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Detection, prevalence and blood safety

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Polyomaviruses in blood donors

Detection, prevalence and blood safety



Sergio Kamminga

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Polyomaviruses in blood donors
Detection, prevalence and blood safety

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Chapter 1

General introduction

Blood safety

Many different blood safety measures are implemented in the Netherlands to prevent disease in recipients of blood products and components. These measures start with donor selection based on a physical check and risk assessment questionnaires, and ends with post-transfusion information, which means an infection is detected after a transfusion and triggers a lookback procedure to identify or prevent further infections. In between, there are many different steps to limit risks for a recipient, such as diversion bags against bacterial infection, serology and nucleic acid testing (NAT), leukodepletion and pathogen reduction (1).

The implementation of these methods (Figure 1) has led to a dramatic decrease in transfusion-transmitted infections (TTI). For example, the transfusion-associated hepatitis incidence, caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), has dropped from 33% at the end of the 60's to virtually zero today (2). A similar trajectory was seen for human immunodeficiency virus (HIV), where identification of the virus and the availability of an anti-HIV test reduced the prevalence in blood donors from 0.04% to almost zero, with the exception of window period donations before the availability of NAT testing (2). Nevertheless, before HIV screening was available, approximately 90% of severe hemophiliacs, 74% of factor VIII deficient persons and 39% of factor IX deficient persons were anti-HIV positive, stressing the importance of adequate safeguards in the blood supply chain (2).

Currently all blood donors in the Netherlands are routinely screened using serologic tests for HBV, HCV, HIV1/2, and *Treponema Pallidum* and NAT for HBV, HCV, HIV1/2 and Hepatitis E virus (HEV) (1,3). In addition, new donors are tested serologically for human T-lymphotropic virus 1/2. Furthermore, blood products and components tested for Parvovirus B19 are available for patients with a high risk for parvovirus related complications. On indication, additional tests are performed for cytomegalovirus, malaria or Q-fever (during an outbreak). Starting in May 2020, selected blood products (based on donor travel) will be screened with NAT for the presence of West-Nile virus (WNV). Between 2007 and 2017, application of these diagnostics in approximately 8.8 million blood donations identified 149 proven infections in repeat donors (3).

Immunocompromised recipients

The adequate screening of blood products and components is of special importance when these are transfused to immunocompromised recipients, such as solid organ

transplant recipients and patients with neoplasms, including haemato-oncological diseases, which often occur in children (4). For example, platelets in the Netherlands are primarily administered to leukemia and lymphoma patients (5). These patients form a large part of the recipients of blood products and components (4,6) and are more susceptible for (fulminant) infection. In addition, the advent of monoclonal antibody therapy can lead to an acquired immunodeficiency and is accompanied by an increased susceptibility to infectious disease (7,8).

Special measures are currently in place to protect the immunocompromised patient population from complications due to transfusion transmitted infections. These opportunistic viruses can cause severe disease in the immunocompromised host. For example, Hepatitis E virus can cause severe hepatitis in immunocompromised patients (9) and cytomegalovirus disease is common among kidney transplant patients, even when using prophylactic treatments (10). NAT is used to detect Hepatitis E virus in blood donations and leukoreduction and/or serology tests are used to reduce chance of cytomegalovirus infection in immunocompromised recipients (11-13).

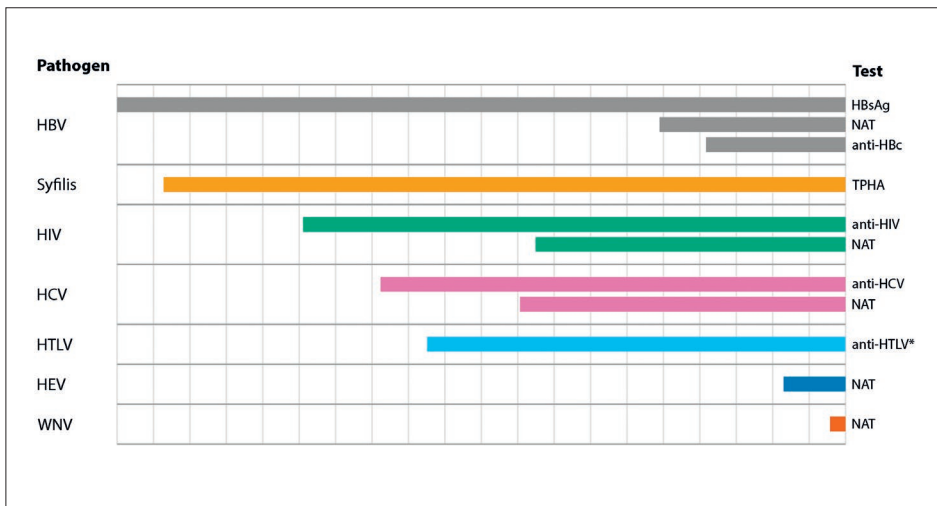


Figure 1. Screening tests routinely performed on blood donations in the Netherlands between 1970 until 2020.

Abbreviations: HBV: hepatitis B virus; HIV: human immunodeficiency virus; HCV: hepatitis C virus; HTLV: human T-cell lymphotropic virus; HEV: hepatitis E virus; WNV: West Nile virus; HBsAg: hepatitis B surface antigen; NAT: nucleic acid testing; anti-HBc: hepatitis B core-antigen antibody; TPHA: *Treponema pallidum* haemagglutination; anti-HIV: HIV antibody; anti-HCV: HCV antibody; anti-HTLV: HTLV antibody.

*HTLV testing switched from testing all donations to only donations from new donors in 2013.

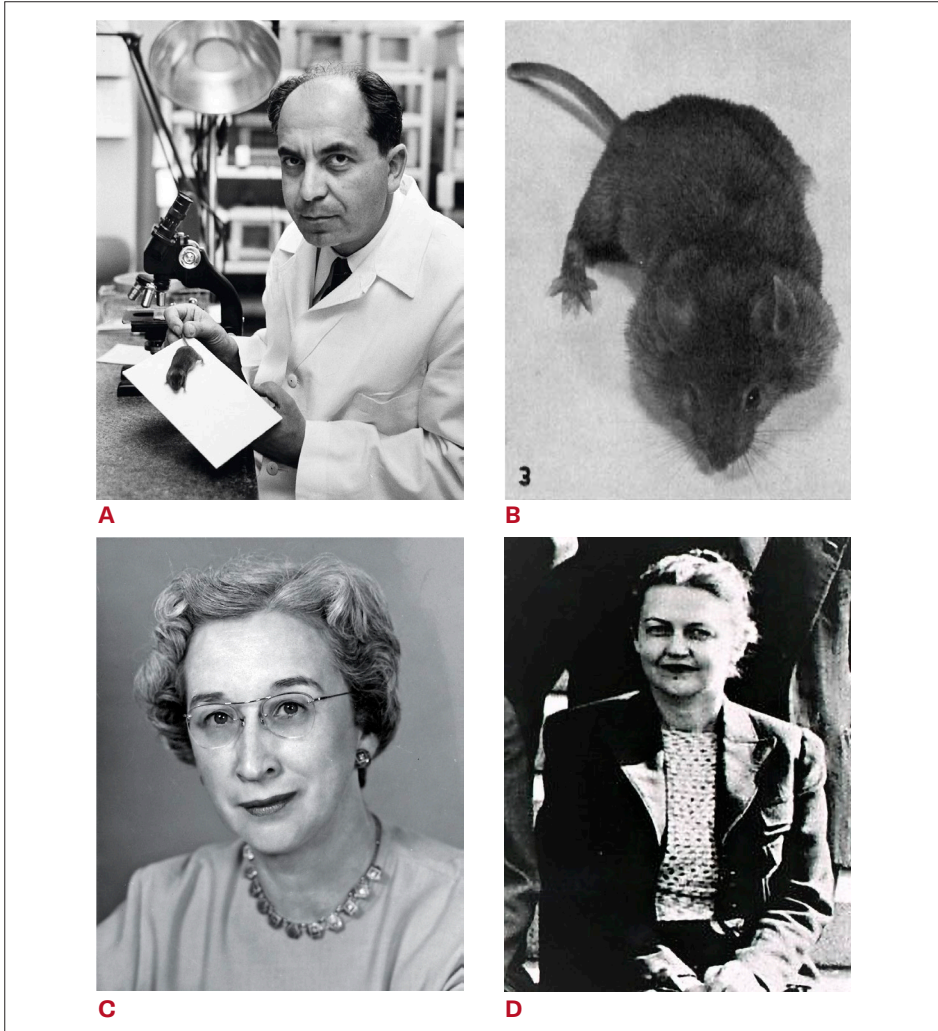


Figure 2. Short history of polyomaviruses.

A. Ludwik Gross was the first to discover a filterable agent capable of inducing parotid gland tumours in mice. **B.** One of the mice of Gross with parotid gland tumours. **C.** Sarah Stewart and **D.** Bernice Eddy who coined the term polyomavirus. Images reprinted with permission (15-17,20)

Similar to cytomegalovirus, human polyomaviruses can cause disease in immunocompromised patients. In the past 15 years, due to advancement of nucleic acid detection techniques, many new human polyomaviruses have been discovered (14). To what extent human polyomaviruses are present in Dutch blood donors and whether polyomaviruses are transfused through the blood supply chain is currently unknown. Because many recipients of blood products and components are immunocompromised, it is important to gain further understanding of these opportunistic pathogens in the blood supply chain.

A short history of polyomaviruses

The first polyomavirus was discovered in 1953 by Ludwik Gross (Figure 2) when he discovered that a preparation of filtered murine leukemia virus (MLV) was capable of not only inducing leukemia, but also parotid gland tumours (15), suggesting presence of a virus beside MLV. These findings would later be confirmed and expanded by Sarah Stewart and Bernice Eddy (Figure 2), who named the virus Stewart-Eddy (SE) polyomavirus, for its ability to induce many (poly-) tumours (-oma) and is an important milestone for the then fledgling field of oncogenic viruses (16,17). Bernice Eddy suggested that a similar virus could be found in monkey kidney cells, which were then used to produce polio vaccines (18). Her suspicion proved right, but between 1955 to 1963 tens of millions of Americans were already administered a poliovirus vaccine contaminated with Simian Virus 40 (SV40) polyomavirus (19) and caused serious public health concerns, since SV40 polyomavirus can induce tumours in rodents. Many cohort studies looked into a relationship between SV40 contaminated polio vaccine and cancer, but no association was ever found (19).

The first two polyomaviruses with humans as natural hosts were discovered simultaneously and independently in 1971 (21,22), called BK polyomavirus and JC polyomavirus, after the initials of the patients these were discovered in. BK polyomavirus was discovered in a kidney transplant patient with ureteric obstruction and JC polyomavirus in a Hodgkin's disease patient with progressive multifocal leukoencephalopathy (PML). For decades, these were the only known HPyVs, however due to the advance of molecular biological techniques, many more have been found since 2007 (14). This started with Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV), both found in respiratory samples (23,24). In 2008, the Merkel Cell polyomavirus (MCPyV) was found out to be the cause of the Merkel Cell Carcinoma (MCC) and integrated in the host genome, proving polyomavirus related oncogenesis in humans (25). HPyV6 and -7 were found in healthy human skin

and are considered part of the skin microbiome (26). The Trichodysplasia Spinulosa polyomavirus (TSPyV) was discovered in a heart transplant patient in 2010 (27). Since then, HPyV9, Malawi polyomavirus (MWPyV), Saint Louis polyomavirus (STLPyV), HPyV12, New Jersey polyomavirus (NJPyV), Lyon IARC polyomavirus (LIPyV) and recently Quebec polyomavirus (QPpyV) were discovered in samples acquired from humans (28–34). For HPyV12, NJPyV and LIPyV the natural host is probably not human, based on serology and metagenome analysis (34,35). HPyV12 may rather be a shrew polyomavirus (36) and LIPyV a feline polyomavirus (37).

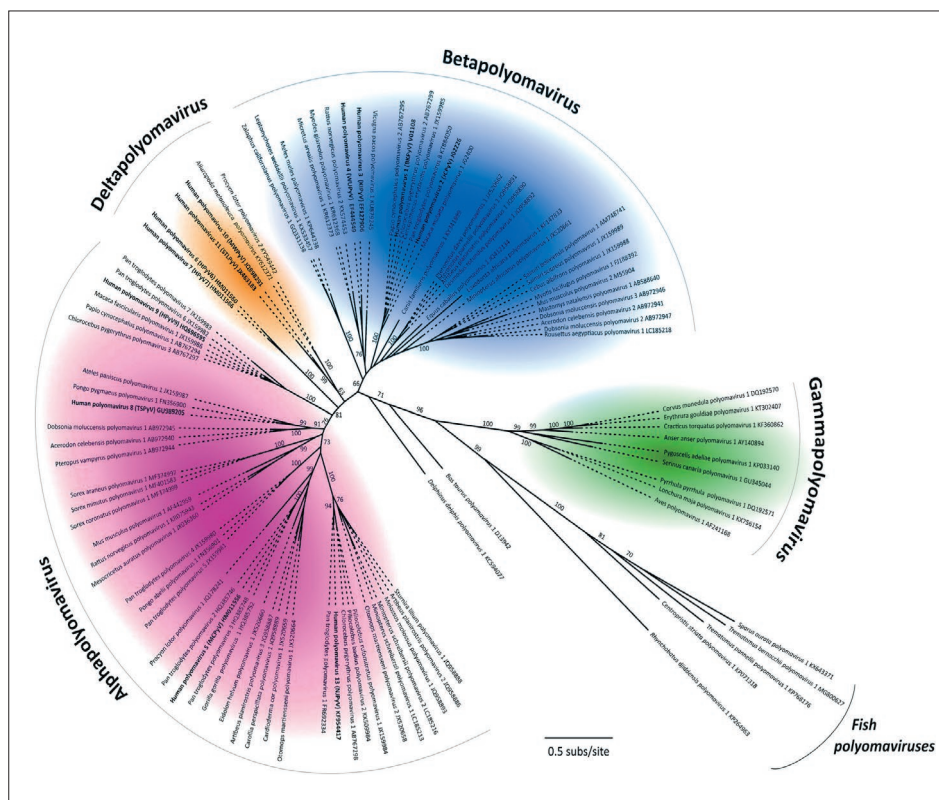


Figure 3. Phylogenetic tree of polyomaviruses.

Unrooted phylogenetic tree for the family *Polyomaviridae*, based on the LT protein amino acid sequences, according to ICTV classification(41). Unassigned species are shown in italics and human polyomaviruses are shown in bold. Figure adapted with permission (38).

Taxonomy and genome organization

The *Polyomaviridae* can infect a broad spectrum of hosts, including many birds and fishes, but also arthropods (38–40). The family currently contains close to 100 different species, which are assigned to 4 genera called the *Alpha-*, *Beta-*, *Gamma* and *Deltapolyomavirus* (41) (Figure 3). The *Alpha-* and *Beta-* and *Deltapolyomavirus* genus contain polyomaviruses from a variety of hosts, whereas the *Gammampolyomavirus* genus contains only avian polyomaviruses. The human polyomaviruses are present in *Alpha-*, *Beta-* as well as *Deltapolyomaviruses*. Distinction between species is made based on the nucleotide sequence of the LT antigen coding sequences. Either at least 15% genetic distance in the LT antigen coding sequence is required for a new species, or less than 15% genetic distance and a critical biological difference, such as disease association or host specificity (41).

The *Polyomaviridae* family consists of small, non-enveloped viruses with dsDNA genomes around 5000 base pairs (bps) in size, with an icosahedral capsid between 40–45 nm in diameter (42). The genome is generally around 5000 bps and is separated in three functional regions (43), called the early region, the late region and the non-coding control region (NCCR) (Figure 4). The early region, which is expressed early in the infection stage through alternative splicing, encodes for non-structural proteins called the large T (LT) and small T (ST) antigens. LT is the major regulatory protein and is essential to drive the host cell into S-phase to support viral replication, by binding tumor suppressor proteins such as p53 and pRB (44). In addition, LT functions as a helicase and facilitates the switch from early transcription to replication and late transcription, via the NCCR (43,44). The ST antigen is involved in large T-stabilization and cellular transformation, but is not essential for viral replication (43,45). The late region is transcribed after the start of viral DNA replication and encodes the structural proteins called Viral Protein (VP) 1, VP2, VP3 and sometimes VP4. VP1 is the major capsid protein and the virion consist of 360 VP1 proteins arranged in 72 pentamers, that are connected by the C-terminal arm of VP1. The virion is further stabilized by calcium ions and disulfide bond between the pentamers (43). Next to virion structure, VP1 plays a major role in both the antigenicity as well as receptor specificity of polyomaviruses (46). The role of VP2 and VP3 is less clear, but these are expressed on the inside of the viral particle on each pentamer (47). In addition, some polyomaviruses can express VP4, which is not part of the virion, but can act as a viroporin (48). Altogether, the viral particle is very stable and relatively resistant to heat inactivation and lipid solvents (49,50).

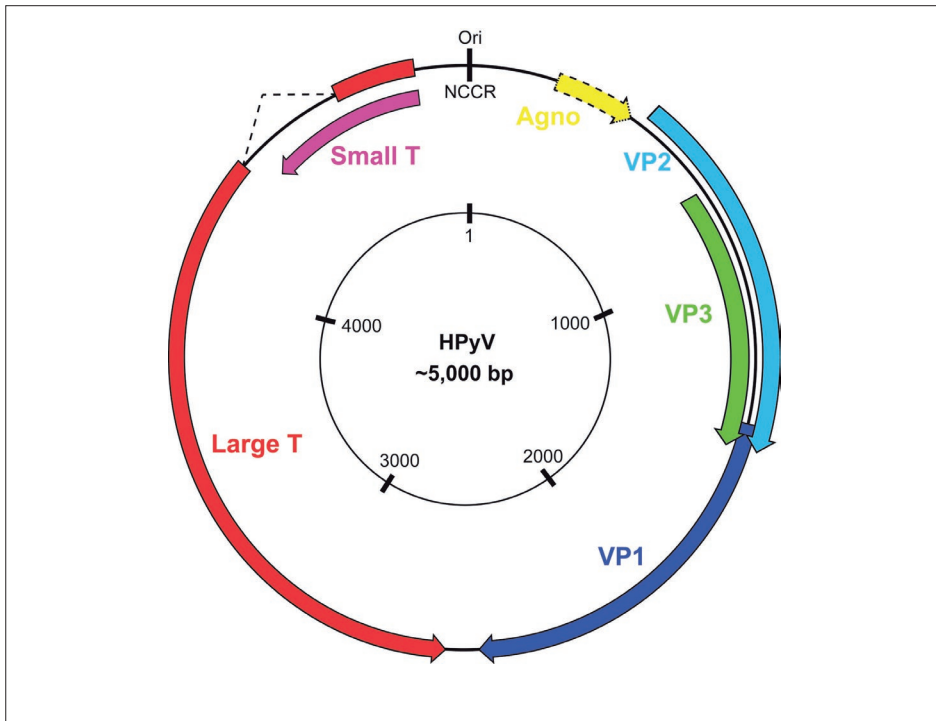


Figure 4. Schematic representation of a human polyomavirus genome.

A circular, double-stranded DNA genome of a human polyomavirus. Regions that code for proteins are indicated by colored arrows. Agno protein is not present in every HPyV genome. Ori: origin of replication; NCCR: non-coding control region; VP: viral protein. Figure adapted with permission (14).

Polyomavirus-related diseases

BKPyV and JCPyV were both discovered and associated with disease simultaneously in 1971 (21,22). BKPyV was determined as the cause of ureteric obstruction in a kidney transplant recipient (Table 1). Afterwards, BKPyV would prove to be an opportunistic pathogen in immunocompromised patients and is now the known cause of BKPyV associated nephropathy (BKPyVAN) (51), hemorrhagic cystitis (52) and ureteric obstruction (21,53) and has been implicated in many other diseases, such as bladder cancer (46,54). JCPyV causes a severe brain disease called PML (Figure 5), which occurred primarily with lymphoproliferative disease and in HIV patients. Since the advent of antiretroviral therapy, the PML burden shifted away from HIV patients, towards patients with drug-induced PML, such as multiple sclerosis patients taking natalizumab (55,56). The risk of PML has led to increased vigilance for MS patients on

natalizumab and these patients are now screened and risk-stratified based on JCPyV serology (57). The first polyomavirus proven to cause tumour development in humans is the MCPyV, which causes MCC (25). MCC is an aggressive, neuroendocrinal skin cancer that often presents already with loco-regional metastases (Figure 5), and as such has a high case-fatality rate (58). This disease is not only found in immunocompromised populations, but also in the elderly. The incidence may be increasing, but is about 50-fold lower than melanoma (58). MCC is difficult to treat, but in recent years therapy has improved due to immune checkpoint inhibitors (58). HPyV6, -7 and TSPyV also cause skin diseases. HPyV6 and -7 can both cause pruritic and dyskeratotic dermatosis in immunocompromised patients (59). Recently, HPyV6 has also been implicated as the cause (or contributing factor) of Kimura disease and angiolymphoid hyperplasia with eosinophilia (60). TSPyV is the cause of Trichodysplasia Spinulosa, a benign but disfiguring skin disease in immunocompromised patients (Figure 5), characterized by follicular papules and keratin spines (27). NJPyV has been shown to cause retinal blindness, necrotic plaques of skin and weakness in a pancreatic transplant patient, who had to flee through floodwater during superstorm Sandy (32). Since the initial report, no new cases have been described. The other HPyVs currently have no clear disease association.

Table 1. Nomenclature and origin of HPyVs.

Species	Virus (abbreviation)	Disease association(s)	Seroprevalence % (31,35,4,6,61-68)	Sample identified	Year identified
HPyV1	BK polyomavirus (BKPyV)	Polyomavirus-associated nephropathy Hemorrhagic cystitis (59) Ureteric stenosis (70) Progressive multifocal leukoencephalopathy Bladder cancer (54)	80-100	Urine	1971 (21)
HPyV2	JC polyomavirus (JCPyV)	Progressive multifocal leukoencephalopathy (71,72) Polyomavirus-associated nephropathy (71,72) Granule cell neuroinopathy (71,72) (Meningo-)Encephalitis (71,72)	40-80	Brain	1971 (22)
HPyV3	Karolinska Institutet polyomavirus (KIPyV)	Respiratory disease (73)	60-90	Nasopharynx	2007 (23)
HPyV4	Washington University polyomavirus (WUPyV)	Respiratory disease (74)	40-100	Nasopharynx	2007 (24)
HPyV5	Merkel cell polyomavirus (MCPyV)	Merkel cell carcinoma (25)	40-80	Skin	2008 (25)
HPyV6	Human polyomavirus 6 (HPyV6)	Pruritic and dyskeratotic dermatoses (56) Keratoacanthoma (75,76) Kimura disease (60,75) Angiolympoid Hyperplasia with Eosinophilia (60,75)	70-85	Skin	2010 (26)
HPyV7	Human polyomavirus 7 (HPyV7)	Pruritic and dyskeratotic dermatoses (56)	35-65	Skin	2010 (26)
HPyV8	Trichodysplasia spinulosa polyomavirus (TSPyV)	Trichodysplasia Spinulosa (27,77)	70-85	Skin	2010 (27)
HPyV9	Human polyomavirus 9 (HPyV9)	None	20-50	Serum	2011 (78,79)
HPyV10	Malawi polyomavirus (MWPyV)	None	40-100	Stool	2012 (28,29)
HPyV11	Saint Louis polyomavirus (STL-PyV)	None	65-95	Stool	2012 (30)
HPyV12	Human polyomavirus 12* (HPyV12)	None	5-20	Liver	2013 (31)
HPyV13	New Jersey polyomavirus (NJPyV)	Retinal blindness, necrotic plaques, vasculitic myopathy (32)	5	Muscle	2014 (32)
HPyV14	Lyon IARC polyomavirus (LIPyV)	None	5	Skin	2017 (33)

*Human polyomavirus 12 is now designated as a shrew polyomavirus by ICTV

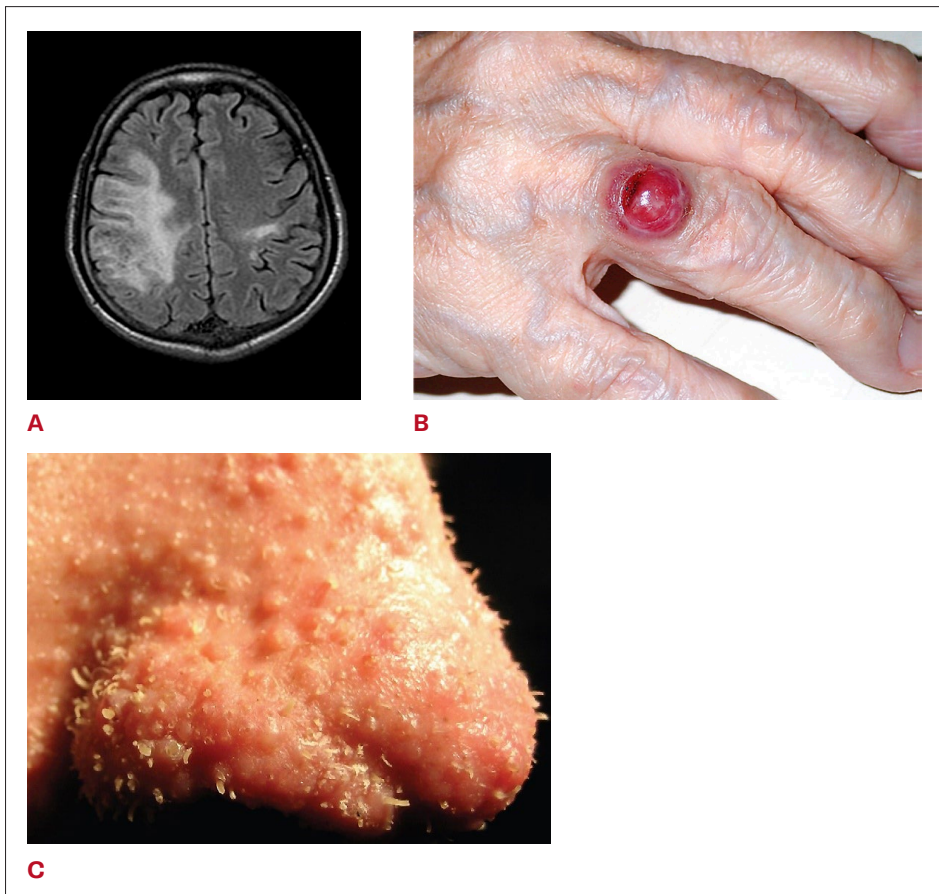


Figure 5. Examples of polyomavirus disease.

A. Axial MRI image of the brain of a patient with PML. **B.** Merkel cell carcinoma on the finger of an elderly patient **C.** Nose of a heart transplant with trichodysplasia spinulosa with numerous papules and spicules. Images reprinted with permission (27,80), PML case courtesy of Assoc Prof Frank Gaillard, Radiopaedia.org, rID: 22071 Merkel cell case reproduced with permission from (81), Copyright Massachusetts Medical Society.

DNA- and seroprevalence

Polyomavirus infections are ubiquitous in the general population and usually infect healthy humans without causing disease. Primary infection with many polyomaviruses occurs early in childhood and is followed by a persistent infection. HPyVs are detected in many different tissues, such as skin (26), urine (82), respiratory samples (83) and tonsillar tissue, (84) using NAT. Despite detection in many different types of tissue, the genuine host cell of polyomaviruses remains unclear (46).

After primary infection, a humoral immune response will develop, leading to the development of IgM and IgG antibodies directed against the major capsid protein VP1. The IgG response will remain over time and is generally used for the assessment of seroprevalence in populations of interest. The seroprevalence can be measured with several methods, generally using the VP1 major capsid protein either expressed as a glutathione-S-transferase (GST) fusion protein or by creation of a virus-like particle (VLP). These are then subsequently bound to either polystyrene beads or ELISA plates (35,61-63,67,68,85,86). Both the VP1 and the VLP method yield very similar seroprevalence results, as was shown for instance for BKPyV (87,88).

Since HPyVs cause a persistent infection, seropositivity for any of these suggests that a seropositive individual is a carrier of that HPyV. A carrier of HPyVs is capable of shedding these viruses, as is demonstrated by detection of BKPyV and JCPyV in urine of healthy individuals (63). The exact route of transmission for HPyVs is often unclear, but for kidney transplant patients it is clear that the donor kidney can function as a vehicle for the virus (88-90). This is evidenced by an increased risk for BKPyV viremia after kidney transplantation from a donor with a high BKPyV IgG seroreactivity (91). Since blood components are often administered to immunocompromised patients, it is important to determine whether these HPyVs are potentially transmitted through blood components.

Scope of thesis

The purpose of the research described in this thesis was to determine the (sero-)prevalence of human polyomaviruses in the Dutch blood donor population, in order to obtain more insight into the risk of blood-transmitted polyomavirus infections. To meet this goal, laboratory tools were developed and adapted to detect these viruses through molecular and serological means. We applied these tools to determine the (sero-)prevalence in a representative cohort of blood donors. Furthermore, we provide a method to monitor these molecular tools over time, in order to identify when an adjustment is necessary.

In **Chapter Two** the development of a tool to measure antibodies specifically for each human polyomavirus is described

In **Chapter Three** the seroprevalence of fourteen human polyomaviruses in a representative cohort of blood donors is determined

In **Chapter Four** quantitative polymerase chain reactions (qPCRs) to detect DNA of fourteen human polyomaviruses are described and the prevalence is determined in the same blood donor cohort

In **Chapter Five** a novel method to monitor these qPCRs in time is described and applied for these qPCRs as well as qPCRs acquired from literature

In **Chapter Six** we provide evidence that JCPyV is transplanted together with the kidney allograft in kidney transplant recipients

In the **General discussion** the implications for blood safety are discussed as well as suggestions for future research



Chapter 2

Development and evaluation of a broad bead-based multiplex immunoassay to measure IgG seroreactivity against human polyomaviruses

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Abstract

Introduction The family of polyomaviruses, which cause severe disease in immunocompromised hosts, has expanded substantially in recent years. To accommodate measurement of IgG seroresponses against all currently known human polyomavirus (HPyV), including the Lyon IARC polyomavirus (LIPyV), we extended our custom multiplex bead-based HPyV immunoassay and evaluated the performance of this pan-HPyV immunoassay.

Methods VP1 protein of fifteen HPyVs belonging to 13 polyomavirus species were expressed as recombinant glutathione S-transferase (GST) fusion proteins and coupled to fluorescent Luminex beads. Sera from healthy blood donors and immunocompromised kidney transplant recipients were used to analyse seroreactivity against the different HPyVs. For BK polyomavirus (BKPyV) the GST-VP1 fusion protein-directed seroresponses were compared to those obtained against BKPyV VP1 virus-like particles (VLP).

Results Seroreactivity against most HPyVs was common and generally high in both test populations. Low seroreactivity was observed against HPyV9, HPyV12, New Jersey PyV and LIPyV. The assay was reproducible (Pearson's $r^2 > 0.84$, $P < 0.001$) and specific. Weak but consistent cross-reactivity was observed between related HPyV6 and HPyV7. Seroresponses measured by the GST-VP1-based immunoassay and the VP1 VLP-based ELISA were highly correlated (Spearman's $\rho = 0.823$, $P < 0.001$).

Conclusions The bead-based pan-HPyV multiplex immunoassay is a reliable tool to determine HPyV-specific seroresponses with high reproducibility and specificity and is suitable for seroepidemiological studies.

Introduction

The *Polyomaviridae* family is a group of double-stranded DNA viruses that infect a broad spectrum of hosts, including humans. The number of identified human polyomaviruses (HPyVs) has substantially increased over the recent years, and currently includes thirteen *polyomavirus species* that are listed in **Table 1**, including full virus names and abbreviations (92). A novel polyomavirus recently identified in human skin samples named Lyon IARC polyomavirus (LIPyV), has not been assigned to a polyomavirus species yet (33).

Several HPyVs are associated with severe disease, such as BK polyomavirus (BKPyV) with nephropathy and haemorrhagic cystitis; JC Polyomavirus (JCPyV) with progressive multifocal leukoencephalopathy (PML); TS polyomavirus (TSPyV) with a dysplastic hair follicle disorder called *trichodysplasia spinulosa*; and MC polyomavirus (MCPyV) with Merkel cell carcinoma (25,51,93,94). An association between HPyV6 and HPyV7 and pruritic and dyskeratotic dermatosis has recently been proposed (95). In addition, HPyV7 might be involved in thymomagenesis (96,97). New Jersey polyomavirus (NJPyV) likely can cause vasculitis, myositis and retinal blindness (32).

The seroprevalence of well-studied polyomaviruses, for instance BKPyV and JCPyV, is generally high and comparable among geographically different populations (61,62,65,67,68,98). Primary HPyV infections usually occur in childhood, which are followed by asymptomatic persistent infection throughout life, sometimes accompanied by little virus shedding (65). Though HPyV infection is widespread and its pathology diverse, symptomatic or manifest HPyV infections are rare and usually limited to the immunocompromised and the elderly (14). For most HPyVs, symptomatic infection occurs when the persistent virus is no longer controlled by the immune system, a phenomenon often referred to as virus reactivation. However, for some HPyVs primary infection coincident with severe immunosuppression has been proposed as the driver of symptomatic disease (77).

