SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Prevalence and *in vitro* study of human polyomaviruses

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DEBRECEN, 2025

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The PhD Defense takes place at the Lecture Hall of the Department of Internal Medicine Building ”A”, Faculty of Medicine, University of Debrecen on the 7th of April 2025 at 13:00.

List of abbreviations

BKPyV	BK polyomavirus
E	early region
9 HPyV9	reference sequence
9_E HPyV9	reference sequence early promoter activity
9_L HPyV9	reference sequence late promoter activity
EV	empty pcDNA3.1+ vector containing no insert
HPyV	Human polyomavirus
HPyV9	Human polyomavirus 9
JCPyV	JC polyomavirus
L	late region
LTAg	large tumour antigen
MWPyV	MW polyomavirus
NCCR	non-coding, control region
OD	optical density
PyV	polyomavirus
RLU	relative luciferase unit
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
STLPyV	STL polyomavirus
UF-1_E	HPyV9 UF-1 isolate early promoter activity
UF-1_L	Late promoter activity of HPyV9 UF-1 isolate
VP1	virus protein 1

Introduction

Currently, 13 viruses within the *Polyomaviridae* family are classified as human pathogens, but only four of these are linked to disease. The first discovered BK and JC polyomaviruses (BKPyV and JCPyV) are ubiquitous in the population, although the way of transmission and the portal of entry is still not clarified. Both establish latent infection, and their reactivation poses a serious risk in immunosuppressed patients. Their genetic variants show geographic distribution.[1,2] Merkel cell polyomavirus (MCPyV) is a tumour virus that causes Merkel cell carcinoma, a rare but extremely poor prognosis skin tumour. Seroprevalence data suggest that the virus infects the majority of humans, but the exact mechanism of oncogenesis is not yet fully understood. DNA prevalence studies suggest transmission by skin-to-skin contact, respiratory secretions, saliva and the faecal-oral route. However, it is still unclear exactly in which cells it replicates.[3] Trichodysplasia spinulosa associated polyomavirus (TSPyV) has been identified from a rare skin disease described exclusively in immunosuppressed patients. Interestingly, not only reactivation of the virus can cause clinical symptoms, but also primary infection. Although serological data suggest that the virus is widespread in the population, the disease it causes is rarely diagnosed.[4] In addition to pathogenesis, the exact mode of transmission and site of replication remain open questions. For the other human polyomaviruses, such as human polyomavirus 9, MW polyomavirus and STL polyomavirus, which were studied in this thesis, even less information is available. To clarify the basic virological questions, we need to know when primary infection may occur, how widespread they are, whether there are geographical differences, and possibly whether there are patient groups that are more susceptible to infection. Seroprevalence studies can help answer these questions. DNA prevalence studies can help to find modes of transmission and sites of replication. Genome sequencing and sequence analysis can determine whether these viruses have different genotypes and whether they are geographically distinct. Analysis of variance in non-coding control regions, followed by functional analysis of these regions in vitro, may help to reveal possible biological differences and identify potential target cells for the viruses.

Literature review

The first member of the *Polyomaviridae* family was isolated from mice in the 1950s.[5] Polyomavirus (PyV) is a Greek name referring to the tumorigenic property of the virus: it is a compound of the words poly (=many) and oma (=tumour),. The International Committee for Taxonomy of Viruses (ICTV, https://ictv.global) currently lists 118 species in the family *Polyomaviridae* that infect mammals, including humans, birds and fish.

