the cells were collected. Before DNA and RNA were extracted, the resulting pellet was recovered, resuspended, and treated with DNase (Allander et al., 2001). Extracted DNA and RNA were amplified separately by random PCR (Froussard, 1992, Allander et al., 2001, Allander et al., 2005, Allander et al., 2007a). The nucleic acids served as a template for random PCR, and the products were separated by gel electrophoresis, cloned, and sequenced. This resulted in several sequence reads that were subsequently trimmed, clustered, and sorted by use of dedicated software. Finally, BLAST (Basic Local Alignment Search Tool) searches were performed in search of similarities with known viral sequences listed in GenBank (Allander et al., 2005, Allander et al., 2007a). Following these findings, various biological specimens and body compartments were screened in an attempt to establish a pathogenetic role for KIPyV in the respiratory tract or in other human diseases (Table 3).

#### **KIPyV genome and structure**

The genomic organization of KIPyV is typical for a member of the family *Polyomaviridae*, with an early region encoding regulatory proteins (LTag and sTag) and a late region coding for structural proteins, separated by a noncoding regulatory region (Figure 7) (Allander et al., 2007a). The genome size falls within the range of the polyomaviruses. The KIPyV genome is circular and 5,040 nucleotides in length (Allander et al., 2007a). Whereas the nonstructural proteins have substantial sequence similarity to those of the other primate polyomaviruses, the structural proteins have a very low degree of similarity to those of other known polyomaviruses (Allander et al., 2007a).

The early proteins show similarities to other members of the polyomavirus family, primarily BKPyV, JCPyV, SV40, and simian agent 12 (SA12). Alignment with the LTag

of other polyomaviruses shows that most regions characteristic of LTag are present also in KIPyV (Allander et al., 2007a).

In the late region of the genome are putative ORFs for capsid proteins VP1, VP2, and VP3 (Figure 7). As in all polyomaviruses, VP3 is encoded by the same ORF as VP2 by the use of an internal start codon. An overlap exists between the C terminus of VP2/3 and the N terminus of VP1, as is the case in other polyomaviruses. It can be noted that both VP2 and VP3 of KIPyV are large in comparison with those of other members of the polyomavirus family (400 and 257 aa, respectively) (Allander et al., 2007a).

The degree of aa identity between the VP1 proteins of the HPyVs has been reviewed (Moens et al., 2013) and the highest degree of identity is found between BKPyV and JCPyV (78%), HPyV6 and HPyV7 (69%), and KIPyV and WUPyV (66%). The degree of similarity of KIPyV VP1 with other VP1 proteins is markedly low. VP1 has only 30% identity with its closest counterparts (those of JCPyV and MPyV). In KIPyV VP1, the only region that shows a relatively high degree of similarity to those of other polyomaviruses is the sequence that in MPyV VP1 binds calcium, corresponding to approximately aa 237 to 248 in VP1 of KIPyV (Allander et al., 2007a).

The VP2/VP3 gene shows even lower similarity to its counterparts in other polyomavirus species. In fact, neither a nucleotide nor a translated BLAST search with this gene sequence generated any significant matches in the public databases. Thus, the identity of this ORF is indicated only by its position in the genome. VP2 and VP3 of all other polyomaviruses contain a conserved VP1-binding domain. No corresponding sequence is found in KIPyV. In KIPyV, the region between the start codons of VP2 and of ST/LT is long (513 bp); this could possibly indicate the presence of an agno gene. However, no corresponding ORF is present in this region (Allander et al., 2007a).

There is no evidence hinting at a virus structure for the KIPyV and other newly discovered HPyVs differing from other polyomaviruses. However, because their virions have not been displayed by EM, no evidence exists for the precise virion structure at this stage.

### **KIPyV epidemiological aspects**

Seroepidemiological studies show that the seroprevalence of KIPyV in the adult population is high, suggesting that primary infection occurs early in childhood (Kean et al., 2009, Nguyen et al., 2009, Moens et al., 2013). Indeed, Nguyen and colleagues, using

an enzyme-linked immunosorbent assay (ELISA) test for human VP1 antibodies, reported a seropositivity of about 70% for KIPyV in the age-group of 50 to 64 years (Nguyen et al., 2009). The primary sequences of KIPyV and WUPyV VP1 are 66% identical with several linear stretches that are completely identical. However, use of plasma samples of patients and fixed Sf9 cells infected with recombinant baculovirus expressing either KIPyV or WUPyV VP1 revealed no indication of antibody cross-reaction in an immunofluorescence assay (Neske et al., 2010). In histidine-tagged VP1-based ELISA both IgG and IgM seem to be specific for KIPyV or WUPyV (Miller et al., 2012). These findings were confirmed by another group who failed to detect antibody cross-reactivity between BKPyV/JCPyV and KIPyV/WUPyV in a VP1-based ELISA (Kean et al., 2009, Nguyen et al., 2009).

KIPyV has a worldwide distribution (Table 3). Detectable by PCR in the respiratory samples of patients from all continents, detection rates range from 0.5 to 8% (Babakir-Mina et al., 2008, Ren et al., 2008, Babakir-Mina et al., 2009b, Mourez et al., 2009). Several real-time PCR methods allow detection of and often distinction between these viruses (Bialasiewicz et al., 2007a, Bergallo et al., 2009, Lindau et al., 2009, Kuypers et al., 2012). Almost all KIPyV-DNA detection has been done by PCR methods. The choice of PCR and target region may affect the final result for KIPyV detection. Conventional PCR has lower sensitivity than does real-time PCR (Lindau et al., 2009). The location of primer and probe sequences in different genomic regions can affect KIPyV and WUPyV detection rates (Bialasiewicz et al., 2007a, Bergallo et al., 2009) and therefore the prevalence of these two viruses in different clinical syndromes. Indeed, primers/probes located in the regulatory or sTag regions of KIPyV have yielded a lower detection rate than have primers/probes located in the VP1 region of KIPyV, when researchers are testing in the same specimen type (Bergallo et al., 2009).

Measuring high KIPyV viral loads (geometric mean of  $6.6 \times 10^6$  copies/cell;  $6.4 \times 10^{13}$  copies/mL) in respiratory samples of immunocompromised patients compared to a control group (geometric mean of  $3.1 \times 10^4$  copies/cell;  $6.9 \times 10^{10}$  copies/mL,  $P = 0.019$ ), led to the hypothesis of an increased pathogenic potential for this virus in immunocompromised patients, particularly in HSCT patients (Rao et al., 2011). However, frequent co-detection with other respiratory viruses makes it difficult to correlate these viral load determinations with disease severity. High viral loads also measured in lymphoid tissue of patients with AIDS (Sharp et al., 2009) imply that diseases associated with KIPyV are more likely to



occur in immunocompromised patients. No correlation between the presence of KIPyV and CD4+ cell counts was, however, observable (Babakir-Mina et al., 2010a).

In a large prospective study, carried out on 2,732 nasal wash samples collected from 222 patients during the first year after allogeneic hematopoietic cell transplantation, the cumulative incidence of KIPyV was 26% (Kuypers et al., 2012). Sputum production and wheezing were associated with KIPyV detection. No association existed, however, between polyomavirus detection and graft-vs-host disease, cytomegalovirus reactivation, neutropenia, lymphopenia, hospitalization, or death.

### **KIPyV disease associations**

KIPyV has been discovered in respiratory tract samples from individuals with acute respiratory tract infections (Allander et al., 2007a), but without conclusive data as to the role of KIPyV as a respiratory pathogen, determination of KIPyV viral loads in diagnostic laboratories is not routine. A causal role for this virus in the respiratory tract is unlikely (Norja et al., 2007). Seroepidemiological surveys show that, similarly to BKPyV and JCPyV, primary infection with KIPyV occurs early in life, probably through the respiratory and oral-fecal routes. The virus remains latent in the body, to become reactivated if immunosuppression occurs. However, differing respiratory tract specimen types tested (nasopharyngeal aspirates, nasal washes, bronchoalveolar lavages, broncoaspirates, throat swabs), non-standardized time-points of specimen collection, and a lack of standardized PCR methods, of a clear clinical cutoff value, of prospective quantitative studies including an asymptomatic control group, all interfere with the use of viral load in management of patients with KIPyV reactivation.

The presence of KIPyV and WUPyV has been described in the brains of patients with and without PML (Barzon et al., 2009b). These results contrast with those reported by other groups that did not detect KIPyV, WUPyV or MCPyV in the CNS (Bialasiewicz et al., 2009b, Giraud et al., 2009). As mentioned, these conflicting results may be linked to ethnic differences in patient populations, different laboratory methods, sequence variations in the viral target, and sensitivity of the PCR methods. Standardization of the methods can help to define the prevalences of the novel polyomaviruses in the CNS.

No KIPyV or WUPyV DNA was detected in the neuroendocrine tumor (Duncavage et al., 2009a), and in another study, all 36 melanoma biopsies examined were also negative for these two viruses (Giraud et al., 2008). Moreover, using an improved RCA technique,

found no KIPyV or WUPyV (Schowalter et al., 2010). Although a small number of individuals have been screened thus far, KIPyV and WUPyV do not seem to possess skin tropism.

Sample type	No. Positive samples /total (%)	Patients Median age (age range, years)	Country	Reference
Respiratory tract sample	6/637 (1)	7 (<0.1–90)	Sweden	(Allander et al., 2007a)
Respiratory tract sample	14/983 (1.4)	– (0.3–34)	UK	(Norja et al., 2007)
Respiratory tract sample	5/486 (1)	0.9 (0.1–5.9)	South Korea	(Han et al., 2007)
Respiratory tract sample	24/951 (2.5)	1.5 (0.1–95)	Australia	(Bialasiewicz et al., 2007b)
Respiratory tract sample	75/2866 (2.6)	1.38 (<0.1–95)	Australia	(Bialasiewicz et al., 2008)
Respiratory tract sample	11/406 (2.7)	0.8 (<0.1–9)	China	(Yuan et al., 2008)
Respiratory tract sample	6/302 (1.9)	1 (<0.1–14)	Thailand	(Payungporn et al., 2008)
Respiratory tract sample	2/415 (0.5)	8.2 (0.1–14)	China	(Ren et al., 2008)
Respiratory tract sample	1/222 (0.5)	27 (0.5–82)	Italy	(Babakir-Mina et al., 2008)
Respiratory tract sample	3/537 (0.6)	1.6 (0.10–2.6)	France	(Foulongne et al., 2008)
Respiratory tract sample	10/371 (2.7)	0.8 (<0.1–79)	UK	(Abedi Kiasari et al., 2008)
Respiratory tract sample	17/265 (6.5)	46 (3.6–85.3)	France	(Mourez et al., 2009)
Respiratory tract sample	7/232 (3)	– (0.1–7.6)	Japan	(Teramoto et al., 2011)
Respiratory tract sample	9/161 (5.6)	4.9 (–)	USA	(Rao et al., 2011)
Respiratory tract sample	7/295 (2.3)	4.9 (–)	USA	(Rao et al., 2011)
Tonsil	11/91 (12)	42 (10–88)	Italy	(Babakir-Mina et al., 2009a)
Lymphoid tissue	3/42 (7.1)	– (–)	UK	(Sharp et al., 2009)
Lymphoid tissue	1/55 (1.8)	– (–)	UK	(Sharp et al., 2009)
Lung tissue	9/20 (45)	68 (40–85)	Italy	(Babakir-Mina et al., 2009b)
Lung tissue	1/30 (3.3)	– (45–77)	Japan	(Teramoto et al., 2011)
Stool	1/192 (0.5)	1 (<0.1–17)	Sweden	(Allander et al., 2007a)
Stool	1/193 (0.5)	1.3 (<0.1–11.8)	Australia	(Bialasiewicz et al., 2009b)
Stool	10/86 (11.6)	7.1 (1–27) 54 (17–75)	Italy	(Babakir-Mina et al., 2009b)
Stool	1/9 (11.1)	46 (3.6–85.3)	France	(Mourez et al., 2009)
Plasma HIV-1 positive	2/62 (3.2)	45.5 (37–54)	Italy	(Babakir-Mina et al., 2009a)
Plasma HIV-1 positive	4/153 (2.6)	41.9 (33.8–47.3)	Italy	(Babakir-Mina et al., 2010a)
Plasma HIV-1 negative	4/130 (3.1)	41 (32–47.5)	Italy	(Babakir-Mina et al., 2010a)
Blood HIV-1 positive	1/100 (1)	46 (23–82)	Italy	(Barzon et al., 2009a)
Plasma	3/640 ~ (0.5)	40 (–)	France	(Touinssi et al., 2013)
Respiratory tract -HIV infected and uninfected	91/1460 (6.2)	-(<2)	South African	(Nunes et al., 2014)

Table 3. Detection of KI polyomavirus in various biological specimens.

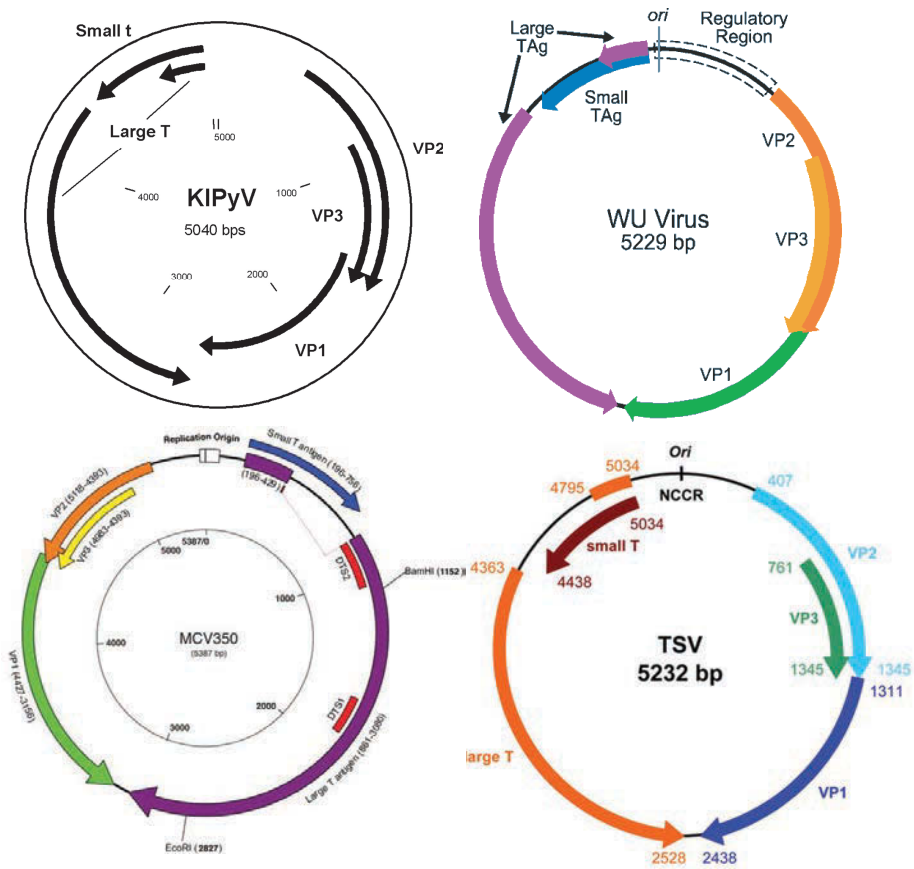


Figure 7. Genome map of KI, WU, MC, and TS polyomaviruses Indicated are the five identified ORFs representing the putative “early” genes encoding small and large T antigen, and the putative “late” genes encoding VP1, VP2, and VP3. The NCCR is placed on top and contains the putative ori. Nucleotide position 1 was chosen within the NCCR in the large T binding region. Each map reproduced with permission from its source: (Allander et al., 2007a, Gaynor et al., 2007, Feng et al., 2008, van der Meijden et al., 2010).

## 2.4. Washington University polyomavirus (WUPyV)

Discovery of WUPyV resulted through a large-scale molecular virus screening system in 2007 created by David Wang's research group at Washington University, in the USA. They reported the identification of a novel WU polyomavirus present in respiratory secretions from human patients with symptoms of acute respiratory tract infection (Gaynor et al., 2007). This virus was initially detectable in a nasopharyngeal aspirate from an Australian 3-year-old child diagnosed with pneumonia. A random library was generated from nucleic acids extracted from the nasopharyngeal aspirate and was analyzed by high-throughput DNA sequencing. The multiple DNA fragments they cloned possessed limited similarity to known polyomaviruses. They also used library construction and shotgun sequencing techniques. For sample preparation, NPA was thawed and directly treated with DNase. Total nucleic acid was extracted and randomly amplified. RNA in the total nucleic acid preparation was converted to cDNA by reverse transcription with the first primer. Two rounds of random priming with the first primer and extension with sequenase enabled second-strand cDNA synthesis as well as random priming of the DNA originally present in the total nucleic acid sample. Amplicons were then generated via PCR by use of a second primer. The second primer-amplified material was TOPO cloned into pCR4.0 and transformed into bacteria, and white colonies were placed into 384-well plates. DNA was purified by magnetic bead isolation and sequenced. Product analysis of shotgun sequences and complete genome amplification revealed limited homology to known polyomaviruses. They subsequently sequenced the entire virus genome of 5,229 bp, referred to as "WU virus". It had genomic features characteristic of the family *Polyomaviridae* (Gaynor et al., 2007).

Thereafter, WUPyV have been detectable worldwide (Table 4) and provide serological evidence of infection. Like KIPyV, the status of this virus as a human respiratory pathogen is a topic of debate because of its detection in samples from healthy individuals and its frequent co-detection with other respiratory viruses.

### **WUPyV genome and structure**

WUPyV DNA sequence analysis has revealed genomic features characteristic of polyomaviruses. The genome size of WUPyV, 5,229 bp, is quite comparable to those of the other primate polyomaviruses (Figure 7) (Gaynor et al., 2007). The WUPyV genome shows the highest sequence identity with that of KIPyV. Phylogenetic analysis

demonstrates that WUPyV, along with its sibling KIPyV, defines a novel clade within the family *Polyomaviridae* (Johne et al., 2011). The genome organization includes an early region coding on one strand for sTAg and LTA<sub>g</sub>, and a late region coding on the opposite strand for the capsid proteins VP1, VP2, and VP3 (Figure 7). These two regions are separated by a regulatory region that contains typical polyomavirus features (Gaynor et al., 2007). Unusual features in the WU regulatory region include two partially overlapping LTA<sub>g</sub> binding sites and slightly variant spacing between the LTA<sub>g</sub> binding sites when compared to that of SV40, BKPyV, and JCPyV (Gaynor et al., 2007).

### **WUPyV epidemiological aspects**

The first prevalence data for WUPyV was based on PCR detection of virus DNA in NPA samples (Gaynor et al., 2007). Now it is obvious that this virus is widespread globally (Table 4). Its DNA prevalence in respiratory tract samples of healthy individuals has ranged from 0.4 to 9%, a difference no higher for those of patients with respiratory symptoms. Its prevalence has increased in immunosuppressed individuals in some, but not in all reports (Ren et al., 2008, van der Zalm et al., 2008, Rao et al., 2011). In parallel with this, other studies used similar methods to look at the prevalence of WUPyV at sites other than the respiratory site, e.g. in urine, blood, and stool (Abedi Kiasari et al., 2008, Dalianis et al., 2009, Ren et al., 2009). WUPyV genomic DNA monitored for by PCR is seldom detectable in the urine of normal or immunosuppressed individuals, or in their blood, but WUPyV has occurred at very low frequency (<1%) in stool samples (Allander et al., 2007a, Gaynor et al., 2007, Ren et al., 2009). Moreover, WUPyV has been absent from the CSF of patients with or without PML (Dang et al., 2011).

No WUPyV DNA has been detected in plasma, urine or respiratory samples from pregnant and non-pregnant women. KIPyV DNA appeared in two plasma samples from non-pregnant women (2%) but was undetectable in other samples from pregnant or non-pregnant women (Csoma et al., 2012). The epidemiology of KIPyV and WUPyV in healthy populations in France, by examination of 640 plasma samples by a conserved real-time PCR detection system, resulted in three KIPyV but no WUPyV sequences found (Touinssi et al., 2013).

Furthermore, ELISA tests for VP1 antibodies show that exposure to WUPyV is common (55–98%), and suggests that the initial exposure occurs during childhood (Carter et al., 2009, Dalianis et al., 2009, Kean et al., 2009, Nguyen et al., 2009).

### **WUPyV disease associations**

Since the time that WUPyV were first identified in St. Louis, in the USA, viral sequences have been regularly detected in respiratory materials worldwide (Table 4), suggesting that viruses are widespread among humans with respiratory tract diseases, but none of these studies revealed a clear correlation between WUPyV and respiratory disease (Babakir-Mina et al., 2011).

Often KIPyV and WUPyV occur together with other known pathogens and not in loads high enough to suggest any causal relationship. Nonetheless, the pathogenicity of KIPyV and WUPyV remains speculative (Norja et al., 2007). Further, the link between KIPyV and WUPyV infection and respiratory disease is complicated by their high rate of co-infection with other respiratory viruses (Allander et al., 2007a, Bialasiewicz et al., 2007a, Bialasiewicz et al., 2007b, Gaynor et al., 2007, Han et al., 2007, Abedi Kiasari et al., 2008, Babakir-Mina et al., 2008, Neske et al., 2008). However, in some cases, KIPyV or WUPyV were the only pathogens detected, despite wide screening including for viruses, bacteria, and fungi (Han et al., 2007, Mourez et al., 2009). Nasopharyngeal aspirate samples from 1,980 pediatric patients with suspected acute respiratory tract infection, and 82 samples from healthy subject controls in South China were analyzed by multiplex PCR and results showed 53 (2.7%) patients as being positive for WUPyV, while none of the 82 healthy controls were positive. One of the most common pathogens in co-infected samples was WUPyV (54.9%) (Cai et al., 2014).