Although knowledge of the prevalence of HPyV infections is increasing, little is known about the incidence and transmission of infection, in particular of the recently identified HPyVs such as Saint Louis Polyomavirus (STLPyV), HPyV12, NJPyV and LIPyV. One way of filling this knowledge gap is to develop HPyV species-specific serology.

In general, two Viral Protein 1 (VP1) antigen expression and presentation methods are used to measure HPyV seroreactivity. One is based on insect cell-expressed VP1 assembled into VP1 virus-like particles (VLP). The other, used in this study, is

based on bacterially expressed glutathione S-transferase (GST)-VP1 fusion proteins. Here we aimed to extend our present HPyV bead-based immunoassay measuring IgG seroresponses against the VP1 major capsid protein of HPyVs belonging to *Human polyomavirus* species 1 (BKPyV), 5 (MCPyV), 6 (HPyV6), 7 (HPyV7), 8 (TSPyV) and 9 (HPyV9) (65), with HPyVs belonging to species 2 (JCPyV), 3 (KIPyV), 4 (WUPyV), 10 (Malawi polyomavirus (MWPyV) and HPyV10), 11 (STLPyV) 12 (HPyV12), 13 (NJPyV) and LIPyV. MWPyV and HPyV10 that belong to the same species were both included, because they differ on eight amino acid positions in VP1, of which three might be located in immunogenic loops important for antigen recognition (46,62).

The performance of this new pan-HPyV multiplex immunoassay is evaluated in this study by measuring seroreactivity in two pilot populations and by determining the reproducibility and specificity of the assay. The GST-VP1 fusion protein bead-based assay is also compared with a VP1 VLP-based serological assay for BKPyV.

Table 1. Nomenclature, origin and accession numbers of HPyVs used in the multiplex immunoassay.

Species	Virus (abbreviation)	Original tissue (disease)	Accession	Reference
HPyV1	BK polyomavirus (BKPyV)	Urine	JF894228	(21)
HPyV2	JC polyomavirus (JCPyV)	Brain (PML)	NC_001699	(22)
HPyV3	Karolinska Institutet polyomavirus (KIPyV)	Nasopharynx	NC_009238	(23)
HPyV4	Washington University polyomavirus (WUPyV)	Nasopharynx	NC_009539	(24)
HPyV5	Merkel cell polyomavirus (MCPyV)	Skin (Merkel cell carcinoma)	JF812999	(25)
HPyV6	Human polyomavirus 6 (HPyV6)	Skin	NC_014406	(26)
HPyV7	Human polyomavirus 7 (HPyV7)	Skin	NC_014407	(26)
HPyV8	Trichodysplasia spinulosa polyomavirus (TSPyV)	Skin (TS spicule)	NC_014361	(94)
HPyV9	Human polyomavirus 9 (HPyV9)	Serum	NC_015150	(79)
HPyV10	Malawi polyomavirus* (MWPyV)	Stool	NC_018102	(28)
HPyV10	Human polyomavirus 10* (HPyV10)	Skin (anal condyloma)	JX262162	(29)
HPyV11	Saint Louis polyomavirus (STLPyV)	Stool	NC_020106	(30)
HPyV12	Human polyomavirus 12 (HPyV12)	Liver	NC_020890	(31)
HPyV13	New Jersey polyomavirus (NJPyV)	Muscle	NC_024118	(32)
unassigned	Lyon IARC polyomavirus** (LIPyV)	Skin	NC_034253	(33)

* MWPyV and HPyV10 belong to the same species

** LIPyV is not classified

Materials and methods

Human polyomavirus serology assays

IgG seroreactivities against VP1 were measured using a customized Luminex xMAP assay, as previously described, albeit expanded to include all currently known HPyVs (**65,85,98**). In short, synthetic DNA sequences of VP1 (**Table 1**) (gBlocks, IDT, San Jose, CA, USA), either wild type (JCPyV, KIPyV, WUPyV, HPyV12, NJPyV, LIPyV) or codon-optimized (MWPyV, HPyV10, STLPyV), were cloned into pGEX-5x-3 vectors (GE Healthcare Life Sciences, Chicago, IL, USA) and expressed as GST-VP1.tag fusion proteins in BL21 Rosetta bacteria. Expression of each newly expressed GST-VP1 fusion protein was analysed by glutathione-sepharose 4B purification, SDS-PAGE (10%) separation followed by Coomassie staining.

The GST-VP1.tag fusion protein is subsequently coupled to glutathione-casein linked polystyrene beads (Bio-Rad Laboratories, Hercules, CA, USA.) Each bead is colour-coded by fluorescent dyes, which allows distinction between the different analytes in a single well. The coupling of the complete GST-VP1.tag fusion protein to the bead is verified on the Bio-plex apparatus using mouse- α -tag antibodies (1:100, kind gift from M. Pawlita) followed by α -mouse-phycoerythrin for detection (1:250 Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) (incubated for 30 minutes each in the dark at room temperature).

In the HPyV multiplex immunoassay, serum samples (1:100) were incubated for 1 hour in blocking buffer (1 mg/ml casein, 0.5% polyvinylalcohol, 0.8% polyvinylpyrrolidone, 2.5% Super ChemiBlock (Chemicon International, Billerica, MA, USA) and 2 mg/ml GST bacterial lysate in PBS) to suppress potential non-specific binding to the beads or to GST (**85,99**). In the meantime, the GST-VP1 fusion proteins were coupled to glutathione-casein linked polystyrene beads and the serum samples were subsequently incubated with the mixture of GST-VP1 beads (one hour in the dark at room temperature). For detection of a VP1-directed human IgG response biotinylated goat-antihuman IgG (H+L) was used (1:1000 Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), followed by streptavidine-R-phycoerythrin (SAPE) (1:1000 Invitrogen, Waltham, MA, USA) (incubated for 30 minutes each in the dark at room temperature). As a positive control, a serially diluted mixture of four serum samples with known seroreactivity against various polyomaviruses was included in each test run (**65**). The seroreactivity was measured in a Bio-Plex 100 analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Specific seroreactivity was defined by subtracting the median fluorescence intensity (MFI) values of both a blank sample and of beads coupled to an irrelevant GST fusion protein (SV40 small T-antigen).

For a comparison between the in-house GST-VP1 based immunoassay and the VP1-VLP ELISA, 396 serum samples were analysed in both assays for BKPyV IgG detection, as described previously (100). Our assay uses the VP1 protein from BKPyV genotype Ib1, while the VLP ELISA uses the VP1 protein from BKPyV genotype Ib2 (98.6% VP1 amino acid similarity between Ib2 and Ib1). (100)

HPyV12 and NJPyV VP1 seroreactivity confirmation

To demonstrate the antigenicity of the HPyV12 and NJPyV GST-VP1 antigens used, two synthetic peptides (HPyV12-VP1: VPKSVTDVTAKIQC; NJPyV-VP1: SIHPNDIAKLPEED) were generated (Genscript, Nanjing, China) and used to immunize rabbits. These peptides were chosen based on expected antigenicity in VP1 (46,62) and low amino acid similarity compared with other HPyV VP1 proteins. The polyclonal rabbit antisera raised against these peptides were used in a 1:100 dilution for the recognition of GST-HPyV12 and NJPyV VP1 coupled beads (incubated for 30 minutes in the dark at room temperature). Detection was performed with α -rabbit biotin (1:1000, Dako, Santa Clara, CA, USA) and SAPE (incubated for 30 minutes each in the dark at room temperature).

Competition analysis

To gain further insight into cross-reactivity, VP1 antigen-competition experiments were performed, as described previously (65). Serum samples with known seroreactivity were serially diluted from 1:100 to 1:409600 and incubated with regular blocking buffer containing either GST or GST-VP1 fusion proteins (~2 mg/ml). For this purpose only serum samples were selected with measured seroreactivity above 5000 MFI at a 1:100 serum dilution.

Study population

For evaluation of the HPyV multiplex serology assay, anonymized serum samples of a cohort of 87 healthy blood donors (HBD) (101) and a cohort of 65 immunocompromised kidney transplant recipients (KTR) (66) were tested. Participants gave written informed consent and the study adhered to the Declaration of Helsinki Principles.

Statistical analysis

Squared Pearson correlation coefficients (r^2) were calculated to determine intertest reliability. Correlation between assessed HPyVs was further examined by calculating Spearman rank correlation coefficients (ρ). Statistical analysis was performed in IBM SPSS Statistics 23. When necessary, the significance level ($\alpha = 0.05$) was adjusted according to the Bonferonni method for multiple comparisons.

Results

Expression and coupling of HPyV VP1 to polystyrene beads

To extend the in-house multiplex immunoassay with all currently known HPyVs, VP1 genes of JCPyV, KIPyV, WUPyV, MWPyV, HPyV10, STLPyV, HPyV12, NJPyV and LIPyV were individually cloned and expressed as GST-VP1 fusion protein. Expression of glutathione-purified GST-VP1 fusion proteins was checked by Coomassie-stained SDS-PAGE and found to be comparable for all HPyVs (**Figure 1A**). GST-VP1 containing crude bacterial extracts were purified and coupled to the glutathione-casein

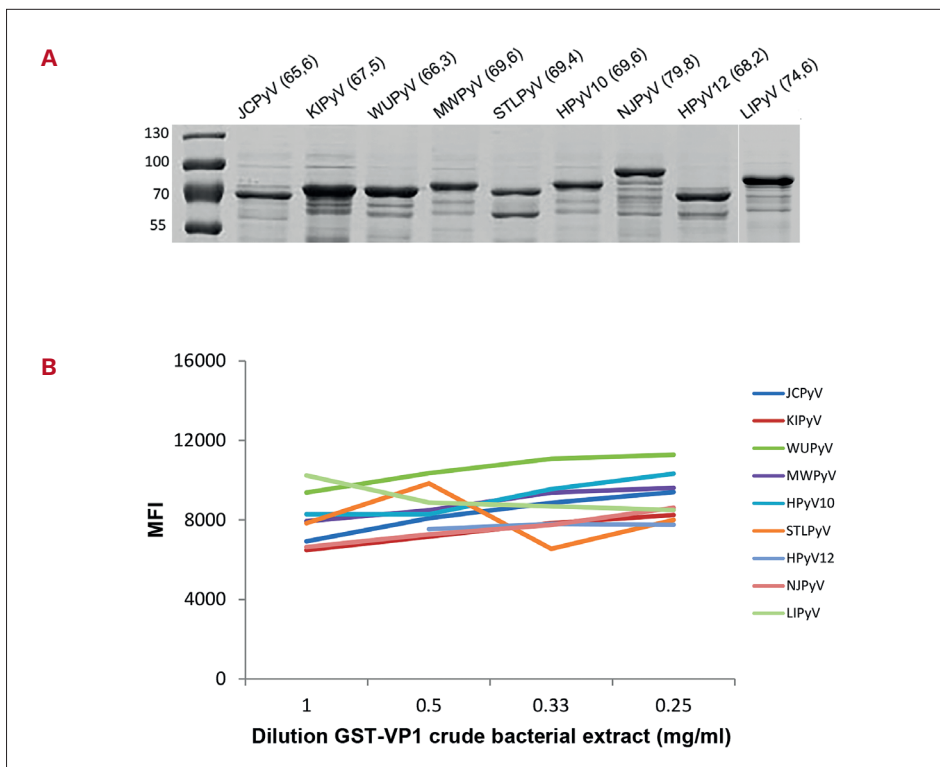


Figure 1. Expression and coupling of HPyV VP1 to polystyrene beads.

Panel **A**: Coomassie-stained SDS-PAGE gel showing glutathione-purified GST-VP1 bacterial lysates of JCPyV, KIPyV, WUPyV, MWPyV, STLPyV, HPyV10, NJPyV, HPyV12 and LIPyV. Numbers between parentheses display molecular mass of the GST-VP1 fusion protein in kilodalton (kDa). Molecular mass in kDa of the pageruler prestained protein ladder (Thermofisher Scientific, Waltham, MA, USA) is indicated on the left. The lane for LIPyV was added at a later date. Panel **B**: purification and coupling of GST-VP1 tag fusion proteins of JCPyV, KIPyV, WUPyV, MWPyV, STLPyV, HPyV10, NJPyV, HPyV12 and LIPyV to glutathione-casein cross-linked beads. GST-VP1 containing crude bacterial extracts were serially diluted (1–0.25 mg/ml). GST-VP1.tag coupling, detected by using α -tag followed by α -mouse-phycoerythrin antibodies, is depicted as median fluorescence intensity (MFI) measured in the Bio-Plex 100 analyzer.

cross-linked beads. A tag-sequence was included at the C-terminus of each GST-VP1 fusion protein to check for efficient antigen binding and saturation of the beads. This was shown in a dilution series of GST-VP1 containing crude bacterial extracts (**Figure 1B**). For convenience, it was decided to use a dilution of ~1 mg/ml of each GST-VP1 crude extract in the HPyV VP1 multiplex immunoassay.

Antigenicity of GST-VP1 in the HPyV multiplex immunoassay

Serum samples of HBD and immunocompromised KTR were tested to analyse the performance of the HPyV multiplex immunoassay. A broad range of seroreactivities was observed that spanned the entire dynamic range of the assay (0 - 25.000 MFI units). Overall comparable results were obtained for both sample sets (**Figure 2A** and **2B**). Measured seroreactivities against HPyV9, HPyV12, NJPyV and LIPyV were generally lower than against most other HPyVs, with the exception of some outliers.

To ensure antigenicity of the HPyV12 and NJPyV VP1 preparations, polyclonal rabbit antisera were raised against specific HPyV12 and NJPyV-derived immunogenic peptides. These rabbit antisera recognized the relevant HPyV VP1 antigen, (**supplementary Figure 1**) demonstrating the ability of our assay to detect HPyV12 and NJPyV antibody reactivity.

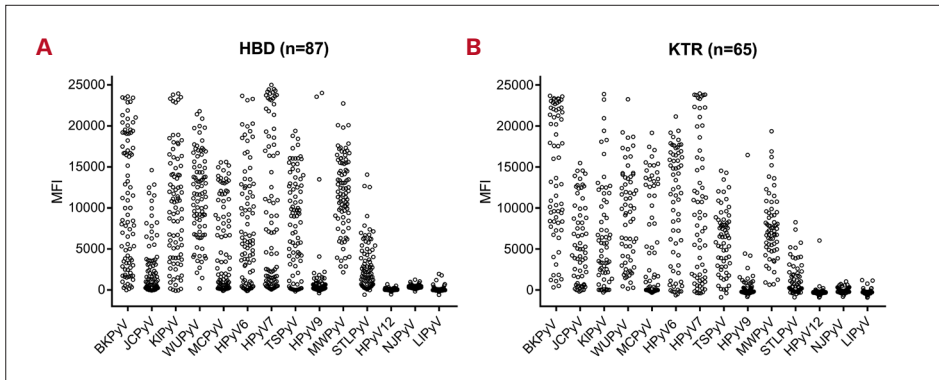


Figure 2. Seroresponses against each GST-HPyV VP1 antigen measured in the multiplex immunoassay.

Seroreactivity was measured in a cohort of healthy blood donors (HBD, n=87, panel A) and a cohort of kidney transplant recipients (KTR, n=65, panel B). Results are depicted as median fluorescence intensity (MFI) measured in the Bio-Plex 100 analyzer, each circle represents one serum sample.

Reproducibility of the HPyV multiplex immunoassay

The reproducibility of the assay was determined by calculating the squared Pearson’s correlation coefficients between repeated measurements while using

beads independently coupled to VP1 fusion proteins. These analyses were highly reproducible with r^2 values ranging from 0.84–0.98 (**supplementary Figure 2A–J**). Furthermore, we compared the use of different fluorescent beads for the same GST-VP1 fusion protein, which was tested for three HPyVs (BKPyV, KIPyV and HPyV10) and revealed reproducible results with r^2 values ranging from 0.77–0.95 (**supplementary Figure 3A–C**). A historical comparison between seroresponses obtained in 2013 for six of the current HPyV targets with the HBD population revealed highly reproducible results (r^2 range 0.71–0.97, **supplementary Figure 4A–F**) (65).

Specificity of the HPyV multiplex immunoassay

Due to VP1 amino acid sequence similarity between different HPyV species varying between 21.6% to 78.5% (**Figure 3**, indicated in the right triangle in red), one might expect epitope-sharing and therefore a certain degree of cross-reactivity among (related) HPyVs. To evaluate this, a correlation-matrix of the HPyV seroresponses was generated (**supplementary Figure 5**) and Spearman rank correlation coefficients were calculated for each HPyV combination, for the HBD population (**Figure 3**, indicated in the left triangle in blue). The KTR population showed comparable data (not shown). The lack of measured seroreactivity against HPyV9, HPyV12, NJPyV and LIPyV did not allow for a meaningful correlation analysis and these viruses were therefore excluded from this analysis. Overall we observed little correlation between seroreactivity determined against the individual HPyVs. A moderate

		VP1 sequence similarity (%)													
		BKPyV	JCPyV	KIPyV	WUPyV	MCPyV	HPyV6	HPyV7	TSPyV	HPyV9	MWPyV	STLPyV	HPyV12	NJPyV	LIPyV
Spearman correlation coefficient (ρ)	BKPyV		78.5	28.2	29.1	43.9	27.7	26.4	54.1	53.8	43.3	41.4	53.8	47.4	44.3
	JCPyV	-0.14		28.4	28.3	46.6	26.3	26.5	52.4	54.4	45.0	40.6	55.1	45.4	42.4
	KIPyV	-0.02	-0.13		66.1	27.2	33.5	36.9	23.9	26.2	24.4	26.3	25.4	22.7	24.2
	WUPyV	0.06	0.16	0.03		29.5	37.4	37.8	24.6	27.1	28.3	26.4	27.6	21.6	26.9
	MCPyV	0.02	0.20	0.08	0.14		27.5	25.1	56.1	57.4	39.1	40.0	56.2	49.3	54.8
	HPyV6	0.00	-0.01	-0.02	0.06	0.03		68.0	27.2	27.7	24.9	29.1	29.9	22.7	26.6
	HPyV7	-0.12	0.23	-0.03	0.12	0.11	0.49		25.7	27.8	26.3	30.1	27.7	24.1	23.9
	TSPyV	0.09	0.24	0.23	0.21	0.16	-0.06	0.01		60.2	43.9	43.2	60.3	55.1	46.3
	HPyV9	nd	nd	nd	nd	nd	nd	nd	nd		46.0	46.7	59.3	57.0	50.1
	MWPyV	0.14	0.09	0.14	0.20	0.17	-0.07	-0.04	0.29	nd		54.8	45.7	39.1	41.7
	STLPyV	-0.12	0.20	0.15	0.25	0.07	0.14	0.17	0.02	nd	0.23		45.2	36.2	40.1
	HPyV12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		54.8	52.5
	NJPyV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		46.1
	LIPyV	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
	VP1 sequence similarity (%):				<50	50-60		60-70		>70					
Spearman correlation coefficient (ρ):				<0.2	0.2-0.4		0.4-0.6		>0.6						

Figure 3. Summary of observed cross-reactivity between individual HPyV VP1 antigens.

The upper right triangle shows the percentage VP1 sequence similarity based on pairwise alignment using Geneious software version 10.0.9 with default ClustalW settings. The lower left triangle shows Spearman correlation coefficients (ρ) calculated on seroresponses measured against VP1 of the HPyV types tested in the HBD cohort. HPyVs with nd: not determined

correlation was observed between HPyV6 and HPyV7 in both the HBD (Spearman's $\rho = 0.49$; $P < 0.001$), as well as the KTR (Spearman's $\rho = 0.44$; $P < 0.001$) population. Despite 78.5% VP1 amino acid sequence similarity between BKPyV and JCPyV, no correlation between these types was measured (Spearman's $\rho = -0.14$, $P = 0.19$). Between species (*HPyV10*) members MWPyV and HPyV10 a high correlation was observed (Spearman's $\rho = 0.92$, $P < 0.001$), which can be explained by their high VP1 amino acid sequence similarity (98%) (**Figure 4**).

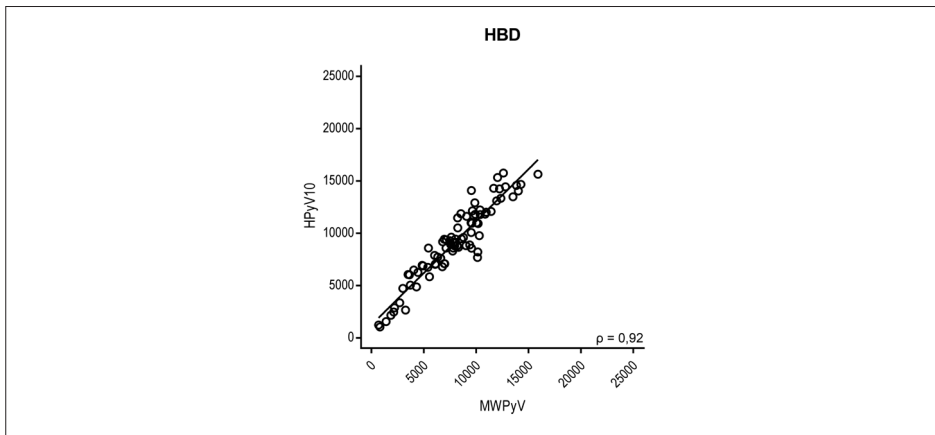


Figure 4. Seroreactivity comparison between MWPyV and HPyV10, both belonging to polyomavirus species 10.

MWPyV and HPyV10 seroreactivities were measured in a cohort of healthy blood donors (HBD). Results are depicted as median fluorescence intensity (MFI) values measured in the Bio-Plex 100 analyzer for MWPyV on the X-axis and for HPyV10 on the Y-axis, respectively. Spearman correlation coefficient is depicted in the figure. Each circle represents one serum sample and the line represents results of linear regression analyses.

To gain more insight into cross-reactivity, antigen competition experiments were performed, in which reactive serum samples were titrated and pre-incubated with soluble GST-VP1 of various HPyVs before being exposed to antigenic beads coated with the relevant HPyV VP1. **Figure 5** shows some examples of these analyses. A complete overview of the selected sera tested in this way can be found in **supplementary Figure 6**. Overall, little competition was observed between VP1 antigens from HPyVs belonging to different species. Pre-incubation with JCPyV-VP1 did not show a reduction in BKPyV seroreactivity in three out of four experiments (**Figure 5A**, **supplementary Figure 6A4** and **6A5**), however, in one competition experiment, a substantial reduction was seen (**supplementary Figure 6A3**). Vice versa, pre-incubation with BKPyV-VP1 reduced JCPyV seroreactivity in two out of four competition experiments (**supplementary Figure 6B1** and **6B3**). Between

closely related species HPyV6 and HPyV7, partial antigen competition was observed indicative of limited cross-reactivity (**Figure 5F** and **5G**, **supplementary Figure 6F1**, **6F2** and **6G2**). As expected, *HPyV10* species members MWPyV and HPyV10 showed high levels of cross-reactivity in this analysis (**Figure 5J** and **5K**). Interestingly, in three out of six HPyV10 competition experiments, pre-incubation with MWPyV-VP1 did not block HPyV10 seroreactivity (**supplementary Figure 6K1**, **6K4**, and **6K5**). A summary of the competition experiments is shown in **Table 2**.

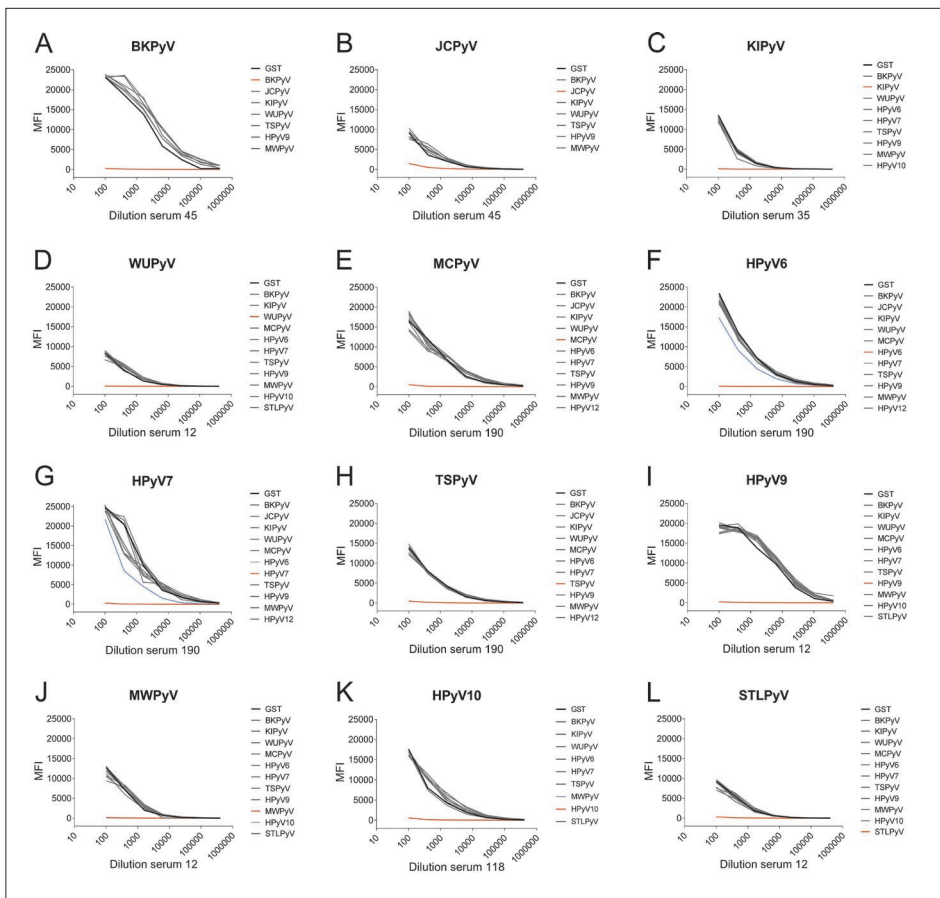


Figure 5. Cross-reactivity analysis of polyomavirus seroresponses by VP1-specific competition.

Titrated serum samples were pre-incubated with crude bacterial extract containing GST-alone (in black), with GST-VP1 of the autologous HPyV (in orange) or with the non-target heterologous HPyVs (in grey). Blue lines indicate competition of VP1 other than the target analyte. Results are depicted as median fluorescence intensity (MFI) measured in the Bio-Plex 100 analyzer and shown for the seroresponses measured for BKPyV (A), JCPyV (B), KIPyV (C), WUPyV (D), MCPyV (E), HPyV6 (F), HPyV7 (G), TSPyV (H), HPyV9 (I); MWPyV (J), HPyV10 (K) and STLPyV (L).

Table 2. Summary of observed cross-reactivity among HPyV VP1 antigens in individual serum samples, as observed in experiments shown in Figure 5 and supplementary Figure 6.

	BKPyV	JCPyV	KIPyV	WUPyV	MCPyV	HPyV6	HPyV7	TSPyV	HPyV9	MWPyV	HPyV10	STLPyV
BKPyV-VP1	++++*	++	-	-	-	-	-	-	-	-	-	-
JCPyV-VP1	+	++++	-	-	-	-	-	-	-	-	-	-
KIPyV-VP1	-	-	++++	-	-	-	-	-	-	-	-	-
WUPyV-VP1	-	-	-	++++	-	-	-	-	-	-	-	-
MCPyV-VP1	-	-	-	-	++++	-	-	-	-	-	-	-
HPyV6-VP1	-	-	-	-	-	++++	++	-	-	-	-	-
HPyV7-VP1	-	-	-	-	-	++	++++	-	-	-	-	-
TSPyV-VP1	-	-	-	-	-	-	-	++++	-	-	-	-
HPyV9-VP1	-	-	-	-	-	-	-	-	++++	-	-	-
MWPyV-VP1	-	-	-	-	-	-	-	-	-	++++	+++	-
HPyV10-VP1	-	-	-	-	-	-	-	-	-	++++	++++	-
STLPyV-VP1	-	-	-	-	-	-	-	-	-	-	-	++++

* Arbitrary interpretation of observed VP1-competition
 - No reduction observed
 + Slight reduction
 ++ Moderate reduction
 +++ High reduction
 ++++ Complete reduction

Comparison between the GST-VP1 based HPyV multiplex immunoassay and a VP1 VLP based ELISA

To learn more about the antigenicity of the GST-VP1 fusion proteins that we use, we compared seroresponses measured for BKPyV in our method to those obtained with a VLP-based ELISA. Although differences in especially the presentation of conformational epitopes were anticipated, the BKPyV seroreactivities measured by both methods were quite similar (**Figure 6**) (100). A high Spearman correlation coefficient ($\rho = 0.823, P < 0.001$) was observed between the OD values obtained with the VP1 VLP ELISA and the MFI values obtained with the GST-VP1 immunoassay.

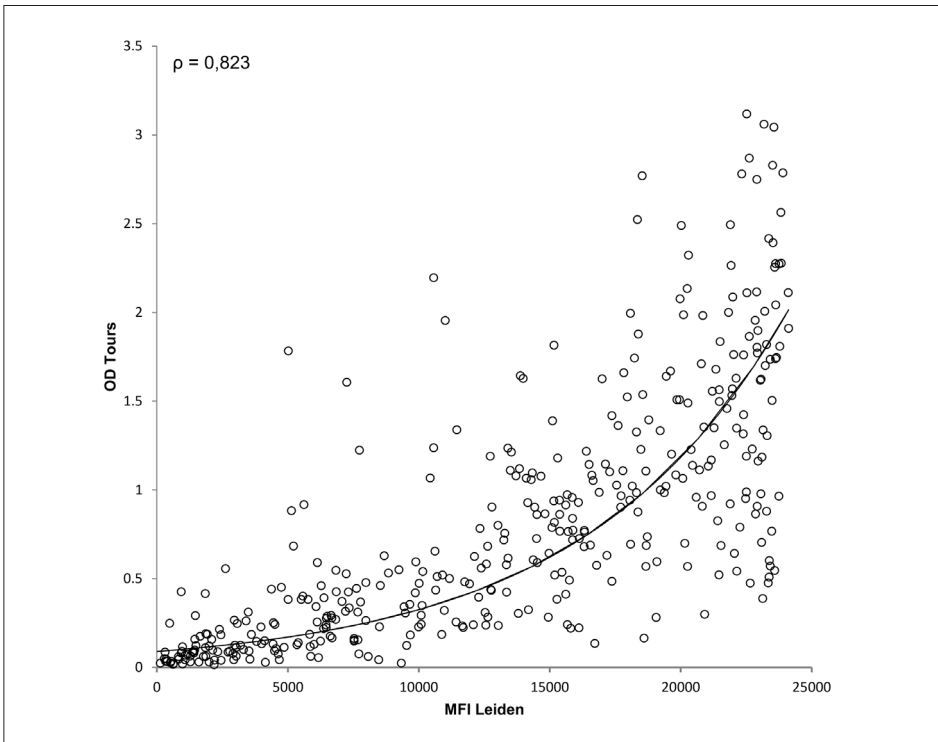


Figure 6. Comparison between the GST-VP1 bead-based assay and the VLP ELISA assay for BKPyV.

Seroreactivity of kidney transplantation donors ($n=396$) were measured by both the bead-based GST-VP1 immunoassay and the VP1 VLP ELISA for BKPyV. Each circle represents one serum sample and the black line indicates correlation between the bead-based measurement (MFI) and the ELISA (OD). This figure is adapted with permission from Wunderink et al (100).

Discussion

Based on the performed evaluation, the broad HPyV multiplex immunoassay described in this report provides highly reproducible and *species*-specific serological data. Between related *HPyV species* sometimes cross-recognizing antibody detection is seen, especially between HPyV6 and HPyV7, which was observed in other studies as well (86). The mean correlation calculated between JCPyV and BKPyV seroreactivity was very low. Nevertheless, some sera clearly demonstrated cross-reactivity between these two, clinically relevant HPyVs. This observation deserves further study, since individual seroresponses against JCPyV, and perhaps against BKPyV as well, (91) are used for patient risk assessment regarding serious complications of

HPyV-induced infection, for example PML (102). However, a limited role for cross-reactivity between HPyV6 and HPyV7 (26) and between JCPyV and BKPyV serology has also been described (46,62,63,103).

Apart from cross-reactivity between two related HPyV species pairs that has been described before for other serological platforms, the GST-VP1 bead-based pan-HPyV assay seems a reliable tool for seroepidemiological HPyV studies. To what extent this assay measures neutralizing antibodies was not investigated in this study, but the high correlation between BKPyV serological data obtained with this assay and those obtained with VP1 VLP-based ELISA suggests that GST-VP1 fusion proteins presented on glutathione-casein coupled beads express conformational epitopes. This was also previously suggested by highly comparable HPyV seroprevalence data obtained worldwide and independently with VP1 VLP and GST-VP1 fusion proteins, for example for TSPyV (61,65,66,104,105).

The high intraspecies cross-reactivity observed between MWPyV and HPyV10 did not come as a surprise, and probably resulted from their high VP1 similarity. Nevertheless, seroreactivity towards HPyV10 was not always abolished after pre-incubation with MWPyV-VP1, indicating a subtle difference between some epitopes of MWPyV and HPyV10, which could be explained by the fact that three of the eight amino acid differences between MWPyV and HPyV10 might be located within the antigenic loops (46,62). The overall high degree of similarity between the seroreactivity profiles of MWPyV and HPyV10, however, suggests no need for separate measurements for both viruses when testing larger cohorts. The lack of seroreactive samples against HPyV9, HPyV12, NJPyV and LIPyV did not allow a thorough analysis of potential cross-reactivity for these HPyVs. As a general remark, cross-reactivity by antibodies against yet unknown HPyVs cannot be excluded.