The first human pathogenic viruses, BK and JC polyomaviruses, were described in 1971. BKPyV was isolated from the urine of a renal transplant patient with ureteral stenosis and JCPyV from the brain tissue of a Hodgkin's lymphoma patient with progressive multifocal leukoencephalopathy.[1,2] Both KI and WU polyomaviruses (KIPyV and WUPyV) were described from nasopharyngeal samples of children with respiratory disease in 2007. Despite research since then, their pathogenic role has not been identified.[6,7] In 2008, MCPyV was identified from a rare but very poor prognosis skin tumour, Merkel cell carcinoma. To date, it is the only human pathogenic polyomavirus that is clearly recognised as a tumour virus.[3,8] Human polyomaviruses 6 and 7 (HPyV6, HPyV7) were described from skin samples taken from healthy individuals in 2010.[9] Some case reports suggest that both viruses can cause skin symptoms associated with hyperkeratosis in immunosuppressed individuals. TSPyV was also described in 2010 from a spicular lesion in the skin of a transplant patient with trichodysplasia spinulosa. The virus is the cause of the skin disease that gives the virus its name.[4] A year later, the following virus, human polyomavirus 9 (HPyV9), was published from the serum of a kidney transplant patient.[10] In 2012, the MW polyomavirus (MWPyV),[11] one year later, STL polyomavirus (STLPyV) was described from stool samples.[12] In 2014 New Jersey polyomavirus (NJPyV) was identified from a muscle biopsy of a transplant patient,[13] and in 2017, LI polyomavirus (LIPyV) was sequenced from skin samples.[14] None of the last five viruses can currently be linked to disease. A few years ago, two new suspected human pathogenic polyomavirus genomes were described, in 2019 Quebec polyomavirus (QPyV) was described from faeces,[15] and in 2023 HPyV16 was described from skin samples.[16] These have not yet been classified by ICTV as part of the virus family. The genome of human polyomavirus 12 (HPyV12) was sequenced from liver tissue in 2013.[17] As an almost identical sequence has been published from shrews, the host species of the virus is currently in question and it is not listed as a human pathogen.[18]

Polyomaviruses are small, 45–50 nanometres in diameter, without envelope and with an icosahedral symmetry. Their capsid is composed of 72 capsomeres, the main component of which is the VP1 (viral protein 1) protein, which forms pentamers. The stability of the capsomeres is ensured by linkages between C-terminal regions, which are further reinforced by disulfide bonds and calcium ions. Each capsomer is linked to the inside of the virion by a VP2 or VP3 protein. Sequence analysis suggests the presence of VP4 protein in BKPyV and JCPyV, similar to some animal polyomaviruses.[19] Since the VP1 protein is located on the outer surface of the capsid, it determines the receptor specificity of the virus, i.e. the cells to which it can bind. Neutralising antibodies are produced against it in those who have been infected.[20] Polyomaviruses have a circular, double-stranded DNA genome. The functional units of the

genome are the early (E), the late (L) and the non-coding control region (NCCR) in between. Interestingly, transcription from both strands occurs in opposite directions. The early region encodes tumour antigens (TAg). The large T antigen (LTAg) has conserved motifs and has several functions: it plays a key role in regulating gene expression and viral genome replication. The late region encodes structural proteins that form the capsid, and microRNAs are also synthesized from this region. In addition, agnoprotein is also produced from the late region of BKPyV and JCPyV, which stimulates viral DNA replication and virion assembly.[21] The NCCR region plays a key role in the regulation of cell tropism and gene expression in polyomaviruses, determining the cell types in which the virus can replicate. It contains the replication origin, LTAg binding site(s), promoters and enhancers in both directions. The NCCR sequences of different viral species are very different, and as hypervariable regions, show considerable variability within species.[22]