These preliminary studies led to the hypothesis that these new viruses do play an etiological role in childhood respiratory disease. Although both viruses occur mainly in respiratory-tract samples of immunocompromised patients, no clear causative link yet exists with respiratory disease (Allander et al., 2007a, Gaynor et al., 2007). Indeed, absence of specific clinical or radiological findings, frequent co-detection with other respiratory pathogens, detection in subjects without signs or symptoms of respiratory disease, and variability in viral loads have prevented any definitive conclusions. Prospective studies with large sample sizes including both immunocompromised and immunocompetent patients with and without respiratory symptoms will be vital.

Standardized quantitative real-time PCR (qPCR) methods, definition of a clear clinical cut-off value, and the identical timing in the collection of respiratory samples are also crucial to understand the pathogenic role, if any, of KIPyV and WUPyV in human pathology.

Sample type	No. Positive samples /total (%)	Patients Median age (age range, years)	Country	Reference
Respiratory tract sample	37/1245 (3)	2 (0.3–53)	Australia	(Gaynor et al., 2007)
Respiratory tract sample	6/890 (0.7)	3 (0.3–51)	USA	(Gaynor et al., 2007)
Respiratory tract sample	34/486 (7)	0.9 (0.1–5.9)	South Korea	(Han et al., 2007)
Respiratory tract sample	3/72 (4.2)	1.2 (0.1–6.5)	South Korea	(Han et al., 2007)
Respiratory tract sample	2/79 (2.5)	1.1 (1.1)	Canada	(Abed et al., 2007)
Respiratory tract sample	5/78 (6.4)	1.8 (1.1–2.0)	Canada	(Abed et al., 2007)
Respiratory tract sample	10/983 (1.0)	– (0.3–34)	UK	(Norja et al., 2007)
Respiratory tract sample	17/406 (4.2)	2.2 (<0.1–8)	China	(Yuan et al., 2008)
Respiratory tract sample	62/1277 (4.9)	1.6 (<0.1–22)	Germany	(Neske et al., 2008)
Respiratory tract sample	19/302 (6.3)	1 (<0.1–14)	Thailand	(Payungporn et al., 2008)
Respiratory tract sample	10/415 (2.4)	0.8 (0.1–14)	China	(Ren et al., 2008)
Respiratory tract sample	128/2866(4.5)	1.38 (<0.1–95)	Australia	(Bialasiewicz et al., 2008)
Respiratory tract sample	13/537 (2.4)	0.11 (0.2–4)	France	(Foulongne et al., 2008)
Respiratory tract sample	4/371 (1)	0.8 (<0.1–79)	UK	(Abedi Kiasari et al., 2008)
Respiratory tract sample	1/278 (0.35)	3 (<0.1–5)	China	(Lin et al., 2008)
Respiratory tract sample	2/265 (0.75)	46 (3.6–85.3)	France	(Mourez et al., 2009)
Respiratory tract sample	3/98 (3)	– (<3)	Germany	(Kleines et al., 2008)
Respiratory tract sample	38/232 (16.4)	– (0.1–7.6)	Japan	(Teramoto et al., 2011)
Respiratory tract sample	5/161 (3)	4.9 (–)	USA	(Rao et al., 2011)
Respiratory tract sample	14/295 (5)	4.9 (–)	USA	(Rao et al., 2011)
Tonsils	4/91 (4.4)	42 (10–88)	Italy	(Babakir-Mina et al., 2009a)
Lymphoid tissue	3/42 (7.1)	– (–)	UK	(Sharp et al., 2009)
Stool	2/ 377 (0.5)	0.9 (0.1–13)	China	(Ren et al., 2009)
Stool	7/193 (3.6)	1.3(<0.111.8)	Australia	(Bialasiewicz et al., 2009b)
Stool	2/221 (0.9)	41.8(0.197.6)	Australia	(Bialasiewicz et al., 2009b)
Stool	7/86 (8.1)	7.1 (1–27) 54 (17–75)	Italy	(Babakir-Mina et al., 2009b)
Cerebral spinal fluid	1/60 (1.6)	44 (4–88)	Italy	(Barzon et al., 2009b)
Plasma HIV-1 positive	1/62 (1.6)	45.5 (37–54)	Italy	(Babakir-Mina et al., 2009a)
Serum HCV positive	2/79 (2.5)	– (–)	USA	(Miller et al., 2009)
Plasma HIV-1 positive	7/153 (4.6)	41.9(33.8–47.3)	Italy	(Babakir-Mina et al., 2010a)
Plasma HIV-1 negative	1/130 (0.8)	41 (32–47.5)	Italy	(Babakir-Mina et al., 2010a)
Blood HIV-1 positive	1/100 (1)	46 (23–82)	Italy	(Barzon et al., 2009a)
Plasma	0/640 (0)	40(–)	France	(Touinssi et al., 2013)
Respiratory tract -HIV infected and uninfected	156/1460(10.7)	-(<2)	South African	(Nunes et al., 2014)

Table 4. Detection of WU polyomavirus in various biological specimens.

## 2.5. Merkel cell polyomavirus (MCPyV)

MCPyV is the first polyomavirus linked to human cancer. Its recent discovery helps explain many of the ambiguous features of MCC (Feng et al., 2008). MCPyV is clonally integrated into MCC tumor cells, which, to survive, require continued MCPyV oncoprotein expression. The integrated viral genomes have a tumor-specific pattern of tumor antigen gene mutation that incapacitates viral DNA replication (Feng et al., 2008). MCPyV is the first human cancer virus that is mostly harmless viral flora in human skin (Foulongne et al., 2010, Schowalter et al., 2010), but is believed to initiate cancer in a host with specific susceptibility factors, such as age and immune suppression if it acquires a precise set of mutations. Identification of this tumor virus has led to new opportunities for early diagnosis and targeted treatment of MCC (Chang and Moore, 2012).

Digital transcriptome subtraction (DTS) is the new molecular technique developed by Feng et al. to discover MCPyV associated with human cancers (Feng et al., 2007, Feng et al., 2008). MCC was known as a rare but aggressive skin cancer common in the elderly and the immunocompromised, and it also occurred among organ transplant recipients (Koljonen et al., 2009b). Based mainly on the link between MCC and immunosuppression, they attempted to determine whether MCC has an infectious origin (Feng et al., 2008). Representational difference analysis (RDA) was used to create representations of the DNA genome of normal or diseased tissue. RDA is a technique used to find sequence differences between two genomic or cDNA samples. Genomes or cDNA sequences from a cancer sample and a normal sample are PCR amplified, and differences are analyzed using subtractive DNA hybridization.

To search for viral genomes in MCC, they built on the principles of RDA and modernized the technique by combining it with the availability of sequences from the entire human genome. The product was a related but distinct method known as DTS (Feng et al., 2008). They generated two cDNA libraries from a total of four anonymized MCC tumors, an approach requiring precise discrimination between human and nonhuman cDNA sequences. Database comparisons show a high likelihood that small viral sequences can be successfully distinguished from human sequences. The work on this approach began over a decade ago, prior to the release of the full human genome. It has become a practical approach to new-virus discovery only with the generation of highly-verified human genomic sequence databases and the development of low-cost, high-throughput sequencing (Feng et al., 2007). After cDNA is created from RNA extracted from fresh



tumor tissues through reverse transcription, this library is then exhaustively sequenced to generate a high-fidelity transcriptome database of the tumor. Precise discrimination between human and non-human cDNA sequences then occurs, and known human sequences that align to GenBank human reference sequences are then computationally subtracted. Careful screening and selection of high-quality sequences prior to computational subtraction is necessary to increase chances of discovering a true candidate. The non-matching non-human sequences remaining are candidate pathogen cDNAs. This smaller candidate database can then be explored using low-stringency alignment to virus databases or by experimental extension to identify novel viral sequences (Feng et al., 2008).

### **MCPyV genome and structure**

Similar to other polyomaviruses, MCPyV has a 5,387 bp genome that is divided into early and late coding regions by a noncoding regulatory region (NCRR) (Figure 7). The MCPyV NCRR contains a 71-bp core sequence that constitutes the viral replication origin, which efficiently sustains T antigen-directed DNA replication (Chang and Moore, 2012). NCRR contains regulatory elements and transcriptional promoters for early and late viral gene expression that are bidirectionally coordinated with viral DNA replication (Feng et al., 2011). The MCPyV early region encodes an LT, an sT, and a 57 kT, all of which share a 78-aa N terminus (Figure 7). MCC-derived MCPyVs have tumor-associated missense mutations or deletions in the portion of the T antigen gene encoding LT. These mutations eliminate the C-terminal replication functions of this protein but do not interfere with N-terminal LT domains, including the LXCXE motif that targets cellular retinoblastoma and related pocket proteins, or with expression of the MCPyV sT protein, which plays a more critical role in cell transformation than does SV40 sT (Feng et al., 2008).

The late region encoding VP1, VP2, and VP3 is expressed after the start of viral DNA replication to self-assemble into ~55-nm-diameter viral particles that demonstrate the icosahedral symmetry characteristic of polyomaviruses (Tolstov et al., 2009). MCPyV structural proteins are relatively dissimilar to those of BKPyV and JCPyV, so antibodies against MCPyV VP generally do not cross-react with those of these viruses (Pastrana et al., 2009).

A study based on analysis of a 1284 bp-long fragment of MCPyV generating three distinct MCPyV genomic fragments of LTag, VP1, VP2, and NCCR demonstrated the

existence of five major geographically related MCPyV genotypes, which are European/North American/Caucasian, African (Sub-Saharan), Oceanian, South American/Amerindian, and Asian/Japanese (Martel-Jantin et al., 2014). In addition, complete and partial sequences of sTag and LTag from 32 Japanese MCPyV-infected MCC revealed that each Japanese patient harbored a specific MCPyV strain with some synonymous or silent mutations and stop codons or deletions, but functional domains of T antigen had no aa changes. All stop codons were localized after the retinoblastoma protein-binding domain. These Japanese MCPyV strains were very closely interrelated. Phylogenetic analysis of nine Japanese sequences and 70 other sequences for the sT and LT genes registered in GenBank indicated that Japanese or Asian MCPyV strains formed distinct clades from the Caucasian clade, and a phylogenetic tree only of the sT gene formed a characteristic three clades and showed that all Japanese or Asian strains were included in the dominant clade (Matsushita et al., 2014).

### **MCPyV epidemiological aspects**

Early studies identified low copy numbers of the MCPyV genome in various tissues from healthy individuals and from non-MCC patients. Although most MCC tumors have high copy numbers of the viral genome, MCPyV DNA appears at trace levels in other tissues, particularly in skin, which is consistent with MCPyV's being part of the healthy human flora (Foulongne et al., 2010, Schowalter et al., 2010, Foulongne et al., 2012). Detection of MCPyV has been reported on environmental surfaces as well (Bofill-Mas et al., 2010, Foulongne et al., 2011). Serologic studies have confirmed this finding and have revealed that MCPyV causes a common if not ubiquitous human infection. Polyomavirus serology has been based largely on antibodies reacting with the late structural proteins VP1 and, to a lesser extent, VP2. Serum reactivity to isolated MCPyV VP1 was first reported as a 25 to 42% prevalence in MCPyV antibody positivity (Kean et al., 2009). An improved multiplex antibody-binding assay has been used to detect levels of MCPyV VP1 in sera and it is found in control subjects from the general population, at a prevalence of 59% (Carter et al., 2009). Immunoblotting of VP1 reveals, however, that conformational epitopes present in assembled viral particles provide higher sensitivity and specificity for measuring past MCPyV exposure (Chen et al., 2011a). Expression of MCPyV VP1 and VP2 in cells generates self-assembling virus-like particles (VLPs) that can serve as antigens for immunoassays. Use of this approach revealed an age-related increase in

MCPyV seroprevalence up to age 50, at which age, 80% of healthy North American blood donors showed evidence of past MCPyV exposure (Tolstov et al., 2009). Use of MCPyV VLPs provided similar results (Touze et al., 2010). Antibody responses to MCPyV VLPs are not diminished by cross competition with other polyomavirus antigens, which indicates that they are highly specific for MCPyV infection (Pastrana et al., 2009).

Thus, similar to other HPyVs, MCPyV appears to be a common infection of childhood, and primary infection may be asymptomatic. The rate of seroprevalence in children younger than age 4 is 9%, which by 4 to 13 years of age increases to 35% (Chen et al., 2011a). In a longitudinal study of healthy adult gay and bisexual men aged 18 and up, recruited and followed for signs and symptoms related to HIV and AIDS, 79% had antibodies to MCPyV at study entry, and subsequently, over a 4-year follow-up period, 31 (5.5%) of the men seroconverted to MCPyV positivity (Tolstov et al., 2011). No apparent signs, symptoms, or common laboratory test results were significantly associated with primary MCPyV infection among these men.

MCPyV DNA has been detectable among non-tumor samples at a variable frequency (Table 5), such as in nasopharyngeal aspirates (NPAs) (Goh et al., 2009) and respiratory tract samples from Australian patients with upper respiratory symptoms (Bialasiewicz et al., 2009a). MCPyV DNA has been amplified in lower-respiratory-tract samples from hospitalized Italian patients with lower-respiratory-tract symptoms (Babakir-Mina et al., 2010b).

Studies have shown T-cells to be important mediators of MCPyV-specific immune surveillance (Iyer et al., 2011, Kumar et al., 2011a, Kumar et al., 2011b, Kumar et al., 2012). A study of the associations between immune cell counts, MCPyV DNA, patient and tumor characteristics, and patient outcome, showed the numbers of T cells to be generally higher in MCPyV-positive than in MCPyV-negative MCC (Sihto et al., 2012).

Sample	No. Samples	DNA-Positive %	Reference
Respiratory tract samples	526	1.3	(Bialasiewicz et al., 2009a)
Respiratory tract samples	635	4.2	(Goh et al., 2009)
Respiratory tract samples	305	3.27	(Abedi Kiasari et al., 2011)
Lymph nodes	29	10.3	(Toracchio et al., 2010)
Lymphoid Tissue	55	1.8	(Sharp et al., 2009)
Sera	23	39	(Fukumoto et al., 2013)
PBMC	21	14	(Shuda et al., 2009)
Buffy coat	60	22	(Pancaldi et al., 2011)
Lung	43	26	(Loyo et al., 2010)
Saliva	10	100	(Loyo et al., 2010)
Saliva	135	30	(Baez et al., 2013)
Bladder	10	60	(Loyo et al., 2010)
Liver	37	54	(Loyo et al., 2010)
Colon	50	18	(Loyo et al., 2010)

Table 5. Detection of MCPyV DNA among non-tumor materials at a variable frequency.

### MCPyV disease associations

MCPyV is linked to a human cancer, Merkel cell carcinoma. Merkel cells are large, clear, oval cells in touch-sensitive areas, such as palmar aspects of the hands and plantar aspects of the feet, lips, and buccal mucosa of vertebrates. The vast majority of Merkel cells are intimately associated with a nerve terminal. They were first described by Friedrich Sigmund Merkel (1845 – 1919), a German anatomist and histopathologist, in 1875 and were referred to as “Tastzellen” or “touch cells” (Halata et al., 2003, Koljonen, 2006). Merkel cells are localized in the basal layer of the skin and mucosa either as single cells or in clusters. In 1972, Toker first described a trabecular carcinoma of the skin (Toker, 1972). However, whether the Merkel cell carcinoma truly derives from the Merkel cell is still to date strongly debated. Without any doubt, more studies are needed to elucidate the origin of Merkel cell carcinoma, because systemic therapy in patients with disseminated disease would probably have a significantly higher impact on survival and on disease-free rates, due to modifications based on the origin of the cancer cells.



*Figure 8. Merkel cell carcinoma on the right eyebrow of a 82-year old patient. Reproduced with permission from Bachmann, J., et al., 2005. Pancreatic metastasis of Merkel cell carcinoma and concomitant insulinoma: case report and literature review. World Journal of Surgical Oncology 3, 58.*

Since the discovery of polyomaviruses, they have been implicated as causative agents of human cancer. *In vitro* studies and experimental animal models have shown that the early viral proteins LTag and sTag of SV40, BKPyV, and JCPyV possess oncogenic potential (Barbanti-Brodano et al., 2006). The novel viral sequences have been found in four Merkel cell carcinoma tumor tissues (Feng et al., 2008). Similar to SV40 and murine polyomaviruses, MCPyV encodes a multiply spliced tumor antigen protein complex that targets several tumor-suppressor proteins (Shuda et al., 2008). Within infected tumors, the T antigen is expressed only in tumor cells, as is predicted for a direct viral carcinogen (Shuda et al., 2009). The LT antigen acts mainly by blocking the function of the cellular tumor-suppressor proteins pRb and p53 and by inducing chromosomal aberration and instability in the host-cell genome. The sTag binds the regulatory and catalytic subunits of the protein phosphatase PP2A, thereby constitutively activating the  $\beta$ -catenin pathway which drives the cells into proliferation (Moore and Chang, 2010).

Further studies show a prevalence of 40 to 100% of the MCPyV in Merkel cell carcinoma specimens (Agelli et al., 2010). The Chang-Moore Laboratory observed monoclonal viral integration in five of ten (50%) patient samples and, interestingly, primary and metastatic MCC tissues from the same patient showed an identical viral integration pattern, indicating that the integration of MCPyV preceded the metastatic spreading of the cancer (Feng et al., 2008).

Given these features, the strongest evidence to support MCPyV as causing MCC comes from its random clonal integration into Merkel cell tumors and from knockdown studies showing that T antigen expression is required for the survival of virus-positive Merkel cell lines (Feng et al., 2008, Sastre-Garau et al., 2009, Houben et al., 2010). Although skin carriage of the virus is common, no other tumors except MCC have yet been convincingly linked to MCPyV infection (Schowalter et al., 2010, Moens et al., 2011).

MCPyV, which has tumor-specific truncation mutations, illustrates common features among the human tumor viruses involving immunity, virus replication, and tumor-suppressor targeting (Figure 9). Although MCPyV is a common infection, loss of immune surveillance through aging, AIDS, or transplantation (Koljonen et al., 2009b), and subsequent treatment with immunosuppressive drugs may lead to resurgent MCPyV replication in skin cells (Pastrana et al., 2009). If a rare integration mutation into the host cell genome occurs (Feng et al., 2008), the MCPyV T antigen can activate independent DNA replication from the integrated viral origin that will cause DNA strand breaks in the proto-tumor cell (Shuda et al., 2008). A second mutation that truncates the T antigen, eliminating its viral replication functions but sparing its RB1 tumor suppressor targeting domains, is required for the survival of the nascent Merkel tumor cell. Exposure to sunlight (ultraviolet, UV, radiation) and other environmental mutagens may enhance the sequential mutation events that turn this asymptomatic viral infection into a cancer virus (Mogha et al., 2010).

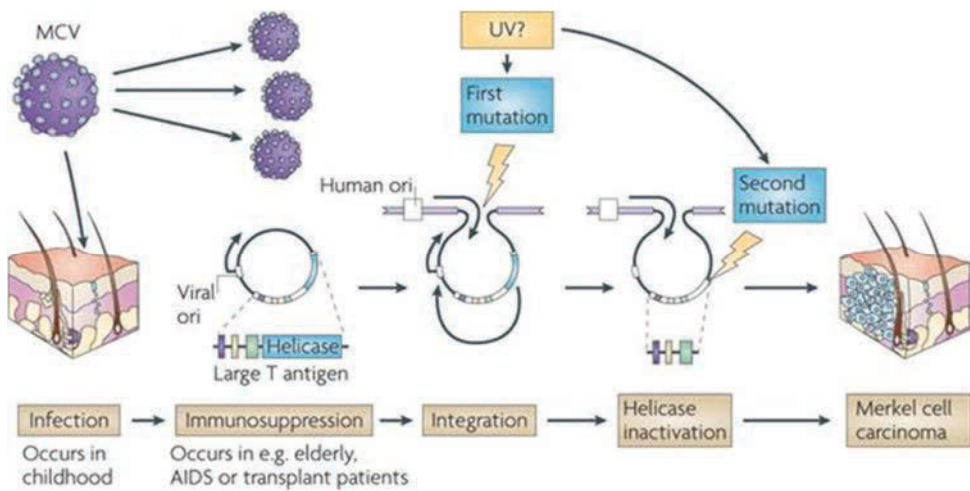


Figure 9. Merkel cell polyomavirus (MCPyV), which has tumor-specific truncation mutations, illustrates common features among the human tumor viruses involving immunity, virus replication and tumor suppressor targeting. Reproduced with permission from Moore, P.S., Chang, Y.A., 2010. Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nat Rev Cancer* 10, 878-889.

Thus, strong evidence exists that MCPyV is the etiological agent responsible for most cases of MCPyV. Based on the evidence accumulated to date, the WHO International Agency for Cancer Research recently classified MCPyV as “probably carcinogenic to humans” (Group 2A) (Bouvard et al., 2012).