The aim of this study was to evaluate the abilities of the assay and not to determine seroprevalence. As such, no seronegative cut-off determination was performed. Seroreactivity against most HPyVs was high in both the immunosuppressed KTR and the HBD cohorts. The observed seroreactivity profile of HPyV9 is lower compared to other polyomaviruses, in line with previous publications including ours (61,65,67,106).

We observed limited seroreactivity against HPyV12, NJPyV and LIPyV. For comparison, to date no other serological data are present for NJPyV and LIPyV. For HPyV12, one study reported a seroprevalence of 15-33% in healthy adults (31). Based on our observed seroreactivity against HPyV12, presented in **Figure 2**, we assume that in Dutch populations the seroprevalence of HPyV12 is low. Based on

VP1 amino acid sequence alignment it was recently suggested that the translation initiation of HPyV12 VP1 is located 48 nucleotides (16 amino acids) downstream of the 5' end of the VP1 open reading frame (107). We also analysed the antigenicity of this shorter GST-HPyV12 VP1 fusion protein and noticed no difference in HPyV12 seroreactivity (not shown). [After submission of this manuscript, the discoverers of HPyV12 published data that convincingly show that HPyV12 is in fact a shrew-derived virus (36), suggesting that HPyV12 does not circulate among humans and explaining the lack of HPyV12 seroreactivity found in our cohorts.]

To our knowledge, infection with NJPyV has been described only once, in an immunocompromised kidney-pancreas transplantation patient fleeing through sewage water following superstorm Sandy (32). Supported by the prompt recognition of NJPyV VP1 by the rabbit polyclonal serum raised against NJPyV VP1 peptides, we are confident that our assay is capable of measuring seroresponses against NJPyV. Therefore, we interpret the lack of detectable seroresponses as an indication that this polyomavirus does not represent a human, but rather a zoonotic polyomavirus that was introduced into man under exceptional conditions. Alternatively, the lack of NJPyV seroresponses could suggest a difference in geographical spread for NJPyV between America and Europe, which is rather unusual for (human) polyomaviruses. LIPyV also showed a low seroreactivity profile, suggesting the possibility of environmental contamination of LIPyV in the original skin sample. A larger seroprevalence study can help to elucidate this issue.

The comparison between a VLP-based ELISA and the bead-based assay showed a clear monotonic relationship, despite the different methods in which conformational epitopes are presented by both assays (Figure 6). A close look at the kinetics of each assay reveals a large dynamic range of the bead-based assay with seemingly increased sensitivity compared to the ELISA to detect seroresponses in the lower reactivity range. For the purpose of seroepidemiology, we believe serological testing using HPyV VP1 expressed as GST fusion protein or as VP1 VLP yields equally useful results. For individual use, for instance to predict the risk of developing polyomavirus-related disease such as PML, additional analyses and assay validation are necessary.

In conclusion, the custom made pan-HPyV multiplex immunoassay is a reliable tool for determination of HPyV-specific seroprevalences. It measures HPyV-specific IgG seroreactivities with high reproducibility and specificity, and can easily be extended in case of new HPyV discoveries, and potentially combined with other (viral) antigens of interest.

Supplemental information

Supplemental information can be accessed online: <https://doi.org/10.1371/journal.pone.0206273>

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Chapter 3

Seroprevalence of fourteen human polyomaviruses determined in blood donors

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Abstract

The polyomavirus family currently includes thirteen human polyomavirus (HPyV) species. In immunocompromised and elderly persons HPyVs are known to cause disease, such as progressive multifocal leukoencephalopathy (JCPyV), haemorrhagic cystitis and nephropathy (BKPyV), Merkel cell carcinoma (MCPyV), and trichodysplasia spinulosa (TSPyV). Some recently discovered polyomaviruses are of still unknown prevalence and pathogenic potential. Because HPyVs infections persist and might be transferred by blood components to immunocompromised patients, we studied the seroprevalence of fourteen polyomaviruses in adult Dutch blood donors. For most polyomaviruses the observed seroprevalence was high (60-100%), sometimes slightly increasing or decreasing with age. Seroreactivity increased with age for JCPyV, HPyV6 and HPyV7 and decreased for BKPyV and TSPyV. The most recently identified polyomaviruses HPyV12, NJPyV and LIPyV showed low overall seroprevalence (~5%) and low seroreactivity, questioning their human tropism. Altogether, HPyV infections are common in Dutch blood donors, with an average of nine polyomaviruses per subject.

Introduction

The *Polyomaviridae* family comprises non-enveloped double-stranded DNA viruses that infect a broad spectrum of hosts. After primary infection, usually in childhood, polyomaviruses cause asymptomatic persistent infection accompanied by low-level replication and shedding, for instance in urine (14,63). Since 2007 the number of identified human polyomaviruses (HPyVs) has greatly increased. They are currently grouped in thirteen species, including the 'classic' BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) (14,43). A novel polyomavirus called the Lyon IARC polyomavirus (LIPyV) that was identified in 2017 has not been assigned to a polyomavirus species yet (33). The major capsid protein VP1 forms the exterior of the viral particle and is immunodominant (46). Polyomaviruses do not cause disease in healthy hosts; however, they can reactivate and cause disease in individuals who are immunocompromised.

BKPyV is the main cause of polyomavirus-associated nephropathy (PVAN) that occurs in up to 10% of kidney transplant patients (108). Haemorrhagic cystitis, also caused by BKPyV, complicates between 6-30% of hematopoietic stem cell transplantations (109). JCPyV causes progressive multifocal leukoencephalopathy (PML), a potentially lethal, demyelinating brain disease, which is found in HIV-infected AIDS patients, immunosuppressed transplantation patients, and nowadays especially in multiple sclerosis (MS) patients treated with immunomodulatory drugs, such as natalizumab (55). The incidence of PML in natalizumab-treated MS patients can be as high as 20 per 1000 patients (110,111). Merkel cell polyomavirus (MCPyV) is an important cause of Merkel cell carcinoma (MCC). The incidence of MCC is low, approximately 0.4 per 100,000 person-years, though this appears to increase (112). Less is known about the incidence of diseases caused by other polyomaviruses, for example *trichodysplasia spinulosa* caused by the trichodysplasia spinulosa polyomavirus (TSPyV) (14,113). Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV) have been implicated in respiratory disease (73,114,115), HPyV6 and HPyV7 in dyskeratotic dermatosis, and HPyV7 in thymomagenesis (59,96,97,116). Furthermore, the New Jersey polyomavirus likely caused a unique but severe case of vasculitis resulting in blindness, dermatitis and myositis. Altogether, the polyomaviruses are a significant cause of disease in the immunocompromised population.

Blood components (red blood cells, platelets and fresh frozen plasma) are administered to haematological, transplant, and other immunocompromised patients in huge numbers. Therefore, it is important to understand the epidemiology of polyomaviruses among healthy adults and potential blood donors, including HPyVs that have been recognized just recently and of which still very little is known. In this study



Figure 1. Geographic distribution of blood donors.

The geographic origin of 1050 collected serum samples in the Netherlands is shown in a map by the location of the collection centres involved. Samples were collected over a period of two weeks to ensure the inclusion of blood donation centres from all regions of the Netherlands. The number of samples from each location is visualized by increasing circle size parallel to the number of samples from that location, with a minimum of 1 and a maximum of 51 samples from individual centres. This image is used with permission from Microsoft and was created using Microsoft Excel 2016.

the seroprevalence and seroreactivity were determined of fourteen polyomaviruses identified thus far in humans, in 1050 Dutch blood donors subdivided into age categories. HPyV serology was performed using a custom bead-based immunoassay which was recently validated for this purpose (117).

Materials and methods

Study population

The study population consisted of serum samples from 1050 Dutch blood donors. Donors were included using weighted random selection from Dutch blood donations to obtain groups of equal size in terms of age and sex. Serum samples from eighty blood donation centres were collected over a period of two weeks to ensure an even geographic distribution over the Netherlands (Figure 1). Every blood donation in the Netherlands is routinely screened for presence of human immunodeficiency virus, hepatitis B and C virus and syphilis, only samples with a negative result were included in this study. Sex and age characteristics are summarized in Table 1. The donors were divided in five age categories: 18 - 29, 30 - 39, 40 - 49, 50 - 59 and 60 - 69 years of age. In total, 529 males and 521 females were included.

Table 1. Demographics of study population.

		Sex		Total
		Male	Female	
Age category	18-29	103	105	208
	30-39	106	103	209
	40-49	106	102	208
	50-59	110	105	215
	60-69	104	106	210
Total		529	521	1050

The study involves anonymous 'left over' samples from blood donors who gave permission to use this material for studies into blood-borne agents. Hence Sanquin's scientific board, and the secretary of Sanquin's Ethical Advisory Board, decided that for this study permission from the Ethical Advisory Board is not applicable.

Human polyomavirus multiplex immunoassay

A customized, recently described Luminex xMAP assay was used to measure IgG seroreactivity against the VP1 major capsid protein of BKPyV, JCPyV, KIPyV, WUPyV,

MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, Malawi polyomavirus (MWPyV), Saint Louis polyomavirus (STLPyV), HPyV12, NJPyV and LIPyV (65,85,117). As described, each GST-VP1 fusion protein was expressed in BL21 Rosetta bacteria and coupled to uniquely colored, glutathione-casein cross-linked magnetic fluorescent polystyrene beads, which allows distinction between several analytes in a single well.

In the multiplex immunoassay the 1:100 diluted serum samples were incubated for one hour in blocking buffer to suppress non-specific binding (85,99). Biotinylated goat- α -human IgG (H+L) (1:1000 Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, catalogue number: 109-065-088, antibody registry number: AB_2337628) followed by streptavidin-R-phycoerythrin (SAPE) (1:1000 Invitrogen, Waltham, MA, USA, catalogue number: S866) were used to detect IgG responses against the individual VP1 antigens. A serially diluted mix of four serum samples with known seroreactivity against various polyomaviruses was included in each plate to measure intertest variability (65,117), which was low. Samples were then measured in a Bio-Plex 100 analyzer (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Bio-Plex Manager 6.1 software (Bio-Rad Laboratories, Hercules, CA, USA). The intraclass correlation coefficient for the 1:100 diluted controls was 0.91 (95% confidence interval: 0.81-0.97; $P < 0.001$). Specific seroreactivity was calculated by subtracting the median fluorescence intensity (MFI) values of both a blank sample and of beads coupled to an irrelevant GST fusion protein, in this case GST-SV40 small T-antigen. Serum samples with a high response against GST-SV40 small T-antigen (resulting in specific seroreactivity below or equal to minus 1000 MFI), were excluded for further analysis ($n=6$). This is likely due to aspecific antigen binding, although a genuine seroresponse against GST-SV40 small T-antigen cannot be excluded.

Determination of the cut-off value

For each HPyV a cut-off value for seropositivity was determined based on seroresponses of Dutch children ($n=36$) between 10 and 15 months old, as previously described (65), because children of this age have lost maternal antibodies and have a low chance of being yet HPyV exposed. To determine a seronegative population, a frequency distribution analysis with a bin width of 250 MFI was performed and samples in bins with a frequency percentage above 10% were used in the calculation of the cut-off score. The cut-off value is calculated by the mean seroresponse of the seronegative population and adding three times the standard deviation. This resulted in the following cut-off values, expressed as MFI, for BKPyV 391, JCPyV 349, KIPyV 341, WUPyV 403, MCPyV 509, HPyV6 322, HPyV7 1069, TSPyV 346, HPyV9 446, MWPyV 325, STLPyV 357, HPyV12 326, NJPyV 994, and LIPyV 438.

Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics 23. Intraclass correlation coefficient was calculated based on a single measures form, absolute-agreement and 2-way mixed-effects model. Associations between categorical variables (e.g. sex, age categories) and seropositivity were analysed by χ^2 test (for trend) or Fisher's exact test where appropriate. Mann-Whitney U test was used to analyse differences in seropositivity numbers between the different age categories per donor. Seroreactivity was not normally distributed and was therefore analysed by a non-parametric test, Jonckheere's trend test for ordinal variables (in this case the association between age and seroreactivity in seropositive samples).

Results

Human polyomavirus seroprevalence

In 1050 Dutch blood donors, the seroprevalence of each polyomavirus was determined by calculating the proportion of serum samples with seroreactivity above the established MFI cut-off points. For the majority of polyomaviruses, the overall seropositivity was high, at least 60% (**Figure 2** and **Table 2**). However, for HPyV9 and especially for HPyV12, NJPyV and LIPyV the overall seropositivity was low, 19.2%, 4.0%, 5.2% and 5.9% respectively. When the seroprevalences were analysed in relation to age, a significant positive association was observed for KIPyV (χ^2 test for trend: $P < 0.001$), HPyV6 ($P < 0.001$), HPyV7 ($P < 0.001$) and TSPyV ($P = 0.04$). For MCPyV, a negative association between seroprevalence and age was observed ($P = 0.013$). Due to low numbers of seropositives, age comparisons were not performed for HPyV12, NJPyV and LIPyV. For all HPyVs no significant differences in seropositivity were observed related to sex.

All blood donors were seropositive for at least four polyomaviruses. The mean number of infections per donor (\pm SD), based on seropositivity, was 8.7 ± 1.6 per subject (**Figure 3**). Participants in the lowest age category (18-29) had a mean of 8.2 ± 1.6 infections, which was significantly lower (Mann-Whitney U test: $P \leq 0.001$) than the other age categories, which showed a mean number of infections as follows: 30-39 years: 8.8 ± 1.7 , 40-49 years: 8.7 ± 1.5 , 50-59 years: 8.7 ± 1.5 , and 60-69 years: 8.9 ± 1.6 . No differences regarding the mean number of infections per donor were observed between the sexes.

Table 2. Seropositivity numbers and seroprevalence.

Polyomavirus type	Total (N=1044 ^a) No. (%)	Sex		Age category				
		Male (N=527) No. (%)	Female (N=517) No. (%)	18-29 (N=206) No. (%)	30-39 (N=207) No. (%)	40-49 (N=207) No. (%)	50-59 (N=215) No. (%)	60-69 (N=209) No. (%)
BKPyV	1033 (98.9)	523 (99.2)	510 (98.6)	204 (99.0)	205 (99.0)	204 (98.6)	213 (99.1)	207 (99.0)
JCPyV	660 (63.2)	336 (63.8)	324 (62.7)	120 (58.3)	138 (66.7)	128 (61.8)	133 (61.9)	141 (67.5)
KIPyV**	957 (91.6)	483 (91.7)	474 (91.7)	164 (79.6)	191 (92.3)	196 (94.7)	202 (94.0)	204 (97.6)
WUPyV	1033 (98.9)	521 (98.9)	512 (99.0)	204 (99.0)	204 (98.6)	205 (99.0)	213 (99.1)	207 (99.0)
MCPyV*	855 (81.9)	429 (81.4)	426 (82.4)	178 (86.4)	168 (81.2)	178 (86.0)	170 (79.1)	161 (77.0)
HPyV6**	875 (83.8)	444 (84.3)	431 (83.4)	156 (75.7)	175 (84.5)	173 (83.6)	184 (85.6)	187 (89.5)
HPyV7**	749 (71.7)	391 (74.2)	358 (69.2)	117 (56.8)	154 (74.4)	155 (74.9)	154 (71.6)	169 (80.9)
TSPyV*	831 (79.6)	431 (81.8)	400 (77.4)	156 (75.7)	160 (77.3)	163 (78.7)	183 (85.1)	169 (80.9)
HPyV9	200 (19.2)	91 (17.3)	109 (21.1)	44 (21.4)	43 (20.8)	40 (19.3)	36 (16.7)	37 (17.7)
MWPyV	1039 (99.5)	523 (99.2)	516 (99.8)	205 (99.5)	205 (99.0)	207 (100.0)	215 (100.0)	207 (99.0)
STLPyV	677 (64.8)	342 (64.9)	335(64.8)	118 (57.3)	145 (70.0)	139 (67.1)	143 (66.5)	132 (63.2)
HPyV12	42 (4.0)	15 (2.8)	27 (5.2)	11 (5.3)	10 (4.8)	5 (2.4)	4 (1.9)	12 (5.7)
NUPyV	54 (5.2)	22 (4.2)	32 (6.2)	8 (3.9)	4 (1.9)	4 (1.9)	16 (7.4)	22 (10.5)
LIPyV	62 (5.9)	33 (6.3)	29 (5.6)	10 (4.9)	14 (6.8)	10 (4.8)	14 (6.5)	14 (6.7)

a. 6 samples were excluded due to GST-SV40 small T reactivity.

* P < 0.05 ** P < 0.001 χ^2 test for trend for differences between age categories.

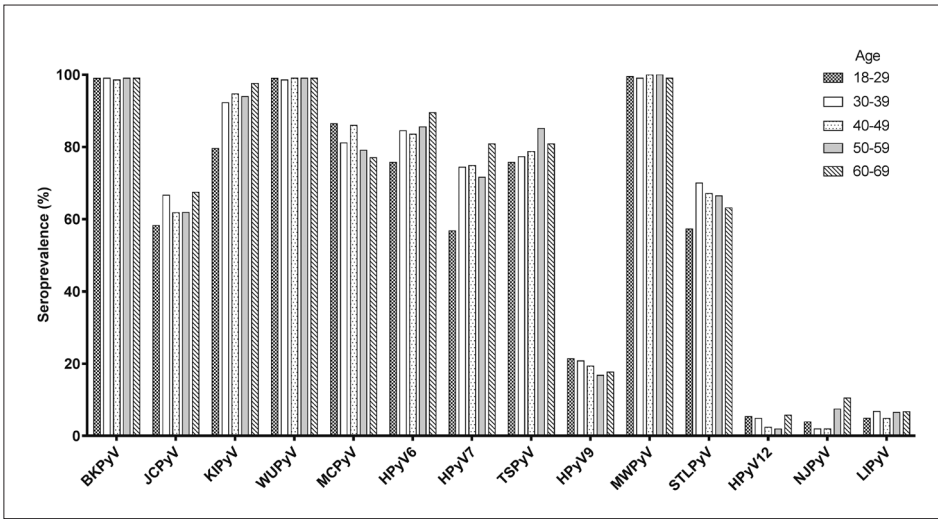


Figure 2. Seroprevalence of indicated polyomaviruses in Dutch blood donors.

The percentage seropositivity of each polyomavirus is shown for the donor age categories 18-29 (checkers pattern, N=206), 30-39 (solid white bars, N=207), 40-49 (dots pattern, N=207), 50-59 (light grey bars, N=215) and 60-69 (diagonally striped pattern, N=209).

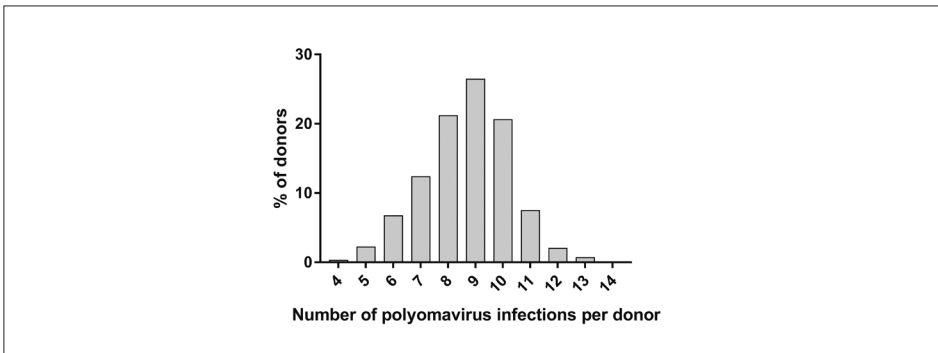


Figure 3. Distribution of the cumulative number of polyomavirus infections per donor.

The distribution of the number of infecting polyomaviruses is shown among the tested blood donors, as indicated by seropositivity.

Human polyomavirus seroreactivity

Seroreactivity detected in seropositive donors differed between the analysed HPyVs. The highest median MFI-values were measured for BKPyV (**Figure 4**). Intermediate values were measured for KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV and MWPyV. Low to intermediate median MFI values were measured for JCPyV, HPyV9

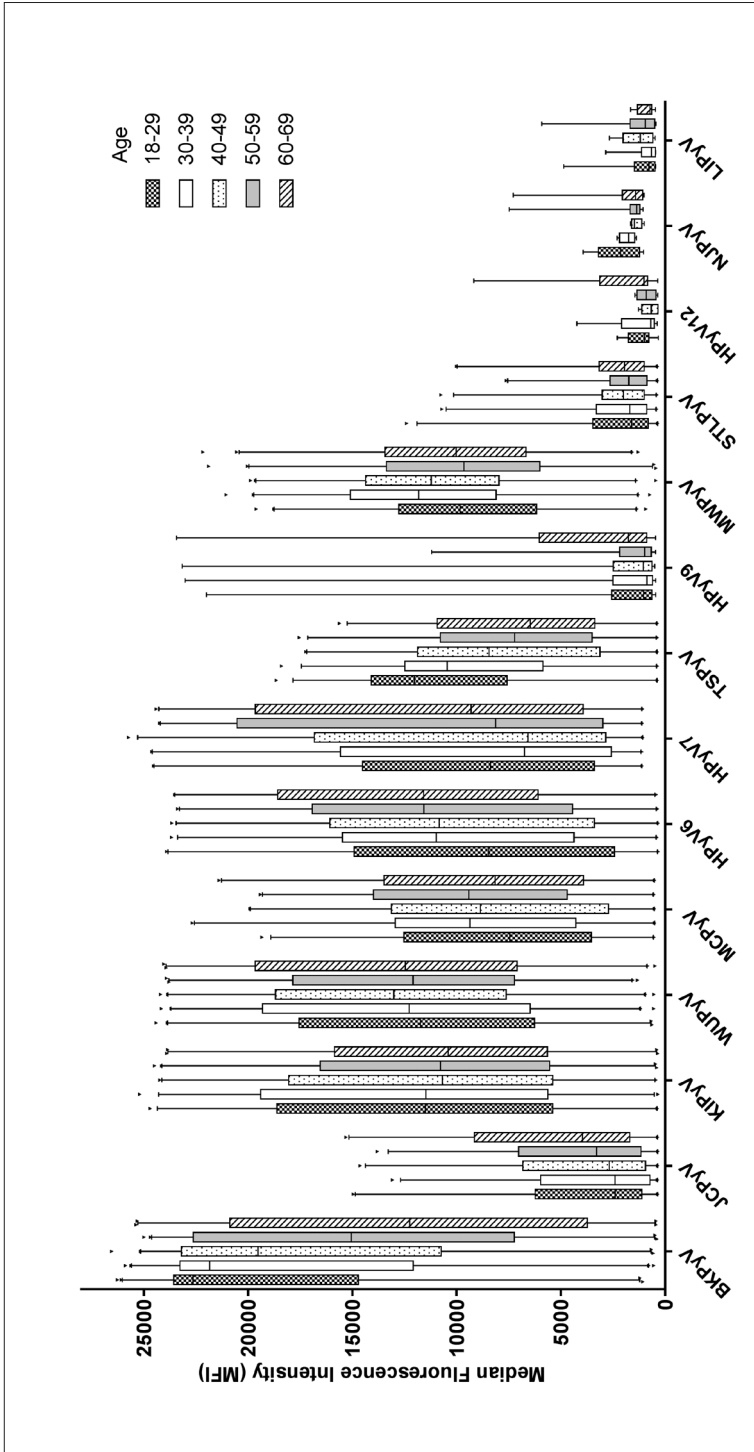


Figure 4. Seroreactivity in seropositive donors categorized by age.

Box plots with whiskers represent 1-99th percentiles. Outliers are indicated by triangles. Age categories shown are 18-29 (checkers pattern), 30-39 (solid white bars), 40-49 (dots pattern), 50-59 (light grey bars) and 60-69 (diagonally striped pattern). Only MFI values from seropositive donors are shown. The total number of seropositives was for BKPyV: 1033; JCPyV: 1033; WUPyV: 957; MCPyV: 1033; HPyV6: 875; HPyV7: 749; TSPyV: 831; HPyV9: 200; MMPyV: 1039; STLPyV: 677; HPyV12: 42; NJPyV: 54; LIPyV: 62.

and STLPyV, although some highly reactive serum samples were noted for HPyV9. The seroresponses against HPyV12, NJPyV and LIPyV were generally very low.

Seroreactivity was further analysed in relation to age by investigating potential trends within the five age-categories shown in **Figure 4**. A significant age-dependent increase of seroreactivity was observed for JCPyV, HPyV6 and HPyV7 (Jonckheere's trend test: $P < 0.001$, $P < 0.001$ and $P = 0.047$, respectively). For HPyV9 a substantial increase was seen for the 60-69 age category. In contrast, for BKPyV and TSPyV a significant decrease of seroreactivity was observed in the higher age categories (Jonckheere's trend test: $P < 0.001$ for both viruses). No significant trends were observed for the other HPyVs. The analyses were not performed for HPyV12, NJPyV and LIPyV, due to low numbers of seropositives.

In general, no differences in seroreactivity between the sexes were observed, but for HPyV7 the overall median seroreactivity among seropositives was higher in men (9250 MFI in men vs. 6464 MFI in women, Mann-Whitney U test: $P = 0.018$).

Discussion

In this study we determined the seroprevalence and seroreactivity of all currently known human polyomaviruses in a large Dutch blood donor cohort. Our findings indicate that seropositivity for many polyomaviruses is common in this healthy population. At the same time, for some recently identified HPyVs the seroprevalence was low.

With regard to most HPyVs that have been serologically analysed before, the seroprevalences reported here are in line with previous seroepidemiological studies in immunocompetent populations from different continents (61,62,65,67,86,113,117-119), therefore we assume our findings to be representative for most other immunocompetent populations. For KIPyV, HPyV6, HPyV7 and TSPyV an increase in seroprevalence with higher age was noticed, which has also been reported previously (61,62,65,67,86). This could reflect continuous viral exposure throughout life or frequent reactivation of persistent infection, which can boost HPyV seroresponses as well (91). Furthermore, we observed a decrease in seroprevalence with age for MCPyV, which was not published before to our knowledge, (61,62,65), and could represent a cohort effect. HPyV infections with a stable seroprevalence in adult life are probably acquired during childhood, as previously indicated by a rapid increase in

seropositivity during the first years of life (62,65,120). Due to the age restrictions on becoming a blood donor in the Netherlands, we did not investigate the HPyV seroreactivity patterns in individuals under 18 years of age or older than 69.

The seroreactivity of seropositive individuals differed with age between the HPyVs, with decreasing intensity for BKPyV and TSPyV, increasing intensity for JCPyV, HPyV6 and HPyV7, and stable intensity for KIPyV, WUPyV, MWPyV and STLPyV. Comparable trends were obtained in healthy Australian, Czech and Italian populations (65–68,86), though MCPyV seroreactivity did not increase with age in our cohort. The decrease in seroreactivity for BKPyV and TSPyV suggests gradually less immunological boosting, possibly related to a decrease in environmental exposure or diminished reactivation of these HPyVs, while the increase in seroreactivity seen for JCPyV, HPyV6 and HPyV7 might reflect continuous exposure or reactivation (91).

The serological profile of HPyV9 is unique compared to other polyomaviruses, with a small subset of seropositive individuals that display very high seroreactivity in a background of weak seroresponders. It was previously shown that HPyV9 has unique receptor binding properties, and preferentially binds to a ligand which cannot be synthesized by humans, but can be acquired through diet (red meat and milk) (121). The necessity for a dietary ligand might explain why this virus is less prevalent among humans than most other HPyVs. Whether highly HPyV9-seroreactive subjects indeed ingest more dairy and meat-containing products could be the subject of further study.

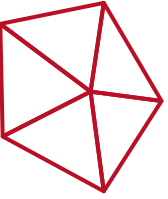
For HPyV12, NJPyV and LIPyV we detected a very low seroprevalence, approximately 5%, with low seroreactivity for all three. In a pilot study, we obtained similar results and confirmed the antigenicity of the used HPyV12- and NJPyV-VP1 antigens by specific polyclonal antibody recognition (117). Therefore, we believe the observed low seroprevalence of these polyomaviruses to be genuine, and we consider the possibility that these polyomaviruses do not frequently circulate in humans, and perhaps do not represent human polyomaviruses at all. For HPyV12, this would fit with recent observations suggesting that HPyV12 represents a shrew rather than a human polyomavirus (36). For NJPyV it could very well be that the only published patient was infected from an animal reservoir under exceptional circumstances, when fleeing from flooding during hurricane Sandy (32). LIPyV was identified in a skin swab sample and subsequently detected in a small subset of oral fluids (2%), skin swabs (2%) and eyebrow hair follicles (0.2%) (33). In this case, the measured low seroprevalence might reflect the LIPyV detection rate, though more studies are needed to further clarify the epidemiology of LIPyV.

In contrast to our data, a very recent study reported 90% seroprevalence for HPyV12, in an Italian adult population (122). This percentage is considerably higher than our finding and the 20% seroprevalence obtained previously for HPyV12 by Ehlers and co-workers using recombinant VP1 and VP1-based VLP ELISA (31,36). Since HPyV VP1-based and VLP based assays generally obtain comparable results, as we have recently demonstrated for BKPyV (117), we have no explanation thus far for this large discrepancy except differences in cut-off value determination and striking geographic differences in virus exposure. Suboptimal HPyV12 antigen recognition, resulting from the use of a premature translation initiation site in HPyV12 VP1, causing a 16 amino acids longer version of VP1 (107), was experimentally ruled out (S1 Fig). Also for NJPyV a much higher seroprevalence was found in the Italian population (50%) than in our population (5%) (122). Overall, more (sero)epidemiological studies are needed to solve these discrepancies and to define the natural host(s) of these viruses, for example by studying seroprevalence in different geographic regions while using comparable serological methods.

In conclusion, by analysing a large group of Dutch blood donors we showed that most HPyV infections are common, although we found little indication of HPyV12, NJPyV and LIPyV circulation in humans. Considering that blood donors are persistently infected with, on average, nine different polyomaviruses and assuming that episodes of viremia sometimes occur, the consequences for the safety of blood transfusion, especially for immunocompromised recipients, remains to be established.

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Chapter 4

Prevalence of DNA of fourteen human polyomaviruses determined in blood donors

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Abstract

Background Human polyomaviruses (HPyVs), like herpesviruses, cause persistent infection in a large part of the population. In immunocompromised and elderly patients, polyomaviruses cause severe diseases such as nephropathy (BKPyV), progressive multifocal leukoencephalopathy (JCPyV) and skin cancer (MCPyV). Like cytomegalovirus, donor-derived polyomavirus can cause disease in kidney transplant recipients. Possibly blood components transmit polyomaviruses as well. To study this possibility, as a first step we determined the presence of polyomavirus DNA in Dutch blood donations.

Study design and methods Blood donor serum samples (N=1016) were analysed for the presence of DNA of 14 HPyVs using HPyV species-specific quantitative PCRs. PCR-positive samples were subjected to confirmation by sequencing. Individual PCR findings were compared with the previously reported polyomavirus-serostatus.

Results MCPyV DNA was detected in 39 donors (3,8%); JCPyV and TSPyV DNA in five (both 0.5%), and HPyV9 DNA in four donors (0.4%). BKPyV, WUPyV, HPyV6, MWPyV and LIPyV DNA was detected in one or two donors. Amplicon sequencing confirmed the expected product for BKPyV, JCPyV, WUPyV, MCPyV, HPyV6, TSPyV, MWPyV, HPyV9 and LIPyV. For JCPyV a significant association was observed between detection of viral DNA and the level of specific IgG antibodies.

Conclusion In 5.4% of Dutch blood donors polyomavirus DNA was detected, including DNA from pathogenic polyomaviruses such as JC polyomavirus. As a next step, the infectivity of polyomavirus in donor blood and transmission via blood components to immunocompromised recipients should be investigated.