Human polyomavirus 9

Based on studies published to date on HPyV9, the adult seroprevalence ranges from 11% to 47%. In addition to the considerable variation in the data, there is also variation in the positivity rates in different age groups. Increasing rates of seropositivity with age have been reported, while other research groups have seen a stable seroprevalence in adulthood or even a decrease in older people. Others have reported low levels that vary little with age.[23-26] Differences in data may be explained by differences in methodology, differences between study groups, or even geographical differences. HPyV9 DNA has been detected at low prevalence in blood,[10,27-31] urine,[10,28] respiratory tract specimen,[28,31] skin sample,[28] tonsils[32] and also from lung tissue.[28] Others have not detected the virus in blood,[23,33] urine,[23-25] skin sample,[27,34] respiratory tract specimen,[23,35,36] tonsils,[37-39] stool[36] or liquor. [36,40] The mode of transmission of HPyV9 remains a mystery, and the investigation of transmission via the respiratory route, oral route, or skin-to-skin contact is warranted. The presence of HPyV9 in various tumours has been tested, all tests were negative.[39,41-46] It has been suggested that immunosuppression may increase susceptibility to viral infection and, if latent infection develops, reactivation. In one study, a significantly higher rate of viraemia was detected in kidney transplant recipients compared to healthy blood donors, and a higher rate of seroconversion was also confirmed compared to the control group.[11] However, others have not found similar results in either kidney transplant[47] or allogeneic stem cell transplant patients.[48] In a case series analysis, HPyV9 DNA and even mRNA were detected in several samples from three transplant patients, but no other known pathogens were detected. All three patients initially developed skin symptoms and died within a year of multiple organ failure.[28]

In vivo cell tropism of HPyV9 is unknown.[49]

MW polyomavirus

The pathogenic role of the virus, the mode of transmission, the site of replication are not known, and relatively little DNA and seroprevalence data are currently available.

Seroprevalence studies are rather inconsistent, with adult seropositivity rates of 41–99% reported so far.[34,50-54] In this case, methodological differences may also be behind the different data from different parts of the world, but geographical variation in prevalence cannot be ruled

out. Indeed, there may even be geographically different viruses, genotypes or serotypes. MWPyV DNA has been detected in 0.5–12.8% of stool samples from healthy children and adults with gastrointestinal symptoms, suggesting that the virus may be transmitted via the faecal-oral route.[55,56] It was also detected in 0.7–9.2% of respiratory tract samples, mostly in children.[56,57] This suggests that respiratory transmission may also be suspected. Previously our team did not detect the genome of the virus in lung tissue samples.[58] However, the presence of MWPyV DNA has also been confirmed in tonsil and adenoids.[38,39] The virus has also been detected in condyloma[59] and other skin samples.[60,61] Blood, urine and cerebrospinal fluid samples were analysed to investigate the dissemination within the body, but MWPyV DNA was detected in blood samples from only one healthy blood donor.[30] Thus, it is currently questionable whether the virus will disseminate in the body after penetration and replication in the target cells, and whether a persistent, latent infection will develop.

STL polyomavirus

We have even less data on STLPyV. STLPyV seroprevalence studies have been carried out by only three research groups. Similar data have been published from the USA, the Netherlands and Italy. The overall seropositivity rate was found to be between 68% and 98%, suggesting that STLPyV is widespread in the human population.[62-64]

The possibility of fecal-oral transmission has been raised. The virus was detected in 0.26–4.4% of stool samples from children with asymptomatic and diarrhoeal symptoms.[65,66] The research team who described the virus did not detect the virus in nasopharyngeal and plasma samples from kidney transplants, and only confirmed the presence of the viral genome in one urine sample.[12] However, in a follow up study of renal transplant recipients, an Australian team detected STLPyV DNA in nasal and throat swab from one patient, but no STLPyV DNA was detected in either blood or urine.[67] A Chinese research team has identified STLPyV DNA in 2% of tonsils from children,[38] but a German team did not detect STLPyV in either tumour or tumour-free tonsil tissues.[60] The link between the virus and the skin was investigated by two groups. STLPyV DNA was detected from the condyloma of an immunosuppressed patient. Viral DNA was detected in 7.3% and 8.3% of skin samples from the forehead and hands of healthy adults.[62-64]

Aims

The research was designed along three main lines.

1. To investigate the seroprevalence of HPyV9, MWPyV and STLPyV the followings were planned:

    - Collecttion of serum samples from immunocompetent children and adults;
    - Production of VP1 proteins in a bacterial protein expression system;
    - Design and optimization of an ELISA method;
    - Perform and analyse seroprevalence assays.