## **2.6. Human polyomavirus 6 (HPyV6)**

Rolling-circle amplification (RCA) was performed on forehead swab-samples of healthy individuals to explore the possibility that non-integrated MCPyV genomes and other polyomaviruses commonly inhabit healthy human skin (Schowalter et al., 2010). They applied some adjustments to increase RCA sensitivity, such as DNA exonuclease treatment to digest cellular DNA (Schowalter et al., 2010).

Among the newly available techniques for whole-genome amplification, the RCA technique has been involved in the discovery of several new members of the polyomavirus family (Schowalter et al., 2010, van der Meijden et al., 2010, Buck et al., 2012, Siebrasse et al., 2012b, Yu et al., 2012b). RCA using the DNA polymerase of bacteriophage phi29 is showing great value for the analysis of viral DNA genomes. This polymerase possesses several features such as strand-displacement activity, proof-reading activity and generation of very long synthesis products, which make it most suitable for the efficient amplification of circular DNA molecules from complex biological samples (Johne et al., 2009). Therefore, phi29 polymerase-dependent RCA has been applied to the study of several virus families possessing circular DNA genomes.

As specific primer sequences are not required for this technique, many novel viruses have been discovered. Moreover, one-step whole genome amplification by RCA has enabled rapid sequencing of viral genomes and generation of infectious genomic clones. This approach has revealed the identity of a new polyomavirus, HPyV6, on the skin of a healthy individual (Schowalter et al., 2010). DNA from the skin swab specimens extracted and then subjecting these to random hexamer-primed RCA (Schowalter et al., 2010). Their initial study goal was to isolate full-length wild-type MCPyV genomic DNA from swabs drawn across the surface of the human skin. RCA reactions performed on DNA extracted from skin swabs were analyzed by digesting the finished reaction with restriction enzymes that would be expected to cut known MCPyV isolates to unit length. Agarose gel analysis of an initial set of samples showed a smoothly distributed smear of products, suggesting that the majority of RCA products were derived from random fragments of linear cellular DNA, as opposed to discrete circular DNA templates. Therefore, to overcome this problem, the authors incorporated a pre-processing step in which the extracted DNA was digested with exodeoxyribonuclease V, an enzyme that digests linear DNA molecules but spares double-stranded circular DNA molecules. This pre-processing step was augmented by the inclusion of a restriction enzyme that they reasoned would be unlikely to digest



MCPyV DNA. The full genomic sequence of the previously unknown polyomavirus species of human origin was amplified by PCR and cloned, and named in the order of its appearance, HPyV6.

### **HPyV6 genome and structure**

The novel HPyV6 shares a genome organization that is typical among polyomaviruses (Figure 4). Phylogenetic comparison of the complete genomes of HPyV6 to other polyomavirus species shows that these occupy a distinct clade (Schowalter et al., 2010). It also occupies a longer common branch with WUPyV and KIPyV (Figure 2), an apparent affiliation attributable primarily to the extreme divergence of the late regions (VP1 or VP2 capsid genes) of this virus from all other polyomaviruses. In contrast to the late regions, the early regions of HPyV6 are quite distinct from those of WUPyV and KIPyV (Schowalter et al., 2010).

### **HPyV6 epidemiological aspects**

Since the first reports on this novel PyV, a number of studies have evaluated its occurrence in healthy subjects and in immunocompetent and immunosuppressed patients suffering from a variety of diseases (Table 6). By high-throughput metagenomic sequencing, HPyV6 was evident in skin swabs of normal-appearing forehead skin from three of six individuals (Foulongne et al., 2012).

One study analyzed over 300 skin swabs from different groups of healthy volunteers who were free of skin diseases; it used type-specific qPCRs and detected HPyV6 DNA in 28.7% of the swabs. The same authors consecutively collected swabs from over 100 healthy individuals, and in these volunteers, persistent cutaneous infections were evident in 24% (Ehlers and Wieland, 2013).

The presence of some known HPyVs was investigated with qPCR in 716 clinical specimens of 32 children receiving a transplant (11 bone marrow, 11 liver, 5 heart, 2 kidney, 2 lung, and 1 liver/lung transplants). HPyV6 was detectable in the feces of a lung and a BMT recipient and in the nasopharyngeal swab of a heart transplant patient (Siebrasse et al., 2012a).

Another study investigated detection rates and DNA loads by qPCR in various skin cancers from Japanese patients. MCPyV, HPyV6, and HPyV7 were detectable at low copy

numbers in 22.2%, 3.2%, and 1.6% of squamous cell carcinomas, 18.0%, 2.0%, and 4.0% of basal cell carcinomas, and 19.1%, 4.3%, and 4.3% of melanomas, (Imajoh et al., 2013).

Schwalter and colleagues found antibodies against VP1 of HPyV6 in 69% of blood donors, identical to that of MCPyV (Schwalter et al., 2010). A very recent study on the seroprevalence of HPyV6, HPyV7, HPyV9, TSPyV, and MCPyV detected HPyV6 antibodies in 37.5% of 1- to 4-year-old children. Their prevalence rose to 67% in 30- to 39-year-old adults, and to 98% in those 80 and older (Nicol et al., 2013b).

### HPyV6 disease association

HPyV6 has not yet been clearly associated with any disease.

Sample type	DNA prevalence (%)	Patients, age (years)	Country	Reference
Skin swabs	14	35 volunteers, ages not reported	USA	(Schwalter et al., 2010)
Forehead skin swabs	50	5 healthy adults (32–63y), 1 patient with previous MCC (75 y)	France	(Foulongne et al., 2012)
NP swabs	0.6	160 NP swabs, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
Urines	0	169 urines, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
Feces	1.6	122 feces, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
Plasma	0	265 plasma, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
Forehead skin swabs HIV-infected	39.0	210 men, median age 43 y	Germany	(Wieland et al., 2014)
Forehead skin swabs HIV-uninfected	27.6	239 men, median age 48 y	Germany	(Wieland et al., 2014)
Squamous cell carcinomas (SCC)	3.2	63 SCC, median age 84 y (62–100 y)	Japan	(Imajoh et al., 2013)
Basal cell carcinoma (BCC)	2.0	50 BCC, median age 78 y (51–92 y)	Japan	(Imajoh et al., 2013)
Malignant melanoma (MM)	4.3	47 MM, median age 70 y (25–92 y)	Japan	(Imajoh et al., 2013)

Table 6. Detection of HPyV6 in various biological specimens.

## **2.7. Human polyomavirus 7 (HPyV7)**

To detect HPyV6- and WUPyV-like sequences, an additional novel polyomavirus was found in these healthy skin samples and was called HPyV7. Biochemical experiments show that polyomavirus DNA is shed from the skin in the form of assembled virions. Since it did not appear to be possible to design universal primers capable of binding a broad range of polyomavirus species, they designed partially degenerate primers targeting sequences conserved between specific pairs of polyomaviruses. A primer pair designed to bind both HPyV6 and WUPyV successfully amplified a unique polyomavirus-like sequence. The complete genome of HPyV7 identified was amplified by PCR and was found to be 68% identical to HPyV6 at the nucleotide level (Schowalter et al., 2010).

### **HPyV7 genome and structure**

Phylogenetic studies of the HPyV genomes indicate that KIPyV and WUPyV are closely related, as are HPyV6 and HPyV7 (Van Ghelue et al., 2012). Whereas VP1s of HPyV classified on different phylogenetic branches display ~26 to 55% aa identity, VP1s of HPyV on the same phylogenetic branch possess high identity: KIPyV and WUPyV: 65.5%, HPyV6 and HPyV7: 68.5%, LPyV and HPyV9: 84.1%, BKPyV and JCPyV: 78.2%. However, the predicted overall structure of all HPyV VP1 proteins is similar in containing exposed loops (Van Ghelue et al., 2012).

The novel HPyV7 shares a genome organization that is typical among polyomaviruses (Figure 4). The early region encodes the regulatory proteins sTag and LTag, and the late region encodes the structural proteins VP1, VP2, and VP3. These regions are separated by a NCCR. An open reading frame encoding the agnoprotein present in the human BKPyV and JCPyV was not identified in the HPyV7. In combined phylogenetic analysis of LTag, VP1, and VP2 proteins, HPyV6 forms a clade with HPyV7, HPyV10 with MWPyV (and MXPpyV), and HPyV9 with the LPyV; a polyomavirus of African green monkeys) and other non-human primate PyVs (Figure 2).

### **HPyV7 epidemiological aspects**

HPyV7 was first identified in skin swabs of healthy individuals (Schowalter et al., 2010). By high-throughput metagenomic sequencing, HPyV6 appeared in skin swabs from three of six individuals, and HPyV7 was detectable in one skin swab (Foulongne et al., 2012).

Forehead swabs, 449 from 210 HIV-positive men and 239 healthy male controls, were analyzed by five type-specific qPCR for the presence of HPyV7; HPyV7 DNA was detectable in 21.0% of the forehead skin swabs of the HIV-positive men, compared to 13.4% of the healthy controls (Wieland et al., 2014).

The seroprevalence of HPyV6 and HPyV7 was investigated in two studies, Schowalter and colleagues found antibodies against VP1 of HPyV6 in 69% and against HPyV7 in 35% of 95 blood donors, compared with 69% for MCPyV. Of the blood donors, 28% had antibodies against both HPyV6 and HPyV7, and 16% against all three cutaneous PyVs (Schowalter et al., 2010). In another study, seroprevalence for HPyV7 was lower than for HPyV6. Only 10% of the 1- to 4-year-old children were seropositive. In adults aged 20 or older, HPyV7 seroprevalence increased with age from 45 to 86% (Nicol et al., 2013b).

Van der Meijden et al. investigated the serological behavior of cutaneous HPyVs in a general population. They analyzed 799 sera from immunocompetent Australian individuals from newborns to adults of 87 with a Luminex xMAP technology-based immunoassay for the presence of VP1-directed IgG antibodies against MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, and used BKPyV as a control. Except for HPyV9, the overall seropositivity was high for the cutaneous polyomaviruses (66-81% in adults), and gradually increased with age. Children below 6 months displayed seropositivity rates comparable to those of the adults, indicative of maternal antibodies. These profiles indicated heterogeneity among cutaneous polyomaviruses and probably reflected differences in exposure and in the pathogenic behavior of these viruses (van der Meijden et al., 2013).

### **HPyV7 disease association**

HPyV7, discovered at the same time as the skin-tropic HPyV6, has, similarly, not yet been clearly associated with any disease.

<b>Sample type</b>	<b>DNA prevalence (%)</b>	<b>Patients, age (years)</b>	<b>Country</b>	<b>Reference</b>
<b>Skin swabs</b>	11	35 volunteers, age not reported	USA	(Schowalter et al., 2010)
<b>Forehead skin swabs</b>	17	5 healthy adults (32–63y), 1 patient with previous MCC (75 y)	France	(Foulongne et al., 2012)
<b>NP swabs</b>	0.6	160 NP swabs, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
<b>Urines</b>	0.6	169 urines, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
<b>Feces</b>	0	122 feces , 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
<b>Plasma</b>	0	265 plasma, 32 children mean 5.8 y; median 3.1 y	USA	(Siebrasse et al., 2012a)
<b>Forehead skin swabs HIV-infected</b>	21	210 men, median age 43 y	Germany	(Wieland et al., 2014)
<b>Forehead skin swabs HIV-uninfected</b>	13.4	239 men, median age 48 y	Germany	(Wieland et al., 2014)
<b>Squamous cell carcinomas (SCC)</b>	1.6	63 SCC, median age 84 y ( 62–100 y)	Japan	(Imajoh et al., 2013)
<b>Basal cell carcinoma (BCC)</b>	4.0	50 BCC, median age 78 y ( 51–92 y)	Japan	(Imajoh et al., 2013)
<b>Malignant melanoma (MM)</b>	4.3	47 MM, median age 70 y (25–92 y)	Japan	(Imajoh et al., 2013)

*Table 7. Detection of HPyV7 in various biological specimens.*

## 2.8. TS-associated polyomavirus (TSPyV)

In 2010, use of RCA allowed the identification and reporting of a new HPyV in plucked facial spiny papules of a heart-transplant patient with *trichodysplasia spinulosa* (TS), a rare skin disease exclusively seen in immunocompromised patients (van der Meijden et al., 2010). TSPyV is a new species of the family *Polyomaviridae* that was discovered from a skin lesion. Thereafter, EM, immunohistochemistry, and viral load measurements have indicated an etiological role for active TSPyV infection in the development of this disease.

The TSPyV genome was amplified through RCA in the following manner, described herein brief. The TempliPhi 100 RCA Kit (GE Healthcare, UK Limited) was used following the manufacturer's instructions with some slight modifications. The isolated total DNA was diluted in sample buffer, denatured at 95°C, and cooled down slowly to 4°C to allow primer annealing. Meanwhile a premix of 5 ml reaction buffer, 0.2 ml TempliPhi enzyme (bacteriophage Q29 DNA polymerase), and an extra 450 mM of each dNTP was prepared and added to the denatured DNA in sample buffer. The RCA reaction was performed at 30°C for 16 hours followed by inactivation of the enzyme at 65°C for 10 minutes. The RCA product was stored at -20°C for genomic sequencing, assembly, sequence alignments, and phylogenetic analysis (van der Meijden et al., 2010).

### TSPyV genome and structure

TSPyV has a genomic orientation similar to that of the other polyomaviruses (Figure 7). It consists of a relatively small genome of 5,232 bp that contains five open reading frames, transcription of which is regulated by the NCCR (van der Meijden et al., 2010). Although TSPyV transcription patterns have not been described yet, analogous with other polyomaviruses, upon infection probably the first (early) genes are expressed that encode LTag and sTag. They initiate at the same start codon, but are subject to alternative splicing. As a result, TSPyV sTag and LTag share an N-terminal region of approximately 80 aa which contains some highly conserved regions important for polyomavirus replication and transformation. Furthermore, sTag contains a putative PP2A-binding motif, whereas LTag contains motifs that putatively interact with the tumor suppressor- and cell cycle-regulatory proteins pRb and p53 and may inhibit their function. (van der Meijden et al., 2010).

The opposing strand of the TSPyV genome encodes the structural proteins VP1, VP2, and VP3 that form the viral capsid. VP1 is the major protein of the pentameric viral capsomere that furthermore incorporates one copy of either VP2 or VP3 (Stehle et al., 1996). The icosahedral capsid is constructed of 72 such capsomers. Serologically, VP1 is considered the immunodominant polyomavirus protein, and therefore TSPyV VP1 represents an important antigen in measuring host antibody reactivity against TSPyV (van der Meijden et al., 2011).

### **TSPyV epidemiological aspects**

TSPyV DNA prevalence has been studied by several groups (Table 8). Studies that have looked at TSPyV prevalence in various samples (skin swabs, skin biopsies, plucked eyebrow hairs, serum/plasma, urine) have found less than 5% DNA-positivity in asymptomatic individuals, regardless of their immune status (Kazem et al., 2013a). A high TSPyV prevalence (100%) and high viral loads were reported only in TS patients (Kazem et al., 2012), which suggests that TSPyV is the causative agent of the disease. Hypothetically, at some stage TSPyV infecting the skin might cause disease there in a small minority of immunocompromised hosts. Whether, however, the skin represents the major organ of TSPyV persistence is far from clear, as the majority of infected (seropositive) individuals are skin-TSPyV negative. What remains unknown at the moment is whether TS is a manifestation of TSPyV reactivation from an as yet unidentified transplanted organ reservoir, or an unfortunately timed primary infection in the midst of immunosuppression.

A study both of healthy control children and of children and adults undergoing diagnostic testing showed HPyV6, 7, 9, and TSPyV also to be detectable in respiratory specimens and fecal specimens at low prevalence (Rockett et al., 2013). It should also be noted that HPyV6, 7, and TSPyV have all been detected on human skin of healthy subjects, and that the possibility of contamination from the skin during collection of the respiratory and fecal samples must be considered.

The serological behaviors of cutaneous HPyVs in a general population were analyzed with a Luminex -based immunoassay for the presence of VP1-directed IgG antibodies; TSPyV seroreactivity levels were strongly increased after age 2 and waned later in life, comparable to BKPyV, whereas MCPyV, HPyV6, and HPyV7 seroreactivity remained rather stable throughout (van der Meijden et al., 2013). Another study, by applying

comprehensive serodiagnostic methods (IgM, IgG, and IgG avidity) and PCR, has found that primary infections with TSPyV and MCPyV are asymptomatic in childhood (Chen et al., 2014).

Epidemiological studies looking at polyomavirus seroprevalence in humans indicate that HPyV infections of the general population are highly prevalent and occur at an early age, probably without clinical manifestations. Detection of serum antibodies against TSPyV VP1 has been described in two independent studies using either a multiplex serology method based on Glutathione-S-transferase (GST)-VP1 fusion proteins (van der Meijden et al., 2011) or on VP1 VLP-based antibody detection by ELISA (Chen et al., 2011b). In both of these studies, a seroprevalence of about 70% was measured, including respectively a total of 528 Dutch and of 371 Finnish individuals, all healthy. Furthermore, age-distribution analysis of TSPyV VP1 seroprevalence revealed an increase from 41% in children aged 0–9 years to 75% in adults aged 30 years and older (van der Meijden et al., 2011).

A higher TSPyV VP1 seroprevalence of 89% has already been shown in a renal-transplant patient population compared with 70% in healthy individuals (van der Meijden et al., 2011). How to interpret the putative increase in TSPyV seropositivity concomitant with immunosuppressive treatment is not fully understood. Hypothetically, this may be explained by an increase in humoral immunity in response to TSPyV reactivation, possibly viremia, and this would result from iatrogenic suppression of cellular immunity (van der Meijden et al., 2010). In short, seroepidemiological data indicate that TSPyV infection is common and occurs primarily at a young age, with a drop in antibody levels at later stages of life. One study suggests that TSPyV Th-cell responses correlate with TSPyV serological responses, but can sometimes also be detected in TSPyV-seronegative individuals (Kumar et al., 2012).

Fifty-five mucosal melanomas were analyzed by a Luminex assay for the presence of DNA of 10 HPyVs, including TSPyV and two primate viruses (SV40 and LPyV), but none of the analyzed HPyVs were detected, suggesting they are unlikely to play a major role in the development of this tumor type (Ramqvist et al., 2014).

Cutaneous (449 forehead swabs) DNA prevalence and viral loads of HPyV6, HPyV7, TSPyV, HPyV9, and HPyV10 in HIV-infected men were compared to healthy male controls by HPyV-specific real-time PCRs. HPyV6, HPyV7, TSPyV, and HPyV10 were found significantly more frequently on the skin of HIV-infected than of HIV-negative



men. No HPyV9 was detectable. In contrast to HPyV6, 7, and 10, TSPyV and HPyV9 do not seem to be a regular part of the human skin microbiome (Wieland et al., 2014).

Sample	No. Samples	DNA-Positive Lesions (%)	Reference
Plucked eyebrows	69	4	(van der Meijden et al., 2010)
Plucked eyebrows	81	2	(Kazem et al., 2013b)
Forehead skin swabs	249	2	(Kazem et al., 2012)
Nasopharyngeal swabs	32	3	(Siebrasse et al., 2012a)
Fecal	32	3	(Siebrasse et al., 2012a)
Fecal	160	1	(Kazem et al., 2013b)
Fecal	38	0	(Scuda et al., 2011)
Urine	179	1	(Scuda et al., 2011)
Urine	17	0	(Kazem et al., 2013b)
Sera	88	0	(Scuda et al., 2011)
Sera	19	0	(Kazem et al., 2013b)
Various skin disorders	193	0	(Scola et al., 2012)
Pilomatricoma	10	0	(Kanitakis et al., 2011)

Table 8. TSPyV DNA detected among non-TS materials at variable frequencies.

### TSPyV disease associations

TS is a rare skin disease; worldwide, some 30 cases have been described, as a cutaneous side effect of cyclosporin-A use, first reported by Izakovic and colleagues in 1995 (Izakovic et al., 1995). In 1999, Haycox and colleagues fully described the disease and introduced the term “*trichodysplasia spinulosa*” (Haycox et al., 1999). They showed for the first time the presence of virus particles and suggested a viral etiology. Subsequent reports confirmed the typical histological findings of hyperplastic hair bulbs and the presence of polyomavirus or papillomavirus-like particles (Wyatt et al., 2005, Tan and Busam, 2011). Attempts to identify the nature of this virus failed until TSPyV was identified with the help of RCA in 2010 (van der Meijden et al., 2010).

Thus far, TS has been observed in immunosuppressed organ-transplant patients and occasionally in chronic and acute lymphocytic leukemia patients. Despite the disparity in TS terminology, in all cases the disease was clinically characterized by spiny follicular papules distributed largely on the face and ears, and to a lesser extent on the extremities, trunk, and scalp. In most patients, non-scarring alopecia of the eyebrows is evident, in which small hyperkeratotic white-yellowish spicules start to protrude from the skin. At the same time, these features also manifest themselves on the nose and ears (Figure 10). As the disease progresses, the skin of eyebrows, ears, and nose thickens to cause

disfigurement of facial appearance in combination with the conspicuous spines (Schwieger-Briel et al., 2010, van der Meijden et al., 2010).

To date, the presence of TSPyV in *trichodysplasia spinulosa* lesions has been confirmed in patients from western Europe, North America, and Australia (Matthews et al., 2011, Fischer et al., 2012, Kazem et al., 2012).