Introduction

Human polyomaviruses (HPyVs) cause asymptomatic persistent infection in healthy humans, (43) whereas they can cause severe disease in immunocompromised patients and elderly persons. Latter groups increasingly receive blood components, though the presence of HPyVs in blood donors has not been studied extensively. Transfusion-transmitted HPyV infection has not been reported, which can be explained by lack of such transmissions, or by an erroneous assumption that HPyV-related disease in immunocompromised patients always is caused by reactivation of their own, hitherto silent infection. In kidney transplant patients a substantial proportion of BKPyV infections and pathology is donor-derived. (123,124)

Polyomaviruses are ubiquitous viruses that frequently infect human beings. During childhood the seroprevalence of most HPyVs rapidly increases, sometimes reaching 100%. (35,61,62,65) Polyomaviruses can be detected in healthy persons, for example in skin, (26) urine, (82) tonsillar tissue (84) and respiratory samples. (83) Despite the persistence of these viruses, little is known about the occurrence of viremia in the healthy population, especially regarding the recently discovered HPyVs. In immunocompromised patients, HPyVs can be found also in blood and cerebrospinal fluid. (91,125) Polyomavirus-associated diseases are increasingly relevant in the immunocompromised population. Two well-known examples of polyomavirus-associated disease are BK polyomavirus (BKPyV)-associated nephropathy (70) and JC polyomavirus (JCPyV)-associated progressive multifocal leukoencephalopathy (PML). (126) Nowadays these severe conditions are primarily seen, respectively, in immunosuppressed kidney transplant recipients and patients on immunomodulatory drugs, such as multiple sclerosis patients taking natalizumab. (126) In the past decade, with the identification of at least ten novel HPyVs, (23-33,78,79) the number of polyomavirus-associated diseases has increased and now includes Merkel cell carcinoma (MCC) and trichodysplasia spinulosa (TS). MCC, caused by Merkel cell polyomavirus (MCPyV), is an aggressive, potentially lethal tumour that occurs in the elderly and in immunocompromised patients. (25) TS, caused by trichodysplasia spinulosa polyomavirus (TSPyV), is a dysplastic and disfiguring skin disease that is especially found in solid organ transplant patients and lymphocytic leukaemia patients. (27) HPyV6 and -7 cause pruritic and dyskeratotic dermatoses in immunocompromised patients. (59) KIPyV and WUPyV were first detected in human nasopharyngeal aspirates from patients with respiratory infection. (23,24) MWPyV and STLPyV were found in stool samples of healthy children. (28,30) HPyV9 was discovered in the serum of a kidney transplant

patient.⁽⁷⁹⁾ HPyV12, NJPyV and LIPyV were all identified in human samples,⁽³¹⁻³³⁾ however, seroprevalence of these viruses is low. Vaccination or proven effective antiviral therapy are not available for human polyomaviruses.

HPyVs are non-enveloped viruses, 40-50 nanometre in diameter, with circular double-stranded DNA genomes. It can be expected that common pathogen reduction techniques used in blood banking have limited efficacy against HPyVs, because these viruses are non-enveloped. HPyVs have been isolated from lymphocytes and hence leukoreduction of blood donations might decrease the presence of HPyVs in donated blood, but the extent of this reduction is unknown.⁽¹²⁷⁻¹²⁹⁾ It is uncertain whether higher levels of specific HPyV-antibodies decrease potential infectivity by neutralization. On one hand, kidney transplant recipients with a high antibody titre against BKPyV have a lower risk of developing BKPyV viremia compared to recipients with low antibody titres, but on the other hand kidney transplant patients have an increased risk of developing BKPyV viremia after receiving a kidney from a donor with high BKPyV antibody levels.^(88,124) No group is fully protected and as such it seems likely that a seropositive transfusion recipient isn't necessarily protected against polyomavirus infection.

Since latent, persistent polyomavirus infections bear a risk for the immunocompromised, one can wonder about the contribution of blood components as a vehicle for HPyV transmission. To start answering this question, we recently determined the seroprevalence of all known, thus far fourteen, HPyVs in a large group of blood donors, and estimated that each blood donor is persistently infected with on average nine HPyVs.⁽³⁵⁾ To further explore the risk from these potentially blood-transmitted viruses, in the current study we analysed the same blood donor cohort by HPyV-specific PCRs for the presence of circulating genomic DNA of all currently known HPyVs.⁽³⁵⁾

Materials and methods

DNA extraction

Nucleic acid extraction was performed on a MagNA Pure LC instrument (Roche Diagnostics, Rotkreuz, Switzerland) using the MagNA Pure LC DNA Isolation Kit Large Volume, according to the manufacturer's instructions, with an input volume of 1000 µL and an output volume of 65 µL. Extraction efficiency and PCR inhibition was controlled by adding a fixed concentration of phocine herpesvirus (PhHV) DNA to the lysis buffer that was added to each sample. ⁽¹³⁰⁾

Polyomavirus DNA detection

Each sample was analysed for the presence of HPyV genomic DNA with the help of three real-time multiplex qPCRs (Multiplex 1, 2 and 3), developed to detect fourteen PyVs (**Table 1**). The PCRs for BKPyV, HPyV6, HPyV7, TSPyV and HPyV9 were previously designed and described. (77,101,131) The PCRs for JCPyV, WUPyV and MCPyV were developed by other research groups. (132-134) Novel primers and probes were designed for KIPyV, MWPyV, STLPyV, HPyV12, NJPyV and LIPyV using Geneious software 10.2.4 (Biomatters, Auckland, New Zealand) (**Table 1** and **Supplementary Table 1**). Multiplex 1 was developed to detect MCPyV, HPyV6, -7, TSPyV and HPyV9; Multiplex 2 to detect BKPyV, WUPyV, MWPyV, NJPyV (and the internal control PhHV), and Multiplex 3 to detect JCPyV, KIPyV, STLPyV, HPyV12 and LIPyV.

The PCR mix (total volume 25 µl) consisted of Qiagen HotStarTaq Master Mix kit (Qiagen, Venlo, the Netherlands), MgCl₂, primers, probes (see **Supplementary Table 1** for concentrations) and 10 µL of input DNA isolate. Cycling conditions for the PCRs were as follows: 95°C for 15 minutes, followed by 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. qPCRs were performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of the qPCR data was performed using Bio-Rad CFX Manager version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Baseline threshold values were determined separately for each target and fluorescence drift correction was applied.

PCR efficiency and analytical sensitivity of each PyV PCR were determined on replicates of serial dilution series of 10.000 to 1 copy per reaction of a plasmid containing a single cloned copy of the PyV target gene (VP1 or Large T-antigen), and was defined as the ability of the assay to detect the target concentration with a probability higher than 95% in a number of replicates (Supplementary Tables 2 and 3).

PCR product sequencing

PCR products amplified with a Ct value below 40 were analysed by Sanger sequencing for confirmation, with a maximum of 10 positive samples (amplicons) per HPyV. The generated PCR products were run on a 2% agarose gel. Bands of the expected size (74-150 bp) were isolated using the Bioline Isolate II PCR and Gel kit (Bioline Reagents, London, United Kingdom), ligated and cloned in *E. coli* using TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. For each successful ligation, three colonies per plate were picked and plasmid DNA was isolated with NucleoSpin Plasmid EasyPure isolation kit (Macherey-Nagel, Düren, Germany). Sanger sequencing was performed on an ABI3730xl system (Thermo Fisher Scientific, Waltham, MA, USA) using M13 forward primer.

Table 1. PyV PCR primers and probes

Multi-plex	Target species Refseq no.	Gene	Product length (bp)	Sense primer sequence (5'-3')	Probe sequence (5'-3')	Antisense primer sequence (5'-3')
1	MCPyV NC_010277	LT	149	CCACAGCCAGAGCTCTCCT	CY5-TCCCAGGCTTACAGACTCCCA*	TGGTGGTCTCCTCTCTGCTACTG
1	HPyV6 NC_014406	VP1	150	GTAGGGTATGCTGGTAAC	YAK-CTCTCCTCTGTCTGAAAGTGAACCTCTAA	CAGGAAATTGCTAAACATCATATC
1	HPyV7 NC_014407	VP1	116	GTGCTGATATGGTTGGAA	TXR-AGCCTGTACTGTTCTCTGGTTACT	TCTGCAGTGGACTCTAAA
1	TSPyV NC_014361	VP1	104	GAGCTAAGGACAACTATGG	Q705-CTTTGCTCTGTGCTACTGCTGTT	CTAGCTGTACTGTAGGTTG
1	HPyV9 NC_015150	VP1	109	CCTGTAAGTCTCTCCTCTTA	FAM-CITGTTTCTCTGGTCTTATGCCTCA	CCTGATAAATTTCTGACTTCTTC
2	BKPyV NC_001538	VP1	90	GAAAAGGAGAGTCCAGGG	FAM-CCAAAAAGCCAAAAGGAAACC	GAACTTCTACTCCTCCTTTTATTAGT
2	WUPyV NC_009539	VP1	74	AACCAGGAAGGTACCCAAAGAAG	TXR-CAACCCACAAAGTGCAAAAGCCTTCC	CTACCCCTCCTTTTCTGACTTGTTT
2	MWPyV NC_018102	VP1	86	GACACCACAATGACAGTTGAG	CY5-CCAAGGATGGCAATGATGTA AAAACA	GGATCACTGTAGCCATACCAT
2	NJPyV NC_024118	VP1	135	CCCACCAAGTAAAGTAAC	YAK- AAGTGTCTTATACCTACTCCAGTGC	CAGAGTCCAATTTCCAGTAGTA
3	JCPyV NC_001699	LT	129	GTCTCCCATACCAACATTAGCTT	YAK-TCTTTCCACTGCACAATCCTCTCATGAATG	GGTTAGGCCAGTTGCTGACTT
3	KIPyV NC_009238	VP1	148	AAGTCCCGGGTACAAACTC	TXR-GGTAGAAGTACTAGCCCGAGTACCACGT	CCATCCTGAGCAGCTGTTGTA
3	STLPyV NC_020106	VP1	101	TTGAAAATGGCTCCAAAAGAAAATCT	CY5- AGATGCACCTCACAGACATGTCCAATGGA	TGGCAGGGATCATATTCACATCT
3	HPyV12 NC_020890	VP1	139	AAGGGCTGAAGAAATCC	FAM-CCAGTATCTGCTCTCCTAACCCAGT	CTCCAAAACCCCTCATATAACC
3	LIPyV NC_034263	VP1	83	TGACAGGTGACAATTCGCCAGG	Q705-AGAGGAAGTACCGGCTCTATGATGGCCAGAG	CCTTGGCAGATCTAACCCCTCC

Abbreviations: LT: Large T; VP1: Viral Protein 1; S: Sense; AS: Antisense

*Probe modified from original article Goh S et al. Emerg Infect Dis. 2009 Mar;15(3):489-91.

Study population

The study population consisted of 1050 serum samples from healthy, Dutch blood donors. The samples were previously used for routine blood donor screening for human immunodeficiency virus, hepatitis B and C virus and syphilis. (35) Out of 1050 samples, 34 were excluded due to insufficient volume for DNA extraction or due to inhibition in qPCR (**Figure 1**). The presence of polyomavirus antibodies in this sample set was determined previously. On average, a donor from this population is seropositive for nine different polyomavirus species and seropositivity ranged from 5% to 100% depending on polyomavirus species. (35) Basic population demographics (age and sex) of the fully screened donor population is shown in **Table 2**. Samples from all regions of the Netherlands were included, as reported previously. (35)

Each blood donor gave permission to use residual blood samples for studies of blood-borne agents. Hence, Sanquin's scientific board and the secretary of Sanquin's Ethical Advisory board decided that for this study additional permission from the Ethical Advisory Board is not needed. The blood donors fulfilled all criteria for blood donation eligibility.

PyV serology

The polyomavirus serostatus of all blood donors was determined and described in a previous study (35), using a multiplex immunoassay as previously described, (87) employing a GST-VP1 fusion protein for each PyV as antibody-binding antigen.

Statistics

Statistics were performed using IBM SPSS Statistics version 23 (Armonk, NY, USA). Chi-squared tests were used to compare PCR results and seropositivity, age category or sex. Mann-Whitney U tests were used to compare seroreactivity results between samples positive or negative in qPCR analysis.

Table 2. Overview of HPyV PCR and sequence results of serum samples from 1016 blood donors

PCR target	No. of PCR-positive donors		PCR product sequencing		No. of positives per sex and age category				Viral load copies/ml	
	Any Ct N (%)	Ct <40 N (%)	Successfully sequenced / total sequenced	HPyV-confirmed / successfully sequenced (%)	Male (n=501)	Female (n=509)	Age category			
					18-29 (n=197)	30-39 (n=201)	40-49 (n=202)	50-59 (n=206)	60-69 (n=204)	
BKPyV	5 (0.5)	1 (0.1)	1/1	1/1 (100)	0	1	1	0	0	55
JCPyV	14 (1.4)	5 (0.5)	5/5	5/5 (100)	2	3	1	2	1	9-37
KIPyV	2 (0.2)	1 (0.1)	1/1	0/1	1	0	0	0	0	46
WUPyV	2 (0.2)	2 (0.2)	2/2	1/2 (50)	1	1	1	0	1	8-30
MCPyV	63 (6.2)	39 (3.8)	9/10	7/9 (78)	23	16	12	7	10	24-452
HPyV6	4 (0.4)	1 (0.1)	1/1	1/1 (100)	0	1	0	1	0	13
HPyV7	7 (0.7)	0	-	-	0	0	0	0	0	-
TSPyV	7 (0.7)	5 (0.5)	5/5	5/5 (100)	4	1	2	1	0	9-81
HPyV9	6 (0.6)	4 (0.4)	3/4	1/3 (33)	3	1	1	1	0	7-68
MWPyV	3 (0.3)	1 (0.1)	1/1	1/1 (100)	1	0	0	0	0	15
STLPyV	1 (0.1)	1 (0.1)	1/1	0/1	1	0	1	0	0	39
HPyV12	11 (1.1)	10 (1.0)	10/10	0/10	6	4	3	4	1	2-13
NJPyV	3 (0.3)	1 (0.1)	1/1	0/1	1	0	1	0	0	123
LIPyV	6 (0.6)	2 (0.2)	2/2	2/2 (100)	2	0	1	1	0	13-31
Any PyV*	111 (10.9)	64 (6.3)	-	55 (5.4) †	45	28	21	17	13	55 ‡

* Codetection of multiple polyomaviruses in a single donor: counts as one for the total no. of positive donors

† Total number of true positive donors based on positive PCR results with Ct value below 40 and sequence confirmation of at least one PCR product

‡ Median viral load

Abbreviations: HPyV: human polyomavirus; Ct: cycle threshold

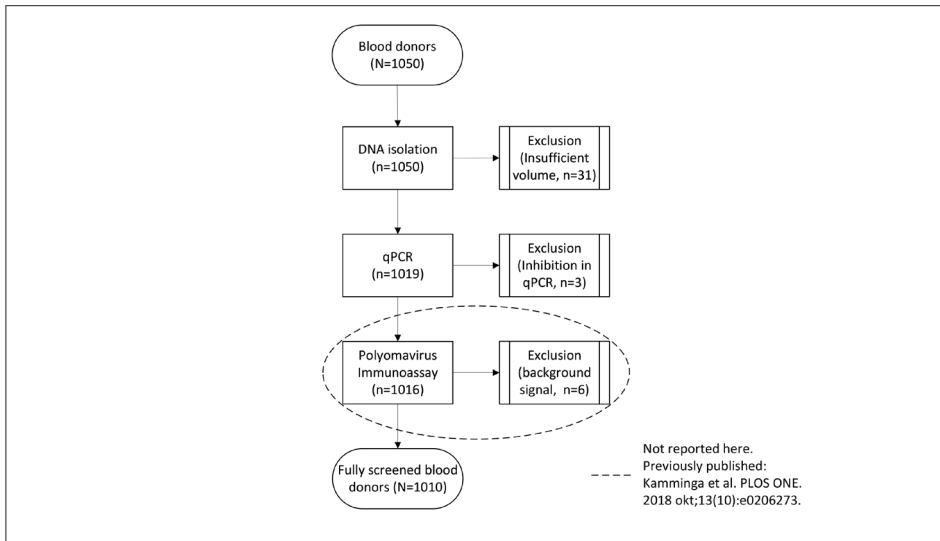


Figure 1. Flow chart for study population.

Numbers between brackets indicate serum samples that were successfully isolated, PCR-amplified and assessed with the immunoassay. Boxes on the right side of the figure state reasons for exclusion of samples.

Results

Polyomavirus PCR validation

The analytical sensitivity was 10-15 copies/reaction for all PCRs, except for the MCPyV PCR, which reliably detects 100 copies/reaction, although the dilution with 10 copies/reaction was detected in 90% of cases (**Supplementary Table 2**). High concentrations of non-target polyomavirus DNA with a cycle threshold (Ct)-value between 25-30) did not inhibit the PCR (**Supplementary Table 4A-N**). In addition, a panel of common double-stranded DNA viruses containing herpes simplex virus 1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus and adenovirus was tested negative in each HPyV PCR (data not shown). In short, all HPyV PCRs detect their target in a sensitive and specific manner.

Presence of polyomavirus DNA in blood donors

Serum samples from 1016 blood donors were analysed for the presence of HPyV DNA using three multiplex PCRs. In **Table 2** the PCR results are summarized. MCPyV DNA was the most prevalent, detected in 39/1016 (3.8%) donors, with a viral load ranging between 24-452 genome equivalent copies/ml. Sequencing confirmed the presence

of the MCPyV DNA in the PCR product in most samples (78%) (**Table 2**). JCPyV, TSPyV and HPyV9 were detected, respectively, in 5 (0.5%, range 9-37 copies/ml), 5 (0.5%, range 9-81 copies/ml) and 4 (0.4%, range 7-68 copies/ml) donors. Sequencing confirmed the presence of virus-specific DNA in 100% of cases for JCPyV and TSPyV, and in 33% for HPyV9. When sequencing was successful but HPyV-specific sequences were not present, especially human genomic DNA and primer-dimers were detected (**Table 2**). For example, the HPyV12 positive findings in 10 donors with a low range of 2-13 copies/ml, could not be confirmed at all by sequencing. The other HPyVs were detected in only one or two donors, while HPyV7 was not detected at all. Summarizing, we found 64 donors to be HPyV PCR-positive (6.3%), of which the detection of specific viral DNA was confirmed (in part) for BKPyV, JCPyV, WUPyV, MCPyV, HPyV6, TSPyV, HPyV9, MWPyV and LIPyV in 55 blood donors (5.4%). HPyV codetection was observed in four donors (0.4%) and involved TSPyV, HPyV9 and LIPyV (all sequence-verified); WUPyV, TSPyV and HPyV9 (WUPyV and TSPyV sequence-verified); TSPyV, KIPyV and NJPyV (TSPyV sequence-verified); TSPyV and HPyV9 (TSPyV sequence-verified), respectively. The distribution of polyomavirus detection over sex and age category is summarized in **Table 2**. For none of the HPyVs a correlation was found between the detection in serum and sex or age category of the donor.

Previously we analysed every sample included in this study serologically for HPyV infection, (35) which enabled us to compare the HPyV PCR findings with HPyV serostatus (seropositivity) and seroreactivity (the median seroresponse given as median fluorescence intensity value) (**Table 3**). In case of BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6, TSPyV and MWPyV, 77-100% of the positive PCR findings were obtained in donors seropositive for the detected HPyV. The HPyV9, STLPyV, HPyV12, NJPyV and LIPyV DNA-positive samples, however, were all from donors seronegative for the HPyV that was detected. No significant correlation was found between presence of polyomavirus DNA and seropositivity. Seroreactivity was comparable between DNA-positive and negative samples for all HPyVs, except JCPyV where significantly higher seroresponses were measured in the JCPyV DNA-positive samples (Mann-Whitney U test, $P=0.005$) (**Table 3**).

Discussion

In this study we determined the presence of polyomavirus DNA in serum samples taken from healthy, Dutch blood donors. Our results show that the prevalence of polyomavirus DNA varies from 0% to 3.8% depending on HPyV species. Importantly,

we detected DNA from known pathogenic polyomaviruses, BKPyV (0.1%), JCPyV (0.5%), MCPyV (3.8%) and TSPyV (0.5%), which suggests that these viruses may be present in blood components.

Table 3. Overview of polyomavirus serostatus and DNAemia in fully screened population (N=1010)

PyV	Seroprevalence*	Seropositives among PCR positives (%)	Median seroreactivity in MFI among PCR positives	Median seroreactivity in MFI among PCR negatives	p-value†
BKPyV	99	1/1 (100)	22519	18936	0.635
JCPyV	62	5/5 (100)	8382	828	0.005
KIPyV	92	1/1 (100)	11956	9755	0.775
WUPyV	99	2/2 (100)	20796	12170	0.108
MCPyV	82	30/39 (77)	9799	6297	0.239
HPyV6	83	1/1 (100)	3022	8140	0.575
HPyV7	71	-	-	-	-
TSPyV	79	4/5 (80)	716	6575	0.391
HPyV9	19	0/4 (0)	-231	-69	0.101
MWPyV	100	1/1 (100)	11023	10425	0.909
STLPyV	65	0/1 (0)	-263	873	0.126
HPyV12	4	0/10 (0)	-288	-259	0.596
NJPyV	5	0/1 (0)	367	204	0.466
LIPyV	6	0/2 (0)	-143	-224	0.514

* Overall percentage seroprevalence, as previously described by Kamminga et al. PLOS ONE. 2018 okt;13(10):e0206273.

† P-value calculated with Mann-Whitney U test

Abbreviations: HPyV: Human polyomavirus; MFI: Median Fluorescence Intensity

The prevalence of polyomavirus DNA (5.4%) was based on PCR amplification (with Ct values below 40) and sequence-confirmation of at least one amplicon per HPyV, which was obtained for BKPyV, JCPyV, WUPyV, MCPyV, HPyV6, TSPyV, HPyV9, MWPyV and LIPyV. Since blood donors in the Netherlands are selected on optimal health and minimal risk exposure (amongst others to infectious diseases), we believe that our estimation of HPyV presence is a minimum estimator of HPyV prevalences in the general, adult Dutch population, and probably in other western populations as well, as little differences in PyV seroprevalence are observed between these populations. **(35,61,62,65)**

A strength of this study is the inclusion of many blood donors and all fourteen currently described HPyVs. Except for MCPyV (see below), the most likely explanation for our findings is that the implicated blood donors were viremic at the time of blood collection. However, HPyV DNAemia as the result of disintegrating persistently infected cells cannot be excluded, which could be relevant for BKPyV and JCPyV that have been found in peripheral blood mononuclear cells from healthy persons. (127,128,135) In a study of 400 plasma samples of American blood donors, BKPyV and JCPyV DNA was not detected, (63) which might be explained by the smaller size of the study. Alternatively, technical or geographic differences could account for the negative outcome in that study.

Since HPyV infections are generally acquired during childhood, (62,65) HPyV-detections probably result from persistent HPyV infections. Although primary infection is a possible explanation for PCR positive-donors that are seronegative. Whether our findings result from continuous HPyV viremia, which occasionally exceeds the lower limit of PCR detection, or from an occasional viremic episode in the background of an otherwise latent infection cannot be deduced from our cross-sectional dataset. Furthermore, infectivity of the suspected HPyV, or loss of infectivity after nuclease treatment, to assess the presence of intact virions, will be difficult because of the detected low viral load levels (median 55 copies/ml serum). Potential infectivity of blood components could be assessed by documented seroconversion or an increase in seroreactivity in the recipient after administration of blood components.

MCPyV was detected in 3.8% of serum samples in our study, which is comparable to other studies. For example, a study of 190 blood donors reported MCPyV in 2.6% of sera (136) and another study of 621 sera from 394 elderly, hospitalized patients above 65 years of age found a prevalence of 9.9% for MCPyV, (137) which suggests that the prevalence may increase with age. MCPyV has been detected in other blood compartments, for example in 22% of buffy coats from blood donors. (138) Interestingly, MCPyV was detected with whole-genome sequencing as part of the blood virome (139) and also with metagenomics in blood components eligible for transfusion. (140,141) KIPyV and WUPyV DNA have previously been reported in plasma from blood donors with prevalence ranging from 0.5%-3.1% for KIPyV and 0.8% for WUPyV. (142,143) This is slightly higher than our finding of 0.1% and 0.2% in serum for KIPyV and WUPyV respectively. Prevalence data in serum from healthy individuals for the other polyomaviruses is currently lacking.

We consider it likely that a substantial part of the relatively high number of MCPyV PCR-positives is explained by the high prevalence (>50%) of MCPyV (DNA) on

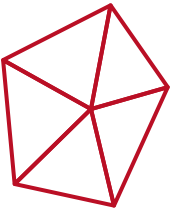
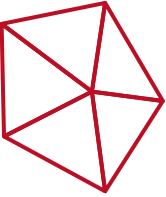
skin of healthy individuals, as reported in several publications. (144–146) During the hollow needle venepuncture, before the blood is actually collected, a small ‘biopsy’ of skin tissue is punched that could act as a source of virus. For TSPyV, however, this scenario is unlikely, as it is hardly found on the skin of asymptomatic immunocompetent and immunocompromised individuals. (144) In addition to the potential ‘contamination’ of donor blood through the skin punch, there is a theoretical risk of MCPyV contamination by blood bank and laboratory personnel, who carry MCPyV as well.

The seroprevalence of each PyV was determined within the same sample set in a previous study. (35) Despite a high concordance ($\geq 77\%$) between DNA positivity and seropositivity for most prevalent PyVs, we found no statistically significant correlation between the two. This lack of association is likely caused by the low number of PCR positives among the generally high number of seropositives. The HPyV9, STLPyV, NJPyV and LIPyV positive donors were seronegative for these polyomaviruses, which could be explained by primary infection or a lack of productive infection. The latter seems likely for NJPyV and LIPyV as these viruses may not have humans as their primary host. (35) For JCPyV we did observe an association between the height of the seroresponse and JCPyV DNA detections. An association between viral load and seroreactivity was previously observed for both BKPyV in kidney transplant patients (88,91) and for JCPyV, where individuals with high seroreactivity had higher viral loads compared to individuals with low seroreactivity. (147) JCPyV serology is also used as risk marker for PML. (148) This suggests that there is an association between JCPyV viral load and JCPyV serology, both in healthy individuals and in patients at risk for PML.

Some limitations of this study include the chance of misclassification by sample contamination and the chance of erroneous detection. In addition, this study shows the presence of viral DNA, rather than the presence of encapsidated, infectious viral particles. The risk of lab-contamination is reduced by storing and preparing reagents in separate rooms, using disposables and using no-template controls. To further limit the chance of erroneous detection, prevalence calculations were based only on positive PCR results with a Ct value below 40. Furthermore, amplicon sequencing of samples (with a maximum of 10) with a Ct value below 40 was performed to check for presence of the expected product. For most PCRs the expected product was detected, though sometimes detection was difficult, for example in case of codetection (TSPyV and HPyV9). Out of curiosity, we analysed several very weak PCR-positive samples and could confirm the presence of JCPyV, HPyV6 and TSPyV-specific DNA in some of those samples (data not shown). For HPyV12, in all 10 PCR

positive samples human genomic DNA was detected, which is probably amplified in a non-specific manner, because of the absence of specific DNA template. Furthermore, the finding of a polyomavirus similar to HPyV12 in shrews (36) combined with a reported low seroprevalence (35) suggests little circulation of this virus in humans.

In summary, DNA of HPyVs was detected in 5.4% of serum samples from a large cross-section of Dutch blood donors. The detection of polyomavirus DNA in these samples suggests that polyomaviruses are present in blood components eligible for transfusion, which should be further investigated using infectivity assays and a donor-recipient transmission study.



Chapter 5

Translating genomic exploration of the family Polyomaviridae into confident polyomavirus detection

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Summary

The family *Polyomaviridae* are ubiquitous dsDNA viruses that establish persistent infection early in life. Screening for human polyomaviruses (HPyVs), which comprise 14 diverse species, relies upon species-specific qPCRs whose validity may be challenged by accelerating genomic exploration of the virosphere. We explored this premise by testing 65 published HPyV qPCR assays *in silico* against the currently known 1784 PyV genome sequences that were divided in targets and non-targets, based on anticipated species-specificity of each qPCR. We identified several cases of problematic qPCR performance that were confirmed *in vitro* and corrected through using degenerate oligos. Furthermore, our study ranked four out of 52 tested BKPyV qPCRs as remaining of consistently high quality in the wake of recent PyV discoveries and showed how sensitivity of most other qPCRs could be rescued by annealing temperature adjustment. This study establishes an efficient framework for ensuring confidence in available HPyV qPCRs in the genomic era.

Introduction

Polyomaviruses are ubiquitous dsDNA viruses that are transmitted early in life and establish asymptomatic persistent infection (62,65). On average, a healthy individual is infected with nine different HPyVs during life-time (35,61). In elderly and immunocompromised patients, HPyVs can cause symptomatic infection. For example, BK polyomavirus (BKPyV) is the causative agent of BKPyV-associated nephropathy in kidney transplant recipients and of hemorrhagic cystitis in hematopoietic stem cell transplantation recipients (149). JC polyomavirus (JCPyV) causes progressive multifocal leukoencephalopathy in HIV patients and in multiple sclerosis patients on immunomodulatory medication (55). Merkel cell polyomavirus (MCPyV) causes approximately 80% of Merkel cell carcinomas (25). Trichodysplasia spinulosa polyomavirus (TSPyV) causes a rare, dysplastic hair follicle disorder in immunocompromised patients called trichodysplasia spinulosa (27). Karolinska Institute polyomavirus (KIPyV), Washington University polyomavirus (WUPyV), human polyomavirus 6 (HPyV6), human polyomavirus 7 (HPyV7), and New Jersey polyomavirus (NJPyV) have been associated with respiratory, skin and vascular diseases, respectively (24,32,59,150). Infections with human polyomavirus 9 (HPyV9), Malawi polyomavirus (MWPyV), Saint Louis polyomavirus (STLPyV), human polyomavirus 12 (HPyV12) and Lyon IARC polyomavirus (LIPyV) have not been associated with disease in humans so far. The human polyomaviruses mentioned above, except for HPyV12, are classified into 13 species, whose name includes “*Human polyomavirus*” followed by a number from 1 to 14 in italic (Table 1). For instance, BKPyV belongs to *Human polyomavirus 1* within this taxonomic framework. HPyV12, which recently turned out almost identical to Shrew polyomavirus 1, remains to be reclassified (36,41).

Humans are tested for the presence of HPyVs using ‘diagnostic’ virus-specific PCRs. These are usually validated and certified at the time of design and thereafter periodically through external quality assessment (EQA). Both *in silico* PCR oligo design and *in vitro* assessment are defined by available and selected target viruses. Sampling varies considerably for different viruses, including polyomaviruses, due to the biased knowledge about natural variation of the respective virus at the time of the PCR design and availability of the viral genomes and panel samples. Pathogenic strains, of JCPyV for example, are more likely to be sequenced and thus overrepresented in sequence repositories compared to persistent, avirulent strains. The same is true for strains that resemble previously identified strains that provided the basis for currently used PCR primer and probe sets. Ideally, it would be desirable to link updates of diagnostic PCRs to the continuous advancement of our knowledge about natural polyomavirus variation due to expanded genome sequencing (Figure 1),

but this may be impractical to do *in vitro* in a time and cost-wise manner. Use of *in silico* approaches may present a viable solution to this persisting problem.

For HPyV detection, our laboratory currently uses 14 qPCRs, of which nine were developed in-house and five adopted from literature (151). EQA has been performed on a regular basis only for the qPCRs that target the most common polyomaviruses: BKPyV and JCPyV. To address this gap and assess whether these 14 HPyV qPCRs remain as good in the face of expanding genome sequencing as they were at the time of design, here we performed *in silico* testing of each HPyV qPCR against all currently, publicly available polyomavirus genome sequences, including those of non-human origins using a previously described approach (152). For this purpose, the PyV genome sequences are divided into target and non-target groups for each qPCR to facilitate calculation of *in silico* sensitivity and selectivity for each its target HPyV. We used conventional qPCR analysis to test a fraction of genomes that were poorly recognized *in silico* and associated with mismatches between oligos and their annealing genome sites. Results of the qPCR and the *in silico* analysis were in agreement. Furthermore, utilization of target and non-target datasets facilitated adjustment of annealing temperature (T_a) for 14 in-house qPCRs and 52 published BKPyV qPCRs *in silico* in a PCR-specific manner.

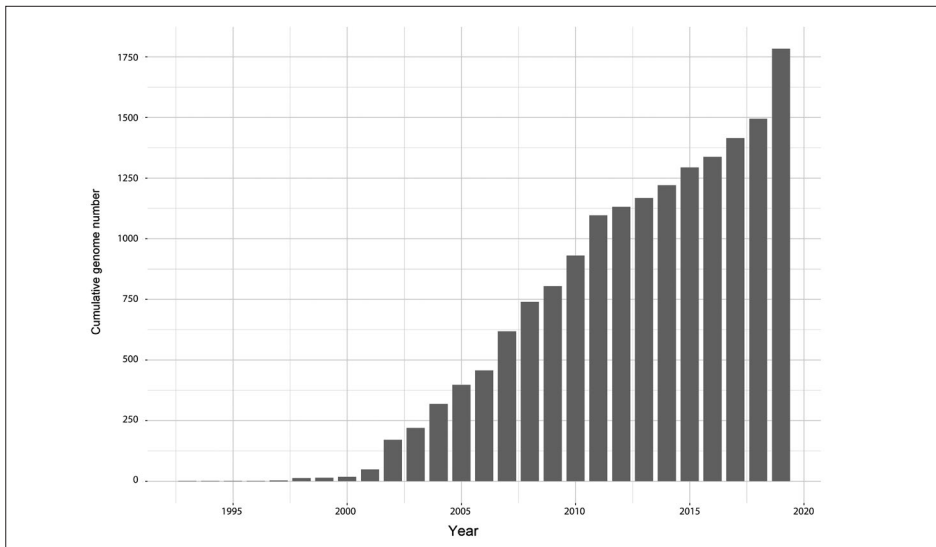


Figure 1. Dynamic of accumulation of complete polyomavirus genome sequences.

Shown is annual dynamic of accumulation for complete genome sequences of the family Polyomaviridae in GenBank until October 10th 2019, according to HAYGENS (<https://veb.lumc.nl/HAYGENS/>). Genome sequences are dated according to their GenBank entries, which may deviate from the first date of the public sequence release.