2. We planned to study the possible respiratory transmission of HPyV9, MWPyV and STLPyV by DNA prevalence study, with the following objectives:

    - Collection of respiratory tract secretions (nasopharyngeal and pharyngeal swab samples), tonsil, adenoid and lung tissues, then isolation of nucleic acid from the samples;
    - Develope a real-time PCR method, perform the PCR tests, evaluate the results;
    - Sequencing of polyomavirus genomes and genome fragments from PCR positive samples, analysis of sequences.

3. *In vitro* functional genome analysis of HPyV9, to investigate the activity of promoters with significantly different sequences in different cells was also the aim. To perform this, our objectives were:

    - Cloning HPyV9 NCCR sequences into a bidirectional luciferase reporter vector;
    - Cloning HPyV9 LTAg coding sequence into an expression vector;
    - Transfection of the vectors into different cells, including airway and intestinal epithelial, endothelial and fibroblast cells;
    - Investigating the activity of promoters and the effect of LTAg on it in different cell types.

Materials and methods

Seroprevalence studies

Samples

The serum samples for seroprevalence study were collected between 2016 and 2021 sent for rotuine diagnostic tests to the Medical Microbiology, University of Debrecen.

A total of 1038 serum samples from individuals aged 0.7 to 93 years were used for HPyV9 seroprevalence study, of which 718 samples were from adults (>18 years) and 320 from children (<18 years). The MWPyV and STLPyV seroprevalence studies were performed with the same sample population of 618–618 serum samples. Samples were collected from children (n=235) and adults (n=383) aged 0.8 to 90 years.

Production of VP1 antigens

For all viruses tested, the gene encoding the VP1 protein was synthesized based on reference sequences. The MWPyV and HPyV9 sequences were codon optimized, the STLPyV sequence was not codon optimized, and a 6xHis tag sequence was inserted into the N-terminal ends. The gene were cloned into pTriEx-4 Neo (Novagen) expression vector. HPyV9 and MWPyV VP1 proteins were expressed in *Escherichia coli* Origami B(DE3)pLacI (Novagen), while STLPyV VP1 was expressed in *Escherichia coli* Rosetta-gami B(DE3)pLacI (Novagen) competent bacteria. Purification of proteins was performed by affinity chromatography (Protino NI-TED Packed Columns, Macherey Nagel) using the 6xHis tag under denaturing conditions. Urea was removed by dialysis. Proteins were stored at -20 °C until use. Quantitative and qualitative analysis of purified proteins was performed by BCA assay, Western blot and Coomassie Brilliant Blue staining.

ELISA

We designed and optimized an indirect, colorimetric ELISA method for the detection of IgG antibodies against polyomavirus capsid proteins. ELISA measurements were performed with 50 ng VP1 protein for HPyV9 and 100 ng VP1 protein for MWPyV and STLPyV. Optical density (OD) was measured by spectrophotometer (MultiSkan Sky Microplate spectrophotometer, Thermo Fisher Scientific) at 450 nm wavelength, corrected by the values measured at 620 nm. To determine the OD of each serum sample, the OD of the blind control was subtracted from the values obtained and the results of the duplicates were averaged. The OD values were sorted in ascending order and a trend line with an appropriate coefficient of determination (r^2) was fitted to the data series. The function was used to calculate the inflection point, which served as the cut-off value for the ELISA. A sample with OD value/cut-off > 1.1 was considered seropositive.