Fischer and colleagues reviewed the clinical, histopathologic, ultrastructural, and molecular findings in 25 cases of TS to date. The age range at diagnosis was 5 to 70 years, with 13 male and 12 female patients. Clinical findings were similar across all racial and ethnic groups represented, with all patients experiencing follicular papules of the central face, followed in frequency by the extremities, with infrequent involvement of the scalp. Most of the patients demonstrated spiny excrescences protruding from some of the papules. The papules were asymptomatic in almost two-thirds of cases, whereas others experienced mild pruritus (Fischer et al., 2012).



Figure 10: Clinical appearance of a *trichodysplasia spinulosa* patient. Reproduced with permission from van der Meijden, E., Janssens, R.W., Lauber, C., Bouwes Bavinck, J.N., Gorbalenya, A.E., Feltkamp, M.C., 2010. Discovery of a new human polyomavirus associated with *trichodysplasia spinulosa* in an immunocompromized patient. *PLoS pathogens* 6, e1001024.

## 2.9. Human polyomavirus 9 (HPyV9)

In 2011, a novel polyomavirus which closely resembled African green monkey-derived lymphotropic polyomavirus (LPyV) was identified by use of degenerative primers independently by two research groups one, in human serum of a kidney transplant patient, and one, in skin surface of a patient with a MCC, and was tentatively called HPyV9 (Sauvage et al., 2011, Scuda et al., 2011).

For identification of HPyV9, generic nested PCR targeting of a short fragment of the VP1 gene (approximately 260 bp) was performed by using two pairs of degenerate and deoxyinosine-substituted primers. All primers were designed to bind sites within the VP1 gene identified as being conserved among a wide range of PyVs. They were derived from the sequence of MCPyV in order to bias amplification toward sequences from PyVs related to MCPyV. Nested specific primers were derived from the sequences amplified with the generic PCR. They were designed tail-to-tail for the amplification and sequencing of the remaining parts of the genomes. The genome of the virus was completely amplified and sequenced. In phylogenetic analyses, it appeared as the closest relative to the African green monkey-derived LPyV (Leendertz et al., 2011). Several serological studies had already shown the presence of cross-reactive antibodies in a percentage of human sera that recognized LPyV (Viscidi and Clayman, 2006, Kean et al., 2009), which was isolated from an African green monkey.

In the other report, DNA isolated from healthy skin swabs was subjected to high-throughput sequencing, which resulted in millions of reads and several contigs that matched MCPyV, HPyV6, and HPyV7, as well as many HPVs. Some contigs showed the highest homology with LPyV, and after filling of the contig sequence gaps by designing additional primers, a virus genome emerged that coincided with HPyV9. It was called the IPPyV strain of HPyV9 (Sauvage et al., 2011).

Degenerate primers are used to detect viruses, including novel viruses, from existing sufficiently homologous virus families. Novel viruses infecting humans are detectable with this technique (Leendertz et al., 2011, Scuda et al., 2011, Yu et al., 2012b). An alternative PCR method, degenerate PCR, uses primers designed to anneal to highly conserved sequence regions shared by related viruses. Because these regions are almost never completely conserved, primers generally include some degeneracy that permits binding to all or the most common known variants on the conserved sequence. The overall aim is to achieve a balance between covering all possible viral variants within a family

(i.e. primers with high degeneracy) and creating an unwieldy number of different primers. At high levels of degeneracy, only a small proportion of primers are able to prime DNA synthesis, whereas a large proportion of the remaining primers will be able to anneal, but because of sequence mismatches, will be refractory to PCR extension (Bexfield and Kellam, 2011).

### **HPyV9 genome and structure**

HPyV9, like other novel PyVs, shares a genome organization that is typical among polyomaviruses (Figure 4). The early region encodes the regulatory proteins sTag and LTag, and the late region encodes the structural proteins VP1, VP2, and VP3. These regions are separated by an NCCR. An open reading frame encoding the agnoprotein present in BKPyV and JCPyV (Khalili et al., 2005) was not identified in the novel PyVs. In combined phylogenetic analysis of LTag, VP1, and VP2 proteins, HPyV9 forms a clade with the lymphotropic polyomavirus (Figure 2) (Van Ghelue et al., 2012).

### **HPyV9 epidemiological aspects**

HPyV9 DNA sequences were amplified from the serum of a kidney transplant patient by use of degenerate primers against conserved regions in the PyV VP1 gene. Re-screening of the analyzed panel of clinical specimens with specific nested PCR revealed the presence of HPyV9 in four additional specimens (plasma and urine from kidney transplant patients, serum from a patient with leukoencephalopathy, and whole blood from a patient suffering from acute myeloid leukemia) (Scuda et al., 2011).

Skin swabs from 8 patients with MCC also showed 2 with HPyV9 DNA, but only 1 among 111 non-MCC patients. An additional 92 respiratory specimens from patients with respiratory tract disorders and 92 fecal samples from children hospitalized for gastroenteritis were all negative for HPyV9 DNA (Sauvage et al., 2011). These findings may indicate that HPyV9 is less common than are the other known HPyVs.

Another study analyzed 449 forehead swabs by HPyV-specific real-time PCRs for cutaneous DNA prevalence of HPyV9 in HIV-infected men compared to healthy male controls. No HPyV9 DNA was detectable in any of the samples. This may indicate that cutaneous re-exposure or reactivation of TSPyV and HPyV9 occurs rarely or that the skin is not the primary site of reactivation/persistence (Wieland et al., 2014). Serum samples from 101 kidney transplant patients in the Netherlands and seroreactivity were screened

for HPyV9 DNA and its high prevalence in serum samples (21%) suggested that HPyV9 causes a systemic rather than a skin infection (van der Meijden et al., 2014).

Sera from immunocompetent Australian individuals from newborns to age 87 underwent analysis for the presence of VP1-directed IgG antibodies against novel PyVs, with BKPyV as a control. Findings were that, except for HPyV9, overall seropositivity was high for the cutaneous polyomaviruses (66–81% in adults), and gradually increased with age. This might be explained by lower circulation of HPyV9 in Queensland, Australia, compared to Europe and the USA or by possible local serotype variation or by differences in the manner of exposure (van der Meijden et al., 2013).

LPyV is a primate virus isolated from a lymphoblastoid cell line of African green monkey cells (zur Hausen and Gissmann, 1979). Recently, LPyV DNA was amplified in blood from healthy blood donors, and about 10 to 15% of the adults tested showed antibodies against LPyV (Viscidi and Clayman, 2006, Kean et al., 2009, Pastrana et al., 2009, Delbue et al., 2010). However, LPyV and HPyV9 VP1 share 84.1% aa identity, so that antibodies may cross-react, and the actual LPyV seropositivity may be lower as underscored by the lower prevalence of LPyV and HPyV9 DNA in human specimens or in urban sewage (Bofill-Mas et al., 2010, Sauvage et al., 2011, Scuda et al., 2011). What remains unknown is whether LPyV causes any human disease.

### **HPyV9 disease association**

Whether HPyV9 is a human pathogen remains to be established.

Sample type	DNA prevalence (%)	Patients, age (years)	Country	Reference
<b>Serum</b> <b>Plasma</b> <b>Whole blood</b>	1.4 (blood samples)	Serum (n = 88) Plasma (n = 176) Whole blood (n = 21)	Germany	(Scuda et al., 2011)
<b>Bronchoalveolar lavage</b>	0	(n = 21)	Germany	(Scuda et al., 2011)
<b>Urine</b>	0.6	(n = 179)	Germany	(Scuda et al., 2011)
<b>CSF</b>	0	(n = 74)	Germany	(Scuda et al., 2011)
<b>Stool</b>	0	(n = 38)	Germany	(Scuda et al., 2011)
<b>Facial skin swabs</b>	0.9 (skin controls) 25 (MCC patients)	111 controls (19–96 years) 8 MCC (57–86 years)	France	(Sauvage et al., 2011)
<b>Respiratory tract samples</b>	0	46 bronchoalveolar lavage 46 nasopharyngeal aspirates	France	(Sauvage et al., 2011)
<b>Faeces</b>	0	92 children with gastroenteritis	France	(Sauvage et al., 2011)
<b>Plasma</b>	4	100 pregnant (16.5–41.9 years) 100 non-pregnant (18–44.3 years)	Hungary	(Csoma et al., 2012)
<b>Urine</b>	2.5	100 pregnant (16.5–41.9 years) 100 non-pregnant (18–44.3 years)	Hungary	(Csoma et al., 2012)
<b>Throat swabs</b>	2	100 pregnant (16.5–41.9 years) 100 non-pregnant (18–44.3 years)	Hungary	(Csoma et al., 2012)
<b>Facial skin swabs</b>	17	(n = 6) 5 healthy adults (32–63 years) 1 patient (75 years) with previous MCC	France	(Foulongne et al., 2012)
<b>NP swabs</b>	0	(n= 160) , mean age 5.8 years; median age 3.1 years	USA	(Siebrasse et al., 2012a)
<b>Urine</b>	0.6	(n= 169), mean age 5.8 years; median age 3.1 years	USA	(Siebrasse et al., 2012a)
<b>Feces</b>	0	(n= 122) , mean age 5.8 years; median age 3.1 years	USA	(Siebrasse et al., 2012a)
<b>Plasma</b>	0	(n= 265), mean age 5.8 years; median age 3.1 years	USA	(Siebrasse et al., 2012a)
<b>Blood</b>	0	27 CLL cases (49–93 years) and 18 healthy donors (27–55 years)	USA	(Imajoh et al., 2012)
<b>Serum</b>	21	101 kidney transplant patients (Mean age, 47 (21–74))	Netherlands	(van der Meijden et al., 2014)

Table 9. Detection of HPyV9 in biological specimens.

## **2.10. Malawi polyomavirus (MWPyV) / human polyomavirus 10 (HPyV10) / Mexico polyomavirus (MXPyV)**

The tenth HPyV, by number and name, was discovered almost simultaneously in 2012 by three different research groups and provisionally designated Malawi polyomavirus (MWPyV) or HPyV10, or Mexico polyomavirus (MXPyV) (Buck et al., 2012, Siebrasse et al., 2012b, Yu et al., 2012a).

The whole-genome sequence of MXPyV is nearly identical to that of the recently described gut-associated polyomavirus MWPyV (St. Louis strain) or HPyV10, sharing a respective 99.8% or 99.7% identity (Figure 11). Multiple sequence alignment analysis revealed MWPyV, HPyV10, and MXPyV to be different variants of the same species (Yu et al., 2012b).

MWPyV was identified by shotgun pyrosequencing of DNA purified from virus-like particles (VLP) in stool of a 1-year-old healthy child from Malawi (Siebrasse et al., 2012b). This virus was discovered by suspending a small amount of feces that subsequently was centrifuged and filtered through 0.45 and 0.22  $\mu\text{m}$  pores. After chloroform extraction, free DNA was removed by DNase treatment. The solution that still contained encapsidated DNA was SDS- and proteinase-K treated, and DNA was isolated. Finally, this DNA was subjected to RCA and pyrosequencing. Hundreds of reads were obtained, sequenced and aligned to sequences within the GenBank database. This revealed several polyomavirus-like reads, and with the use of a primer walking strategy, the complete MWPyV genome was achieved. Furthermore, the Siebrasse group sequenced two complete genomes of MWPyV, one from the index child in Malawi and one from a child in St. Louis in the USA. A high degree of strain variation (5.3%) existed between these two MWPyV strains (Siebrasse et al., 2012b).

In the second half of 2012, in an effort to identify previously unknown viruses, Buck et al. purified virions from a patient with a rare genetic disorder known as “warts, hypogammaglobulinemia, infections, and myelokathexis” (WHIM) syndrome (Buck et al., 2012). This syndrome is marked by the patients’ relative inability to effectively control HPV infections (Kawai and Malech, 2009). For HPyV10 identification, the warts were minced and treated with detergents and nucleases, and out of the resulting extract, virions were purified by ultracentrifugation. DNA was extracted from the purified virions and subjected to random-primed RCA. Restriction fragments of the RCA product were subjected to plasmid-based cloning. This cloning-based approach revealed the presence of

three viral species: HPV6 and HPV124, as well as an almost identical virus to MW polyomavirus called HPyV10. This last one was identified in an anal wart of an immunocompromised patient with WHIM syndrome with the help of RCA (Buck et al., 2012).

Using random PCR and an unbiased deep sequencing approach, Mexico polyomavirus (MXPvV) was identified, like MWPvV in stool samples from children suffering from diarrhea in Mexico (Yu et al., 2012b), as follows: viral particles were purified from stool samples by generating a suspension in a centrifuge, and recovering the aqueous supernatant. Supernatants were then passed through a 0.45 mm filter and treated with a cocktail of nucleases prior to nucleic acid extraction. Sample cDNA libraries were prepared from extracted nucleic acid by a random PCR amplification method, were separately barcoded, and sequenced. Raw Illumina sequences consisting of 75 bp paired-end reads were filtered to exclude low-complexity, homopolymeric, and low-quality sequences, and then processed through an automated pipeline for pathogen identification. Sequences corresponding to MXPvV were identified on the basis of viral BlastX homology as the tenth polyomavirus (Yu et al., 2012b).

In the category of sequence-independent amplification techniques among new molecular methods, one alternative technique is known as “Random” PCR (Froussard, 1992). This method is common in amplifying and labeling probes with fluorescent dyes for microarray analysis but has also served for identification of novel viruses. Random PCR has no requirement for an adaptor ligation step and compared with conventional PCR, which utilizes a pair of complementary forward and reverse primers to amplify DNA in both directions, random PCR utilizes two different primers and two separate PCR reactions. The single primer used in the first PCR reaction has a defined sequence at its 5' end, followed by a degenerate hexamer or heptamer sequence at the 3' end. A second PCR reaction is then performed with a specific primer complementary to the 5' defined region of the first primer thus enabling amplification of products formed in the first reaction (Bexfield and Kellam, 2011).

Random PCR has been used extensively for the detection of both DNA and RNA viruses and is currently the molecular method most commonly used to identify unknown viruses. Random PCR has also proved successful in detecting novel viruses infecting humans, including a parvovirus (Allander et al., 2005, Allander et al., 2007a), a



coronavirus (Fouchier et al., 2004), and a bocavirus in patients with diarrhea (Kapoor et al., 2009).

### **HPyV10 genome and structure**

The complete genome of MWPyV is 4,927 bp, and its genome structure (Figure 11) follows that of other polyomaviruses (Siebrasse et al., 2012b). Maximum likelihood analysis of the VP1, VP2, and LTag proteins demonstrates MWPyV to be highly divergent from all other known polyomaviruses (Figure 2). Analysis of VP1 sequences shows that MWPyV is midway between the *Wukipolyomavirus* and *Orthopolyomavirus* genera (Figure 2). In contrast, based on VP2 and LTag sequences, MWPyV clusters with the clade containing; HPyV9, LPyV, HaPyV, MPyV, TSPyV, MCPyV, ChPyV, and the orangutan polyomaviruses. These discordant phylogenetic relationships suggest that MWPyV may be derived from an ancestral recombination event (Siebrasse et al., 2012b). Sequence variation between the St. Louis (WD976 strain) and Malawi (MA095) isolates diverged by 5.3% at nucleotide level. Strain WD976 has two insertions (11 bp and 1 bp) in the NCCR, which result in a genome size of 4,939 bp. The vast majority of the polymorphisms in the coding regions result in synonymous mutations. One notable mutation changes the size of the sTag ORF. The predicted TAA stop codon identified in the MA095 strain is mutated to AAA in WD976, resulting in a protein prediction of 206 aa, seven aa longer than the index genome's sTag (Siebrasse et al., 2012b).

### **HPyV10 epidemiological aspects**

The initial discovery of MWPyV was in a stool specimen collected from a healthy child in Malawi (index case, strain MA095). Further screening by real-time PCR demonstrated the presence of the virus in 12 stool samples collected from a cohort of patients in St. Louis, Missouri (St. Louis case, strain WD976). These data demonstrate that MWPyV is geographically widespread in human populations and can be found on two continents (Siebrasse et al., 2012b).

MWPyV infection is relatively common, since a seroprevalence of 41.8% has been observed in adulthood (Nicol et al., 2013a). MWPyV seroprevalence is similar to the seroprevalence of 39.4% reported for HPyV9 (Nicol et al., 2013b), but lower than the seroprevalence reported for MCPyV, HPyV6, HPyV7, and TSPyV (63.6% to 87.0%) (Nicol et al., 2013b). The relatively low seroprevalence observed for MWPyV (39.4%)

correlates with the detection of its DNA in only 2.3% of human stools (Siebrasse et al., 2012b). Prevalence rates and tissue tropism for MWPyV (strains MA095, WD976), HPyV10, and MXPpyV were tested by real-time PCR targeting a conserved region of VP1 in feces, urine, blood, respiratory swabs, and CSF both from healthy control children and children and from adults undergoing diagnostic testing. Results were the following: viral DNA in 1.5% of respiratory specimens from symptomatic patients, 9.8% of respiratory samples from healthy control children, 5.9% of fecal specimens from patients suffering gastrointestinal illness, and in 15.3% of feces from healthy control children. The tenth HPyV occurred only in respiratory and fecal specimens from children, the oldest being 9 years old. This findings suggests that the tenth polyomavirus can result in a subclinical infection with persistent or intermittent shedding, particularly in young children (Rockett et al., 2013). Another study detected DNA from HPyVs TSPyV, MWPyV, HPyV6, 7, and 9 in 55 primary mucosal melanomas, analyzed by a Luminex assay (Ramqvist et al., 2014).

#### **HPyV10 disease association**

No disease is associated with the tenth HPyVs yet.

Virus	Sample type	DNA prevalence (%)	Patients, age (years)	Reference
<sup>1</sup> HPyV10	Stool	2.3	514 children (0–18 years) with diarrhea	(Siebrasse et al., 2012b)
	Stool	3.4	834 children, with or without diarrhea	(Yu et al., 2012b)
	Nasal washes	0.7	136 nasal washes from children with pneumonia	(Yu et al., 2012b)
	Plasma	0	480 plasma samples	(Yu et al., 2012b)
	Urine	0	480 urine samples	(Yu et al., 2012b)
	Stool	2.2(children) 0 (adults) 1.5 (cases) 1.3(controls)	514 (USA, 0–18 y) 237 (USA, adult) 722 (Gambia (Africa) 0- 5 y) (cases, n = 332) and without diarrhea (controls, n = 390)	(Lim et al., 2013)
	Plasma	0	261 plasma, from adult	(Lim et al., 2013)
	NP swabs	0	261 NP swabs from adult	(Lim et al., 2013)
	Urine	0	373 urine samples from adult	(Lim et al., 2013)
STLPyV	Stool	1.1(children) 0 (adults) 0 (cases) 0.3 (controls)	514 (USA, 0–18 y) 237 (USA, adult) 722 (Gambia (Africa) 0- 5 y) (cases, n = 332) and without diarrhea (controls, n = 390)	(Lim et al., 2013)
	Plasma	0	261 plasma, from adult	(Lim et al., 2013)
	NP swabs	0	261 NP swabs from adult	(Lim et al., 2013)
	Urine	0.3	373 urine samples from adult	(Lim et al., 2013)
HPyV12	Stool	1.8	56 (patients' age not reported)	(Korup et al., 2013)
	Plasma/serum	0	99 (patients' age not reported)	(Korup et al., 2013)
	Urine	0	152 (patients' age not reported)	(Korup et al., 2013)
	Oral fluids	0	30 (patients' age not reported)	(Korup et al., 2013)
	BAL	0	22 (patients' age not reported)	(Korup et al., 2013)
	CSF	0	25 (patients' age not reported)	(Korup et al., 2013)
<sup>1</sup> HPyV10 (and its isolates MWPyV and MXPpyV)				

Table 10. Detection of HPy10, HPy11 (STLPyV1, HPy12, in biological specimens.

## **11. Human polyomavirus 11 or STL polyomavirus (STLPyV)**

The virus-hunter group of David Wang in the USA in 2013 performed shotgun 454 pyrosequencing of DNA amplified by multiple displacement amplification (MDA) from the stool of a healthy 15-month-old child in Malawi, and the novel polyomavirus recovered from the fecal microbiota of that child was provisionally called STL polyomavirus (STLPyV) (Lim et al., 2013).

MDA is a recently described method of whole-genome amplification that has proven efficient in the amplification of small amounts of DNA, including DNA from single cells. This method is based on the annealing of random hexamers to denatured DNA, followed by strand-displacement synthesis at a constant temperature, resulting in DNA products of high molecular weight (up to 12 kb). For discovery of STLPyV prior to DNA extraction, the stool was processed by CsCl ultra centrifugation to generically enrich for viral particles. DNA was purified from the fecal sample, amplified by rolling circle amplification and subjected to FLX Titanium pyrosequencing (Reyes et al., 2010).

The complete genomes of the STLPyV MA138 and WD972 strains were PCR amplified in three overlapping fragments, cloned and sequenced. DNA from the samples was initially subjected to rolling circle amplification using the Illustra GenomiPhi V2 kit (GE Healthcare) prior to PCR amplification (Lim et al., 2013). Furthermore, use of random-primed RCA combined with deep sequencing has uncovered, a divergent variant of STLPyV, STLPyV-like isolate (11ww), in a disinfected human skin wart specimen (Pastrana et al., 2013).