Materials and methods

In-house HPyV qPCRs

For this study, we selected 14 HPyV-specific qPCRs used in our laboratory to detect BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, MWPyV, STLPyV, HPyV12, NJPyV, and LIPyV respectively (151). **Table 1** lists these qPCRs along with information on the targeted viral gene, amplicon length, oligo sequences, and reference to its first description in literature (77,101,131–133,150,151). All HPyV qPCRs had their primer and probe oligos perfectly matched to the respective HPyV genome sequence regions at the time when they were designed.

Literature compiled BKPyV qPCRs

In August 2019, we compiled a dataset of BKPyV qPCRs reported in literature with the help of text-mining. Biopython 1.73 was used to search through PubMed's Entrez Database (153) (**Figure 2**, top panel). The case-insensitive search terms used were “polyoma AND qPCR AND human”. The result (n=453 articles) was then parsed using R 3.6.1 software and its readxl, readr, and Tidyverse packages (154–157). Subsequently, abstracts of these articles were scanned for the presence of the following terms: “bk” and at least one of “qpcr”, “real-time pcr”, “taqman”. The extracted articles with these terms (n=96) were further screened individually for inclusion in this study. Finally, we considered 52 Taqman-based qPCRs (the most common qPCR method) targeting viruses belonging to the species *Human polyomavirus 1*, including BKPyV, and collected information about primer and probe oligo sequence, concentration, polarity, dye linkage and inclusion of modified bases. Concentration of Mg²⁺ and other variables of the qPCR buffer, cycling conditions and the reaction volume were also documented whenever available. When neither of the latter was reported, default values of our qPCR test-system (see below) were used in subsequent analyses. Each qPCR was assigned with a unique name that included the last name of the first author of the paper, year of the publication, qPCR virus target name and, if necessary, an additional identifier when multiple qPCRs were described in a single paper.

Sequence database of polyomavirus genomes: target and non-target groups

In August 2019, complete polyomavirus genome sequences were downloaded from the National Center for Biotechnology Information (NCBI) GenBank and RefSeq into the VirAlis platform (158), using the HAYGENS tool (version 2.4, available at the <http://veb.lumc.nl/HAYGENS>) (**Figure 1**). In brief, HAYGENS combined results of homology search in GenBank for polyomavirus genomes using a hidden Markov model HMM profile (HMMER tool version 3.2.1. <http://hmmer.org>) of the alignment of 1199 PyV Large T-antigens (LT) (159) with queries of GenBank entries on the presence

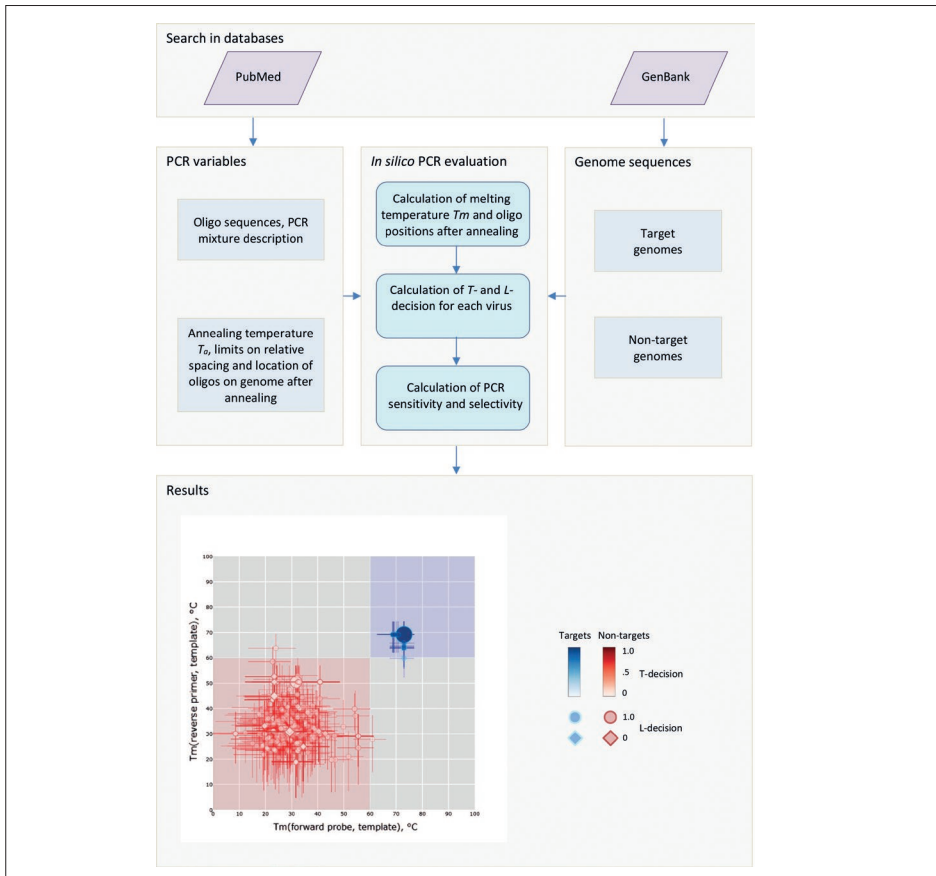


Figure 2. Schematic workflow of *in silico* PCR testing and example of results visualization.

Presented are main stages of *in silico* testing of a publicly available HPyV qPCR using genome sequences of polyomaviruses (top two panels), as well as an example of results visualization (bottom panel). This pipeline is also applied to analysis of in-house HPyV qPCRs, which provided PCR variables. All calculations are performed for each genome sequence and PCR oligos set and are detailed in the M&M. Results of *in silico* evaluation of the qPCR in respect to T-decision ranges of qPCR oligos annealed to target (blue) and non-target (red) templates are presented using a T_m -map (static and interactive versions of 2D- and 3D T_m maps for the data presented in this study are available ([online supplement](#))). Each T_m map is divided into four non-overlapping orthogonal zones delimited by two boundaries set at temperature corresponding to the T_a of the presented qPCR, and distinguished by three background colors. Light blue zone: T-decision ranges favorable for both oligos to facilitate qPCR; light red zone: T-decision ranges unfavorable for both oligos to facilitate qPCR; two light grey zones: one of two T-decision ranges is unfavorable for an oligo to facilitate qPCR. When the coordinates of the calculated qPCR product conforms to the sequence location boundaries delimited by the corresponding L-decision=1, T_m values of a pair of oligos annealed to the respective sequence are labelled with a circle, otherwise they are a diamond. Two labels may be separate or overlap, partly or fully, on the map, and the opacity of each label corresponds to its T-decision value within the respective target or non-target color gradient. Size of circle or diamond label is proportional to the number of labels, when they fully overlap. Each label maps T_m of two oligos vs a template under the T-decision equal to 0.5. T_m ranges for these oligos and their template corresponding to the T-decision [0.95-0.05] intervals are delimited with vertical (bottom-to-top) and horizontal (left-to-right) bars around each label, respectively (see M&M).

of the term “polyomavirus”. Using lengths of 85 polyomavirus genomes from RefSeq and the location of LT in these genomes as entries for training, HAYGENS separated full and partial genome sequences of other entries. With this approach, 1784 PyV genomes (1523 of human and 261 non-human origin) were recognized as (nearly) complete and their sequences were used in this study. For evaluation of each qPCR, the PyV genome sequences were divided into PCR-specific target and non-target groups, respectively, based on their description and taxonomy annotation. For evaluation of each HPyV qPCR, the target group includes HPyV genome sequences belonging to the respective polyomavirus species; all other polyomavirus genomes were considered non-target for this qPCR.

In silico evaluation of polyomavirus qPCR and results visualization

General aspects. The *in silico* evaluations performed here estimate the ability of HPyV qPCR to discriminate targeted from non-targeted HPyV genome sequences, using a modified computational procedure that we applied previously to astroviruses (152). Briefly, this procedure analyzed oligo/template complexes for a given set of oligos at all possible genomic sites under specified concentrations (160) to locate sites with maximal melting temperatures (T_m) of the oligo/template annealing complexes. It followed a nearest-neighbor approach, with T_m being the temperature at which the oligo and template molecules are equally probable to be separated or annealed (161). For degenerate oligos with degeneracy d , T_m value for each oligo/template combination was calculated assuming that concentration of each unique oligo is $1/d$ of the total oligos concentration, and T_m of the reaction was at the maximum value of T_m for the considered oligo/template combinations. For oligos conjugated with a minor groove binder, T_m value was increased by 15°C, due to the average value of such increase estimated previously (162,163). The same concentration of target DNA (1 nM) was used in each qPCR during the *in silico* evaluation. For convenience, a schematic overview of major steps of *in silico* qPCR evaluation is shown in the second panel of **Figure 2**, and further explained in its legend. As detailed below, it involves calculation of several characteristics, including T- and L-decisions, for each genomic sequence to assess sensitivity and selectivity of qPCR.

Temperature (T)-decision. Each PCR specifies annealing temperature T_a that is selected within a range of 50 °C to 60 °C, and commonly set to 60 °C (standard T_a). It must be substantially below the respective T_m , that ensures target recognition (true positives), but high enough to avoid recognition of non-targets (false positives). To facilitate comparison of T_a -related results for all analyzed sequences of a PCR, we introduced a continuous T-decision function that changes from 0 to 1 and equals to

Table 1. Overview of lab-developed, in-house used HPyV qPCRs

HPyV target Name abbreviation (HPyV species #)	GenBank ID Target Gene	Expected product length (bp)	Sense primer sequence (5'-3')	Probe sequence (polarity) (5'-3' for Sense and 3'-5' for Antisense)	Antisense primer sequence (3'-5')	Year of design Reference
BKPyV (1)	NC_001538 VPI	90	GAAAAGGAGAGTGTCCAGGG	FAM-CCAAAAAGCCAAAAGGAAACCC (S)	GAACITCTACTCTCTCTCTTTTATTAGT	2003 Meijden et al. 2014
JCPyV (2)	NC_001689 LT	129	GTCTCCCCATACCAACATTAGCTT	YAK-TCTTTTCCACTGGCACAATCTCTCATGAATG (S)	GGTTTAGGCCAGTTGCTGACTT	2006 Pal et al. 2006
KIPyV (3)	NC_009238 VPI	148	AAGITTCOCGGGTACAAAATC	TXR-GGTAGAAGTACTAGCCGAGTACCACACTGT (S)	CCATCTCTGAGCAGCTGTGTA	2016 Kamminga et al. 2019
WUPyV (4)	NC_009539 VPI	74	AACAGGAAGTCCACCAAGAAG	TXR-CAACCCACAAGAGTGCAAAAGCCCTCC (S)	CTACCCCTCCTTTTTCTGACTTGTTT	2011 Rao et al. 2011
MCPyV (5)	NC_010277 LT	149	CCACAGCCAGAGCTCTTCT	CY5-TCCACGGGCTTCAGACTCCCA* (S)	TGGTGGTCTCTCTCTCTGCTACTG	2009 Goh et al. 2009
HPyV6 (6)	NC_014406 VPI	150	GTAGGGTATGCTGGTAAC	YAK-CTCTCTCTCTGTCTGAAGTGAACCTCTAA (AS)	CAGGAATTGCTAAACATCATATC	2012 Purdie et al. 2018
HPyV7 (7)	NC_014407 VPI	116	GTGCTGATATGTTGGAA	TXR-AGCCCTGTACTGTCTCTGGTACT (AS)	TCTGCCAGTGGACTCTAAA	2012 Purdie et al. 2018
TSPyV (8)	NC_014361 VPI	104	GAGTCTAAGGACAACATATGG	Q705-CTTGCTCTCTGGTCACTGCTGTT (AS)	CTAGCTGTACTGTAGGTTG	2012 Meijden et al. 2016
HPyV9 (9)	NC_015150 VPI	109	CCTGTAAAGCTCTCTCTTA	FAM-CTTGTCTCTGGTCTTATGGCTCA (S)	CCTGATAAATCTGACTTCTTC	2012 Meijden et al. 2014
MWPyV (10)	NC_018102 VPI	86	GACACCACAATGACAGTTGAG	CY5-CCAGGATGGCCAATGATGTAACAACA (S)	GGATCACTGTAGCCATACCAT	2016 Kamminga et al. 2019
STLPyV (11)	NC_020106 VPI	101	TTGMAAATGGCTCCAAAAGAATACT	CY5-AGATGCACTCACACACATGCCAATGGA (S)	TGGCAGGATCAATATCACATCT	2016 Kamminga et al. 2019
HPyV12/SAPyV**	NC_020890 VPI	139	AAGGCTGTAAAGAATCC	FAM-CCAGTATCTGCTCTTACCAGT (S)	CTCCAAACCCCTATATACC	2015 Kamminga et al. 2019
NJPyV (13)	NC_024118 VPI	135	CCACCACAAGTAAGTAAC	YAK-AGTGTCTCTATACCTCTCCAGTGC (S)	CAGAGTCAATTTTCAGTAGTA	2015 Kamminga et al. 2019
LIPyV (14)	NC_034253 VPI	83	TGACAGGTGACAATTCGAGG	Q705-AGAGGAAGTACGGCTCTATGATGGCAGAG (S)	CCITGGCAGATCTAACCCCTCC	2017 Kamminga et al. 2019

* Probe modified from original article. Abbreviations: LT: Large T; VPI: Viral Protein 1; S: Sense; AS: Antisense

** Sorex araneus polyomavirus 1 (https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=201904426). No species name assigned yet

0.5, when $T_a = T_m$. T-decisions were calculated for each pair of DNA template, target and non-target, and PCR oligonucleotide, primer and probe, which then were used to calculate a cumulative T-decision for a DNA template as a product of its T-decisions for all individual PCR oligos. Finally, average T-decision of a qPCR for a polyomavirus species was calculated by averaging over cumulative T-decisions for genome sequences of the species.

Length (L)-decision. Template recognition by PCR depends also on proper spacing of its annealing sites for PCR primer and probe oligos. The latter must conform to product length maximum size and the lack of overlap between annealing sites for certain oligo pairs, collectively forming “length” constraints of qPCR. The maximum amplicon length was set either at 400 nucleotides or at 200 nucleotides (according to the expected maximum length of the PCR product) and minimum distances between 5'- and 3'ends of the corresponding oligos were set to 0. Due to evolutionary considerations, these constraints are most likely fulfilled for targets although not necessarily for non-targets, if those diverged considerably at the expected cognate site(s) of annealing. For these sequences, maximal T_m may be observed at alternative sites, either compatible or not with the product length constraints of the qPCR. We called the respective binary outcome of comparison of the calculated product lengths with permitted size ranges “L-decision”; it equals either 1 or 0 when length constraint was satisfied or not, respectively. L-decision was calculated for each pair of oligos, including forward and reverse primers and a single probe, resulting in three L-decisions for a template, target or non-target. Also, cumulative L-decision for a template was calculated as a product of (three) individual L-decisions under a condition that it was zero if any of individual L-decision is zero.

Sensitivity and selectivity of qPCR *in silico*. Using both T- and L-decision values for all selected PCR oligos, an overall probability (p) of detection of a HPyV genome sequence was calculated; ranging between 0 and 1. The calculated p is true positive (TP), and $1-p$ is false negative (FN) for target sequences; p is false positive (FP) and $1-p$ is true negative (TN) for non-target sequences. Accordingly, cumulative TP+FN of target sequences is always equal to the total number of targets and cumulative FP+TN of non-target sequences is equal to the total number of non-targets. Finally, *in silico* sensitivity and selectivity of qPCR in respect to separate target sequences or an entire species were calculated using respective TP, FN, FP, and TN values under standard T_a , original T_a supplied in publications, or adjusted T_a . The latter corresponds to the temperature that maximized half of the sum of the respective sensitivity and selectivity ($[\text{sensitivity} + \text{selectivity}] \times 0.5$), which was designated as balanced PCR classification rate (BCR). If several sequences were considered,

their sensitivity and selectivity were averaged for the respective group, including species, whose values were used to characterize the respective qPCR, unless specified otherwise.

Visualization of results. The results of a given HPyV qPCR evaluation were visualized using T_m maps generated with the original software and Python package Plotly (164) (Figure 2). T_m was calculated for interaction of oligos (primers or probes) and DNA templates, target and non-target, and plotted using either 2D T_m maps for each pair of oligos (forward primer vs. reverse primer, forward primer vs. probe, probe vs. reverse primer), or a single 3D T_m map for comparisons all three oligos together. 2D and 3D T_m plots of every qPCR *in silico* evaluation are shown online in the **online supplement** (<https://veb.lumc.nl/MANUSCRIPTS/Polyomaviridae2021.cgi>).

In vitro qPCR quality assessment

HPyV qPCRs were evaluated *in vitro* using Bio-Rad CFX Manager version 3.1. Cycling conditions were 95 °C for 15 minutes, followed by 45 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. Baseline threshold values were determined separately for each target and fluorescence drift correction was applied. For qPCR optimization, plasmids containing the relevant HPyV full genome or the Viral Protein 1 (VP1)-coding sequence were used. To obtain HPyV plasmid 10-fold dilution series (10.000 - 1 copy/reaction), total DNA concentration of each target was measured in a Qubit 4 Fluorometer using a Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Plasmids containing a mismatch in the annealing region were created by site-directed mutagenesis, using the QuikChange kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions.

Results

In silico evaluation of in-house HPyV qPCRs

Sensitivity and selectivity of the in-house qPCRs targeting one of the 14 HPyVs either in VP1 or LT genes (12 and 2 PCRs, respectively) (**Table 1**) were analyzed *in silico* using the 1784 complete PyV genome sequences retrieved from GenBank (**Table 2**).

Selectivity was consistently high for all analyzed qPCRs (between 0.98-1), regardless of the use of standard or adjusted T_a , suggesting a high specificity of all original qPCRs in respect to the sequenced HPyV genomes. In contrast, the *in silico* sensitivity under the standard T_a was quite variable (between 0.20-0.97), indicating target-dependence of some qPCRs. T_a adjustment considerably improved qPCR sensitivity, up to the range of 0.96-1. A correlation between the calculated qPCR sensitivity and the presence of mismatches, before and after T_a adjustment, was not observed. Below we will discuss *in silico* analysis of three HPyV qPCRs (TSPyV, JCPyV and BKPyV) in detail and use *in vitro* testing to verify the sensitivity estimations of several targets, and extending it also to oligos with corrected mismatches.

Evaluation and refinement of the TSPyV qPCR

Analysis of the T_m oligo/template plots for the TSPyV qPCR revealed that T-decision [0.95-0.05] ranges of 7 out of 23 (30.4%) analyzed genome sequences were either at or just outside the favorable T_m zone for TSPyV detection under the standard T_a (**Figure 3AB**). Those seven genomes, prototyped by KM007161.1 and sequenced after the original TSPyV qPCR was designed, have a recurrent mismatch in the forward primer annealing site (GAGTCTAAGGA[C→G]AACTATGG), which correlated with a T_m drop from 60.9 °C to 50.9 °C (**Figure 3AB** and **online supplement**).

The predicted detrimental effect of this mismatch on the qPCR sensitivity was analyzed *in vitro*. It caused an 8 Cq-increase and therefore drop in analytical sensitivity of the qPCR toward the respective sequences compared to the original TSPyV sequence (**Figure 3C**). Inclusion of the degenerate base in the forward primer (GAGTCTAAGGASA AACTATGG) increased the T_m of its complex with respective targets to 59.3°C *in silico* (**Figure 3AB** and **online supplement**), and, accordingly, almost entirely rescued the TSPyV qPCR analytic sensitivity towards KM007161.1 at the standard T_a (**Figure 3D**). These results revealed excellent agreement between the *in vitro* and *in silico* results.

According to *in silico* evaluation shown in **Table 2**, the sensitivity-rescue caused by the degenerate primer could be realized as well by lowering the T_a , without swapping

bases to overcome any mismatch. This would allow efficient recognition of all TSPyV genomes with the original PCR set of oligos with a predicted sensitivity of 0.96.

Testing and refinement of the JCPyV qPCR

In contrast to TSPyV, none of the analyzed 690 genome sequences of JCPyV were found in a problematic region of three T_m maps under standard T_a , although T-decision [0.95-0.05] ranges of 96 JCPyV genomes partially overlapped with one such region (**Figure 4** and **online supplement**). The latter genome sequences all contained a mismatch within annealing region to a respective primer or probe (**Table 2**). Since the T-decision [0.95-0.05] range of most of these oligo/target complexes were predominantly above the qPCR standard T_a and they accounted only for 14% of targets, these mismatches decreased average sensitivity of this qPCR only to 0.94. Compared to other JCPyV, a mismatch in the forward primer annealing region (GTCTCCCAT[A→G]CCAACATTAGCTT) in 72 JCPyV genome sequences (prototyped by AF015535.1) out of 96 affected (10.5% of total) was associated with comparable decreases in T_m *in silico* (4 °C from 68.4 °C to 64.4 °C, **Figure 4A-C**) and *in vitro* (0.6 from 24.2 to 24.8 at 10^5 copies/reaction, **Figure 4D**). This effect was even smaller for the remaining 24 JCPyV genome sequences *in silico*, which also showed that sensitivity of the JCPyV qPCR could be increased from 0.94 to 1.0 by adjustment of T_a from 60.0°C to 46.7°C (**Table 2**).

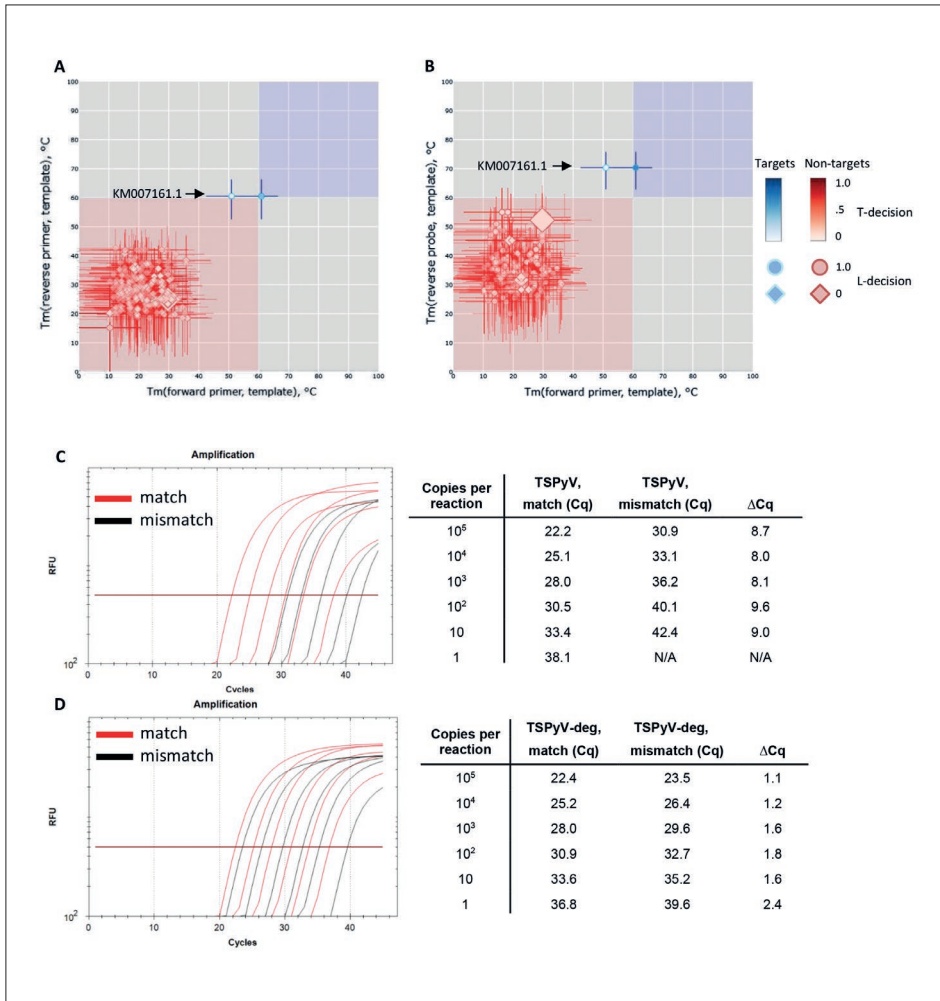


Figure 3. *In silico* and *in vitro* testing and refinement of a TSPyV qPCR.

Results of *in silico* testing of the in-house TSPyV qPCR under standard T_a in respect to T-decision ranges of qPCR oligos annealed to 23 target (blue) and 1758 non-target (red) templates are presented using two T_m maps: forward vs. reverse primer oligo pairs (**A**), forward primer vs. probe oligo pairs (**B**). A T_m shift for oligo/template complex of seven TSPyV genomes, typified by KM007161.1, after including a degenerated base into the forward primer (**Supplemental File 8.2B** and **D**) is shown with the bold arrow. Overall design of the T_m maps is explained in the legend of Figure 2. **C.** *In vitro* dilution series of two TSPyV genomes with either full match (NC_014361) or a mismatch (KM007161.1) to the forward oligo. A relative poor recognition of the mismatch genome is evident. **D.** The same as in (C) except for using forward primer with a degenerate base. Note similar recognition of the two genomes.

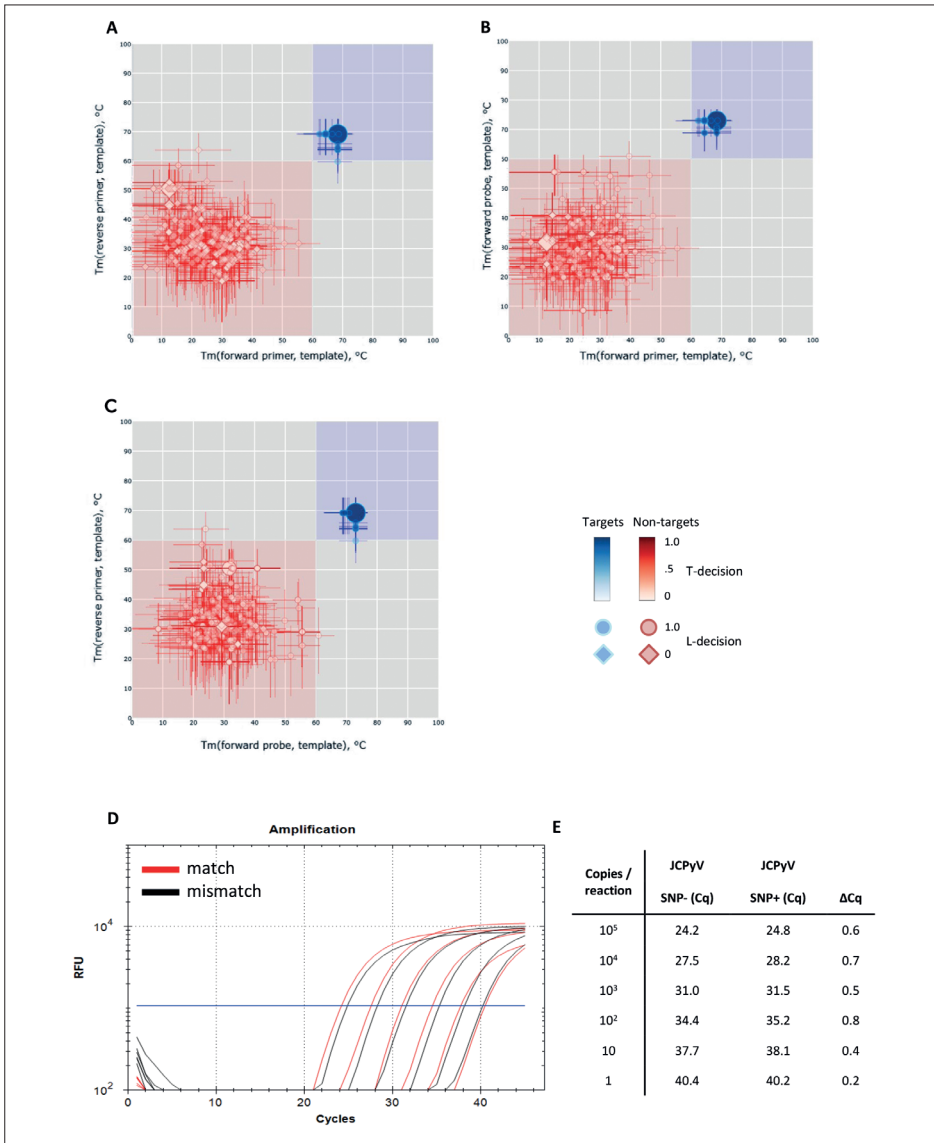


Figure 4. *In silico* and *in vitro* testing of a JCPyV qPCR.

Results of *in silico* evaluation of the in-house JCPyV qPCR under standard T_a in respect to T-decision ranges of qPCR oligos annealed to 690 target (blue) and 1091 non-target (red) templates are presented using three T_m maps: forward primer vs. reverse primer oligo pairs (**A**); forward primer vs. probe oligo pairs (**B**); and reverse primer vs. probe oligo pairs (**C**). Overall design of the T_m maps is explained in the legend of **Figure 2**. Fully interactive versions of these maps and a 3D melting temperature map are available in **Supplemental File 2**. The influence of a common mismatch present in 72/690 JCPyV genomes was tested by comparing the performance of the qPCR on the regular control plasmid without mismatch (Mismatch-) and the plasmid containing the mismatch (Mismatch+) (e.g. AF015535.1, forward primer annealing region: GTCTCCCAT[A→G]CCAACATTAGCTT). A small difference in Cq values is seen when the mismatch is present.

Table 2. *In silico* evaluation of in-house HPyV qPCRs, ranked according to average sensitivity under standard T_a

qPCR Target HPyV (N genomes)	Oligo mismatch (number and location) ¹				Average Sensitivity ²		Average Selectivity ²	
	Number of genomes (%)	5'primer genomes, n	Probe genomes, n	3'primer genomes, n	Standard T_a ³	Adjusted T_a ⁴	Standard T_a ³	Adjusted T_a ⁴
STLPyV (7)	0	0	0	0	0.97	1.00	1.00	1.00
KIPyV (12)	0	0	0	0	0.95	1.00	1.00	1.00
MCPyV (63)	0	0	0	0	0.95	1.00	1.00	1.00
JCPyV (690)	96 (14)	81	10	9	0.94	1.00	1.00	1.00
WUPyV (147)	25 (17)	0	25	3	0.90	1.00	1.00	1.00
MWPyV (21)	1 (5)	1	0	0	0.85	1.00	1.00	1.00
LIPyV (2)	1 (50)	0	1	1	0.80	1.00	1.00	1.00
BKPyV (522)	120 (23)	1	107	12	0.76	0.99	1.00	0.98
HPyV9 (4)	0	0	0	0	0.39	1.00	1.00	1.00
HPyV6 (17)	1 (6)	1	0	0	0.37	0.99	1.00	1.00
HPyV12 (1)	0	0	0	0	0.37	0.99	1.00	1.00
HPyV7 (10)	1 (10)	1	0	0	0.32	0.99	1.00	1.00
TSPyV (23)	7 (30)	7	0	0	0.23	0.96	1.00	1.00
NJPyV (1)	0	0	0	0	0.20	0.99	1.00	1.00
TSPyV-deg (23)	0	0	0	0	0.26	0.99	1.00	1
Total (1784)		94	143	25				

¹ Some sequences may have mismatches with more than one type of the oligo, so the sum of the number of sequences in the first three columns may exceed the number in the Total column

² Average sensitivity and selectivity were calculated by averaging over sensitivity and selectivity of discrimination of respective target versus non-target genome sequences by an in-house qPCR

³ Standard T_a : 60°C

⁴ Adjusted T_a varied between 45-50°C (46.7, 50.8, 45.8, 46.0, 45.0, 45.4, 45.0, 46.3, 45.0, 46.8, 45.0, 45.1, 45.0, 45.0 °C for JCPyV, BKPyV, WUPyV, MCPyV, TSPyV, MWPyV, HPyV6, KIPyV, HPyV7, STL, HPyV9, LIPyV, HPyV12, NJPyV, respectively.

Testing and refinement of the in-house BKPyV qPCR

The most complex results were obtained for the in-house BKPyV qPCR analyzed against 522 target and 1259 non-target genome sequences, as evident from the T_m plots under the standard T_a (**Figure 5A-C**; interactive 2D and 3D T_m plots are shown in the **online supplement**). A small overlap with a non-favorable T_m map zone was observed for the T-decision [0.95-0.05] ranges of oligo complexes with 120 BKPyV genome sequences. Furthermore, the T-decision [0.95-0.05] ranges for oligo complexes with two BKPyV genome sequences (MF627830.1 and AY628231.1) were predominantly outside the favorable zone in two of three oligo/target T_m maps. As a result, average sensitivity of the in-house BKPyV qPCR was 0.76 under the standard T_a .

The above group of 120 genome sequences (23% of the analyzed BKPyVs) include mismatches between oligos and corresponding annealing sites, primarily in the probe target region (107 of 120 genomes; **Table 2**). The most prevalent mismatch (CCAAAAAGCCAAAGGA[A→C]CCC), found in the probe annealing site of 105 genome sequences and prototyped by AB211375.1, caused the estimated probe/template T_m to decrease from 66.48 to 64.28 °C (**Figure 5BC** and **online supplement**). This common mismatch resulted in recurrent decrease of approximately 1 Cq *in vitro* (**Figure 5D**). This decrease was reverted by linking a minor groove binder (MGB) to the probe (**Supplemental Figure 1**), which accordingly increased the T_m of the probe/template complex by approx. 15 °C (**online supplement**).