DNA prevalence studies

Samples

For our DNA prevalence studies, we collected samples from patients who underwent adenectomy and/or tonsillectomy between May and November 2016. Throat swabs (n=146) were collected from all patients immediately prior to surgery, and if the patient had serous otitis media at the time of surgery, middle ear discharge (n=15) were also collected. A total of 100 tonsil and 100 adenoid tissue samples were collected, 54 patients from both types of samples and 46–46 patients from only one of the two types of samples. Nucleic acid was isolated with DNeasy Blood and Tissue kit (Qiagen). During the COVID-19 pandemic, between September 2020 and March 2022, nucleic acid from nasopharyngeal samples sent to Medical Microbiology, University of Debrecen for SARS-CoV-2 PCR testing was collected from children and adults. The prevalence of MWPyV and STLPyV DNA was tested from nasopharyngeal samples from 1130 patients, while 919 nasopharyngeal samples were used for HPyV9 DNA detection. Nucleic acid isolation was performed using MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) or Chemagic Viral DNA/RNA 300 H96 (PerkinElmer) Kit according to the manufacturer's protocol. The presence of HPyV9 DNA was tested in both tumour and non-tumour lung tissue samples. For this purpose, 147 lung tissue samples from 143 patients were analysed, of which 100 samples were from patients with lung carcinoma and 47 control samples were non-tumour tissue. The samples were sent to the Department of Pathology, University of Debrecen, between 2012 and 2016. Nucleic acid isolation was performed with High Pure FFPET DNA Isolation Kit (Roche) following the manufacturer's instructions.

Real-time PCR

Real-time PCR was performed with Applied Biosystems 7500 real-time PCR and QuantStudio 5 (Applied Biosystems) using TaqMan Universal PCR Master Mix. Detection of MWPyV and STLPyV DNA was performed by multiplex real-time PCR (Applied Biosystems) using previously published primers and assays.[11] For HPyV9 DNA detection, we designed the primers and assay. PCR protocols were as follows: 50°C 2 p, 95°C 10 p, 45 cycles: 95°C 15 s and 60°C 1 min. Detection of the fluorescence signal was performed at 60°C. HPyV9 positivity was also confirmed by amplification and sequencing of the LTAg region.

The MWPyV and STLPyV NCCR regions and the whole genome of STLPyV and HPyV9 were amplified from real-time PCR positive samples. After Sanger sequencing of the amplicons, the sequences were analyzed: phylogenetic and haplotype network analysis was performed, and mutations in the NCCR region were analyzed.

In vitro analysis of HPyV9 promoter activities

Vectors

The complete NCCR sequence of the reference HPyV9 genome and the UF-1 isolate was cloned into a pGL4Luc-RLuc (Addgene) bidirectional reporter vector. After insertion of the NCCR into the vector, the polyomavirus bidirectional promoter regulates the expression of the firefly and *Renilla* luciferase genes encoded in opposite directions of the vector. The gene encoding HPyV9 LTAg was cloned into pcDNA3.1+ vector (Thermo Fisher Scientific).

Cell cultures

Our in vitro experiments were performed using different cell types: primary small airway epithelial cell, MRC-5 lung fibroblast cell, A549 lung epithelial cell line, HEK-293 renal epithelial cell, Caco2 colon epithelial cell line and SK-HEP1 endothelial cell line. ATCC-derived cells were maintained in the adequate media.

Transfection

Cells were seeded 24 h prior to transfection in 12-well plates and transfected with 1 µg reporter vector (pGL4Luc-RLuc with or without HPyV9 NCCR) in Lipofectamine 2000 reagent (Thermo Fisher Scientific). In addition to the reporter vector, 200 ng of vector expressing LTAg or empty pcDNA3.1(+) was used for co-transfection. In each case, experiments were performed with three parallel cell cultures and repeated three independent times. Promoter activity was measured after 24 h by Dual-Luciferase Reporter Assay Kit (Promega). Firefly luciferase represented the late (L) and *Renilla* luciferase the early (E) promoter activity, values were normalized to protein concentration. LTAg expression was verified by Western blot.

Statistical and phylogenetic analysis

Tests of normality, Fisher's exact test, chi-square trend test, Mann-Whitney U test, Kruskal-Wallis test, Dunn post hoc analysis and *t*-tests were performed using GraphPad Prism version 9.4.0. The results of statistical tests were considered significant if $p<0.05$. Mega X software was used for phylogenetic analysis and PopART software for haplotype network construction.