### **STLPyV genome and structure**

The STLPyV genome organization and the sizes of its open reading frames is characteristic of known polyomaviruses (Figure 11). Further, the non-coding control region of STLPyV (nucleotide positions 1–352) contains features typical of polyomaviruses (Van Ghelue et al., 2012). In addition, there is a 17-nucleotide stretch of an AT-rich region including the putative TATA box (nucleotide positions 59–75). Complete genome comparisons of two STLPyV strains (MA138 and WD972) (Lim et al., 2013) demonstrated that 5.2% nucleotide divergence and the complete genomic sequence of the new STLPyV-like isolate (11ww) (Pastrana et al., 2013) shares 92% similarity with both the original MA138 and WD972 isolates of STLPyV. STLPyV-11ww shows a pattern of ORFs and splicing signals similar to those of MA138 (Pastrana et al., 2013).

Alternative splicing of the STLPyV early region yielded a unique form of T antigen, which was called 229T, in addition to the expected LTag and sTag. STLPyV has a mosaic genome and shares an ancestral recombinant origin with MWPyV. The discovery of STLPyV highlights a novel alternative splicing strategy and advances our understanding of the complex evolutionary history of polyomaviruses (Lim et al., 2013).

### **STLPyV epidemiological aspects**

STLPyV was detected in human fecal samples collected in Malawi, the United States, and the Gambia at up to 1% frequency, demonstrating that it is geographically widespread (Lim et al., 2013). STLPyV was undetectable in 237 fecal specimens from adult renal-transplant patients. Screening of nasopharyngeal specimens, serum, and urine from these patients yielded a single urine sample positive for STLPyV (Lim et al., 2013). A divergent variant of STLPyV was isolated and reported from condylomas (anogenital warts) surgically removed from the buttocks of a patient suffering from the WHIMS syndrome (Pastrana et al., 2013). Observation of STLPyV in a surface-disinfected tissue specimen strongly suggests that STLPyV productively infects humans and thus can be considered the eleventh known HPyV.

### **STLPyV disease associations**

Questions remain to be answered regarding the potential role of STLPyV in human infection, but STLPyV and the recently described MWPyV are the first two polyomaviruses to be discovered in human stool samples, which raises the question of whether they may have a primary gastrointestinal tropism. Together with the frequent detection of WUPyV and KIPyV in respiratory specimens (Babakir-Mina et al., 2011), and the apparent primary tropism of HPyV6 and HPyV7 for skin (Schowalter et al., 2010), these findings demonstrate the ubiquity of polyomaviruses in various types of human specimens. As disease associations have been established for neurotropic polyomaviruses (JCPyV), renal-tropic polyomaviruses (BKPyV), and skin-tropic polyomaviruses (MCPyV, TSPyV), who can guess whether disease associations for the other novel polyomaviruses will also emerge.

We still do not know whether either STLPyV or MWPyV causes bona fide infection in humans. Assuming that STLPyV actually does infect humans, its lower prevalence than that of JCPyV in adult renal transplant patients may result from one or more of the

following factors: (1) STLPyV infection may be relatively rare; (2) acute STLPyV infection may occur primarily in children; (3) STLPyV may have a distinct tropism that is not reflected by the samples tested; (4) the life cycle of STLPyV may not involve persistence and then reactivation in the context of immunosuppression.

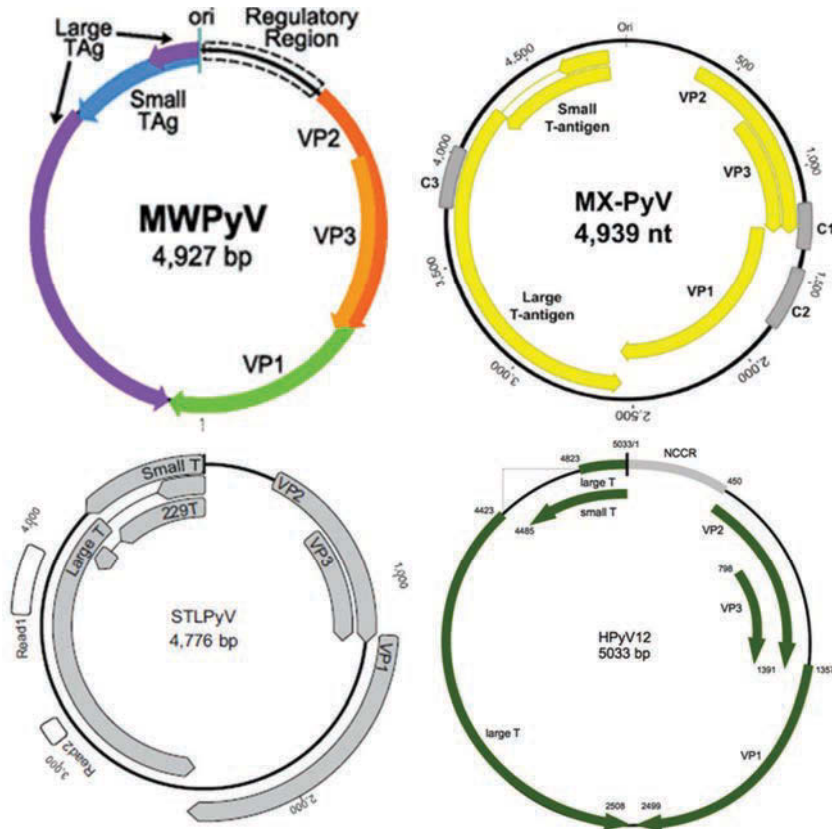


Figure 11. Genome map of MWPyV, MXPyV, STPyVL, and HPyV12. Indicated are the five identified ORFs representing the putative “early” genes encoding small and large T antigen, and the putative “late” genes encoding VP1, VP2, and VP3. The NCCR is placed on top and contains the putative ori. Nucleotide position 1 was chosen within the NCCR in the large T binding region. Each map reproduced with permission from its source: (Siebrasse et al., 2012b, Yu et al., 2012b, Korup et al., 2013, Lim et al., 2013).

## **12. Human polyomavirus 12 (HPyV12)**

The latest HPyV discovered to date is HPyV12 (Korup et al., 2013). With a generic polyomavirus PCR targeting the VP1 major structural protein gene, this polyomavirus was initially identified in resected human liver tissue. HPyV12 is the first HPyV whose LTag lacks any LxCxE motif. As this motif is essential to the retinoblastoma protein-dependent transforming activity of other polyomaviruses, HPyV12 may exhibit reduced transforming potential (Korup et al., 2013).

Discovery of HPyV12 was by means of extraction and purification of DNA and generic polyomavirus PCR carried out as described previously for HPyV9 (Leendertz et al., 2011, Scuda et al., 2011). To obtain additional sequence information on the novel polyomavirus, a 950 bp genome fragment was amplified with nested PCR using two degenerate sense primers targeting the VP3 gene of polyomaviruses (Scuda et al., 2011) and two virus-specific antisense primers derived from the novel VP1 sequence. From the resulting sequence, tail-to-tail primers were derived and used in nested long-distance (LD) PCR for amplification and sequencing of the remaining part of the virus genome.

### **HPyV12 genome and structure**

Analysis of the HPyV12 genome for putative open reading frames (ORFs) revealed a genome structure typical for polyomaviruses. The genome includes an early region, encoding regulatory proteins (sTag and LTag), and a late region, encoding structural proteins (VP1, VP2 and VP3) that are separated by an NCCR (Figure 11). The ORF encoding for the auxiliary agnoprotein (Gerits and Moens, 2012) was not identified. HPyV12 LTag and sTag share 71 aa residues that are encoded at the N-terminus. This region contains the DnaJ domain HPDKGG, which is fully conserved between all known HPyVs identified so far (Van Ghelue et al., 2012). However, HPyV12 LTag lacks the retinoblastoma binding motif LxCxE which is present in LTag sequences of all known HPyVs. The NCCR contains four repeats of the putative GAGGC LTag binding site and AT-rich palindromic sequences. The NCCR possesses potential binding sites for numerous cellular transcription factors, but the functional importance of these sites remains to be proven (Korup et al., 2013).

### **HPyV12 epidemiological aspects**

HPyV12 is a very recently discovered virus and there exists only the original report concerning its epidemiology. Organs of the gastrointestinal tract like the liver, gall bladder, esophagus, stomach, colon, rectum, spleen, and lymph node specimens were tested with generic polyomavirus PCR targeting the VP1 gene, and the HPyV12 sequence was amplified from liver specimens of four individuals (Korup et al., 2013). The 242 samples from gastrointestinal organs, spleens, and lymph nodes were re-evaluated with diagnostic nested PCR and qPCR. The presence of HPyV12 sequences was confirmed in the samples that were originally HPyV12-positive in the initial generic PCR. In addition, 10 liver samples tested positive that had been negative in the generic PCR. In total, of 124 liver samples, 14 (11%) were HPyV12-positive. One positive sample each was also identified among rectum and colon samples. Analysis of gall bladder, esophagus, stomach (cardia), spleen and lymph nodes yielded negative results. Testing of feces revealed one HPyV12-positive sample, but plasma, serum, urine, oral fluids, bronchoalveolar lavage, and CSFs were negative (Table 10). In qPCR, the HPyV12-positive DNA samples revealed genome copy numbers of up to 133/PCR reaction (equivalent to 27 copies/ $\mu$ l DNA from fluid samples; 532 copies/ $\mu$ g DNA from tissue samples). In summary, analysis of 636 clinical samples revealed the presence of HPyV12 only in organs of the gastrointestinal tract and in feces.

Korup et al. also for study of the seroprevalence of HPyV12, performed ELISA to specifically detect VP1 antibodies against HPyV12. A pediatric population of 74 subjects and 299 healthy adults and adolescents were tested, and the data were stratified by age. The seroprevalence of HPyV12 was 12% in children aged 2 to 5 and rose to 26% in the group aged 6 to 11. In young adults aged 21 to 30, the prevalence was 27%. In older adults, the prevalence ranged between 15 and 33%. No difference existed in HPyV12 seroprevalences between male and female adults. This assay thus revealed that healthy individuals are frequently infected with HPyV12 before the age of 20 (Korup et al., 2013).

### **HPyV12 disease associations**

No disease has been yet associated with HPyV12.



## **AIMS OF THE STUDY**

Emerging HPyVs have been discovered since 2007. When this thesis project was initiated, KIPyV and WUPyV were the only new HPyVs detected. The initial aim of the study was therefore to

- I)** express and purify the structural proteins of KIPyV and WUPyV.
- II)** study the antibody and DNA prevalences of these two novel polyomaviruses in young children with respiratory disease.

The subsequent discovery of eight new HPyVs included only MCPyV causing MCC, and TSPyV causing TS, both skin diseases. Because of their novelty, some very basic questions involving molecular and serological prevalence, transmission route, and persistence site require answers. We planned this study to address these questions. Other aims of the study were therefore to

- I)** determine the molecular and serological prevalence of KIPyV, WUPyV, MCPyV, and TSPyV in clinical samples from patients immunocompetent or immunocompromised or both.
- II)** determine the frequency of fetal infections by KIPyV, WUPyV, and MCPyV.
- III)** determine the occurrences and blood levels of MCPyV and TSPyV DNAs among the elderly and any association between the prevalences of their corresponding antiviral IgG antibodies.
- IV)** assess whether DNA from these four viruses is detectable in tonsillar tissue of constitutionally healthy individuals, judged against the corresponding antiviral seroreactivities.
- V)** assess whether the occurrence of these novel virus DNAs in body tissues or fluids plays any role in disease etiology.

# MATERIALS AND METHODS

## 1. Materials

### 1.1. Patients and clinical material

Seven different patient groups (Figure 12) were surveyed in this research project (Studies I, II, III, IV, and V). Clinical materials included in all studies were nasopharyngeal aspirates (NPAs), sera, fecal samples, tonsillar tissue, and formalin-fixed paraffin-embedded (FFPE) tissue samples from human fetuses.

Group 1 (Thesis studies: I, II)	<ul style="list-style-type: none"><li>• <b>51 febrile children with acute lymphoblastic leukemia</b></li><li>• Nasal swabs, sera and fecal samples</li></ul>
Group 2 (Thesis studies: I, II)	<ul style="list-style-type: none"><li>• <b>117 wheezing children</b></li><li>• Nasopharyngeal aspirates (NPAs) and paired sera</li></ul>
Group 3 (Thesis studies: I and V)	<ul style="list-style-type: none"><li>• <b>229 children or adults with tonsillitis or tonsillar hypertrophy</b></li><li>• Matched pairs of tonsillar tissue and sera</li></ul>
Group 4 (Thesis studies: II)	<ul style="list-style-type: none"><li>• <b>5 children hospitalized in Germany</b></li><li>• Sera</li></ul>
Group 5 (Thesis studies: II)	<ul style="list-style-type: none"><li>• <b>25 asymptomatic adults, mostly voluntary staff</b></li><li>• Sera</li></ul>
Group 6 (Thesis studies: III)	<ul style="list-style-type: none"><li>• <b>550 fetuses + 462 corresponding maternal sera</b></li><li>• Placenta, heart, and liver</li></ul>
Group 7 (Thesis studies: IV)	<ul style="list-style-type: none"><li>• <b>394 hospitalized senior citizens</b></li><li>• Blood samples</li></ul>

*Figure 12. Seven different patient groups surveyed in this study.*

### **Group 1 (Thesis Studies I, II), children with acute lymphoblastic leukemia**

The clinical material comprised nasal swabs, sera, and fecal samples from 51 febrile children with acute lymphoblastic leukemia undergoing anticancer treatment. The patients' information has been described elsewhere (Koskenvuo et al., 2008). Briefly, samplings were done between April 1, 2000, and October 31, 2005, at four Finnish university hospitals, including 51 children with acute leukemia. The majority, 42, of the children were followed from the beginning to completion of their chemotherapy regimen. The mean follow-up time was 1.5 years (range 0.1–2.5 years). Two children with high-risk acute myeloid leukemia (AML) and five with high-risk acute lymphoblastic leukemia (ALL) underwent bone marrow transplantation during the study period, with their follow-up completed on the day of transplantation.

During the study period, in these 42 children, 156 febrile episodes were studied. Fever was defined as an axillary temperature  $\geq 38.0^{\circ}\text{C}$ , and the interval between separate febrile episodes had to be  $\geq 7$  days. In 18 episodes, no virologic samples were taken; 138 fever periods were included in the study. In all these cases, a blood culture was also taken, with findings reported separately. A febrile episode was considered to be nosocomial if fever arose 48 hours after admission. Otherwise, the febrile episode was considered to be community acquired. The total white blood cell count, absolute neutrophil and lymphocyte counts, and serum C-reactive protein (CRP) levels were recorded during the first febrile day.

### **Group 2 (Thesis Studies I, II), children with acute expiratory wheezing**

The second group comprised 278 children aged 3 months to 15 years (mean 2.3 years, median 1.6) who were admitted to the Department of Pediatrics of Turku University Hospital (Turku, Finland) from 1 September 2000 through 31 May 2002 for acute expiratory wheezing. These children were observed and described elsewhere (Jartti et al., 2004, Allander et al., 2007b, Kantola et al., 2008).

Briefly, at their admission to the hospital, NPAs for viral diagnosis were obtained by a standard procedure (Jartti et al., 2004). Blood samples were collected at hospital admission and 2 to 3 weeks after hospital discharge, and the paired serum samples were stored at  $-20^{\circ}\text{C}$ . Of the 259 children included in the previous virus etiology study, comprising PCR studies of NPAs for 16 viruses (adenovirus; human bocavirus (HBoV); coronaviruses 229E, OC43, NL63, and HKU1; enteroviruses; rhinoviruses; influenza A

and B viruses; human metapneumovirus; parainfluenza virus 1–4; and respiratory syncytial virus), 49 (19%) were HBoV-positive by PCR (Allander et al., 2007b). Study (I), for DNA detection of MCPyV, WUPyV, and KIPyV, involved used nasopharyngeal aspirates (NPAs) from 140 wheezing children and paired sera from 248. In Study (II), we studied a total of 443 serum samples (201 paired, 41 unpaired) from 242 individuals and 192 nasopharyngeal samples from 192 patients for the presence of KIPyV and WUPyV DNA by polymerase chain reaction (PCR). From the sera tested by PCR, 120 samples (100 unpaired, 10 paired) were selected, on the basis of age ( $\leq 4$  years) or NPA PCR result (KIPyV or WUPyV DNA-positive), for immunoblotting, and 50 ( $\leq 2$  years) for immunofluorescence assays (IFA).

### **Group 3 (Thesis Studies I and V), children or adults with tonsillitis or tonsillar hypertrophy**

The third group included matched pairs of tonsillar tissue and sera from 229 children or adults who had been studied for HPV (Chen et al., 2005). In brief, 212 enrolled patients were operated on in 2001 and 2002, 135 because of tonsillitis and 77 for tonsillar hypertrophy at Helsinki University Central Hospital. After tonsillectomy, a piece of tissue from each child was immediately frozen at  $-70^{\circ}\text{C}$ . A 100- $\mu\text{m}$  cryo-section per tonsil per patient was subjected to DNA extraction. Five-micrometer sections from both ends of the tissue were toluidine blue-stained to verify that the tissue section consisted of at least 30% epithelial cells. After every tenth sample, an optimal cutting-temperature compound including no tonsillar tissue was cryo-sectioned as a negative control for the DNA extraction; none of these sections showed positive  $\beta$ -globin or HPV by PCR. Serum samples were drawn on the day of surgery. Tonsillar exfoliated cells from 189 control subjects (age- and sex-matched to patients) with normal tonsils were collected bilaterally from tonsillar surfaces with a Cytobrush Plus Cell Collector (Medscand Medical). The brushed samples were stored in phosphate-buffered saline at  $-70^{\circ}\text{C}$ . Serum samples were collected on the same day.

### **Group 4 (Thesis Study II), children hospitalized in Germany**

The fourth group consisted of five children aged 0.7 to 5.6 years (mean 3.0 years, median 2.8), hospitalized in Germany and shown to be WUPyV PCR-positive in NPA

(Neske et al., 2008). The sera from three of these children were taken on the same day, for one 4 days and for one, 5 months after the first NPA samples.

### **Group 5 (Thesis Study II), asymptomatic adults**

The fifth group consisted of 25 asymptomatic adults aged 20.5 to 60.7 years (mean 33.5 years, median 27.8), mostly voluntary staff, from whom only serum samples were obtained. These adult sera were immunoblotted without PCR testing.

### **Group 6 (Thesis Study III), fetal autopsy samples**

Formalin-fixed paraffin-embedded (FFPE) tissue samples from 550 fetuses that were collected from July 1992 through December 1995 and from January 2003 through December 2005 at the Helsinki University Central Hospital were retrieved (Riipinen et al., 2008). Briefly, samples from five fetuses were  $\beta$ -globin PCR-negative because of DNA degradation or PCR inhibition and were thus excluded. In twin- or multiple-fetus pregnancies, tissue samples were available mostly from only one fetus. Both fetuses were available from 10 twin pregnancies, and all were found to be parvovirus B19 DNA-negative. For conformity and statistical analysis, we considered each set of twins as one (10 exclusions). Thus, a total of 535 fetuses were included in the final study population. Samples from three organs (placenta, heart, and liver) were pooled from each fetus. The time of fetal death ranged from 11 to 42 gestational weeks. Fetal loss was defined as an intrauterine fetal death (IUFD) when it occurred during or after gestational week 22 and as miscarriage when it occurred earlier (according to the International Classification of Diseases, Tenth Revision). Our study comprised 120 miscarriages and 169 IUFDs. According to official statistical records, a total of 67,858 live births and 290 IUFDs occurred at Helsinki University Central Hospital during the study period. Thus, 169 (58.3%) of the 290 fetuses from IUFDs in the Helsinki region were available for our study. The proportion of miscarried fetuses included in our study, however, is unknown, because not all miscarriages are registered. From induced abortions (performed exclusively for medical reasons), 246 fetuses served as control fetuses.

We furthermore examined for the presence of MCPyV IgG antibodies all serum samples available from the 462 corresponding mothers. These samples had been collected at the municipal maternity centers during antenatal screening around the ninth gestational week (mean 9; median 9; range 2 to 36) and were stored frozen at the Finnish Maternity

Cohort, National Institute for Health and Welfare, Oulu, Finland. The mothers' ages ranged between 18 and 45 years (mean 31, median 31).

**Group 7 (Thesis Study IV), hospitalized elderly individuals with respiratory symptoms**

From 394 hospitalized senior citizens with respiratory symptoms or suspected pneumonia, cardiovascular, or other diseases in the city hospital of Turku, Finland, 621 blood samples were collected between July 2007 and April 2009. The criteria for sampling were age 65 years or older, disease requiring hospitalization, and a written assignment from the patient or trustee. Patients having a short elective operation were excluded. Eligible patients were informed of this study at hospital entry. After signing the consent, the patient or trustee had an interview, and hospital records were reviewed for clinical history. Nasopharyngeal swab samples (flocked swab, 520CS01, Copan, Brescia, Italy) and serum samples were collected at hospital entry and after 2 weeks or at discharge for detection of acute infections. The swabs in dry tubes and serum samples were stored at -80°C. Disposable gloves prevented contamination.