Two other mismatches were in the probe annealing site. They caused a drop in predicted T_m of oligo/target complexes from 66.14°C for the ‘wild type’ genome prototyped by NC_001538.1 to 56.96°C for MF627830.1 (CCAAAAAGCCAAAGGAA[C→T]CC) and to 60.38°C for AY628231.1 (CCAAAAAGCCA[A→G]AGGAACCC) (**Figure 5BC** and **online supplement**). Because the genomes carrying these mismatches represented only 0.4% of the sequenced BKPyV genomes, their detection was not tested *in vitro*.

In silico evaluation of BKPyV qPCRs described in literature

To extend utility of our *in silico* qPCR evaluation strategy, we applied it to a substantial selection of BKPyV qPCRs described in literature. A database of 52 BKPyV-specific qPCRs taken from 32 papers (**132,151,165–194**) was created by searching PubMed with a text-mining approach detailed in the Methods section. Each selected BKPyV qPCR is listed in **Supplemental Table 1** with its reference, original and adjusted T_a values, and degeneracy of its oligos. **Supplemental Figure 2** shows the location of each PCR oligo annealing site on the BKPyV genome, as well as the calculated sensitivity and selectivity with the T_a described in the original paper.

Under the original T_a , both sensitivity and selectivity were better than 0.95 for at least five BKPyV qPCRs, with “Gustafsson 2013 Polyoma ST” being the top PCR (**Figure 6**). Overall, sensitivity varied considerably among the published PCRs, with being < 0.9 and as low as in the range of 0.2-0.5 for 30 and 10 BKPyV qPCRs, respectively. This low sensitivity was primarily due to overlap of T-decision [0.95-0.05] ranges for some oligo/template complexes between target and non-target genomes. Adjusting the individual BKPyV qPCR T_a -values according to the BCR criterion (see M&M) resulted in a T_a drop in most cases, which substantially increased the sensitivity of almost all BKPyV qPCRs, with none of qPCR being below 0.8 after the T_a adjustment. In contrast, only two BKPyV qPCRs displayed a selectivity below 0.9 (“Yamamoto 2015” and “Delbue 2015”) after the T_a adjustment, which was caused by a high similarity between BKPyV and JCPyV at sites complementary to the PCR oligos in a fraction of non-target genomes (**online supplement**) (193-196). This complication was not resolved by the T_a adjustment, which also had minor effect on already high selectivity of other BKPyV qPCR.

As a rule, the T_a adjustment was associated with a temperature decrease, most often in a range from 5°C to 10°C (**Figure 6**). For two PCRs, T_a adjustment was minor ($< 1^\circ\text{C}$) that did not affect either sensitivity or selectivity, which were already good under standard T_a : >0.95 for “Keith 2018” and >0.8 for “Delbue 2015”. For other two PCRs (“Signorini 2014” and “Yamamoto 2015”), adjustment of T_a led to improved selectivity as the result of decreased false positive detection of the closely related non-targets, although it was accompanied by a decrease in PCR sensitivity.

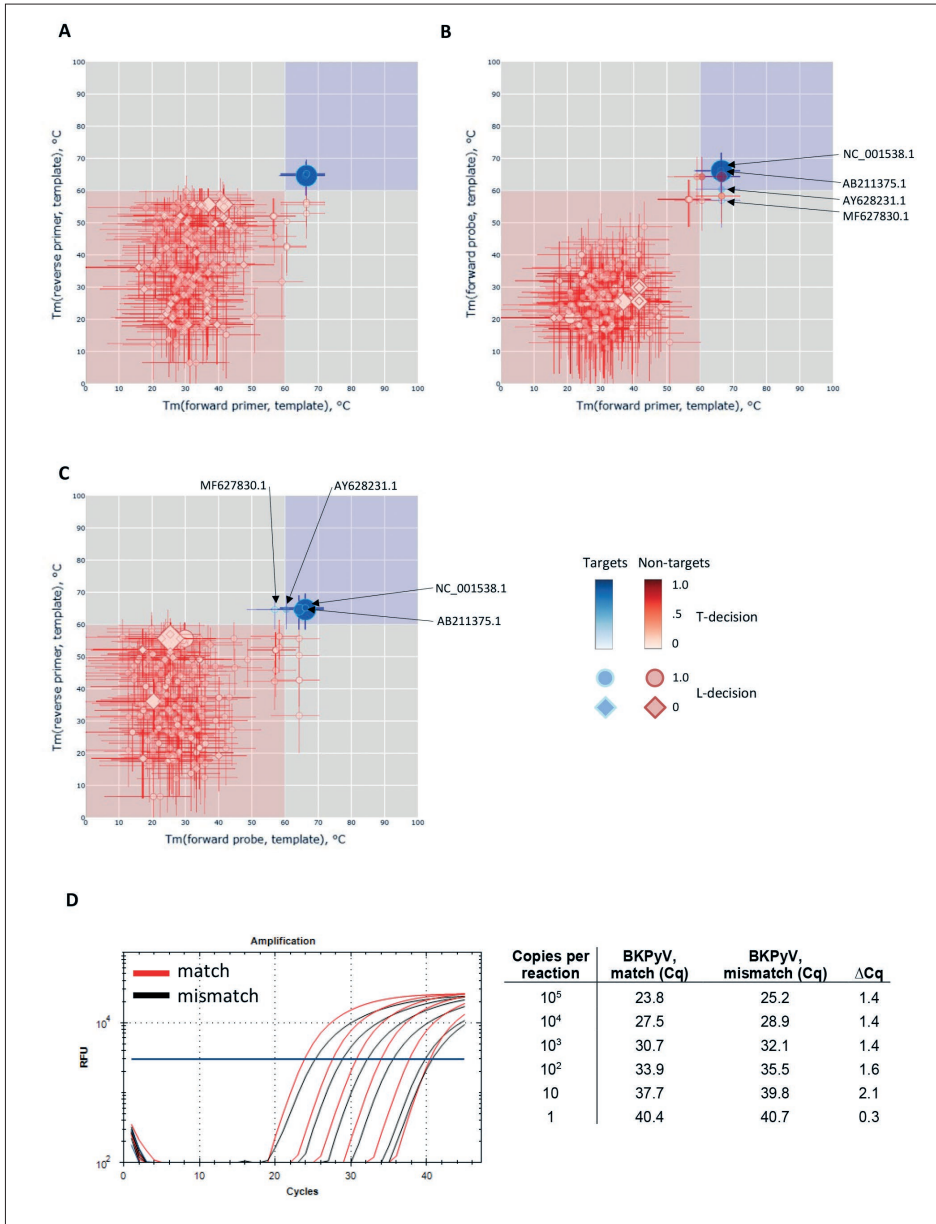


Figure 5. In silico and in vitro testing of a BKPyV qPCR.

Results of in silico testing of the in-house BKPyV qPCR under standard T_a in respect to T-decision ranges of a qPCR oligo annealed to 522 target (blue) and 1259 non-target (red) sequences are presented using three quadrant maps as detailed in legend to **Figure 2**. Selected genome sequences discussed in the text are indicated with arrows accompanied by their GenBank numbers. **D.** *In vitro* evaluation of impact of a common single nucleotide mismatch in the probe-to-target annealing region on the qPCR performance against GenBank ID AB211375.1. An increase of about 1 Cq for the target with the mismatch relative to the matching target was observed.

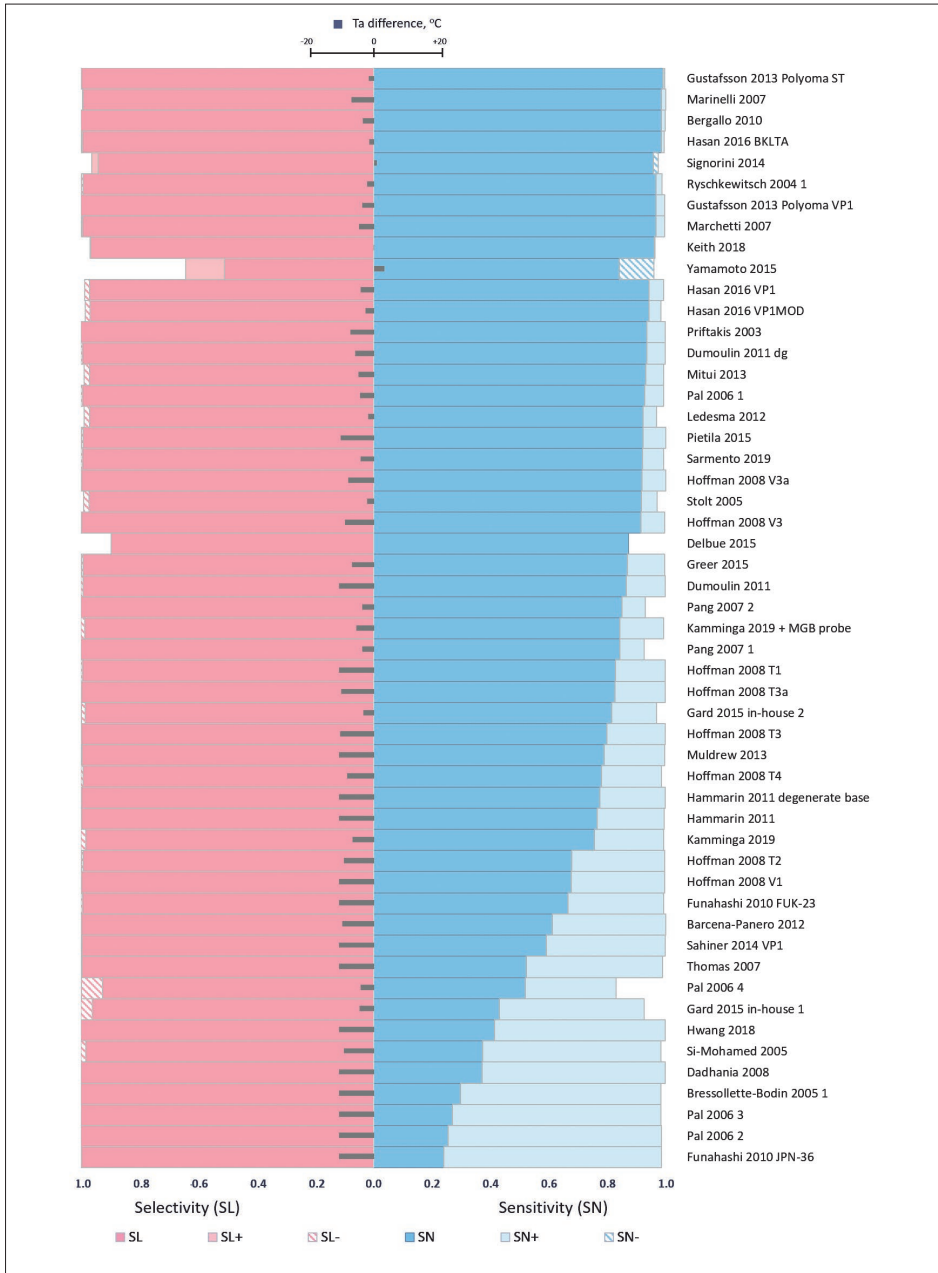


Figure 6. Sensitivity and selectivity for BKPyV qPCRs under original and adjusted T_a .

For each published BKPyV qPCR specified at the right, selectivity and sensitivity are depicted schematically with contrasting colors under original and adjusted T_a along with difference between these T_a . Impact of T_a adjustment is shown as increase (SN+, SL+) or decrease (SN-, SL-) of the corresponding original sensitivity and selectivity values (SN and SL). PCRs are ordered according to the sensitivity under adjusted T_a . T_a difference = (adjusted T_a - original T_a) is shown with grey bars

Discussion

In this report we demonstrated how fast accumulating genome sequences of the family *Polyomaviridae* could be utilized for testing species-specific HPyV qPCRs *in silico* in an efficient manner. This analysis identified qPCRs, which can detect and discriminate all known PyVs, as well as those PCRs that require upgrade. We showed how this improvement could be achieved using either degenerate nucleotides in oligos or through adjustment of T_a in a procedure assisted by the use of non-target PyVs. Below we briefly discuss its premise, main findings, including apparent agreement between *in silico* and *in vitro* results, as well as limitations and challenges of the approach that may be addressed in future research.

Design of conventional PCR involves calculation of key variables that is informed by target sequences and concerning oligos number, size and template location as well as T_m and T_a of oligo/template complexes. This computation-based foundation of the PCR analysis enables running a PCR *in silico*, as we previously demonstrated in an analysis of human astroviruses (152) and used here for HPyVs. Compared to its *in vitro* counterpart, qPCR analysis *in silico* offers scalability in a cost and time effective manner that affords testing 1784 rather than few PyVs as it is common otherwise. Since PyV discovery and characterization are fast pacing and firmly sequence-based, accumulation of target sequences can inform periodic HPyV qPCR testing *in silico* for keeping it up to date. To assist with this task, we provide web access to interactive T_m maps that facilitate visual inspection of all genome sequences of this study by the 66 HPyV qPCRs.

To measure quality of target sequence recognition by species-specific HPyV qPCR, we calculated sensitivity that was averaged over all known target genome sequences of the species. Composition of species target genome sequence datasets reflects sampling and full genome sequencing of the known natural diversity of the respective species, which both may be biased. Accordingly, the obtained sensitivity values could be skewed when they were below the maximum possible value (1). This bias could be partially addressed by sequence weighting and including partial genome sequences where feasible in the analysis. We note in this respect that validation of HPyV qPCR *in vitro* also depends on limited choice of known HPyVs for testing.

Besides sensitivity, we also calculated selectivity of species-specific HPyV qPCR in a similar manner. It measured discrimination of non-target polyomaviruses by the respective qPCR and may serve as a proxy for its expected false positive rate. Non-target sequences included all known PyVs regardless of their host and other

than of the respective target species. New HPyVs continue to be discovered and often they cluster phylogenetically with PyVs of non-human origins that supports the family-wide choice of the non-target datasets in our study. This broad host range of non-target PyVs sequences combined with bias of PyV genome sequencing affects estimation of selectivity, when its values fall below the maximum possible (1.0), like it was discussed for the sensitivity calculation above. This estimation may be adjusted by limiting choice of non-target sequences only to the most closely related to target HPyV species, which might be especially warranted if they co-circulate, like observed for BKPyV and JCPyV.

Sensitivity and selectivity of qPCR depends on choice of T_a , which is commonly selected within the 45-65°C range, as seen e.g. in the dataset of 52 BKPyV qPCRs analyzed *in silico*. The selected T_a must be below the respective T_m of the involved oligo/template complexes of targets and above those of non-targets. By combining sensitivity and selectivity of qPCR into a single BCR characteristic we were able to propose T_a adjustment that led to improved overall quality for most of qPCRs analyzed. This result illustrated benefits of the computational framework for HPyV detection that was informed by the current state of genome sequencing. It may be especially valuable in respect to non-target sequences which are relatively under-characterized compared to target sequences upon conventional *in vitro* evaluation. Besides non-target PyV sequences, DNA of other origins if present in considerable excess to target sequences may be a factor affecting qPCR by depleting PCR oligos through non-specific annealing. This concern, which is pertinent for relatively low T_a could lead to false negative results and may be addressed in future *in vitro* testing.

Our study was prompted by the need to evaluate 14 in-house qPCRs, which we designed or adopted from literature over prior years. We learned that *in silico* five qPCRs had sensitivity 0.9 or higher, three in the range of 0.5-0.9, and six below 0.5, due to uneven recognition of some newly sequenced genomes in most of the cases. For *in vitro* characterization, we chose three qPCR with different sensitivities, 0.94, 0.76 and 0.23, respectively. The most pronounced drop of sensitivity to 0.23 was in TSPyV qPCR due to a primer mismatch with a relatively large fraction of target genomes that were sequenced after the original qPCR was designed. Incorporation of a degenerate base in the forward primer restored the TSPyV qPCR capacity to recognize the full known diversity spectrum of this HPyV, as was shown both *in silico* and *in vitro*. The two assays were also in agreement in respect to two other qPCRs, JCPyV and BKPyV, although we decided against modifying oligos of those PCRs after considering other factors affecting scale of sensitivity gain. For BKPyV qPCR, this decision was informed by low frequency of the poorly recognized

targets, which account to only 0.4% of BKPyV genome sequences in GenBank. Should this number be revised upward significantly in the future, the BKPyV qPCR design could be revisited or another BKPyV qPCR be adopted from literature (see below). Drop of the qPCR sensitivity for poorly compared to properly recognized targets was defined by T_m decrease of target/oligo complexes *in silico* and accompanied by C_q increase *in vitro*. To establish the exact relationship between the changes of T_m and C_q , which may depend on many factors, additional analysis is required.

In silico analysis provided also a unified platform for comparison of numerous BKPyV qPCRs which were designed by different labs in different years against different genomic loci and tested under different conditions using different panels of viruses. The need for this type of comparison was raised in literature but not met (197,198). Our study identified several BKPyV qPCRs which proved to be resilient in the face of the continuing expansion of BKPyV genome sequencing and may be best choice to go forward. We also provide suggestions toward how analytical sensitivity for other published BKPyV qPCRs could be improved by adjustment of T_a . We believe that combined with characterization of the in-house HPyV qPCRs, these results facilitate efficient use of the accumulated PyV genome sequences for the detection of HPyVs.

Acknowledgements

This work was supported and funded in part by Sanquin Blood Supply Foundation, a not-for-profit organization, and the EU Horizon2000 EVAg project (<https://www.european-virus-archive.com/>). IAS and AEG thank Dmitry Samborskiy for help with administrating the VirAliS software.

Online supplement

The online supplement can be found at:

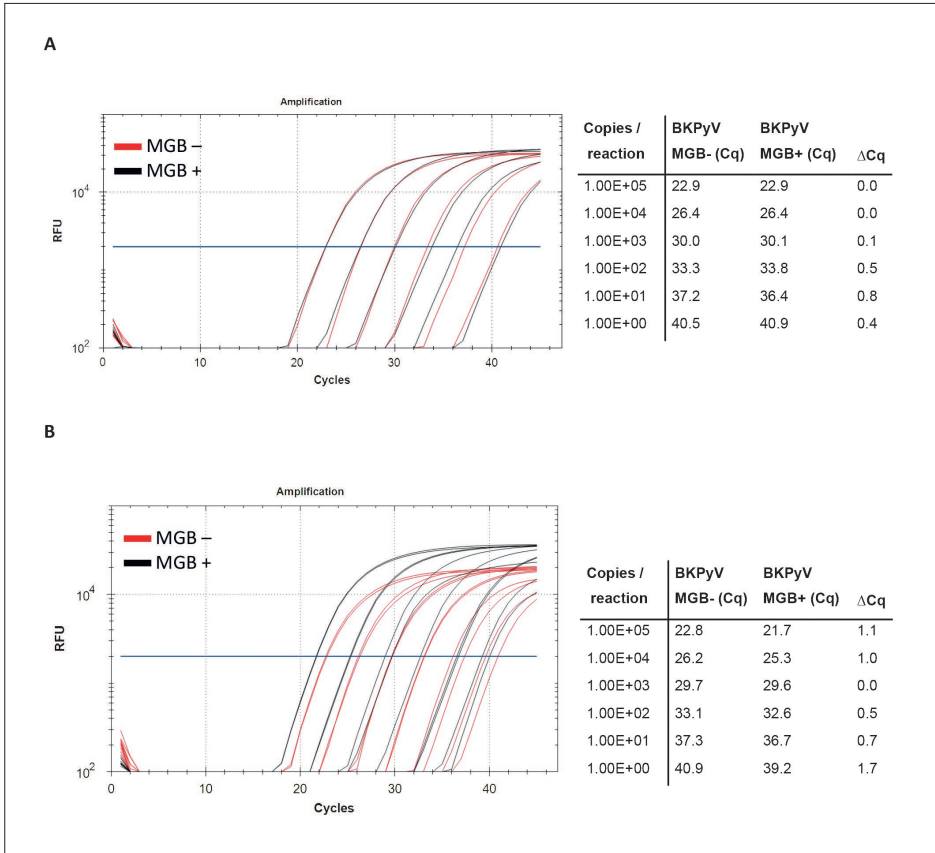
<https://veb.lumc.nl/MANUSCRIPTS/Polyomaviridae2021.cgi>

Supplemental Table 1. Selected BKPyV-specific qPCRs from literature

qPCR name	Reference	Oligos degeneracy†	T _a (°C)	
			original	adjusted
Barcena-Panero 2012 BKPyV	(40)	16/1/4	60	46.3
Bergallo 2010 BKPyV	(39)	1/1/1	50	45.3
Bodin 2005 BKPyV 1	(53)	1/1/1	60	45.0
Dadhania 2008 BKPyV	(38)	1/1/1	60	45.3
Delbue 2015 BKPyV	(63)	1/1/1	60	63.3
Dumoulin 2011 BKPyV	(41)	1/1/1	60	45.0
Dumoulin 2011 BKPyV dg	(41)	3/2/4	60	52.0
Funahashi 2010 BKPyV FUK-23	(47)	1/1/1	60	45.0
Funahashi 2010 BKPyV JPN-36	(47)	1/1/1	60	45.0
Gard 2015 BKPyV in-house 1	(58)	1/1/1	60	56.5
Gard 2015 BKPyV in-house 2	(58)	1/1/1	60	58.3
Greer 2015 BKPyV	(45)	1/1/1	60	50.5
Gustafsson 2013 Polyoma BKV ST	(46)	1/1/1	50	47.8
Gustafsson 2013 Polyoma BKV VPI	(46)	1/1/1	50	45.0
Hammarin 2011 BKPyV	(44)	3/1/2	60	45.0
Hammarin 2011 BKPyV degenerate base	(44)	3/2/2	60	45.3
Hasan 2016 BKPyV BKLTA	(36)	6/1/2	60	58.0
Hasan 2016 BKPyV VPI	(36)	2/1/1	60	54.3
Hasan 2016 BKPyV VPIMOD	(36)	2/1/1	60	56.3
Hoffman 2008 BKPyV T1	(35)	1/1/1	60	45.0
Hoffman 2008 BKPyV T2	(35)	1/1/1	60	47.3
Hoffman 2008 BKPyV T3	(35)	1/1/1	60	45.3
Hoffman 2008 BKPyV T3a	(35)	1/1/1	60	45.8
Hoffman 2008 BKPyV T4	(35)	1/1/1	58	46.5
Hoffman 2008 BKPyV V1	(35)	1/1/1	60	45.3
Hoffman 2008 BKPyV V3	(35)	1/1/1	60	47.5
Hoffman 2008 BKPyV V3a	(35)	1/1/1	60	49.0
Hwang 2018 BKPyV	(72)	1/1/1	60	45.0
Kamminga 2019 BKPyV + MGB probe	(16)	1/1/1	60	52.5
Kamminga 2019 BKPyV*	(16)	1/1/1	60	50.8
Keith 2018 BKPyV	(22)	1/1/1	60	59.8
Ledesma 2012 BKPyV	(55)	1/1/1	60	56.8
Marchetti 2007 BKPyV	(54)	1/1/1	58	45.0
Marinelli 2007 BKPyV	(34)	1/1/1	60	46.0
Mitui 2013 BKPyV	(49)	2/1/1	60	53.3
Muldrew 2013 BKPyV	(43)	1/1/1	60	45.0
Pal 2006 BKPyV 1	(22)	1/1/1	60	54.0
Pal 2006 BKPyV 2	(22)	1/1/1	60	45.0
Pal 2006 BKPyV 3	(22)	1/1/1	60	45.0
Pal 2006 BKPyV 4	(22)	1/1/1	60	54.5
Pang 2007 BKPyV 1	(61)	1/1/1	50	45.0
Pang 2007 BKPyV 2	(61)	1/1/1	50	45.0
Pietila 2015 BKPyV	(37)	1/1/1	60	45.5
Priftakis 2003 BKPyV	(42)	1/1/1	60	50.0
Ryschkewitsch 2004 BKPyV 1	(50)	1/1/1	55	49.3
Sahiner 2014 BKPyV VPI	(57)	1/1/2	60	45.0
Sarmento 2019 BKPyV	(48)	1/1/1	60	54.0
Signorini 2014 BKPyV	(60)	1/1/1	60	61.3
Si-Mohamed 2005 BKPyV	(52)	1/1/1	60	47.0
Stolt 2005 BKPyV	(56)	1/1/1	60	57.3
Thomas 2007 BKPyV	(51)	1/1/1	60	45.0
Yamamoto 2015 BKPyV	(62)	1/1/2	60	62.3

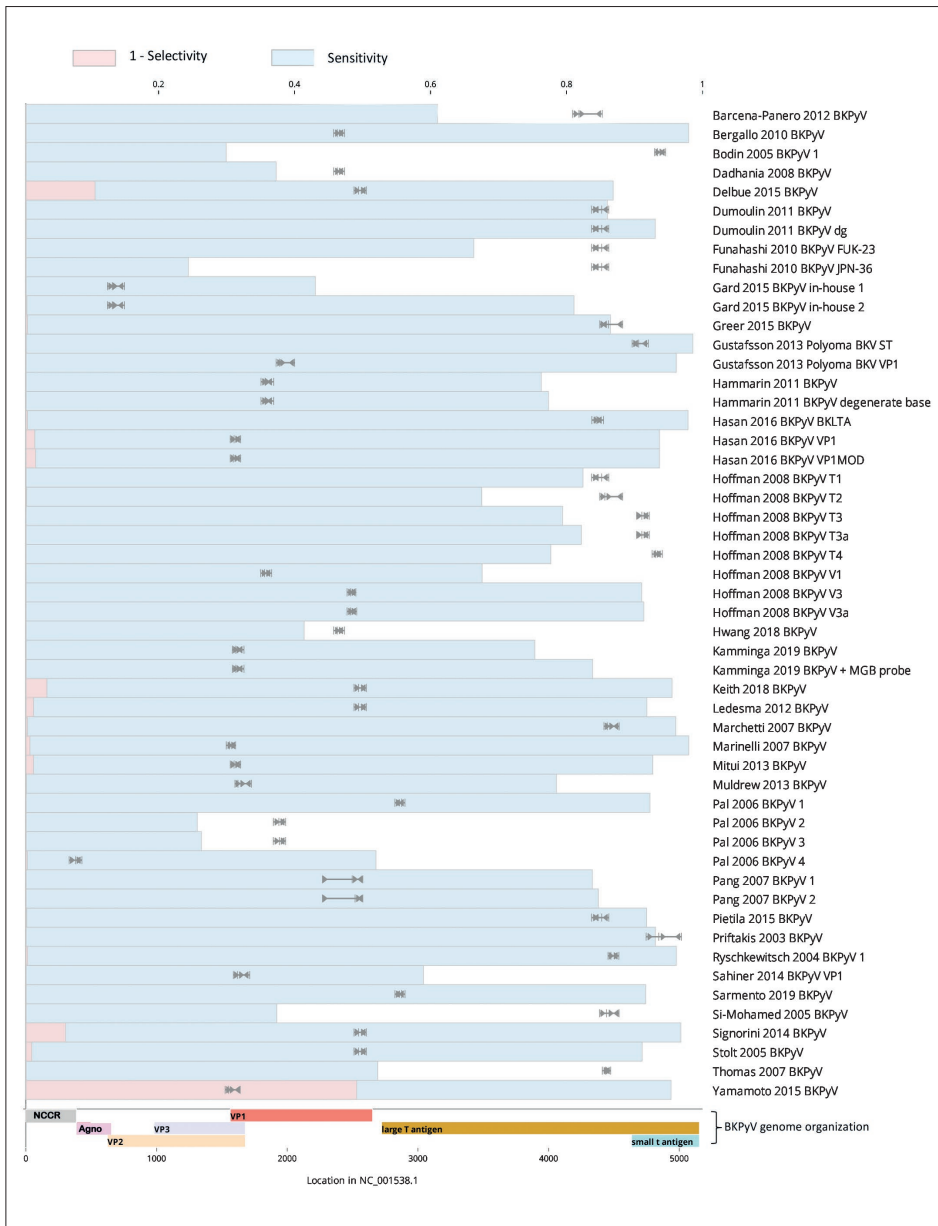
Oligo degeneracy for forward primer/probe/reverse primer

* Our original lab-developed BKPyV qPCR is indicated in bold



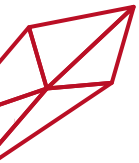
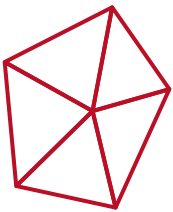
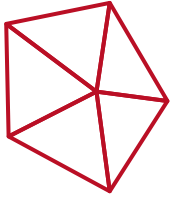
Supplemental Figure 1.

A. Result of adding a minor groove binder (MGB) to the BKPyV qPCR probe on detection of a BKPyV plasmid standard dilution series. The MGB increases probe melting temperature by approximately 15°C. This does not result in better detection of the BKPyV plasmid. **B.** When a mismatch is present in the probe annealing region (e.g. AB211375.1, probe annealing region: CCAAAAAGCCAAAGGAGCCCC), the MGB probe performs slightly better compared to the regular BKPyV qPCR.



Supplemental Figure 2.

Overview of location, sensitivity and selectivity of BKPvV qPCRs selected from literature. Calculated selectivity (pink) and sensitivity (blue) are based on the T_a reported in the original paper. The arrowheads indicate the oligo position and orientation related to the BKPvV genome shown below. qPCRs are sorted by name of first author. Sensitivity and selectivity of the qPCR were evaluated using a common set of target and nontarget genome sequences under concentration of 1 nM, and T_a delivering maximum BCR and with values of other qPCR parameters as described in the corresponding reference.



Chapter 6

Transmission of JC and Human polyoma- virus 9 after kidney transplantation: an exploratory serological cohort study

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Abstract

Introduction Human polyomaviruses (HPyVs) cause disease in immunocompromised patients. BK polyomavirus (BKPyV) for instance persistently infects the kidneys. In kidney transplant recipients, (KTRs) BKPyV can cause allograft nephropathy. JCPyV, MCPyV, TSPyV and HPyV9 reside in the kidneys too, or have been detected in urine. In this study, we investigate exposure to JCPyV, MCPyV, TSPyV and HPyV9 after kidney transplantation by serological means.

Materials and methods Serum samples from 310 KTR collected before and 6 months after transplantation (n= 620), from 279 corresponding kidney donors collected before transplantation, and from blood donor controls collected one year apart (n=174) were assessed for HPyV species-specific IgG responses using a multiplex immunoassay. KTR HPyV IgG kinetics were compared to those of healthy blood donors by linear mixed modelling, and related to those of their donors by linear regression.

Results In the KTR, increased IgG levels during follow-up were observed for JCPyV (14.8%), MCPyV (7.1%), TSPyV (10.6%), and for HPyV9 (8.1%), while blood donor antibody levels remained stable. Seroconversion was observed for JCPyV (6.5%), MCPyV (2.3%), TSPyV (1.3%), and for HPyV9 (6.5%). The linear mixed model analysis showed that antibody increase was significant for JCPyV ($p < 0.001$) and HPyV9 ($p < 0.001$). Post-transplant JCPyV and HPyV9 antibody responses were associated with donor antibody levels against these HPyVs, respectively.

Conclusions KTR are exposed to JCPyV and HPyV9 after transplantation. Whether the allograft serves as the source, as indicated by the donor serostatus association, deserves further study.

Introduction

On average, each individual is persistently infected with nine different human polyomaviruses (HPyVs) (199). In the immunocompetent host, HPyV infections are controlled by the immune system and not accompanied by symptoms and disease. When immunity is compromised, for instance in long-term immunosuppressed solid organ transplant (SOT) recipients (200), HPyVs can freely replicate, damage tissues and cause disease. The *Polyomaviridae* currently contain 13 HPyV species (201).

Most clinical complications are seen with the BK polyomavirus (BKPyV), which causes BKPyV-associated nephropathy (BKPyVAN) in kidney transplant recipients (KTR), and haemorrhagic cystitis in primarily hematopoietic stem cell transplantation patients. The phylogenetically closely related JC polyomavirus (JCPyV) can also cause nephropathy, (71) but is particularly known for causing progressive multifocal leukoencephalopathy (PML) in AIDS patients and in multiple sclerosis patients treated with specific immunomodulatory drugs, such as natalizumab (202,203). Both BKPyV and JCPyV can be detected in blood, CSF and urine of affected patients. The Merkel cell polyomavirus (MCPyV) causes approximately 80% of Merkel cell carcinoma (MCC) in the skin. MCC is rare and found primarily in elderly and sometimes in long-term immunocompromised patients (58,204,205). MCPyV has been detected in blood, albeit at very low amounts (206,207). The Trichodysplasia spinulosa polyomavirus (TSPyV) causes trichodysplasia spinulosa (TS), an extremely rare, dysplastic follicular skin disease seen in severely immunocompromised (often SOT) patients (77,208). TSPyV DNA can be detected in blood, CSF and urine of SOT patients as well, with high viral loads during primary infection. (77) Human polyomavirus 9 (HPyV9) has been identified in blood and urine of KTR (79), but an association with disease in immunocompromised patients has not been established (101,209). For HPyV6 and HPyV7 an association with a skin disorder in severely immunosuppressed has been described, but systemic infection (for instance accompanied by viremia) has not been reported. Similar observations were made for KIPyV and WUPyV related to respiratory infections in severely immunocompromised hosts (23,24,150). For NJPyV one convincing case of combined myositis, retinitis and vasculitis has been described, accompanied by detectable viral loads in blood (210). For the other HPyVs (HPyV10, STLPyV, and LIPyV), disease associations are absent and detection of virus in blood and/or urine is extremely rare.