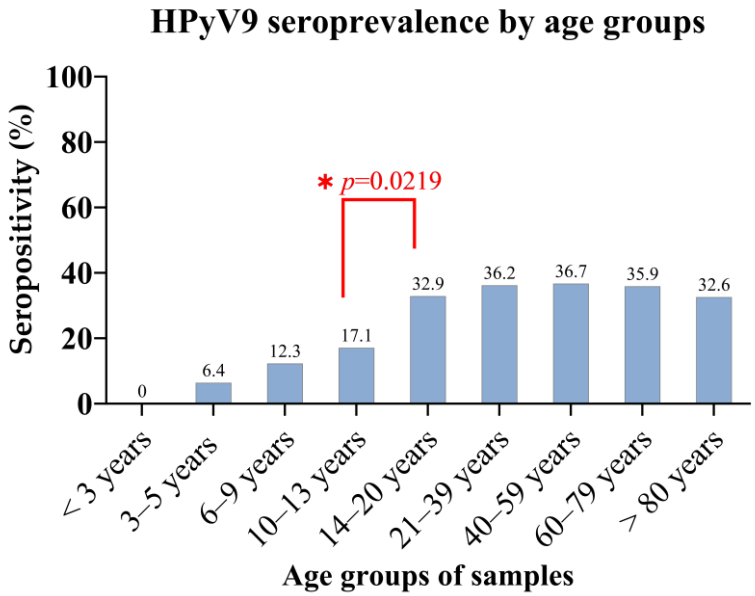
Results and discussion

The antigens were considered suitable for serological reactions for all three polyomaviruses. Specificity tests were performed with homologous and heterologous proteins, based on which ELISA assays were also found to be sufficient.

HPyV9 seroprevalence

Our data were also analysed by age groups. A statistically significant increase in seroreactivity between consecutive age groups was observed only between the 10–13 and 14–20 age groups (p=0.0151; Mann-Whitney U test). According to Kruskal-Wallis, Dunn post hoc analysis, seroreactivity increased statistically with age until young adulthood. HPyV9 seroprevalence was 30.6% in the overall cohort, with 36.2% of adults (>18 years) were seropositive. Despite different testing methods, our HPyV9 seroprevalence is in agreement with the results of several research groups. The seroprevalence, the proportion of seropositive individuals by age group, is shown in **Figure 1**.

Figure 1. : HPyV9 seropositivity by age group

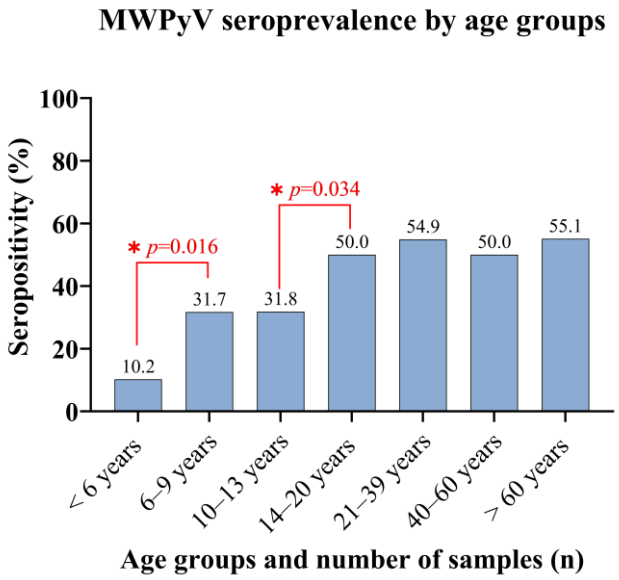


No antibodies against HPyV9 were detected in the youngest age group, under 3 years. The antibody positivity rate increased significantly with age over 3 years up to the age group 21–39 years. Similar to seroreactivity, we observed a significant increase in seroprevalence between the age groups 10–13 years and 14–20 years (p=0.0219; Fisher's exact test), and the increase in the rate of infection until young adulthood was confirmed by X^2 trend test (p<0.0001). No sex differences in transmission rates were detected. Our results suggest that the virus is present in our country, but a significant proportion of adults are susceptible to virus infection.