## 1.2. Plasmid (I, II, III, IV, and V)

In Studies I and III, for use as positive controls and to determine PCRs assay sensitivities by limiting dilution analysis, plasmids containing the VP2 gene of WUPyV (EU693907) and KIPyV (EU358767) and the LT3 amplification product of MCPyV (EU375803) were constructed; the amplification products of the VP2 genes were cloned into pCR8/GW/TOPO (Invitrogen; Carlsbad, CA, USA), whereas the MCPyV LT3 region was synthesized and cloned into pGOv4 by Gene Oracle, Inc. (San Leandro, CA, USA). In the MCPyV PCRs assay, plasmid controls with 30 and the KIPyV/WUPyV assay, 5 copies per reaction were reproducibly positive. Their sensitivities remained unaffected by inclusion of genomic human DNA from cultured 293T cells at 100 ng per reaction (4 ng/l). In a non-nested format with 40 PCR cycles the LT3 primers had a sensitivity 1 log lower than that of the nested assay both in the presence and absence of human genomic DNA.

In Study II, for cloning of the viral protein genes, we used several primers and the plasmids (Table 11).

In Studies IV and V, for detection of MCPyV by real-time PCR, control plasmids were cloned from amplicons of PCR-positive tonsillar samples from Study I by means of the CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific, Inc., Waltham, USA). Serial dilutions of the plasmids allowed determination of assay sensitivity. In each assay, five copies per reaction were reproducibly positive, corresponding to 200 copies/mL of serum.

In TSPyV Studies (IV and V), for use as positive controls and to determine assay sensitivities due to the lack of known positive samples, the TSPyV LT and VP regions were synthesized and cloned into pUC57 by GenScript (Piscataway, NJ, USA). The detection limit of each assay was five target copies per reaction, corresponding to 200 copies/mL of serum.

Screening for the single-copy human *RNase P* gene by real-time quantitative PCR (qPCR) was done by use of the plasmid containing this gene (Study V). This plasmid was a generous gift from Dr Janet S. Butel (Baylor College of Medicine, Houston, Texas, USA).

Gene	Primer sequence (5'-FWD-3', 5'-REV-3')	GenBank accession number	Plasmid
WUPyV VP1	ATGGCCTGCACAGCAAAG TCATCCTGTGTGTTAGTATTGGG	ABQ09289	pENTR-VP1
WUPyV VP2	ATGGGCATATGCTGTCTGTG TCAAACCTCTGTTTCTTCTGACAGTGG	ABQ09290	AF-p002
WUPyV VP3	ATGGCACTGGCACCTATCCC TCAAACCTCTGTTTCTTCTGACAGTGG	ABQ09291	
KIPyV VP1	ATGAGCTGCACCCCGTGC TCACTTTGAAATTTTGTGAGTATT	ABN09917	pGEM-KIPyVP1-2
KIPyV VP2	ATGGGAATATTTCTTCTGTAC TCAAATTCGTCTTCTGACAG	ABN09918	
KIPyV VP3	ATGGCATGGTACCTATCCCIG TCAAATTCGTCTTCTGACAGTGG	ABN09919	
BKV VP1	ATGGCCCCAACCAAAAGAAAAGGAG TCAAAGCATTTTGGTTTGCATTTGT	ABC18005	pBKV
BKV VP2	ATGGGTGCTGCTCTAGCACTTTTGG TCAAACCTCTAGAACTTCTACTCCTC	ABC18003	
BKV VP3	ATGGCTTTGGAAATGTTTAAACCCAG TCAAACCTCTAGAACTTCTACTCCTC	ABC18004	
JCV VP1	ATGGCCCCAACCAAAAGAAAAGGAG TCAACAGCATTTTGTCTGCAACTGT	BAF95594	pJCV
JCV VP2	ATGGGTGCCGCACITGCACT TCAAACCTCTAGAACTTCTACTCCTCCTC	BAF95592	
JCV VP3	ATGGCTTTACAATTAATTAATCCAG TCAAACCTCTAGAACTTCTACTCCTC	BAF95593	

Table 1.1. Primers and plasmids used for cloning of the viral protein genes in study (1).



## **2. Methods**

### **1.2. DNA extraction (I, II, III, IV, and V)**

Total nucleic acids were extracted from the sera, tonsillar tissues, nasal swabs, and NPAs with the DNAMini kit (Qiagen, Crawley, UK), and from the fecal samples with the QIAamp Qiagen DNA Stool kit (Qiagen) according to manufacturer's instructions. In Study V, from the serum samples, DNA was extracted by lysis buffer/proteinase K treatment and phenol-chloroform extraction.

In Study III, the paraffin blocks were punch-biopsied, proteinase –K digested, and the DNA was prepared by salting out, as described (Riipinen et al., 2008). Briefly, tissue lysates were heated at 95°C for 10 min. After centrifugation at 13,200 rpm for 5 minutes (Eppendorf; 4°C), the paraffin then appeared floating on the surface. The liquid phase was removed to a new tube, and sodium chloride was added to achieve a final concentration of 1.2 mol/L, and the sample was mixed for 20 s and recentrifuged. The supernatant was transferred to a new tube, carefully avoiding particles. The DNA in the supernatant was precipitated with absolute ethanol and was redissolved in 60 µL of water. The DNA solution diluted 1 to 10 was stored at - 20°C until use.

### **2.2. DNA screening (III, V)**

#### **Human beta -globin-PCR (III)**

With emphasis on DNA stability and lack of appreciable PCR inhibition, the human  $\beta$  -globin-PCR was done by primers targeting the human  $\beta$ -globin gene (Table 12), and all these DNA preparations were  $\beta$  -globin-PCR-positive.

#### **Human RNase P gene PCR (V)**

All samples were screened for the single-copy human *RNase P* gene by establishing real-time quantitative PCR (qPCR). The target gene for quantifying cell number is the proven single-copy *RNase P* gene. Each sample was subjected to Taq Man PCR for a control gene *RNase P*. Standard curves were constructed for the *RNase P* gene plasmid. Standards were made by preparing serial 10-fold dilutions of the plasmid with copy numbers ranging from 1 to 10<sup>8</sup> copies. PCR amplification reactions were set up in a

reaction volume of 17  $\mu$ l using 10  $\mu$ l TaqMan universal PCR master mix (Applied Biosystems), 2.7  $\mu$ l DNase-RNase free water, 0.9  $\mu$ l of (10 $\mu$ M) each primers, and 0.5  $\mu$ l of probe (Table 12). Amplifications were performed with the Stratagene Mx3005p (Stratagene, La Jolla, CA, USA) by use of the following cycling parameters: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and of 1 min at 60°C. Reactions were considered positive if >10 genome copies/reaction were detectable. This strategy detected potential PCR inhibitors in the DNA preparations, determined the human cell equivalents in each DNA sample, and normalized TSPyV viral loads to human cell numbers.

Virus	Gene	PCR	Forward primers Reverse primers Probes	Amplicons size (bp)
KIPyV & WUPyV	VP2	Nested outer	5-ATCTRTAGTCTGGAGGAGCAGAG-3 5-CCYTGGGGATTGTATCCTGMGG-3	336
KIPyV & WUPyV	VP2	Nested inner	5-RTCAATTGCTGGWTCTGGAGCTGC-3 5-TCCAATTGSACTTCCTGTTGGG-3	277
MCPyV	LT3	Nested outer	5-TTGTCTCGCCAGCATTGTAG-3 5-ATATAGGGCCTCGTCAACC-3	309
MCPyV	LT3	Nested inner	5-TGACGTGGGAGAGTGTITTTG-3 5-GAGGAAGGAAGTAGGAGTCTAGAAAAG-3	155
MCPyV	LT1	Nested inner	5-TACAAGCACTCCACCAAAGC-3 5-TCCAATTACAGCTGGCCTCT-3	440
MCPyV	LT1	Nested outer	5-GGCATGCCTGTGAATTAGGA-3 5-TTGCAGTAATTTGTAAGGGGACT-3	179
MCPyV	VP1	qPCR	5-TGCCTCCACATCTGCAAT-3 5-GTGTCTCGCCAATGCTAAATGA-3 <b>FAM-TGTCACAGGTAATATC-MGBNFQ</b>	59
MCPyV	LT1	Semi nested PCR	5-CTAAGTGCCTGTATTAGCTGTAAG-3 5-ATATAGGGCCTCGTCAACC-3 5-GAGGAAGGAAGTAGGAGTCTAGAAAAG-3	334 229
MCPyV	LT1	qPCR	5-CCACAGCCAGAGCTCTTCCT-3 5-TGGTGGTCTCCTCTCTGCTACTG-3 <b>FAM-TCCTTCTCAGCGTCCCAGGCTTCA-TAMRA</b>	140
TSPyV	LT	qPCR	5-TGTGTTTGAAACCAGAATCATTTG-3 5-TGCTACCTTGCTATTAATGTGGAG-3 <b>FAM-TTCTTCTCCTCCTCATCTCCACCTCAAT-BHQ1</b>	140
TSPyV	VP1	qPCR	5-AGTCTAAGGACAACATGGTTACAG-3 5-ATTACAGGTTAGGTCCTCATTCAAC-3 <b>FAM-ACAGCAGTGACCAGGACAAGCCTACTTCTG-BHQ1</b>	140
TSPyV	VP1	PCR	5-AGTCTAAGGACAACATGGTTACAG-3 5-ATTACAGGTTAGGTCCTCATTCAAC-3	140
Beta globin			5-ACACAACCTGTGTTCACTAGC-3 5-GGTGAACGTGGATGAAGTTG-3	110
RNase P gene			5-GAGGGAAGCTCATCAGTGGGG-3 5-CTTGGAAGGTCTGAGACTAGGG-3 <b>FAM-AGTGCCTCTGCTCACTCCACTC-TAMRA</b>	84
Tail Sequence			AACTGACTAAACTAGGTGCCACGTCTGTAAGTCTGACAAGTGTCTCTGC	50

Table 12. PCR primers used in the studies of this thesis.

## **2.3. DNA amplification**

### **KIPyV and WUPyV detection (I, II, III)**

#### **Nested PCR**

WUPyV and KIPyV detection was performed by primer set A as described (Norja et al., 2007). Briefly, two sets of primers hybridizing to regions of sequence conservation between the virus groups were selected. Set A comprised positions in the published complete genome sequence of KIPyV [NC 009238] yielding amplicon lengths of 336 and 276 bp for first- and second-round amplification reactions (Table 12).

#### **Real-time PCR assay**

Nucleic acid extraction from the NPAs underwent a single-tube dual probe, real-time PCR assay for KIPyV and WUPyV DNA as previously described (Lindau et al., 2009).

### **MCPyV detection (I, III, IV)**

#### **Nested PCR**

For MCPyV detection, nested PCR was performed using as outer primers the previously described LT3 primers (Table 12) (Feng et al., 2008) and as inner primers a pair hybridizing to conserved regions within the LT3 region. To increase the robustness of fecal PCRs, bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA, USA) was included at the final concentration. All samples positive for MCPyV were re-analyzed with the LT1/M1 nested PCR primer set as described (Kassem et al., 2008) and sequenced. Samples positive with the LT3 nested primer set but negative with the LT1/M1 primer pair were analyzed with a third (seminested) PCR. The semi-nested forward primers and LT3 antisense primers were used for the first-round PCR, whereas the internal reverse primers of LT3 with the same semi-nested forward primer used for the second-round PCR. The mastermix and cycling conditions were the same as for the other two PCRs.

### **Real-time PCR assay**

Two published primer sets targeting conserved sequences in the MCPyV genome, the LT gene, and the VP1 gene (Table 12), we used as described (Goh et al., 2009). PCR was done with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) thermal cycler, using the TaqMan universal PCR master mix (PE Applied Biosystems).

As the MCPyV VP1 PCR product was too short for direct sequencing, we added a 40-bp nonspecific nucleotide tail (Table 12) (Binladen et al., 2007) in addition to a poly(C) to the 5' end of the sequencing primers to accomplish a product of 110 bp. The neutral sequence is a randomly generated sequence not matching any sequence in a BLAST search.

### **TSPyV detection (IV and V)**

#### **Real-time PCR assay**

For detection of TSPyV, we applied published qPCRs, with primer pairs targeting the VP1 and LT genes (van der Meijden et al., 2010) but changed the quenching dye TAMRA to BHQ1 on the 3' base of the probes (Table 12). Although annealing was for 15 s at 62°C, the cycling conditions were otherwise identical to those of the MCPyV protocol.

## **2.4. DNA sequencing and sequence analyses (I, II, III, IV, and V)**

The PCR products were purified for automated sequencing with the High Pure PCR product purification kit (Roche, Mannheim, Germany). The resulting DNA sequences were aligned by means of the BLAST against the MCPyV sequences in GenBank.

## **2.5. Statistical analysis (I and IV)**

The ages of the patients with MCPyV or WUPyV DNA in their tonsils were ranked and compared with the unequal variance *t*-test (I). We examined the association between MCPyV DNA positivity in serum and the clinical characteristics of the elderly (IV). Univariable logistic regression served to analyze the association between patient

characteristics and virus etiology. For statistics we used the SAS Enterprise Guide 4.3 (SAS Institute Inc., Cary, NC, USA).

## **2.6. DNase treatment (I)**

Three NPAs testing positive for MCPyV DNA were selected for DNase treatment to determine the encapsidation status of the viral DNA. The NPAs were centrifuged at 16,000×g for 10 min, after which both the supernatants and the pellets (after resuspension and sonication) were diluted 1:2 with molecular biology-grade water and treated with 100 units of DNase I (Roche) or mock treated with water. After 2 h at 37 °C, the enzyme was inactivated with 8mM EDTA and heated at 70 °C for 10 min. DNA was isolated with DNA Mini kit (Qiagen).

## **2.7. Serological assays**

### **Recombinant protein production (II, III, IV, and V)**

In Studies III, IV, and V, VLPs of MCPyV and TSPyV VP1 were used for serology. VLPs were produced in insect cells as described elsewhere (Chen et al., 2011a, Chen et al., 2011b). Briefly, recombinant baculoviruses were generated by the Bac-to-Bac system (Invitrogen) in accordance with the manufacturer's instructions. *Spodoptera frugiperda* (*Sf9*) cells, infected with high titers of recombinant virus, were harvested 4 to 5 days after infection and processed by two-step lysis. The lysate was loaded on a 25% (wt/vol) sucrose shelf and centrifuged in a Beckman SW55Ti rotor at 103,246 g at 4 °C for 2 hours. The resulting pellet was resuspended in 20 mmol/L Tris (pH 7.5) and layered on top of a 1.23–1.36 g/cm<sup>3</sup> cesium chloride gradient. After ultracentrifugation in a Beckman SW55Ti rotor at 103,246 g at 4 °C for 15 hours, fractions were collected, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-page). The fractions showing VLPs in EM were pooled and dialyzed (3 times) against phosphate-buffered saline. For use as an antigen, the VLPs were biotinylated with the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce) as described (Chen et al., 2011a, Chen et al., 2011b).

In Study II, the VP1, VP2, and VP3 genes of the four HPyVs were amplified by PCR using Platinum Taq DNA polymerase (Invitrogen) from partial or full-length KIPyV (Allander et al., 2007a), WUPyV (Gaynor et al., 2007), BKPyV, and JCPyV (Loeber and

Dorries, 1988) clones. All 12 amplified genes were cloned into the pET SUMO vector (Invitrogen) for protein expression in *E. coli*. Protein expression utilized an N-terminal fusion tag, a *Saccharomyces cerevisiae* Smt3 protein (SUMO), an 11-kDa homolog of the mammalian SUMO-1. The tertiary structure of this tag is very accurately recognized by a SUMO protease, which cleaves it such that no aa remain between the cleavage site and the protein of interest (Malakhov et al., 2004). The SUMO expression constructs were transformed into *E. coli* BL21 (DE3) pLysS (Novagen, Gibbstown, NJ, USA), or Rosetta 2 (DE3) pLysS (Novagen) expression strains, and grown overnight in 500 mL Luria broth (LB) with 50 mg mL<sup>-1</sup> kanamycin (BL21 and Rosetta) and 34 mg mL<sup>-1</sup> chloramphenicol (Rosetta).

At an optical density of 0.6 at 600 nm, protein expression was induced with 1mM IPTG for 3 h (Isopropyl  $\beta$ -D-1-thiogalactopyranoside is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator). Inclusion bodies from lysed cells were pelleted by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in 25 mL of lysis buffer (6M GuHCl, 100mM NaH<sub>2</sub>PO<sub>4</sub>, and 10mM Tris Cl; pH 8.0). The rest of the purification was done according to the Ni-NTA matrix manufacturer's (Qiagen Inc., Valencia, CA, USA) instructions. The purified proteins were renatured at 48 °C by a 4-h dialysis against 2M (WUPyV and KIPyV) or 3 M urea (BKPYV and JCPYV) containing 150 mM NaCl, 5% (v/v) glycerol, and 1 mM DTT in 20 mM Tris/HCl, pH 8.0. Cleavage of the SUMO carriers from the partially unstable WUPyV, BKPYV, and JCPYV VP3 proteins was done with 1 U of SUMO protease (Invitrogen) per 2  $\mu$ g of substrate at 30 °C for 2 h. All other proteins were cleaved overnight at 48 °C with 1 U of protease per 10 mg substrate. The cleaved WUPyV and KIPyV proteins were passed twice through a Ni-NTA spin column (Ni-NTA spin kit; Qiagen) to remove the hexahistidine-tagged SUMO protein and the SUMO protease.

The WUPyV VP1, VP2, and VP3 proteins were expressed as native without a fusion tag in Sf9 insect cells. The genes were cloned in the pCR8/GW/TOPO gateway entry vector and transferred to a pDest8 gateway destination vector to create recombinant baculoviruses.

## **Western blotting and immunofluorescence assay (II)**

After expressing and purifying the structural proteins of KIPyV, WUPyV, JCPyV, and BKPyV, we performed immunoblotting to compare the immunoreactivities. The purified proteins VP1, VP2, and VP3 were separated by 12% SDS-PAGE and immunoblotted with human sera and horseradish peroxidase-conjugated anti-human IgG.

NPA PCR-positive children were also tested for IgM as previously described (Kantola et al., 2008). In immunoblotting, a sample was regarded as seropositive if it had an unequivocal band of correct molecular size, and all samples were scored in blinded fashion. An increase in IgG was defined as a significant elevation in band intensity between the acute and convalescence serum samples, when both were tested identically and simultaneously. Borderline sera were retested with a lower dilution of serum (1:30). All samples still (considered) borderline were regarded as negative. The IgG seroprevalences against the SUMO fusion tag, seen with the isolated carrier protein, increased with age from 8% among children to 72% in adults. To avoid false positivity due to the SUMO fusion partner required both cleaved and uncleaved recombinant proteins; those 30 subjects reactive with all three uncleaved antigens were re-examined with the cleaved antigens. To account for the possibility that the prokaryotic origin would affect the antigenicity of the recombinant proteins, all sera from 50 children aged 3 to 4 years old were also immunoblotted with WUPyV VP1- and VP3-expressing insect cells.

To validate our immunoblot (IB) results and to further study the importance of protein folding for the antigenicity of KIPyV and WUPyV VP1, we used an immunofluorescence assay (IFA) (Neske et al., 2010) using baculovirus-infected SF9 insect cells to test the sera of 54 children for WUPyV and KIPyV VP1 IgG. Samples for the IFA assay were selected on the basis of age ( $n=50$ ,  $\leq 2$  years). Briefly, SF9 cells infected with recombinant baculoviruses were collected by centrifugation, washed with PBS, mounted on glass slides, and fixed with acetone. Slides with uninfected SF9 cells and SF9 cells infected with a control baculovirus encoding glutathione S-transferase (GST) were prepared in the same manner to control for anti-cellular and antibaculoviral antibodies. These slides were incubated with 1:10 and 1:40 replica dilutions of each serum for 2 h at 37 °C, washed, and incubated with a 1:150 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human-IgG (Jackson ImmunoResearch West Grove, PA, USA) for 1 h at 37 °C. The slides were read in blinded fashion.

### **MCPyV antibody ELISA (III and IV)**

MCPyV IgG antibodies were measured by ELISA based on virus protein 1 (VP1) VLPs (Chen et al., 2011a). Briefly, the VP1-VLPs expressed in insect cells and purified by CsCl density gradient centrifugation were biotinylated and attached (at 60 ng/well) to streptavidin-coated microtiter plates (Thermo Scientific) and saturated with a sample diluent (Ani Labsystems). The sera (1:200) were applied in duplicate, the bound IgG was quantified with peroxidase-conjugated anti-human IgG (1:2000; DakoCytomation) using H<sub>2</sub>O<sub>2</sub> and OPD substrates (o-Phenylenediamine dihydrochloride), and the absorbances at 492 nm were read after blank subtraction. The ELISA cut-off for IgG positivity was 0.120 OD units.

### **TSPyV antibody ELISA (IV and V)**

TSPyV IgG antibodies were measured by in-house ELISA based on VP1 virus-like particles (VLPs) as described (Chen et al., 2011b). For TSPyV IgG ELISA, the lower cutoff was 0.100, and the higher cutoff was 0.240. Samples with absorbance values exceeding the higher cutoff were considered IgG-positive, and those with values below the lower cutoff, IgG-negative, whereas values between the cutoffs were considered indeterminate (Chen et al., 2011b). The PCR-positive individuals also were tested for IgM by  $\mu$ -capture ELISA and for IgG avidity by the corresponding ELISA, as described (Chen et al., 2014). The cutoff values for IgM were 0.194 and 0.240; and for low and high avidity, 15% and 25%, respectively.