Not much is known about HPyV transmission and infection. Most natural infections occur (early) in childhood and are thought to result from oral ingestion or inhalation. For some HPyVs, for example BKPyV and JCPyV, and possibly TSPyV, KIPyV and

WUPyV, it is believed that after primary infection of the oropharynx, they replicate in tonsils and possibly salivary glands followed by spread via the circulation (211,212). In this way, they can reach the end organ that becomes persistently infected, for instance the kidneys in the case of BKPyV and JCPyV. Whether 'strict' cutaneous HPyVs (for example HPyV6 and HPyV7) are transmitted through direct skin-skin contact is not known.

From studies in KTR, it is very likely that BKPyV can be transmitted via the allograft from donor to recipient (88,90). In a small group of KTR, with the help of viral genome sequencing, it was recently suggested that JCPyV as well can be transmitted through kidney transplantation (213). A serological study in a paediatric KTR kidney transplant population, suggested the same after observing JCPyV seroconversion in about half of the seronegative children (214). Furthermore, SOT is a known risk factor for development of PML (215). At the moment it is unknown whether donor-derived JCPyV, opposed to autologous reactivating JCPyV, is causing these post-transplantation PML cases. Transplantation-related transmission has not been suggested for HPyVs other than BKPyV and JCPyV.

In this study, by analysing a cohort of KTR, we aimed to provide additional, serological evidence for allograft-transmission of JCPyV and a number of other HPyVs with viremic potential. With the help of a multiplex immunoassay, (87) we determined JCPyV, TSPyV, MCPyV and HPyV9-specific antibody responses and seroconversions before and after transplantation in KTR, while BKPyV was included in the analyses for comparison. Healthy blood donor (HBD) sera collected one year apart were included for comparison, as well as pretransplantation kidney donor serum samples when available.

Materials and methods

Population and samples

Two stored serum samples, one collected before transplantation and one approximately six months after transplantation, were analyzed from each of 310 adult KTR transplanted between 2014 and 2018 in the Leiden University Medical Centre (LUMC), with a mean age of 49.9 (range 19.0 - 74.8) and 188 male (60.6%). The date of collection of samples taken before kidney transplantation ranged from 261 days to 1 day before transplant, with a median of 8 days before transplant. Samples taken after transplantation ranged from 33 to 299 days post-transplant, with a median of 177. For 279 kidney transplant patients, serum samples of their respective kidney donor

were also available for analysis. The study adhered to the General Data Protection Regulation, the code of conduct for medical research and the code of conduct for responsible use of human tissue. The data management plan was approved by the data protection officer of the LUMC. The medical ethical committee of the LUMC determined this research was outside the scope of the medical research involving human subjects act (reference: B19.067/ML/1111).

Paired, anonymized HBD serum samples (n=174) were acquired one year (median of 397 days) apart, as described in a previous study, (101) adhering to the code of conduct for responsible use.

Polyomavirus multiplex immunoassay

A customized Luminex multiplex immunoassay was used to assess IgG antibody responses against the major capsid protein Viral Protein 1 (VP1) of JCPyV, MCPyV, TSPyV, HPyV9 and BKPyV. This assay was previously described in detail (87). Briefly, VP1 fusion proteins were expressed in *E. coli* and coupled to uniquely colored, magnetic fluorescent beads (Bio-Rad Laboratories, Hercules, CA, USA). The serum samples were blocked in 1:100 dilution in blocking buffer to suppress non-specific binding. Biotinylated goat- α -human IgG (H+L) (1:1000) followed by streptavidin-R-phycoerythrin (SAPE) (1:1000) were used to detect IgG responses against the individual VP1 antigens. To control for intertest variability, a serially diluted pool of four serum samples with known IgG response was added to each plate. Antibody responses were measured in a Bio-Plex 200 analyzer (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Bio-Plex Manager 6.1 software. Specific antibody responses were calculated by subtracting from each sample the median fluorescence intensity (MFI) values of a blank sample (no serum added) and of beads coupled to GST protein only as a background measurement. Cut-off values for seropositivity were determined as described previously using a seronegative population and a bin-width distribution analysis (87). The thresholds for the KTR, expressed in MFI, were 846 for JCPyV, 550 for MCPyV, 126 for TSPyV, 1000 for HPyV9 and 1079 for BKPyV. The HBD were tested on a different Bio-Plex 200 analyzer and therefore cut-offs were determined separately. The thresholds for HBD were 666 for JCPyV, 747 for MCPyV, 638 for TSPyV, 274 for HPyV9 and 3085 for BKPyV.

Antibody response kinetics

For this study the serological status of an individual was determined by calculating the slope between the sampling time points before and after transplantation as follows: $(\text{MFI after transplantation} - \text{MFI before transplantation}) / \text{number of days between sampling}$. Samples were categorized as 'stable' if the slope remained within

the range of plus or minus two standard deviations of the slope from zero. If the slope was outside of these ranges, individuals were called having either ‘increased’ or ‘decreased’ antibody levels. The same categorization was applied for the KTR and the HBD, although a correction factor (the ratio between the median follow-up of the populations) was applied to account for the difference in follow-up time between KTR and HBD cohorts. In case individual follow-up antibody responses passed the seropositivity cut-off threshold and showed either an increased or decreased response, these were categorized as seroconversion ($- \rightarrow +$) or as seroreversion ($+ \rightarrow -$), respectively.

Statistics

Statistical analysis was performed in RStudio 1.2.1335 (216) and R 3.6.2 (154) with packages Tidyverse (217), lme4 (218), lattice (219), limma (220), and ggplot2 (221). To compare responses between populations (KTR and HBD) and account for the correlation between repeated measurements from the same individual, linear mixed models with random intercepts were applied. These models apply fixed effects (group (KTR and HBD), time (in MFI / day)) and random intercepts (i.e. unique for each subject) to model polyomavirus IgG responses over time. In addition, an interaction term between time and group was used since this improved the model as assessed by a likelihood ratio test (ANOVA). Linear regression models were applied to study factors of influence on post-transplant IgG response.

Results

Serum samples from 310 kidney transplant recipients collected before and after transplantation, and from 87 healthy blood donors collected 12 months apart were assessed for IgG antibody responses to JCPyV, MCPyV, TSPyV, HPyV9 and BKPyV. Compared to the HBD, whose HPyV antibody responses remained largely stable, the KTR showed more dynamic HPyV serologic profiles (Figure 1). Increased antibody levels were observed in 14.8% of KTR for JCPyV, 7.1% for MCPyV, 10.6% for TSPyV, 8.1% for HPyV9 and 11.9% for BKPyV (Table 1). In total, 101 KTR showed increased antibody levels for any HPyV. The majority, 63 (62.4%), increased for just one HPyV (21 solely for JCPyV, 18 for BKPyV, 12 for MCPyV, 9 for TSPyV and 3 for HPyV9), while 38 (37.6%) showed increased antibody levels for more than one HPyV (Figure 2). The most prevalent combinations were JCPyV, HPyV9 and BKPyV; JCPyV and HPyV9; TSPyV and MCPyV, which occurred in five KTR. Most increases for HPyV9 (21/25) occurred in conjunction with increases for JCPyV (19/25) and BKPyV (11/25).

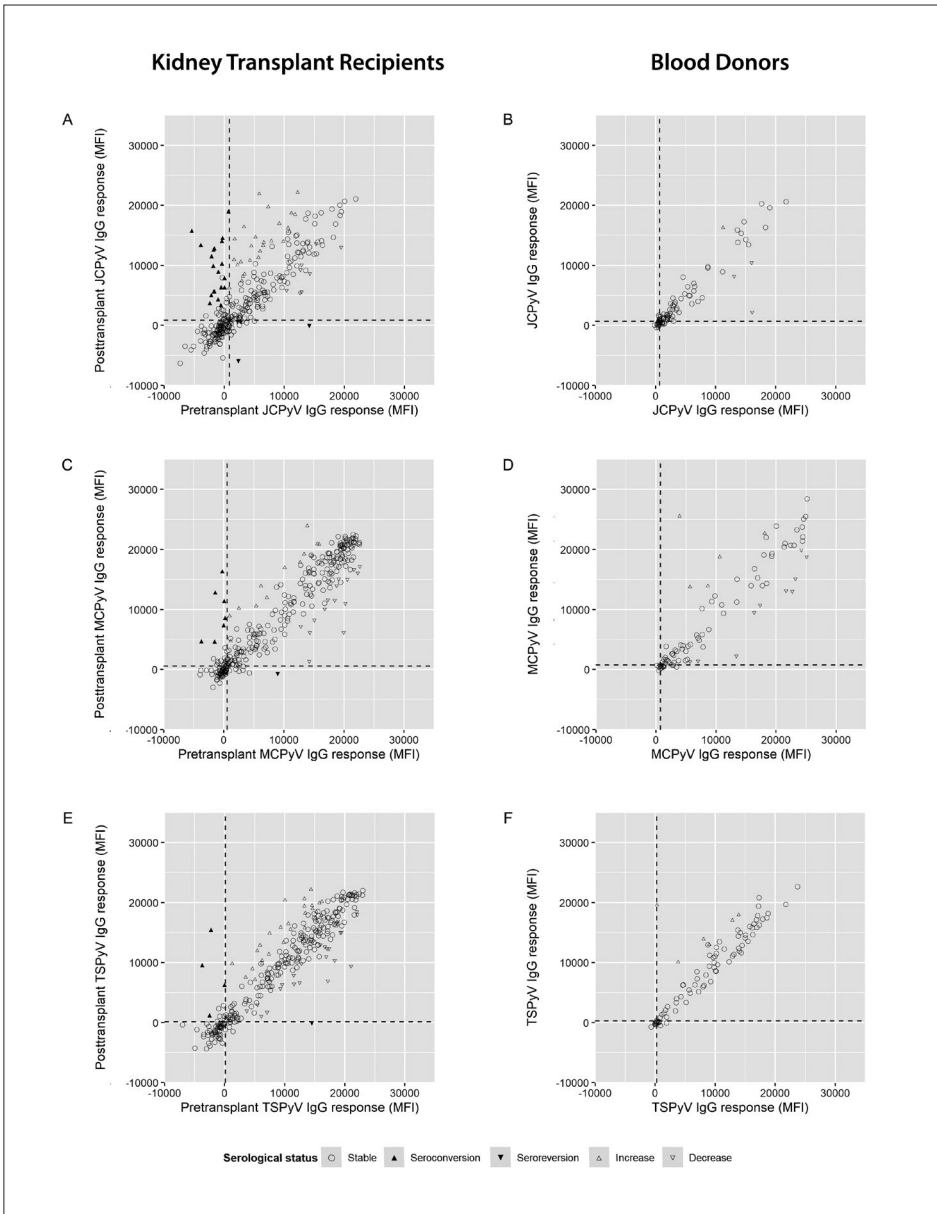


Figure 1. Individual IgG responses of kidney transplant recipients and blood donors during follow-up. (A-F)

Shown are IgG responses for KTR (left panels) and blood donors (right panels) in median fluorescent intensity (MFI) against JCPyV (A, B); MCPyV (C, D); TSPyV (E, F); HPyV9 (G, H) and BKPyV (I, J). First measurement is shown on the x-axis, the second measurement on the y-axis.

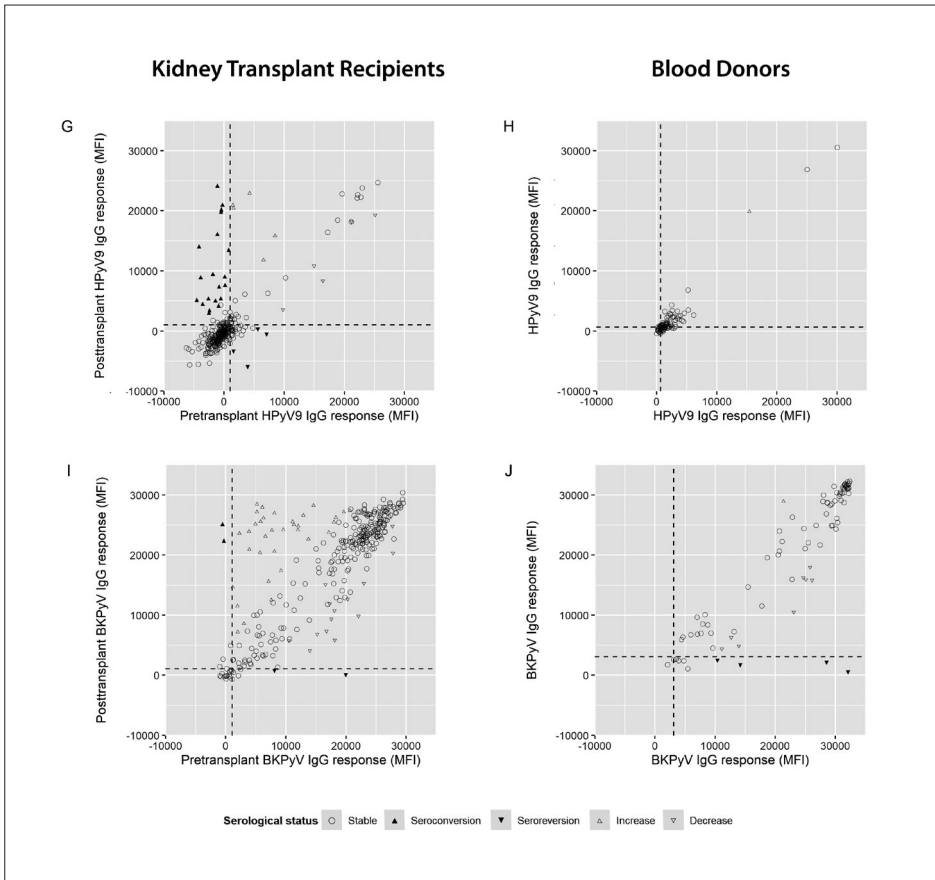


Figure 1 (continued). Individual IgG responses of kidney transplant recipients and blood donors during follow-up. (G-J)

Shown are IgG responses for KTR (left panels) and blood donors (right panels) in median fluorescent intensity (MFI) against JCPyV (A, B); MCPyV (C, D); TSPyV (E, F); HPyV9 (G, H) and BKPyV (I, J). First measurement is shown on the x-axis, the second measurement on the y-axis.

Table 1. Human polyomavirus IgG antibody response kinetics among kidney transplant recipients and blood donors

IgG kinetics	JCPyV N (%)	MCPyV N (%)	TSPyV N (%)	HPyV9 N (%)	BKPyV N (%)
Kidney transplant recipients (N=310)					
Stable	252 (81.3)	272 (87.7)	252 (81.3)	276 (89.0)	256 (82.6)
Increased	46 (14.8)	22 (7.1)	33 (10.6)	25 (8.1)	37 (11.9)
- seroconverted	20 (6.5)	7 (2.3)	4 (1.3)	20 (6.5)	2 (0.6)
Decreased	12 (3.9)	16 (5.2)	25 (8.1)	9 (2.9)	17 (5.5)
- seroreverted	4 (1.3)	1 (0.3)	1 (0.3)	4 (1.3)	2 (0.6)
Blood donors (N=87)					
Stable	83 (95.4)	72 (82.8)	81 (93.1)	86 (98.9)	74 (85.1)
Increased	1 (1.1)	5 (5.7)	1 (1.1)	1 (1.1)	1 (1.1)
- seroconverted	0	0	0	0	0
Decreased	3 (3.4)	10 (11.5)	0	0	12 (13.8)
- seroreverted	0	0	0	0	4 (4.6)

Seroconversion was observed in 6.5% of KTR for JCPyV, 2.3% for MCPyV, 1.3% for TSPyV, 6.5% for HPyV9 and 0.6% for BKPyV (**Table 1**). The percentage seroconverters among the baseline seronegatives was 14.7% (20/136) for JCPyV, 9.6% (7/73) for MCPyV, 6.6% (4/61) for TSPyV, 8.1% (20/247) for HPyV9 and 10% (2/20) for BKPyV. Decreasing HPyV antibody levels were less frequently observed, varying from 2.9 - 5.5%, with seroreversions between 0.3 and 1.3%.

In **Figure 3**, the change in antibody levels over time is shown for the KTR. The antibody response against JCPyV and HPyV9 increased over time following the pattern of that against BKPyV. Antibody responses against MCPyV and HPyV9 remained more or less stable during follow-up. In order to compare trends in HPyV antibody responses between KTR and HBD, linear mixed models with random intercepts were used. Responses at baseline were comparable between KTR and HBD for JCPyV ($p = 0.220$), MCPyV ($p = 0.520$) and TSPyV ($p = 0.444$), but higher in HBD for BKPyV ($p < 0.001$) and HPyV9 ($p < 0.001$) (**Table 2**). As expected, the BKPyV response increased significantly over time after kidney transplantation, while BKPyV response declined slightly in the HBD (8.26, 95% Confidence Interval (CI): 4.87-11.65, $p < 0.001$) (**Table 2**). Interestingly, the JCPyV and HPyV9 antibody response also increased significantly in KTR compared to the HBD (6.61, 95% Confidence Interval (CI): 4.36-8.86, $p < 0.001$, and (4.74, 95% Confidence Interval (CI): 2.59-6.88, $p < 0.001$, respectively). No increase in responses after transplantation was observed for MCPyV and TSPyV.

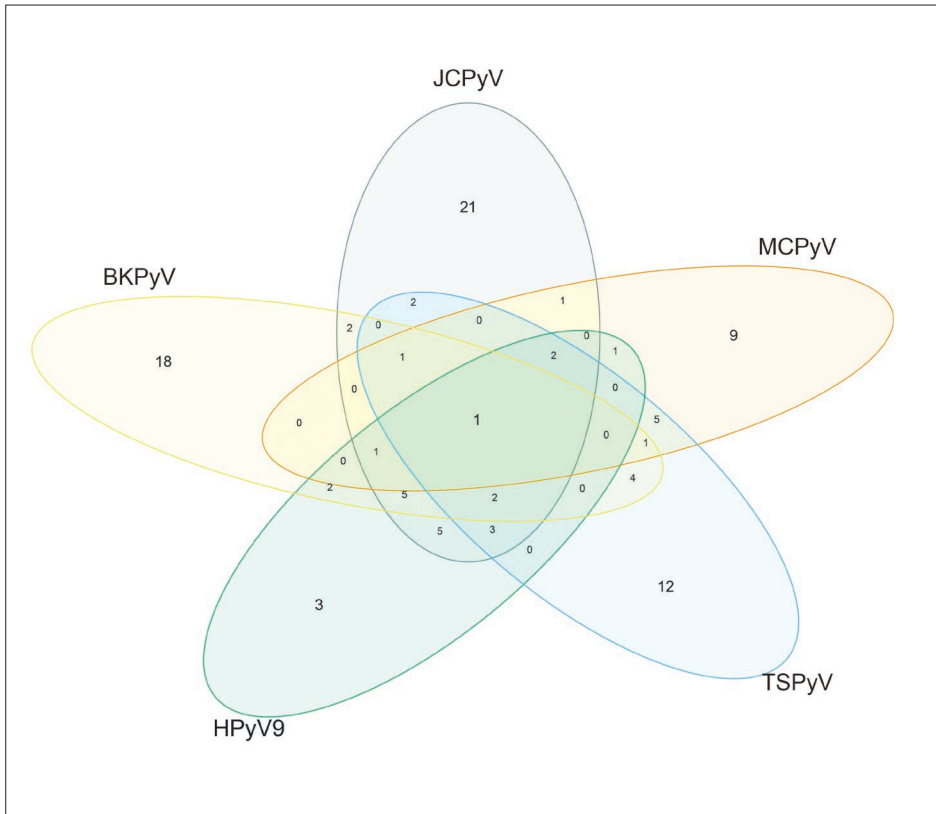


Figure 2. Venn diagram of kidney transplant recipient with increased IgG responses (including seroconversions) against HPyVs.

The numbers in the figure indicate the number of KTRs that showed an increased IgG response to the corresponding polyomavirus(es).

Since the observed increase in JCPyV and HPyV9 posttransplantation antibody response resembled that of BKPyV, we analyzed a possible correlation with donor HPyV response level, as we have demonstrated previously for BKPyV(88). As expected, the pretransplant antibody level was most influential on post-transplant response ($p < 0.001$ for all analyzed polyomaviruses). Furthermore, a high baseline donor antibody level was associated with significant increase in post-transplant levels for JCPyV ($p = 0.037$), HPyV9 ($p = 0.005$) and BKPyV ($p = 0.014$) (Table 3). For HPyV9, the size of the effect depended on the recipient serological status before transplantation, as is evidenced by the interaction term ($p = 0.016$, Table 3), suggesting a high antibody level in the transplant recipient protects against a rise in HPyV9 antibody levels.

Discussion

In this study, we analyzed IgG responses against selected HPyVs with viremic potential in KTR before and after transplantation, in comparison with HPyV IgG responses in HBD over a comparable period of time. Increased IgG responses after kidney transplantation were observed for JCPyV and HPyV9, comparable to what has been shown for BKPyV, while MCPyV and TSPyV IgG responses remained stable in the post-transplantation period, just like all analyzed HPyV IgG responses in the HBD. The observed increase in JCPyV and HPyV9 IgG responses were associated with kidney donor IgG response against the relevant HPyV.

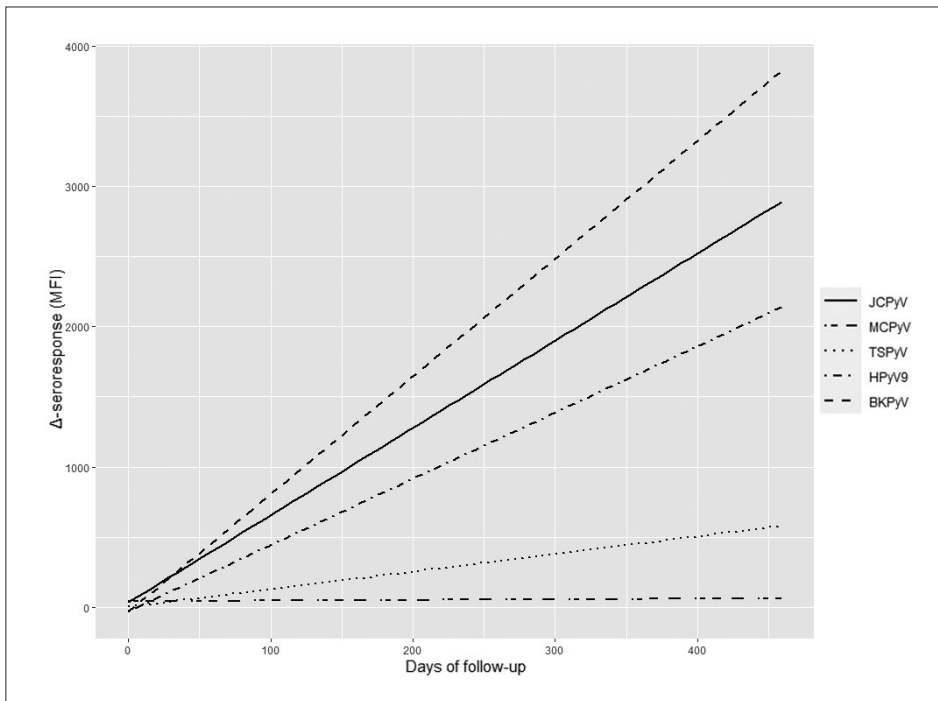


Figure 3. Increase in HPyV IgG response during follow-up for the kidney transplant recipients.

Shown are linear regression lines for IgG responses against the different polyomaviruses with the first timepoint set at zero MFI.

The lack of increased IgG responses against MCPyV and TSPyV after transplantation may not come as a surprise. These HPyVs are generally believed to primarily infect the skin, (222) a superficial organ and possibly less sensitive to fluctuations in central antiviral host immunity. For MCPyV this indeed could be the case, although small

amounts of MCPyV DNA are consistently detected in blood (206,207,223). However, for TSPyV it has been shown to circulate at high loads ($>10^6$ genome equivalent copies/ml blood) in KTR for months, in the presymptomatic phase of TS (224). Therefore, it is likely that TSPyV, next to skin, replicates as well in internal, not yet identified, organ or tissue. In such a context it is difficult to explain why IgG responses against TSPyV remain stable, while those against JCPyV and BKPyV clearly rise, unless the (transplanted) end organ, the kidney in the case of JCPyV and BKPyV, is of pivotal importance here. In this regard, the observed increasing serological trend for HPyV9 in KTR, comparable to JCPyV and BKPyV, might suggest that HPyV9 is nephrotropic. Cross-seroreactivity against HPyV9, BKPyV and JCPyV VP1 antigens to explain the concurrent rise in serum antibodies has been ruled out previously (199,212).

For BKPyV we have previously shown that post-transplantation increase in seroreactivity is related to duration and peak viral load of post-transplantation episodes of viremia (225). Unfortunately we are unaware of JCPyV activity and load after kidney transplantation in our KTR cohort, but it might be expected that JCPyV viremia can be detected in (a proportion of) KTR with increased JCPyV IgG responses, since JCPyV and BKPyV are phylogenetically closely related and share the same end organ. Since the kidney donor IgG response seems to influence both the JCPyV IgG response and the HPyV9 IgG response after transplantation, it appears that JCPyV and HPyV9, similar to BKPyV, are transplanted together with the kidney transplant, in a subset of kidney transplant patients. For JCPyV, this has been suggested previously by molecular comparison of JCPyV genomes before and after transplantation (213). Since JCPyV is a significant pathogen in the kidney transplant population, the influence of acquiring JCPyV through the kidney allograft should be subject of further study, especially for JCPyV seronegative recipients.

Table 2. Linear mixed effects models of polyomavirus IgG levels during follow-up

HPyV	Predictors*	Estimates	95% CI	p-value
JCPyV	Intercept	3770.70	3104.57 - 4436.83	<0.001
	Group	893.47	-532.20 - 2319.14	0.220
	Time	6.61	4.36 - 8.86	<0.001
	Group * Time	-7.39	-10.43 - -4.35	<0.001
MCPyV	Intercept	9428.54	8510.92 - 10346.16	<0.001
	Group	644.79	-1316.83 - 2606.41	0.520
	Time	0.22	-1.76 - 2.20	0.827
	Group * Time	-2.01	-4.69 - 0.66	0.140
TSPyV	Intercept	9334.44	8504.31 - 10164.56	<0.001
	Group	-692.83	-2467.29 - 1081.64	0.444
	Time	1.33	-0.39 - 3.05	0.131
	Group * Time	-0.23	-2.55 - 2.09	0.846
HPyV9	Intercept	483.37	-84.78 - 1051.53	0.096
	Group	2003.58	786.95 - 3220.21	0.001
	Time	4.74	2.59 - 6.88	<0.001
	Group * Time	-5.57	-8.47 - -2.68	<0.001
BKPpyV	Intercept	17122.68	16083.76 - 18161.59	<0.001
	Group	5767.08	3543.90 - 7990.27	<0.001
	Time	8.26	4.87 - 11.65	<0.001
	Group * Time	-14.38	-18.95 - -9.81	<0.001

* Linear mixed models with random intercepts and fixed effects 'Group' (with kidney transplant patients as the reference group, opposed to blood donors) and 'Time' (in MFI / day). 'Group * Time' is the interaction term, which improved the fit of the model (as tested by ANOVA)

Assuming seroconversion results from primary infection, we compared the frequency of seroconversions in the KTR to the HBD controls, to find evidence for transplantation-related transmission of HPyVs. The highest seroconversion rates in KTR were observed for JCPyV and HPyV9, both 6.5%, which clearly differed from the HBD, where no seroconversions were noticed. For HPyV9, the majority of the seroconverters (80%; 20 out of 25, **Table 1**) were actually seroconverters, whereas for JCPyV this constituted 43%. Since BKPpyV seroprevalence in the general population is extremely high (**199,226**), there is no use in comparing the observed seroconversion numbers for this virus. Altogether, these data are suggestive of donor origin of at least a proportion of JCPyV and HPyV9 infections after transplantation in KTR. The actual size of kidney allograft-mediated HPyV transmission could be larger but is difficult to assess serologically, because transmission in seropositive recipients does not result in seroconversion. However, it could result in increased IgG responses, as we observed especially for JCPyV.

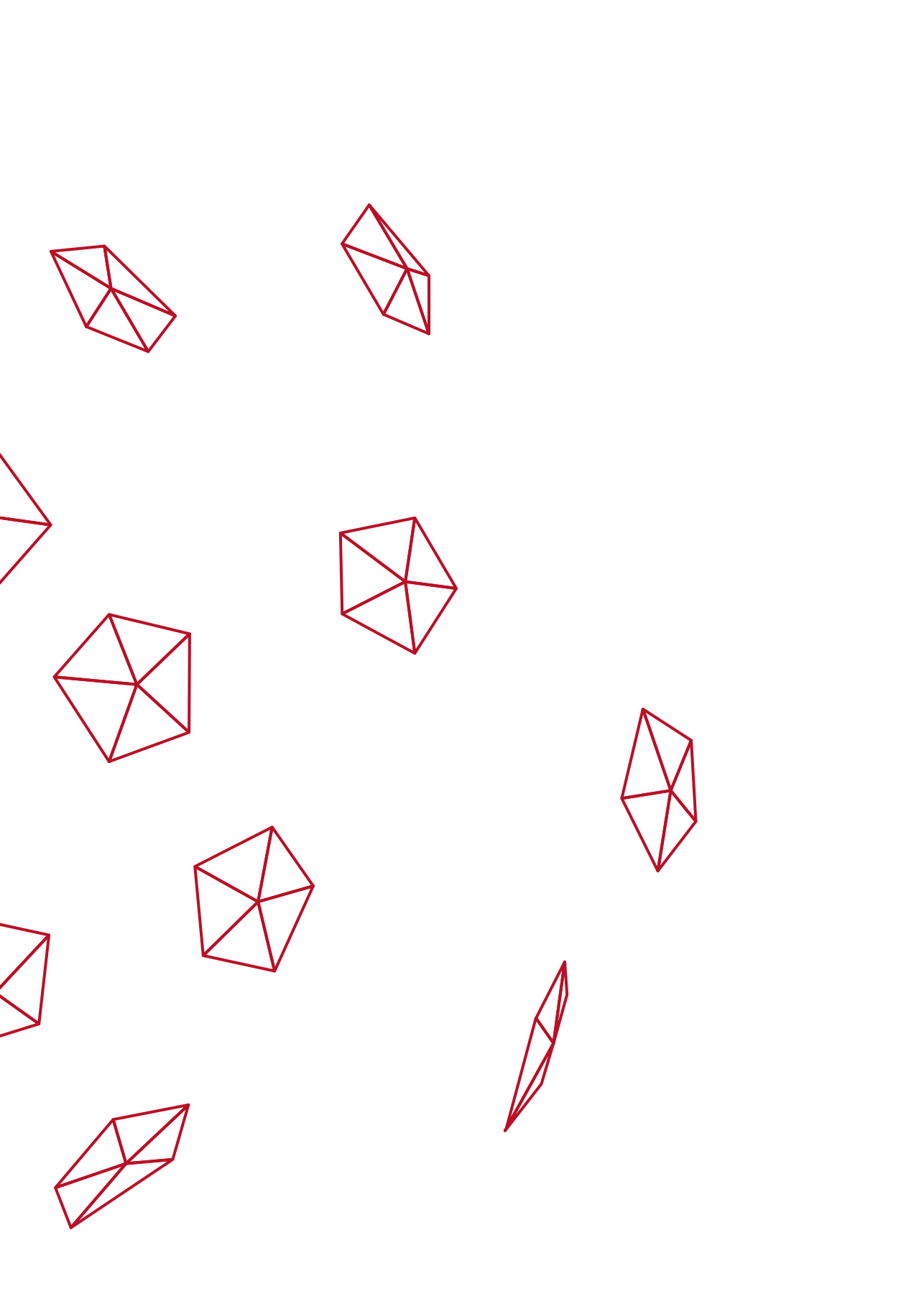
Table 3. Linear regression models of age, sex, donor MFI (per 1000) and MFI before transplant (per 1000).