MWPyV seroprevalence

Significant differences and increases in seroreactivity were detected between the two youngest age groups and between the 10–13 and 14–20 age groups. The seropositivity of MWPyV in the total cohort was 45.6%, with 54% of adults and 31.9% of children having MWPyV infection. Although the sex ratio in the study group did not show a statistically significant difference (51.3% vs. 48.7%), seropositivity was significantly higher in women compared to men (165/317 vs. 117/301; p=0.0012; Fisher's exact test). Also among children, a significantly higher proportion of girls were seropositive (51/119 vs. 24/116; p=0.0003), but amond adults, no such sex difference was confirmed (114/198 vs. 93/185; p=0.182). MWPyV seropositivity rates by age group are shown in **Figure 2**. The antibody positivity rate increased significantly with age until adulthood, as confirmed by the X^2 trend test (p=0.0042). Statistically significant seroprevalence differences were observed between the two youngest age groups and between the 10–13 and 14–20 age groups. Similar to seroreactivity, the antibody positivity rate did not show a significant change (50–55.1%) above the age of 21 years.

Figure 2. : MWPyV seropositivity by age group



Our results are similar to those reported in an Italian study, although they reported higher childhood transmission rates.[68] Another Italian study showed a very high seropositivity rate of 91% among 8–17 year olds.[69] A US study found a 50% seropositivity rate among teenagers and young adults, and the trend of increasing transmission into young adulthood is very similar to ours.[50] However, a higher seropositivity rate of 65.7% was measured among adults (>20 years), which showed a decrease among older people. Some research groups have reported much higher adult seropostitivty rate: in the Czech Republic, adult (>20 years) seroprevalence was 85.6%, while in the Netherlands, the USA and Australia it was even higher, above 97%.[34,51,52,64]

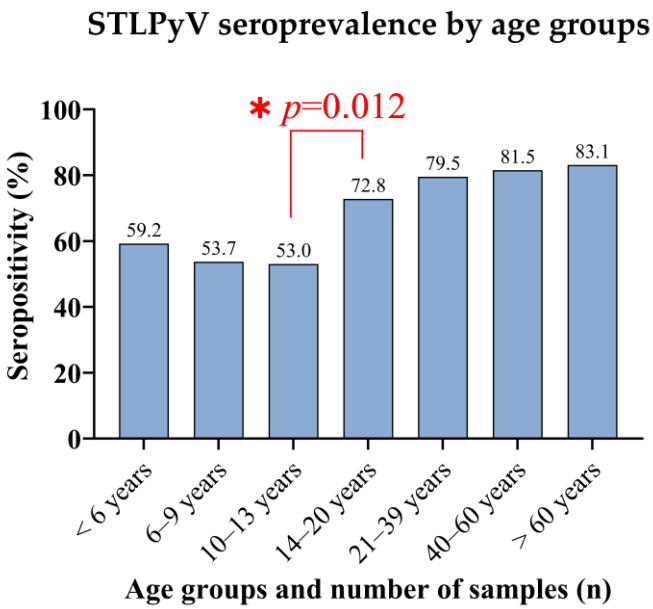
These differences can be explained by differences in the study groups, the antigens used and the methods. Further studies using the same methods are needed in the future to provide more accurate and comparable data. However, it cannot be excluded that there are geographically

different variants of the virus, possibly with real geographical differences in seroprevalence. Knowledge of the mode of transmission would be crucial, as geographically different social and cultural circumstances may also cause differences in transmission.