## **2.8. Ethical aspects (I, II, III, IV, and V)**

The study was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa. Permission for use of fetal tissues came from the National Supervisory Authority for Welfare and Health (Valvira).



## RESULTS AND DISCUSSION

Since 2007, new molecular and bioinformatic techniques have uncovered ten new polyomaviruses. This has revitalized scientific debate on polyomaviruses and their potential contributions to human disease including cancers. What these discoveries show is that polyomaviruses represent a large family with a high degree of divergence (Van Ghelue et al., 2012). Although all ten of the new HPyVs are of a genomic structure and gene organization that are similar to those of SV40, important differences in sequence exist that may impact the life cycle and host cell tropism of the new human viruses (Van Ghelue et al., 2012). That the number of HPyVs continues to increase means that vital questions emerge as to their tropism, transmission route, latency site, and disease-causing potential.

For the newly discovered polyomaviruses, over this short period of time much new knowledge has become available. Similarly to BKPyV and JCPyV, infection by these viral agents is widespread among the human population. So it is hypothesized that a benign primary infection occurs early in childhood, probably by a respiratory or oral-fecal route. This infection will persist in a latent state in a yet unidentified body location, and be reactivated in a setting of immune suppression due to immunosuppressive drugs or other underlying medical conditions.

The association has been shown between two novel polyomaviruses (MCPyV and TSPyV) and two skin diseases (MCC and TS) (Feng et al., 2008, van der Meijden et al., 2010). However, eight of the newly discovered HPyVs still remain orphaned. Given that polyomaviruses retain oncogenic potential, investigation of their occurrence in benign, semimalignant and malignant tumors could uncover no occurrence of these new polyomaviruses in any of samples tested (Dalianis and Hirsch, 2013).

KIPyV, WUPyV, MCPyV, and TSPyV have been detectable in respiratory specimens worldwide, but the number of patients positive to any given virus is still low, and no convincing evidence exists at this time for an etiological role of these viruses in respiratory disease (Babakir-Mina et al., 2013, Coursaget et al., 2013, Kazem et al., 2013b). Furthermore, because these are frequently co-detected with other known human pathogens, and often control groups are lacking, it is difficult to establish a causal link between these newly discovered human viruses and clinical symptoms. It may be possible

that KIPyV, WUPyV, and MCPyV play a role in at least a subset of pneumonia in immunocompromised patients.

Infection by all HPyVs is widespread in the human population, but they show important differences in their tissue tropism and association with disease. Despite our understanding of many of the clinical and biological aspects, still much remains unknown about these new viruses. In this study, we investigated some very basic questions concerning emerging HPyVs, such as gaps in our basic understanding of their epidemiology and occurrence in humans from birth to death. Other examples involve the possibility of detecting these novel polyomaviruses in different types of sample materials from fetuses up to seniors, and determining whether these viruses persist in lymphoid tissue, or if they are associated only with acute infections or cancers. Furthermore, we also studied routes of transmission and the epidemiology of these newly discovered viruses.

In this study we performed immunoblot with purified VP1, VP2, and VP3 proteins of KIPyV, WUPyV and human sera. We found cross-reactivity for the VP2 and VP3 proteins. However, sera with IgG for BKPyV or JCPyV were unreactive with KIPyV and WUPyV VP2 and VP3. There was 72% aa identity for VP2, and 66% identity for VP3 of KIPyV and WUPyV, but only 22 to 33% identity with the corresponding proteins of BKPyV and JCPyV. Interestingly, immunoblotting resulted in >10 times lower seropositivity for VP1 than with VP2 and VP3. However, the immunofluorescence assay of Sf9 insect cells infected with baculovirus encoding VP1, VP2, or VP3 gave comparable seroprevalences for the three antigens. This indicates that serodiagnostics using immunoblotting and VP1 as a target antigen is an inadvisable method. Our results indicate the significance of protein conformation in immunoreactivity of VP1, and show the antigenic importance of the WUPyV and KIPyV minor proteins VP2 and VP3.

In addition, MCPyV, TSPyV, WUPyV, and KIPyV DNAs were detectable in clinical samples of many different types: nasal swabs and nasopharyngeal aspirates, tumor-free tonsillar tissues, and sera. The high IgG prevalence rates observed in this study for these four viruses support the notion that these novel polyomaviruses are widespread and are acquired early in childhood. Whereas these emerging polyomaviruses occur frequently in tissues of many different types, and also in environmental samples, our PCR data from 535 pregnancies suggest that none of these viruses are frequently transmitted vertically.

## **1. Detection of KI, WU and MC polyomaviruses in respiratory samples**

WUPyV was detectable in 1 (0.9%) and KIPyV in 4 (3.8%) nasal swabs (Study I) and of the 192 NPA samples studied by PCR, 7 (3.6%) were positive for WUPyV, and 3 (1.5%) for KIPyV DNA (Study II). Of the 7 children positive for WUPyV and the 3 for KIPyV, 4 and 1, respectively, had a high load of DNA ( $\geq 10^4$  copies/mL of the original sample); the DNA loads in the remaining samples were  $\leq 500$  copies/mL. Three of the MCPyV-positive NPAs (Study I) were treated (before DNA isolation) with DNase I. Two were reproducibly resistant to the enzyme with no change in their levels of viral DNA. One sample was in three repeated assays fully susceptible to DNase I, PCR-negative versus PCR-positive, suggesting that the viral DNA occurred in nonencapsidated form. The encapsidated form of DNA is the result of virus replication, whereas the nonencapsidated DNAs can be integrated DNAs in the human genome, be episomal DNAs, or even result from contamination. The DNA encapsidation signal of the polyomavirus capsid proteins for viral progeny formation, however, has not been determined. Our study indicated that the N-terminal region of the HPyV VP1 may be involved in viral genome encapsidation during progeny maturation (Ou et al., 2001). Consequently, DNA encapsidation provides basic knowledge concerning how viruses assemble into infectious particles and will eventually help to develop the biology and pathogenesis of viral diseases.

Since the time that KIPyV and WUPyV were first identified, viral sequences have been confirmed in respiratory specimens worldwide (Tables 3 and 4), suggesting that both viruses are widespread among human populations. Furthermore, available data support a model in which primary infection with KIPyV or WUPyV occurs during childhood (Bialasiewicz et al., 2007a, Gaynor et al., 2007, Abedi Kiasari et al., 2008). Despite their clear presence in specimens of patients with respiratory illnesses, the pathogenicity of KIPyV and WUPyV remains speculative (Norja et al., 2007). The proposed association of KIPyV and WUPyV with respiratory disease is tenuous because the majority of studies to date include no specimens from asymptomatic patients. In the three studies that include these control groups, viral sequences were detected at similar frequencies in asymptomatic patients (Han et al., 2007, Norja et al., 2007, Abedi Kiasari et al., 2008). The link between KIPyV and WUPyV infection and respiratory disease is further complicated by the high

rates of co-infection with other respiratory viruses that were observed in these studies (Allander et al., 2007a, Bialasiewicz et al., 2007b, Gaynor et al., 2007, Han et al., 2007, Norja et al., 2007, Abedi Kiasari et al., 2008, Neske et al., 2008). Despite the lack of evidence for KIPyV and WUPyV as causing disease, more research is necessary to investigate this possibility, especially considering their similarities to BKPyV and JCPyV in nucleotide sequence and potential route of infection.

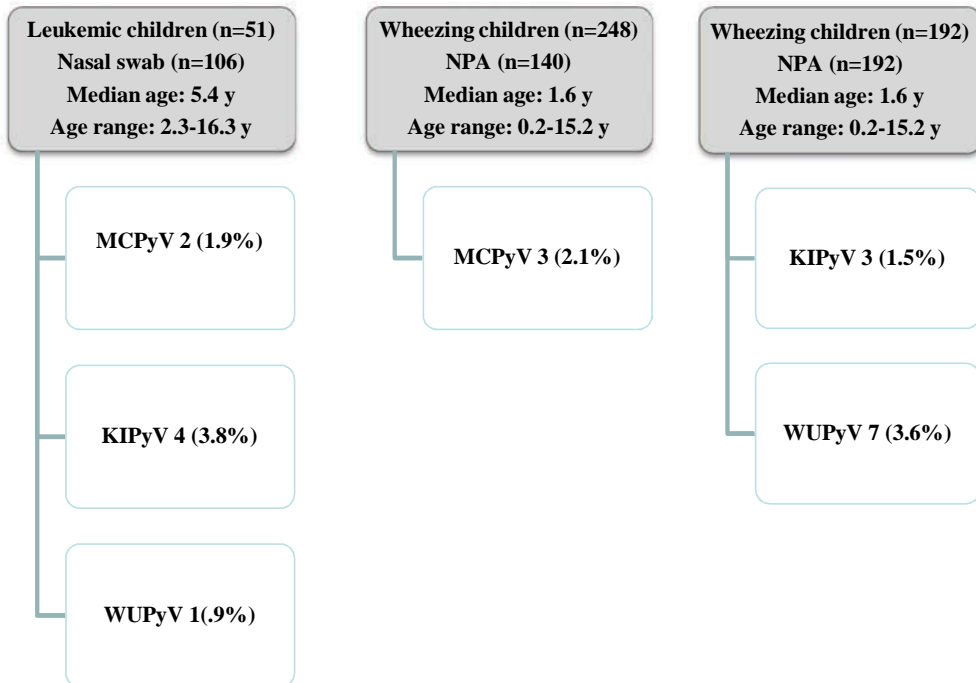


Figure 13. Patient, sample group, and PCR results in the respiratory tract.

MCPyV DNA was detectable in 3 (2.1%) of 140 NPAs, and in 2 (1.9%) of 106 nasal swabs (Study I). The presence of MCPyV in the respiratory tract raises questions about the mode of transmission and respiratory pathogenicity of this newly described polyomavirus.

MCPyV DNA was detected at a variable frequency in 27 (4.25%), of 635 NPAs and in 7 (1.3%) of 526 respiratory tract samples from Australian patients with upper respiratory symptoms (Table 5) (Bialasiewicz et al., 2009a, Goh et al., 2009). MCPyV DNA was amplified in 15 (17.24%) of 87 lower respiratory tract samples from hospitalized Italian patients with lower respiratory-tract symptoms (Babakir-Mina et al., 2010b). Sequence data showed that MCPyV found in respiratory secretions is similar to the virus identified in Merkel cell carcinoma (Babakir-Mina et al., 2010b). Of 305 samples tested, MCPyV DNA was detected in 10 (3.27%) respiratory specimens collected from immunocompetent and immunocompromised patients and they evaluated the possibility of the contribution of this virus in respiratory disease alone, or in combination with other respiratory viruses (Abedi Kiasari et al., 2011).

The presence of MCPyV in respiratory secretions indicates that it is shed into the respiratory tract or is present in cells of the respiratory tract, similar to KIPyV, WUPyV, BKPyV, and possibly JCPyV. Its presence also suggests that the respiratory tract may be a route of transmission and become latent in other sites, such as epidermal tissue, by systemic spread.

MCPyV, KIPyV, and WUPyV have been found worldwide in respiratory specimens along with a high prevalence of codetected viruses, but clear evidence for a causal association with respiratory illness has yet to be identified. These PyVs may be merely transmitted through the respiratory route or be detected during periods of reactivation. Similarly, JC and BK PyVs are suspected of being transmitted by inhalation and are occasionally detectable in respiratory samples, yet patients generally remain asymptomatic or exhibit nonspecific upper respiratory tract symptoms (Wiedinger et al., 2014). Conclusions about their primary target organs and pathogenicity cannot be drawn without epidemiologic support and without further investigation into different sample types.

## 2. Detection of MCPyV in blood samples

In total, 840 sera from 229 tonsillectomy patients in the age-range 1.5 to 72 years, 51 leukemic children, and 248 wheezing children were PCR-negative for KIPyV, WUPyV, and TSPyV DNA (Studies I, II, and V) and the 840 sera studied for MCPyV. Only one serum sample, from a leukemic child, was PCR-positive (Figure 14) (Study I).

Interestingly, our survey revealed no evidence of the occurrence of new HPyV DNA in the serum or plasma of immunocompetent individuals. In contrast, several studies have shown blood cells of immunocompetent individuals to harbor BKPyV and JCPyV DNA. BKPyV and JCPyV DNA has also appeared in the sera or plasma of immunocompromised patients (Dorries et al., 1994, Sundsfjord et al., 1994, Azzi et al., 1996, Ferrante et al., 1998, Dolei et al., 2000, Drachenberg et al., 2007). Plasma samples from 62 HIV-1-positive patients have revealed both KIPyV and WUPyV in two (3.2%) and one (1.6%) cases (Babakir-Mina et al., 2009a). In addition, the prevalence of KIPyV in HIV-1-positive patients was similar to that in healthy blood donors (Babakir-Mina et al., 2010a).

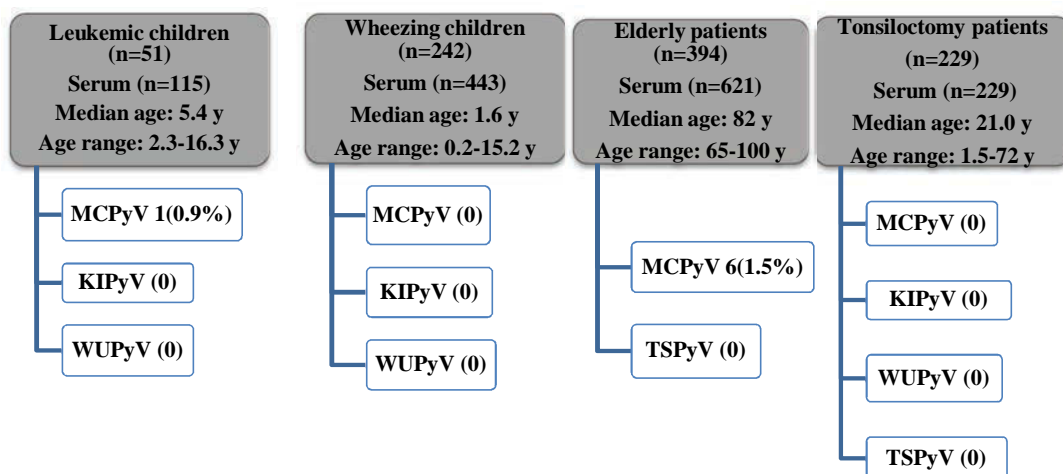


Figure 14. Patient, sample groups, and PCR results in blood.

In Study IV, MCPyV DNA, unlike TSPyV DNA, occurred in low copy numbers in serum samples from a notable proportion of aging individuals. Of the 394 patients, 39 (9.9%) were positive once for MCPyV DNA by the LT PCR, and 33 (8.4%) by the VP1 PCR, while 6 (1.5%) were positive by both PCR assays. In general, the viral DNA copy numbers were low. In sharp contrast, no TSPyV DNA was detectable with qPCRs for the corresponding genomic regions. Whether this reflects enhanced viral replication possibly due to waning immune surveillance and is associated with increased MCC risk deserves exploration. Our results show the occurrence of MCPyV DNA rather commonly in sera from the elderly.

Interestingly, when we examined the association between MCPyV DNA positivity in serum and the clinical characteristics of the elderly (IV), VP1-PCR positivity was significantly and positively associated with chronic respiratory disease ( $p = 0.023$ ). Univariable logistic regression was used to analyze the association between patient characteristics and virus etiology. Statistical significance was established at the level of  $P < 0.05$ . No associations appeared between LT1-PCR positivity and clinical condition.

MCPyV has been detectable in inflammatory monocytes, but not in resident monocytes of patients with MCC, leading to the hypothesis that MCPyV persists in inflammatory monocytes and spreads along the migration routes of inflammatory monocytes (Mertz et al., 2010). Moreover, the presence has also been noted of MCPyV in peripheral blood at low copy numbers (Feng et al., 2008, Shuda et al., 2009). MCPyV sequences have been detected in buffy coats from healthy blood donors (Pancaldi et al., 2011). The prevalence of 22%, as determined in this study, is probably an underestimate, because blood samples contain very low copies of MCPyV DNA, and data suggest that MCPyV is able to persist at a low viral copy number in the peripheral blood mononuclear cells of immune-competent persons (Pancaldi et al., 2011).

Investigation has reported MCPyV sequences in 27.1% of purified malignant cells from human chronic lymphocytic leukemia (CLL) samples (Pantulu et al., 2010). Our MCPyV DNA results in serum samples, together with data from reported MCPyV DNA in buffy coats (Pancaldi et al., 2011), suggest that in a fraction of healthy individuals, MCPyV persists or remains latent in blood cells. In the long-term, viral persistent infection may allow MCPyV to generate mutants that can participate in the cell transformation process. Indeed, a novel truncating MCPyV LTag deletion in CLL cells has been detected (Pantulu et al., 2010). As suggested, this oncogenic process, together with the immune impairment

of the host and other factors, is a well-known multistep cell transformation mechanism used by other DNA tumor viruses such as human papilloma viruses (Levine, 2009), viruses which are closely related to the PyVs.

### **3. Detection of KIPyV in the gastrointestinal tract**

In Study I, WUPyV was detectable in none, but KIPyV in 2 (2.7%) fecal samples. The novel KI and WU polyomaviruses have also occurred in the digestive tract of children with respiratory or gastrointestinal symptoms or both (Gaynor et al., 2007, Ren et al., 2009). KIPyV and WUPyV can occur in the stool of patients with hematological disorders, but often in combination with other viruses involved in gastrointestinal disorders (CMV, adenovirus, BKPyV) (Babakir-Mina et al., 2009b). Because of frequent co-infections, a clear correlation between novel polyomaviruses and clinical symptoms could not be established. However, it was observed that diarrhea occurred frequently in patients infected by KIPyV compared with patients not infected by this virus (Babakir-Mina et al., 2009b). A case-control study to investigate the correlation between the first 10 HPyV species and the occurrence of gastroenteritis, despite its frequent detection of KIPyV, WUPyV, MCPyV, and MWPyV in fecal samples, had no data to support a causative role for HPyV in gastroenteritis (Li et al., 2013).

Rockett et al. (2013) detected MWPyV in 4.4% of fecal specimens collected from patients suffering gastroenteritis and in 12.8% of healthy control children, which is higher than the rates of 2.3%, 3.3%, and 0% from children with diarrhea in St Louis, Missouri, in California (Siebrasse et al., 2012b) and in Chile (Yu et al., 2012b). In contrast to these findings however, Yu et al. (2012) reported a high detection rate of 12.5% in children suffering from diarrhea in Mexico. Rockett et al. also detected HPyV6, 7, and TSPyV in respiratory and fecal specimens, but at low prevalence (<1%), confirming very recent reports of HPyV6, 7, 9, and TSPyV in respiratory, urine, and fecal specimens from immunocompromised children in St Louis, Missouri. (Siebrasse et al., 2012a). HPyV9 was detected in none of the fecal samples (Rockett et al., 2013).

STLPyV has occurred in clinical stool specimens from the USA and Gambia at an up to 1% frequency (Lim et al., 2013). HPyV12 was initially identified in the organs of the gastrointestinal tract (in resected human liver tissue) (Korup et al., 2013). Besides the novel HPyV12, only MCPyV was detected in liver and other organs of the gastrointestinal tract (Loyo et al., 2010). Although many questions are still to be answered regarding the



potential role of these viruses in human infection, STLPyV and the recently described MWPyV are the first two polyomaviruses to be discovered in human stool samples. This makes one ask whether they may have a primary gastrointestinal tropism.

#### **4. Detection of WU, MC, and TS polyomaviruses in tonsil samples**

Until now, the role of the tonsils has been regarded as solely immunologic: it is hypothesized that their position at the entrance of the respiratory and alimentary tract facilitates their functional role in defense against pathogens (Ramos et al., 2013). Tonsillectomy may be indicated when the patient experiences recurrent infections of acute tonsillitis. Viral infections are the most common cause of acute tonsillitis; adenovirus is the most common cause of nonstreptococcal tonsillitis (Chiappini et al., 2012).

In our first (I) and last (V) study, we found MCPyV, WUPyV, and TSPyV DNA in tonsillar tissue. The Monaco group showed that, in addition to human glial cells, JCPyV can infect tonsillar lymphocytes and stromal cells in vitro and in vivo, as well (Monaco et al., 1996, Monaco et al., 1998).

We used PCR amplification (qualitative, quantitative, and nested PCR) to determine whether DNA from nondissected tonsillar tissue of healthy individuals contained newly discovered polyomavirus-specific nucleotide sequences. Of 229 matched pairs of tonsillar tissue biopsies and serum samples from asymptomatic donors; tonsils both from children and adults, 8 (3.5%) tonsillar tissues were positive for the MCPyV, 8 (3.5%) for the TSPyV, and 5 (2.2%) for the WUPyV sequences (Figure 15).

Ages of patients with MCPyV or WUPyV DNA in their tonsils were ranked and compared by the unequal variance *t*-test (I), yielding a statistically significant difference of  $p < 0.001$ . Moreover the difference in median age of the patients with and without MCPyV ( $p = 0.032$ ) or WUPyV ( $p < 0.001$ ) in the tonsils was statistically significant.