HPyV	Predictors*	Estimates	95% CI	p-value
JCPyV	Intercept	1478.88	-527.43 - 3485.20	0.148
	Age	-0.58	-37.15 - 36.00	0.975
	Sex	417.88	-611.43 - 1447.18	0.425
	Donor MFI	80.03	4.80 - 155.26	0.037
	MFI before Tx	786.81	700.19 - 873.42	<0.001
MCPyV	Intercept	-94.62	-1634.34 - 1445.10	0.904
	Age	22.08	-5.19 - 49.36	0.112
	Sex	-67.06	-840.27 - 706.16	0.865
	Donor MFI	23.66	-27.66 - 74.99	0.365
	MFI before Tx	897.82	851.19 - 944.45	<0.001
TSPyV	Intercept	2138.27	584.61 - 3691.94	0.007
	Age	-25.24	-51.46 - 0.99	0.059
	Sex	399.16	-339.14 - 1137.45	0.288
	Donor MFI	7.07	-41.70 - 55.85	0.775
	MFI before Tx	901.62	855.10 - 948.14	<0.001
HPyV9	Intercept	823.64	-1002.89 - 2650.16	0.375
	Age	-1.26	-35.37 - 32.85	0.942
	Sex	217.19	-746.68 - 1181.06	0.658
	Donor MFI	188.46	56.63 - 320.30	0.005
	MFI before Tx	864.39	759.39 - 969.39	<0.001
	Donor MFI * MFI before Tx	-25.92	-47.07 - -4.78	0.016
BKPyV	Intercept	3456.13	359.01 - 6553.26	0.029
	Age	-1.54	-48.89 - 45.81	0.949
	Sex	476.73	-863.24 - 1816.70	0.484
	Donor MFI	89.58	18.45 - 160.71	0.014
	MFI before Tx	783.28	709.80 - 856.77	<0.001

* Interaction terms (Donor MFI * MFI before Tx) were tested for all polyomaviruses, but are only shown here if these improved the fit of the model (as tested by ANOVA). Tx: transplantation.

Further research is necessary to confirm our findings and to determine the clinical relevance of, for example, JCPyV allograft exposure for developing viremia, JCPyV-associated nephropathy, and perhaps even PML after kidney transplantation. A study in adult KTR has previously shown a correlation between kidney donor JCPyV IgG response and JCPyV viruria, suggesting the donor kidney to be the origin of JCPyV viruria in the recipient (227). Recently, transmission of JCPyV through the kidney allograft was demonstrated for a small number of kidney transplantation donor and recipient pairs with the help of metagenomic sequencing (213), comparable to what has been shown for BKPyV (88,124).

In conclusion, this study provides evidence that KTR are exposed to JCPyV and HPyV9 after transplantation (next to BKPyV). The origin of this exposure could lie in the transplanted kidney. Whether donor screening could provide insight in determining KTR risk of developing for instance JCPyV infection and related complications could be subject of further study.



Chapter 7

General discussion

Blood products and components are often administered to immunocompromised patients, including children with haemato-oncological diseases (4). Polyomavirus-related diseases occur mainly in immunocompromised patients, a group that is slowly growing in number. For example, the number of kidney transplant patients has increased worldwide (228) as well as the number of patients on immunomodulatory drugs, such as multiple sclerosis patients on natalizumab (229). Unlike other viral infections in immunocompromised patients, such as cytomegalovirus, no specific, proven effective treatments are available for polyomavirus-related diseases, other than reduction of immunosuppressive regimens. Interestingly, donor polyomavirus serology can help assess risk for BK polyomavirus-associated nephropathy (88) and primary infection is an important mechanism in the development of trichodysplasia spinulosa (77). This means that exposure rather than reactivation plays an important role in the occurrence of disease. Thus, prevention of exposure to polyomaviruses can be an important preventative measure for immunocompromised patients.

The primary purpose of this thesis was to gather prevalence data regarding polyomavirus infections in Dutch blood donors. In **Chapter 2**, an assay was described to measure seroreactivity in serum and in **Chapter 3** this assay was used to determine the seroprevalence of fourteen polyomaviruses in blood donors using a multiplex immunoassay. On average a donor was found to be seropositive for nine human polyomaviruses. Counterintuitively, because antibodies do not protect against polyomaviruses and polyomaviruses persist, the seropositive donors are the population in which viremia can occur (in addition to primary infection). In **Chapter 4** the DNA prevalence of polyomaviruses was determined by screening blood donors with a quantitative PCR. Overall, polyomaviral DNA was detected in 5% of blood donors, albeit at low viral loads. For JCPyV a correlation was observed between JCPyV viral load and JCPyV seroresponse.

Relating to blood safety, we now know that polyomaviruses are present in blood donations, yet the infectivity remains to be established (i.e. whether a PCR positive blood product can cause infection in a recipient). To further elucidate this matter, the determination of the presence of viral particles, rather than only DNA, is important. This can be done for example by inclusion of an endonuclease step before a qPCR test (230). In addition, blood components eligible for transfusion (e.g. plasma, red blood cells, platelets) should be tested, since detection of infectious viral particles in these samples will mean that polyomaviruses are transfused. This could be combined with serology in the blood transfusion recipients. Recently, studies using

metagenomics found evidence of polyomavirus presence in platelets (141), in red blood cell concentrates and fresh frozen plasma (140). Compared to qPCR, metagenomics is still limited in sensitivity and it is likely to find a higher prevalence with qPCR.

Plasma, platelets and to a lesser extent red blood cell concentrates are often treated with pathogen inactivation techniques, such as solvent-detergent or photochemical inactivation. However, polyomaviruses are non-enveloped while many inactivation techniques mainly disrupt the lipid membrane of viruses. As a result, these pathogen inactivation techniques do not affect polyomaviruses, comparable to other non-enveloped viruses, like Parvovirus B19 and Hepatitis A virus (50). Unlike parvovirus and hepatitis A virus, where antibodies present in blood donations protect against infection of blood transfusion recipients, polyomavirus antibodies are a poor predictor of immunity against polyomavirus infection. They merely reflect recent viral activity (replication, increased viral load) and seem to function as a determinant of the viral reservoir, as was shown for Merkel cell polyomavirus (231) and for BK polyomavirus (88). As such, donors with high polyomavirus antibodies may experience a relatively higher viral load in blood donations and pose a higher risk for recipients. Donor deferral based on serology is often used in infectious risk management for hemovigilance, for example for HIV. However, since polyomavirus seropositivity is so common, this would be ill-advised. Furthermore, any risk associated with polyomaviruses would generally be limited to immunocompromised recipients, who are more susceptible to infection from any source, including blood transfusion.

Since blood transfusions are frequently given to immunocompromised patients, it requires special care to protect these patients from infectious risk. In recent years, a prime example of hemovigilance for the protection of immunocompromised patients has been the start of screening for Hepatitis E virus (HEV), specifically genotype 3. HEV is a nonenveloped virus, which is found primarily in pigs and can be acquired by healthy persons through consumption of uncooked or cured meat, such as sausages or gammon. This can lead to an asymptomatic period of HEV viremia in healthy persons (232). Unfortunately, HEV is not inactivated by solvent / detergent treatment. Through blood transfusion, this can then be acquired by immunocompromised patients (233). Recently, the screening of blood donors for the presence of HEV has increased in Europe (9). In the Netherlands, this is currently done by screening all blood donors. Although the risk of a transfusion-acquired HEV infection is low (9), it is apparently high enough to merit the screening of all donors. For polyomaviruses, it may be more sensible to work towards selective thorough

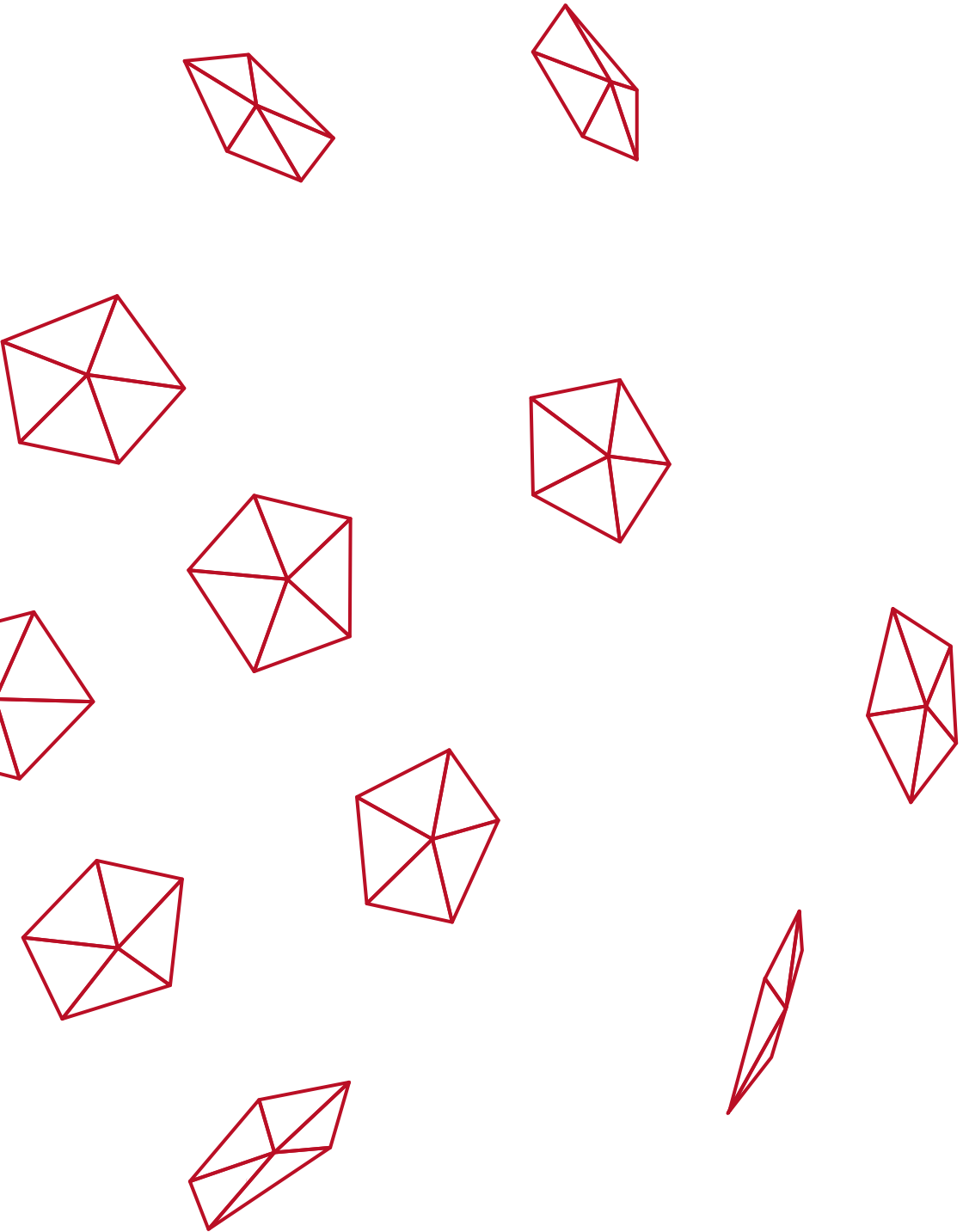
screening for transfusion transmitted infections specifically for blood products that are transfused to immunocompromised patients.

In **Chapter 6**, we describe that kidney transplantation can lead to an increased response to or seroconversion for BKPyV, JCPyV and HPyV9, likely because of the presence of these viruses in the kidney transplant. This is troublesome, because both BKPyV and JCPyV can lead to polyomavirus-associated nephropathy in kidney transplant recipients. Previously, it was demonstrated that pretransplantation serological screening can predict risk of development of BKPyV viremia (88). Similar mechanisms may play a role for JCPyV, although JCPyV-associated nephropathy is rare (234). Furthermore, donor-derived JCPyV was recently demonstrated through metagenomic sequencing in the urine of kidney transplant patients, despite presence of antibodies against JCPyV in the kidney transplant patients (213). Next to JCPyV-associated nephropathy, JCPyV can also cause progressive multifocal leukoencephalopathy in both solid organ transplant recipients and bone marrow transplant recipients (235). Further study of polyomaviruses in immunocompromised patients may lead to better prevention of polyomavirus-related disease. For example, patients at high risk for disease can be screened more intensively, which can lead to earlier diagnosis and better prognosis. While patients at low risk do not require intensive monitoring and thus less frequent controls.

Pathogen detection requires well-validated, specialized tools to adequately estimate infectious risks. In **Chapter 2** the lab-developed immunoassay was extensively validated, whereas in **Chapter 5** the qPCRs were retrospectively analyzed with a novel *in silico* evaluation. In recent years, the sequencing of whole (viral) genomes has become easier, due to advanced sequencing techniques and bioinformatic tools. It has been estimated that hundreds of thousands of viruses are currently unknown in mammals alone (236) and a recent study showed that discovery of several thousand new viruses is already possible with current techniques (237). Inevitably, the number of known viral genomes will increase enormously in the coming years, including variants of known pathogens that may be poorly recognized. Automated determination of probability of detection of new viral genomes by existing qPCRs using *in silico* evaluation will be extremely helpful to keep current diagnostics up-to-date.

Not all challenges relating to human polyomavirus transmission have been solved by the studies performed in this thesis. However, the creation of well-validated molecular tools and the first evidence of polyomavirus transmission will provide

a solid base for follow-up research. Specifically, the identification of infectious particles in different blood components will be vital to further substantiate any risk. Furthermore, developments in next generation sequencing allow easier identification of donor-derived human polyomavirus transmission.



Chapter 8

Addendum

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Summary

Polyomaviruses are a family of viruses with a small genome, wrapped in a viral particle with a diameter of 40-45 nanometres. In 1971, the first two human polyomaviruses were discovered, called BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV), named after the initials of the first patients. Since then, many new polyomaviruses were discovered, both in humans and all kinds of animals, adding up to about a hundred different *species* now. The focus of this thesis is on the human polyomaviruses.

Polyomaviruses are able to persist in the human body. This means that a person, once infected, does not lose the virus despite an immune response. Infection with polyomaviruses starts very early in life, but does not cause any symptoms. Afterwards, polyomaviruses are secreted from time to time in healthy humans, for example through the skin or in urine, without occurrence of any symptoms.

Polyomaviruses however are capable of causing disease, but this is mostly limited to persons with a compromised immune system. For example, BKPyV can cause an infection in kidney transplant patients, called polyomavirus-associated nephropathy (PVAN). Some of these patients will lose the transplanted kidney due to BKPyV. Another disease caused by BKPyV is hemorrhagic cystitis, a severe cystitis occurring primarily in stem cell transplant recipients. JCPyV can cause a severe brain disease, called progressive multifocal leukoencephalopathy (PML). PML used to occur primarily in HIV patients, but the disease burden has shifted to patients on certain immunomodulatory drugs, e.g. multiple sclerosis patients taking natalizumab. These patients are screened using serology, in other words, by detecting the presence of JCPyV antibodies in the blood. Merkel cell polyomavirus is the primary cause of Merkel cell carcinoma, a rare but difficult to treat skin tumour. These tumours are more prevalent in the immune compromised population compared to the healthy population, but also occur in elderly without immune disorders. Trichodysplasia spinulosa polyomavirus (TSPyV) causes the eponymous disease, which has a characteristic skin phenotype of 'spicules' in primarily the face. Next to these, some other diseases have been associated with human polyomaviruses.

Because human polyomaviruses persist and are shedded by healthy humans, there is a possibility that these viruses are also present in blood donations. A large part of blood donations are transfused to the immunocompromised population, who can get

polyomaviruses-related diseases. It is currently unknown whether human polyomaviruses are present in Dutch blood donations and thus contribute to exposure of these patients to polyomaviruses. In this thesis, the presence of polyomaviruses in Dutch blood donors has been determined in two ways, namely by detecting antibodies directed against polyomaviruses (serology) and by detecting DNA of polyomaviruses using quantitative polymerase chain reaction (PCR).

The presence of antibodies against polyomaviruses proves that these donors have been infected with the specific virus. Because polyomaviruses persist, that also means that seropositive donors are capable of shedding these viruses. To measure the antibody status, we have developed an immunoassay that uses the Viral Protein 1 (VP1), which forms the exterior of the polyomavirus particle and is targeted by the immune response. This immunoassay is described in **Chapter 2**, and uses the fourteen different VP1 proteins from the fourteen tested polyomaviruses. Because these VP1 proteins are quite similar to each other, the cross-reactivity was determined. There was some cross-reactivity between human polyomavirus 6 (HPyV6) and HPyV7 and sporadically between BKPyV and JCPyV. This test has then been applied to over one thousand blood donors in **Chapter 3**. In that chapter, it is described that, on average, a blood donor is seropositive for nine different human polyomaviruses and hence a donor carries and can also shed these viruses. In other words, polyomaviruses are highly prevalent in the Dutch blood donor population and it is necessary to determine what this means for the recipients of blood products.

Furthermore, for three polyomaviruses antibodies were almost absent, namely for HPyV12, New Jersey polyomavirus (NJPyV) and Lyon IARC polyomavirus (LIPyV). The absence of antibodies against these polyomaviruses can be explained if humans are not the true hosts of these viruses. Indeed, HPyV12 appears to be a shrew polyomavirus and LIPyV is likely a cat polyomavirus.

In **Chapters 2** and **3**, it is demonstrated that an immune response against polyomaviruses has occurred in blood donors. In **Chapter 4** however, the presence of polyomavirus DNA is determined. DNA detection was performed with quantitative PCR. These tests use small pieces of DNA, called oligos, which match the DNA of the targeted viral genome like pieces of a puzzle. If there is a good match between oligos and the genome, an amplification reaction occurs and is detected by fluorescence. In total, we have found evidence of small quantities of polyomavirus DNA in 5% of the blood donors. In addition, an association was found between the presence of JCPyV DNA and JCPyV antibodies, namely that donors with JCPyV also have an increased JCPyV seroresponse.

In **Chapter 5** the quality of the quantitative PCRs is evaluated, both of our qPCRs and qPCRs acquired from literature. As described earlier, the viral DNA is detected by oligos which match the viral genome like pieces of a puzzle. In this chapter, algorithms were applied to determine the fit of the oligos on the viral DNA and whether these oligos do not accidentally detect other viral genomes, for example animal polyomaviruses. From these calculations, the sensitivity and specificity were calculated. Of special interest were possible adjustments to increase the sensitivity of the qPCR, which was done for the TSPyV qPCR. In conclusion, the digital evaluation of qPCRs provides insight into strong and weak points of qPCRs and is helpful to keep these tests up to par with current knowledge of viral genomes.

Furthermore, in **Chapter 6** we have examined another possible source of exposure to polyomaviruses for kidney transplant patients, namely the kidney allograft. From previous research, we knew that BKPyV is transplanted along with the kidney allograft. In addition, we've determined the serostatus of these patients by applying the immunoassay from **Chapter 2** before and after transplantation, next to the kidney donor. Next to BKPyV, there were a relatively large number of seroconversions and increased seroresponses to JCPyV. Furthermore, kidney donor JCPyV IgG level influenced recipients IgG level after transplantation, which means JCPyV may be transplanted along with the kidney as well.

In conclusion, in this thesis it is shown that the serum of about 5% of blood donors is positive for polyomavirus DNA and on average blood donors are seropositive for nine different polyomaviruses. Blood transfusions are not the only source of exposure, as was demonstrated for BKPyV and JCPyV in kidney transplant patients. Knowledge of all possible sources of exposure can contribute to the prevention of infection. For follow-up, it is necessary to validate these results in blood components, such as red blood cells, plasma and platelets, rather than serum tubes used for diagnostics. Furthermore, it should be determined whether the viruses detected here are infectious viral particles or remnants of viral DNA. The combination of infectious disease occurrence data from immunocompromised patients in combination with blood transfusion data can be of key importance to determine the infectious risk for this group. The availability of new immunomodulatory drugs will increase in the coming years, as will the number of opportunistic infections in patients that are prescribed these drugs. Solid knowledge of polyomavirus prevalence will contribute to prevention of polyomavirus-related diseases in this group.

Samenvatting

Polyomavirussen zijn een familie virussen met een klein genoom, verpakt in een viruspartikel met een doorsnede van 40-45 nanometer. In 1971 werden de eerste twee humane polyomavirussen ontdekt, genaamd BK polyomavirus (BKPyV) en JC polyomavirus (JCPyV), genoemd naar de patiënten waar zij uitkomen. Sindsdien zijn er echter veel nieuwe polyomavirussen ontdekt, zowel in mensen als in allerlei dieren, in totaal zijn er nu ongeveer honderd verschillende *species* bekend. De focus van dit proefschrift ligt bij de humane polyomavirussen.

Eén van de bijzonderheden van polyomavirussen is dat ze in staat zijn om te persisteren in het menselijk lichaam. Dit betekent dat zodra iemand is geïnfecteerd, deze het virus vervolgens niet meer kwijtraakt. Dit begint al op zeer jonge leeftijd, maar gelukkig verloopt deze infectie asymptomatisch. Daarna worden polyomavirussen van tijd tot tijd uitgescheiden door gezonde mensen in bijvoorbeeld de huid of urine. Desalniettemin leidt dit niet tot symptomen en is men zich hier niet van bewust.

Polyomavirussen zijn wel in staat ziektes te veroorzaken, maar dit komt met name voor bij mensen met een stoornis in het immuunsysteem. BKPyV kan bijvoorbeeld een infectie veroorzaken bij niertransplantatiepatiënten, genaamd polyomavirus-associated nephropathy (PVAN). Bij een deel van deze patiënten zal dit leiden tot het verlies van de getransplanteerde nier. Ook kan BKPyV hemorragische cystitis veroorzaken, een ernstige blaasontsteking die met name bij stamceltransplantatiepatiënten voorkomt. JCPyV kan leiden tot een ernstige hersenziekte, progressieve multifocale leukoencefalopathie (PML). PML kwam vroeger met name voor bij HIV patiënten, maar wordt tegenwoordig voornamelijk gezien bij patiënten die gebruik maken van bepaalde immuunsysteem modulerende medicijnen, met name multiple sclerose patiënten die gebruik maken van natalizumab. Deze patiënten worden gescreend op middels serologie, oftewel de aanwezigheid van antilichamen tegen JCPyV in het bloed. Merkel cell polyomavirus (MCPyV) is de voornaamste oorzaak van het Merkelcelcarcinoom, een zeldzame maar moeilijk te behandelen huidtumor. Deze tumoren komen in hogere mate voor bij mensen met een immuunstoornis, maar ook bij ouderen zonder immuunstoornis. Trichodysplasia spinulosa polyomavirus (TSPyV), veroorzaakt het gelijknamige ziektebeeld, wat een karakteristiek huidbeeld geeft van 'spicules' in met name het gelaat. Daarnaast zijn nog enkele andere ziekten in verband gebracht met humane polyomavirussen.

Aangezien humane polyomavirussen persisteren en uitgescheiden worden door gezonde mensen, is er een mogelijkheid dat deze virussen zich ook in bloeddonaties bevinden. Een steeds groter deel van de bloeddonaties komt bij patiënten terecht met een gecompromitteerd immuunsysteem. Dit zijn bij uitstek de mensen die ziek kunnen worden van polyomavirussen. Het is onbekend of polyomavirussen in bloeddonaties voorkomen en dus kunnen bijdragen aan blootstelling van deze kwetsbare patiëntengroep. Op twee manieren is dit in kaart gebracht, namelijk door het meten van antistoffen tegen deze virussen (serologie) en door DNA te detecteren in het serum van bloeddonoren middels kwantitatieve polymerase chain reaction (PCR).

De aanwezigheid van antistoffen tegen polyomavirussen duidt erop dat bloeddonoren geïnfecteerd zijn geweest met het desbetreffende polyomavirus. Aangezien deze virussen persisteren, betekent dit ook dat de seropositieve bloeddonoren dit virus kunnen uitscheiden. Om dit te meten, hebben we een immuniteitstest ontwikkeld die gebruik maakt van het Viral Protein 1 (VP1), wat de buitenkant van het viruspartikel vormt en waar de afweer zich tegen richt. In **Hoofdstuk 2** is deze immuniteitstest beschreven, die gebruik maakt van de veertien verschillende VP1 eiwitten van de veertien geteste polyomavirussen. Aangezien deze VP1 eiwitten op elkaar kunnen lijken, is uitgebreid getest op kruisreactiviteit, waar bij humaan polyomavirus 6 (HPyV6) en HPyV7 en sporadisch bij BKPyV en JCPyV enige sprake van was. Deze test is vervolgens toegepast op ruim duizend bloeddonoren in **Hoofdstuk 3**. Daaruit bleek dat een bloeddonor gemiddeld seropositief is voor negen verschillende polyomavirussen, en deze dus ook kan uitscheiden. Met andere woorden, polyomavirussen zijn veelvoorkomend onder bloeddonoren en het is dus noodzakelijk om uit te zoeken wat dit uiteindelijk betekent voor de ontvangers van bloedproducten.

Daarnaast bleek dat voor drie polyomavirussen zeer weinig antistoffen aanwezig zijn, namelijk voor HPyV12, New Jersey polyomavirus (NJPyV) en Lyon IARC polyomavirus (LIPyV). Later bleek één van deze polyomavirussen spitsmuizen te infecteren in plaats van mensen. Zelf hebben we serum monsters van katten onderzocht en antistoffen tegen LIPyV gedetecteerd, waaruit blijkt dat dit een kattenvirus is.

Waar in **Hoofdstukken 2 en 3** vooral gezocht werd naar bewijs dat het immuunsysteem in contact is geweest met polyomavirussen, wordt in **Hoofdstuk 4** gezocht naar het DNA van het virus zelf. Dit werd gedaan met behulp van kwantitatieve PCR testen. Deze testen maken gebruik van kleine stukjes DNA, ook wel oligo's genoemd, die als puzzelstukjes op het genoom van het virus passen. Indien er een goede match is, wordt het DNA vermenigvuldigd en kan dit met fluorescentie gedetecteerd worden.

In totaal vonden we bij ongeveer 5% van de donoren bewijs van de aanwezigheid van kleine hoeveelheden van polyomavirus DNA. Daarnaast vonden wij een verband tussen de aanwezigheid van JCPyV DNA en JCPyV serologie, namelijk dat donoren positief voor JCPyV DNA, tevens een hogere serologische uitslag hebben.

In **Hoofdstuk 5** is de kwaliteit van de gebruikte PCR testen geëvalueerd, zowel van onszelf als van PCR testen uit de literatuur. Zoals eerder beschreven, wordt het DNA gedetecteerd met oligo's die als puzzelstukjes op het genoom passen. Hier is met algoritmes berekend hoe goed deze puzzelstukjes passen op de gewenste genomen en of deze niet per abuis ook andere polyomavirus genomen kunnen detecteren, bijvoorbeeld van dieren afkomstig. Hieruit werden de sensitiviteit en specificiteit berekend. Van bijzonder belang, waren mogelijke aanpassingen aan de PCR om de sensitiviteit te verbeteren, zoals gedaan werd voor de TSPyV PCR. Concluderend, digitale evaluatie van PCR maakt sterke en zwakke punten inzichtelijk en is behulpzaam bij het up-to-date houden van deze testen.

Verder hebben we in **Hoofdstuk 6** gekeken naar een andere mogelijke bron van blootstelling voor polyomavirussen aan niertransplantatiepatiënten, namelijk de gedoneerde nier. We wisten van eerder onderzoek al dat BKPyV waarschijnlijk overgedragen worden met de gedoneerde nier. Daarnaast hebben we nu ook naar de polyomavirussen JCPyV, MCPyV, TSPyV en HPyV9 gekeken, middels de serologische test uit **Hoofdstuk 2** voor en na transplantatie. Naast BKPyV, waren er een relatief groot aantal seroconversies en stijging van serologische waarden voor JCPyV, wat erop duidt dat JCPyV in de getransplanteerde nier zit.

Concluderend, in dit proefschrift wordt aangetoond dat in het serum van ongeveer 5% van de bloeddonoren het DNA van polyomavirussen gedetecteerd wordt en dat bloeddonoren gemiddeld positief zijn voor negen verschillende polyomavirussen. Bloedtransfusies zijn natuurlijk niet de enige potentiële bron van besmetting, ook bijvoorbeeld een niertransplantaat voor zowel BKPyV als JCPyV. Kennis over alle mogelijke besmettingsbronnen kan bijdragen aan het voorkomen van infecties. In de toekomst is het belangrijk om deze resultaten te valideren in de bloedcomponenten zelf, zoals rode bloedcellen, plasma en bloedplaatjes, in plaats van in de serumbuizen voor de diagnostiek. Verder moet vastgesteld worden of het daadwerkelijk om infectieuze viruspartikels gaat of om 'restanten' DNA. Ook het betrekken van gegevens over infectieziekten bij immuungecompromitteerde patiënten in combinatie met bloedtransfusie data kan een sleutelrol spelen in het verhelderen van infectierisico voor deze groep. Met het beschikbaar komen van nieuwe immuunsysteem modulerende middelen de komende jaren, zal deze groep groter worden en zal dus

het risico op opportunistische infecties ook toenemen. Gedegen kennis over het voorkomen van deze polyomavirussen zal een positieve bijdrage leveren aan het voorkomen van infecties in deze groep.

Portfolio

PhD student: Sergio Kamminga
 PhD period: June 2016 - July 2020
 PhD supervisors: Hans Zaaijer and Mariet Feltkamp

1. PhD training	Year	Workload (Hours)
General courses		
AMC World of Science	2016	19.6
eBROK	2017	28
Crash course for MDs	2017	5.6
Sanquin Science Course	2017	17
Practical Biostatistics	2017	40
Communication in Science	2018	38
Sanquin Personal Development	2019-2020	30
Specific courses		
Virology (Erasmus MC)	2016	50.4
NGS Introduction Course (LUMC)	2017	2.8
Bioinformatics	2019	30
Bioinformatics Sequence Analysis	2019	30
Practical Linux	2018	5
Infectious Diseases	2019	36
Seminars, workshops and master classes		
Dutch annual virology symposium (DAVS)	2018	7
Symposium "Why we study viruses"	2018	7
CMAT Symposium	2018	4
Presentations		
Poster presentation at NVMM spring meeting	2017	2
Oral presentation at ESCV, Stresa, Italy	2017	2
Poster presentation at xMAP connect, Amsterdam	2017	2
Poster presentation Sanquin Science Day	2017	2
Poster presentation at NVMM spring meeting	2018	2
Poster presentation Sanquin Science Day	2018	2
Poster presentation at NVMM spring meeting	2019	2
Oral Presentation at American Society for Virology, Minneapolis, Minnesota	2019	2
Oral Presentation at ESCV, Copenhagen, Denmark	2019	2

1. PhD training (continued)	Year	Workload (Hours)
(Inter)national conferences		
International congress HPV & Polyomavirus in skin cancer, 16-18 november, MECC Maastricht	2016	21
NVMM Spring Meeting, 11-12 April, Papendal	2017	14
European Society for Clinical Virology, 13-16 September, Stresa, Italy	2017	28
NVMM Spring Meeting, 27-28 March, Papendal	2018	14
NVMM Spring Meeting 26-27 March, Papendal	2019	14
American Society for Virology meeting, 20-24 July, Minneapolis, Minnesota	2019	50
European Society for Clinical Virology, 10-14 September, Copenhagen, Denmark	2019	35

2. Teaching	Year	Workload (Hours)
Lecturing		
Sanquin monday afternoon lecture	2018	1
Center Infectious Disease lecture	2019	1
Supervising		
Michael Tadesse	2019	84

3. Parameters of Esteem	Year
Grants	
ESCV Travel grant	2017
ASV Travel grant	2019
NWKV Travel Grant	2019
Awards and Prizes	
Poster prize xMAP connect	2017

4. Publications

Peer reviewed

Kamminga, S., Meijden, E. van der, Wunderink, H.F., Touzé, A., Zaaijer, H.L., Feltkamp, M.C.W., 2018. Development and Evaluation of a Broad Bead-Based Multiplex Immunoassay To Measure IgG Seroreactivity against Human Polyomaviruses. *J. Clin. Microbiol.* 56, e01566-17 *Recommended article on F1000Prime*

Kamminga, S., Meijden, E. van der, Feltkamp, M.C.W., Zaaijer, H.L., 2018. Seroprevalence of fourteen human polyomaviruses determined in blood donors. *PLOS ONE* 13, e0206273.

Kamminga, S., van der Meijden, E., de Brouwer, C., Feltkamp, M., Zaaijer, H., 2019. Prevalence of DNA of fourteen human polyomaviruses determined in blood donors. *Transfusion* 59, 3689-3697.

López-Labrador FX, et al. ESCV Network on Next-Generation Sequencing. Recommendations for the introduction of metagenomic high-throughput sequencing in clinical virology, part I: Wet lab procedure. *J Clin Virol.* 2021 Jan;134:104691. doi: 10.1016/j.jcv.2020.104691. Epub 2020 Nov 18. PMID: 33278791.

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Kamminga, S., van Rijn, A.L., de Brouwer, C.S., Rotmans, J.I., Zaaijer, H.L., Feltkamp, M.C.W., 2021. JC and Human polyomavirus 9 after kidney transplantation: An exploratory serological cohort study. *Journal of Clinical Virology* 143, 104944.

Other

Polyomavirussen en hun rol bij cutane infecties en kanker. VAPvisie Analyse - 03/2019

Curriculum vitae

Sergio Kamminga was born on the 11th of January 1991 in Zoetermeer. After finishing the Gymnasium at the Oranje Nassau College in Zoetermeer, in the directions Nature & Technology and Nature & Health, he proceeded to study Medicine at the Leiden University Medical Centre in Leiden. Fascinated by infectious diseases, he performed a scientific internship with a focus on molecular diagnostics and polyomaviruses at the Department of Medical Microbiology. After obtaining his medical degree in 2016, he started his PhD studies, under supervision of prof. dr. H.L. Zaaijer and dr. M.C.W. Feltkamp, in a collaboration between the Department of Blood-borne Infections of Sanquin and the Department of Medical Microbiology of the LUMC, focused on the prevalence polyomaviruses in blood donors. After his PhD studies, Sergio will continue to work in infectious diseases and medicine as a resident in Medical Microbiology in the LUMC.