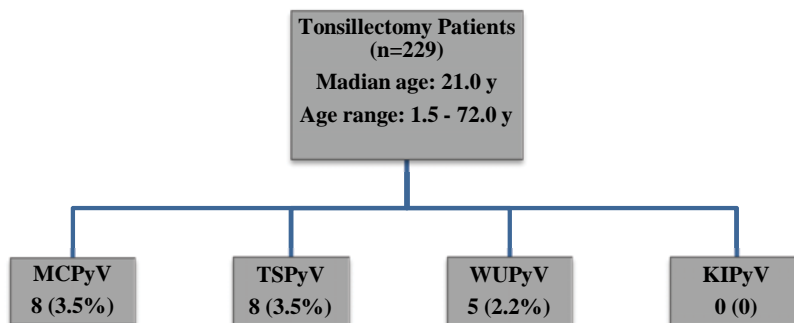


Figure 15. Patient, sample groups, and PCR results in tonsil samples.

HPyVs in general establish persistent infections and undergo periodic reactivations, causing disease in susceptible hosts (Boothpur and Brennan, 2010). Evidence suggests that the lymphoid system plays a role in polyomavirus infections and its persistence (Goudsmit et al., 1982, Monaco et al., 1996, Monaco et al., 1998, Kato et al., 2004). The previously identified HPyVs BK and JC or their DNA have occurred in tonsillar tissue in children, and JCPyV DNA also in the spleen and lymph nodes (Monaco et al., 1996, Monaco et al., 1998, Kato et al., 2004, Meneses et al., 2005). We and others have reported PyVs DNA in tonsillar tissue, suggesting lifelong persistence in lymphoid tissue or mucosa (Monaco et al., 1998, Babakir-Mina et al., 2009a, Kantola et al., 2009, Astegiano et al., 2010, Sadeghi et al., 2014).

Furthermore, we showed occurrence of MCPyV and TSPyV DNA in tonsillar tissue. This may be a general feature of HPyVs, as tonsillar tissues can harbor other polyomavirus DNA, and tonsillar stromal cells have shown a susceptibility to JCPyV infection (Goudsmit et al., 1982, Monaco et al., 1996, Monaco et al., 1998).

Blood cell-derived MCPyV and TSPyV positivity of the tonsils could not be completely ruled out, because from our tonsillectomy patients, only serum samples (all of which tested negative for MCPyV and TSPyV DNA) were obtained at the time of surgery.

However, DNA of several HPyVs (JC, BK, KI, WU, MC, and TS) has been detectable in tonsillar tissue, a possible point of entry (Monaco et al., 1998, Babakir-Mina et al., 2009a, Kantola et al., 2009, Astegiano et al., 2010, Sadeghi et al., 2014). The process by which polyomaviruses gain access to and establish persistent infections in distal body

compartments is not well established. JCPyV has been localized to tonsillar stromal cells and to B lymphocytes, with the latter cell type implicated in circulatory dissemination to other anatomic sites (Monaco et al., 1998). Since all viruses can be detected at increased frequencies in blood and lymphoid tissues during host immunosuppression (Sharp et al., 2009), it is likely that hematolymphoid cells can carry or harbor polyomaviruses.

## **5. Detection of newly discovered polyomaviruses in fetal autopsy**

In Study (III) we attempted to clarify whether vertical transmission of KIPyV, WUPyV, and MCPyV occurs. To the best of our knowledge, ours was the first molecular and serological study of these virus infections of women during pregnancy.

We searched formalin-fixed, paraffin-embedded tissues (placenta, heart, and liver) of 535 fetal autopsy samples for the KI, WU, and MC polyomaviruses. We found no genomic DNAs of KIPyV or WUPyV in any of the stillborn or deceased fetuses. This suggests that during the study period, of these two newly found viruses, neither one often caused miscarriage or IUFD. On the other hand, one pooled sample was positive for MCPyV by PCR. Tissue samples from two sites were available for further study of this fetus. On retesting of the placenta and fetal heart separately, the heart was PCR-negative, and the placenta was PCR-positive for MCPyV.

Transplacental transmission of PyVs after the intraperitoneal inoculation of pregnant hamsters with SV40 and pregnant mice with murine polyoma virus (MuPyV) has been clear in animal models (Zhang et al., 2005, Patel et al., 2009), but human transplacental JCPyV and BKPyV transmission is less clear. A study of 300 pregnant women in their third trimester of pregnancy offered no evidence of the transplacental crossing of PyVs in their offspring (Boldorini et al., 2008), but a study of 15 aborted fetuses found the viral genome in 12 placental and brain samples, and in 9 kidney samples (Pietropaolo et al., 1998). Of 60 samples from 7 fetuses, the BKPyV genome was detected in 22 (36.6%) (Boldorini et al., 2010). Serological evidence of vertical transmission of BKPyV and JCPyV has been shown by evidence of a postnatal rise in IgG level or by transient appearance of an IgM or IgA response in newborns as evidence of infection. Among 19 newborns this has then shown BKPyV and JCPyV infections in 4 (21%) and JCPyV infections in 3 (16%) (Boldorini et al., 2011). Our study indicates that maternal-to-fetal

passage of newly discovered polyomaviruses may occur in humans, although this is not a common means of spread for these viruses.

## **6. DNA sequencing and sequence analyses of PCR-positive samples**

We performed for DNA samples different PCRs using specific primer pairs, generating distinct MCPyV genomic fragments called LTag, sTag, VP1, and VP2 (Table 12). We have obtained positive PCR for fragments and have thus sequenced the fragments. PCR product sequencing verified the specificity of the amplified products and detected possible genomic variants. The products showed 100% identity to each other and also to those of the previously described KIPyV, WUPyV, MCPyV, and TSPyV strain sequences with no deletions or other mutations. In conclusion, based on the analysis of 440 bp, 336 bp, and 140 bp long fragment of MCPyV, of KIPyV and WUPyV, and of TSPyV respectively, we demonstrated no new geographically or pathologically-related genotype. Further analyses based on larger fragments of these viruses, will very probably help to reveal any new genotype existence, as it has been shown for MCPyV (Martel-Jantin et al., 2014).

## **7. Seroepidemiology of the newly discovered human polyomaviruses**

One of the most crucial PyV structural proteins is the major capsid protein VP1. VP1 forms the exterior part of the capsid and is the immunodominant protein; the estimate is that 1 to 10 molecules of the minor capsid proteins VP2 and VP3 occupy the interior part. These proteins are thought to be inaccessible to antibody binding in the context of mature assembled virions. Assays to monitor the presence of BKPyV, JCPyV, and SV40 antibodies or proteins include immunoblotting, hemagglutination inhibition, neutralization assay, complement fixation, immunoelectroosmophoresis, indirect fluorescent antibody, and ELISA (Knowles, 2006). The most common method currently is ELISA using VP1 capsomers or VP1-based virus-like particles (VLPs) as the capture antigen. The advantage of this method is that recombinant VP1 of all HPyVs can be produced in *E. coli*, yeast, or baculovirus/insect cell expression systems. Moens et al. (2013) reviewed the degree of aa identity between the VP1 proteins of the genuine HPyVs; this is presented in (Table 13).

Virus	GenBank	BKPyV	JCPyV	KIPyV	WUPyV	MCPyV	HPyV6	HPyV7	TSPyV	HPyV9	HPyV10	STLPyV	HPyV12
BKPyV	NC_001538	100	78	28	31	43	28	25	53	53	48	44	52
JCPyV	NC_001699		100	28	30	43	27	27	52	54	49	46	54
KIPyV	NC_009238			100	66	28	36	39	26	28	32	31	26
WUPyV	NC_009539				100	29	38	38	27	28	33	33	29
MCPyV	NC_010277					100	29	26	51	54	41	42	50
HPyV6	NC_014406						100	69	29	29	33	33	28
HPyV7	NC_014407							100	26	30	34	64	27
TSPyV	NC_014361								100	61	49	50	57
HPyV9	NC_015150									100	51	67	56
HPyV10	JX262162										100	56	42
STLPyV	JX463183*											100	48
HPyV12	JX308829												100

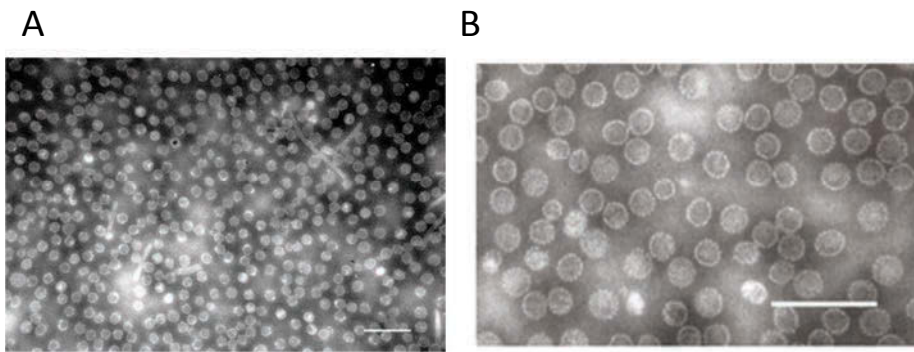
Table 13. Overall amino acid identity between the VP1 proteins of the different human polyomaviruses. The highest degree of identity is found between BKPyV and JCPyV (78%). Serological cross-reactivity between human polyomaviruses. Reviews in Medical Virology 23(4): 250-264.

### KIPyV and WUPyV expression and purification of structural proteins

The structural proteins VP1, VP2, and VP3 of KIPyV, WUPyV, BKPyV, and JCPyV were each expressed with the SUMO expression system in two *E. coli* strains. In addition to this method for the prokaryotic expression, we also did purification of all three structural proteins of each of four HPyVs, including the recently discovered WUPyV and KIPyV, with high yield and >95% purity. We used the SUMO protein as a fusion tag to enhance expression levels. The SUMO fusion partner was efficiently cleaved from the KIPyV and WUPyV VP1, VP2, and VP3 to yield fully tag-free recombinant proteins, and was then depleted by second-affinity purification. Expression levels in the *E. coli* Rosetta strain were, in general, higher, most notably for WUPyV VP1, which was expressed at levels 3 to 4 times as high as with the BL21 (DE3) strain. The protein yield was high in all cases, with 1 L of culture resulting in 20 to 25 mg of purified protein. Due to low solubility, all proteins were purified under denaturing conditions to >95% purity as estimated by SDS-PAGE. Expression of WUPyV VP1 and VP3 in SF9 cells resulted in major bands in SDS-PAGE, whereas VP2 was undetectable.

### **MCPyV and TSPyV expression and purification of structural proteins**

The synthetic MCPyV and TSPyV VP1 gene was cloned into a recombinant baculovirus and expressed in Sf9 cells (Chen et al., 2011a, Chen et al., 2011b). After 4 days of culture, a major protein band of 46KDa was evident in SDS-PAGE of the whole cell lysate, and as the sole constituent of the CsCl gradient-purified material. In EM, the purified VLPs had an icosahedral shape, and a diameter of 42 to 58 nm (Figure 16).



*Figure 16. EM of recombinant MCPyV (A) and TSPyV (B) VP1 virus-like particles. Scale bar = 200 nm. Reproduced with permission from (A) Chen, T., et al. (2011). Serological evidence of Merkel cell polyomavirus primary infections in childhood. J Clin Virol 50(2): 125-129 and (B) Chen, T., et al. (2011). Seroepidemiology of the newly found trichodysplasia spinulosa-associated polyomavirus. J Infect Dis 204(10): 1523-1526.*

## **KIPyV and WUPyV immunoblotting and IFA**

Our immunoblot results showed a strong increase in WUPyV and KIPyV VP2/VP3 seroprevalence with age. In contrast, the VP1-IgG prevalence by the same method was very low, with little difference between age-groups. Of 100 NPA PCR-negative wheezing children aged 4 years, 31 (31%) were positive for WUPyV and 31 (31%) for KIPyV VP2/VP3, compared to only 3 (3%) and 5 (5%) for VP1 of these, respectively. For comparison, the respective WUPyV and KIPyV IgG seroprevalences as determined by immunofluorescence assay (IFA) with nondenatured VP1 were 80% and 54% among 50 NPA PCR-negative children aged  $\leq 2$  years. This difference shows the importance of conformational VP1 antigens.

Of the 25 adults, 52% were IgG-positive in immunoblots for VP2/VP3 of WUPyV and 68% for KIPyV, and 8% and 12% for VP1 of these. Of the 192 NPA samples studied by PCR, 7 (3.6%) were positive for WUPyV, and 3 (1.5%) were positive for KIPyV DNA.

The apparent importance of VP1 protein conformation for antibody binding renders immunoblotting an inadvisable method for VP1-based polyomavirus serodiagnostics, and underlines the requirement of correctly folded VLPs or VP1 pentamers in sensitive and virus-specific antibody detection. Indeed, conformational VP1 pentamers were recently shown to be highly reactive in WUPyV and KIPyV ELISA (Kean et al., 2009). Therefore, compared with VP1, it is interesting that the minor proteins VP2 and VP3 were found in our study to be immunogenic, even in denatured form.

Our IFA data disclosed a ubiquitous IgG prevalence, even in young children, for conformational WUPyV and KIPyV VP1. This contrasts with the immunoblot results, in which the VP2/VP3 seroprevalences increased with age. It is tempting to hypothesize that the KIPyV and WUPyV VP2/VP3 seroreactivities might evolve by repeated exposure to these viruses, possibly including reinfections or reactivations.

## **MCPyV and TSPyV -IgG ELISA**

We examined by VLP ELISA in Studies III, IV, and V and detected an IgG seroprevalence for MCPyV of 45.9% among the pregnant women and 59.6% among the elderly, and an IgG seroprevalence for TSPyV of 67.3% among the elderly. In addition for

80 children and 149 adult asymptomatic donors, TSPyV IgG seroprevalence among the children was 39% and among the adults, 70%. Each of the eight PCR-positive subjects had antiviral IgG of high avidity but not IgM.

For MCPyV, seroprevalences of 40 to 80% have been reported in immunocompetent general populations (Carter et al., 2009, Kean et al., 2009, Tolstov et al., 2009, Touze et al., 2010, Nicol et al., 2013b), and of for TSPyV 70% (Chen et al., 2011b, van der Meijden et al., 2011, Nicol et al., 2013b). Serological studies also suggest that first exposure to MCPyV may occur at a young age (Kean et al., 2009, Tolstov et al., 2009, Chen et al., 2011a). Chen et al. have shown among the northern European pediatric populations a seroprevalence of 9% among 79 wheezing children aged 1 to 2 years, and 35% among those aged 4 to 12. These findings are consistent with other reports on the presence of MCPyV IgG in childhood; though lower than the 20.5% of Kean et al. at 1 to 5 years and the 43% of Tolstov et al. at 2 to 5 years.

Seroepidemiological studies indicate that TSPyV is ubiquitous (Chen et al., 2011b, van der Meijden et al., 2011). Furthermore, we have observed the TSPyV VP1 seroprevalence to increase from 34% to 48% at ages 6 to 10 to 70% in adulthood, and to be 67% among the elderly (Studies IV and V). The eight subjects carrying TSPyV DNA in their tonsils had antiviral IgG of high avidity but not IgM, indicating that tonsillar tissue appears to be a latency site for this virus. Obviously, such data by no means exclude respiratory transmission of this virus, a possibility that deserves exploration.

A common feature of HPyV is that the occurrence of viral associated disease in the population is very rare, and yet antibodies to this virus can be detected in a large percentage of people, indicating that infection is widespread. For example, most human beings have antibodies to BKPyV and JCPyV by their second decade of life, but viremia is rarely detectable except in patients with, respectively, PVAN and PML.

Like JCPyV and BKPyV, the polyomaviruses KIPyV and WUPyV are also rarely detected and seem to be associated with no pathology whatsoever. Seroepidemiological studies show infection to be widespread. In adults over 21, seropositivity rate for KIPyV is reportedly 55% and for WUPyV, 69% (Kean et al., 2009), whereas one study of different age-groups reported increases in rates from ages 1 to 79 years, reaching maximum respective rates of 70% and 80% (Nguyen et al., 2009).



## CONCLUSIONS AND FUTURE DIRECTIONS

This thesis includes studies focusing on four of the ten recently discovered HPyVs: MCPyV, KIPyV, WUPyV, and TSPyV, with respect to determination of their molecular and serological epidemiology, transmission route, and latency site and in finding any association between these novel viruses and diseases.

In the thesis, KIPyV and WUPyV structural proteins were expressed in *E. coli* with SUMO protein as a fusion tag to enhance expression levels. The proteins were immunoblotted for antibodies of wheezing young children and asymptomatic adults, and the children's sera and NPA were examined for KIPyV and WUPyV DNA by PCR.

We employed for MCPyV and TSPyV molecular and serological diagnostic assays including PCR and ELISAs for IgG, for estimation of MCPyV and TSPyV prevalence in clinical samples from immunocompetent or immunocompromised patients.

With the objective of exploring the possibility of transplacental infection by newly discovered HPyVs and identifying any site or tissue prevalence of latency, we searched for KIPyV, WUPyV, and MCPyV DNA sequences in various organs sampled from aborted fetuses; we obtained data to rule out vertical transmission.

We studied the occurrences and blood levels of MCPyV and TSPyV DNAs among the elderly and our results indicate that MCPyV DNA, unlike TSPyV DNA, occurs in low copy numbers in serum in a notable proportion of aging individuals. In addition the serology data showed the IgG antibodies against MCPyV and TSPyV occurred at high rates in serum samples from the elderly.

To answer the questions regarding the possibility that human tonsils represent the initial site of infection or a latency site, or both we investigated the occurrence of KIPyV, WUPyV, MCPyV, and TSPyV DNA in tonsillar specimens and observed MCPyV and TSPyV DNAs particularly often in tonsillitis or hypertrophic tonsillar tissues, unlike the status for KIPyV or WUPyV.

Despite understanding many clinical and biological aspects, future studies may aim at addressing additional issues such as the development of cell culture systems for the newer HPyVs. This is important in order to advance research into their biology, for example, the possibility of isolating and growing the infectious polyomavirus particles in the laboratory or an experimental system.

The role of the new HPyVs in human cancer (if any) is an important area of future research requiring screening of many different types of cancer. Except for MCPyV in MCC, none of the new HPyVs has yet to be implicated in human cancer.

Diarrheal illnesses caused by pathogenic enteric bacteria and viruses remain among the top five causes of death worldwide, especially in developing countries. Many viruses as possible etiologic agents of many diarrheal cases remain unknown. The detection of MWPyV, MXPYV, and STLPyV in stool samples obtained from children with diarrhea, many of whom have no known infectious etiology, raises the possibility that these viruses may play a role in the development of human gastroenteropathy.

Routes of transmission and epidemiology of these newly discovered viruses will need to be determined. The HPyV6, HPyV7, and MCPyV seem to be common in the skin and even show a high tropism for the skin. Their continuous shedding from the skin facilitates their transmission to other individuals and may explain their high seroprevalence, at least that of MCPyV.

Regarding pathogenicity, the majority of the novel HPyVs are thus far without a possible associated disease. More studies are needed to identify and correlate the presence of the novel viral sequences with specific diseases. Because of the nature of polyomaviruses, as well as diseases caused by acute infection, these studies will probably focus on chronic diseases and cancer. Furthermore, as some of the novel HPyV-associated diseases are not exclusively seen in severely immunocompromised hosts, immunocompetent subjects may become the focus of study. In general, more research is needed to understand the role of immunosurveillance in controlling the novel HPyVs, and the role of genetic host factors in this regard.

To study pathogenic mechanisms of the HPyVs in more detail, more knowledge is needed concerning the tropism of each virus. Discrepancies between detection rates and seroprevalence rates in both immunosuppressed and immunocompetent hosts suggest that at least some of the newly identified HPyVs persistently infect the host in a niche not yet well identified or properly sampled. Knowledge about HPyV tropism and reservoirs is indispensable to identify what type(s) of cell is necessary in further cellular and molecular studies. Such studies can guide us in our research strategies to further understand and cope with these viruses.

While many of the new HPyVs have been only very recently discovered through the development of novel molecular biological techniques, we must ask whether additional

viruses lurk in various populations. This also has inspired new interest in previously-identified viruses. Sick individuals' pathogens have been the focus of most of the current virus discovery attempts, but it is possible that viruses exist that are associated with no disease, ones that simply persist and spread without causing clinical symptoms.

Considering the increasing incidence of polyomavirus-associated diseases, particularly the many severe diseases in immunocompromised individuals, one must also consider whether antiviral therapy and vaccine development is plausible. Endlessly, the medical world may be challenged with development of efficient therapies against emerging human viruses with emerging disease associations.

Science itself is continuously evolving, and virology is no exception. What will be the trends in virology in the near future? Clearly, prevention of virus disease in humans is the major aim of virologists. We are continually surprised by the speed and precision with which these technologies are able to tackle a variety of fundamental and fascinating problems, particularly in allowing investigation of non-cultivable viruses and no doubt, will feed back to aim in preparation of better preventive measures. Finally, I would like to end my doctoral book with a quote from Avicenna (973-1037), the Persian physician and philosopher, concerning the subject-matter of medicine in his book entitled *The Canon of Medicine*: "Medicine deals with the states of health and disease in the human body. It is a truism of philosophy that a complete knowledge of a thing can only be obtained by elucidating its causes and antecedents, provided, of course, such causes exist. In medicine it is, therefore, necessary that causes of both health and disease should be determined."

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