STLPyV seroprevalence

Significant increas in seroreactivity was detected between the age groups 10–13 years and 14–20 years (p=0.0006; Mann-Whitney U test). Based on Kruskal-Wallis, Dunn post hoc analysis of OD values by age group, we observed increasing seroreactivity with age until young adulthood and relatively stable seroreactivity above the age of 21 years, similar to a previous study. STLPyV seropositivity was 73.5% in the overall study group. The seropositivity rate was 60.9% in children and 81.2% in adults. In the overall cohort, the age of seropositive subjects (0.8–90 years, median: 35 years) was significantly (p=0.0001; Mann-Whitney U test) higher compared to seronegative subjects (0.9–80 years, median: 16.7 years), but no such age difference was detected when analysing data from children and adults. When analysing the data of all subjects, we found that the proportion of seropositive women was significantly higher than that of men (245/317 vs 209/301; p=0.029), but this sex difference was not observed in children or adults. No similar observations were reported by other research groups. Seropositivity data by age are shown in **Figure 3**.

Figure 3. : STLPyV seroprevalence by age group



Similar to data from other studies, STLPyV seropositivity in the youngest (<6 years) age group was close to 60%, meaning that primary infection is common in childhood.[62,63] A significant increase in the seropositivity rate (p=0.012; Mann-Whitney U test) was observed between 10–13 and 14–20 years age goups. The proportion of those infected increased with age, with the highest rate of 83.1% in the age group over 60 years. Our results are similar to the previously published [62-64] 68%–98% STLPyV seroprevalence data, and support the idea that STLPyV is a ubiquitous virus.

DNA prevalence results

HPyV9 DNA prevalence

Although sufficient quality and quantity of DNA was isolated from lung tissue samples, HPyV9 DNA was not detected in either tumour or non-tumour tissue samples. To date, only one working group has detected HPyV9 DNA in a lung autopsy tissue sample. Moreover, viral mRNA has been detected, suggesting that the virus is able to replicate in these tissues.[28] However, confirming results have not yet been published.

No HPyV9 DNA was detected in either middle ear, adenoid and throat swab samples. However, we did detect the virus in the tonsil of a 6-year-old boy (1/100; 1%). The entire viral genome was amplified and sequenced (GenBank identifier: MH844627). HPyV9 DNA was detected in 5.2% of nasopharyngeal samples collected during the COVID-19 pandemic. We also successfully amplified and sequenced the LTAg sequence fragment of the virus from all real-time PCR positive samples, confirming our results. The prevalence of HPyV9 DNA was significantly (p<0.0001; Fisher's exact test) higher in SARS-CoV-2 RNA negative samples (9.11%) than in SARS-CoV-2 RNA positive samples (0.25%). The age of the HPyV9 DNA positive patient group was significantly (p=0.0139; Mann-Whitney U test) lower compared to the HPyV9 DNA negative group, with 79% (38/48) of HPyV9 positive samples from adults aged 21–40 years. Although a higher proportion of HPyV9 DNA was detected in adult samples (6.16%, 40/649) than in children (2.96%, 8/270), this difference was not statistically significant (p=0.0508; Fisher's exact test). No sex difference was detected. Interestingly, no HPyV9 DNA was detected in 146 throat swabs collected in 2016. The fact that we tested for the presence of the virus in a different sample type (throat swab vs. nasopharyngeal sample) does not explain this, as our group has previously detected HPyV9 DNA in throat swab samples.[31] The fact that we detected a higher prevalence of HPyV9 in samples collected during the COVID-19 pandemic compared to previous results is of particular interest. The samples obtained from a period when the spread of the virus in general was significantly inhibited by restrictive measures, school and nursery closures and curfews. Although our results suggest that HPyV9 may be present in the respiratory tract, in the secondary lymphoid tissue, further studies are needed to understand the mode of transmission and the site of replication.

HPyV9 sequence analysis results

At present, seven complete HPyV9 genome sequences are available in the GenBank database together with our published sequence, which were used for phylogenetic and haplotype network analysis. Based on both phylogenetic tree and haplotype network analysis, four clusters of genomes have been distinguished. European isolates formed one cluster. The NCCR regions of the sequences differ significantly.

MWPyV DNA prevalence

No MWPyV DNA was detected from either nasal or pharyngeal tonsils. In contrast, two groups of researchers confirmed the presence of MWPyV DNA in only a few samples, but in 2% and 6% of tonsils and 1% of adenoids.[38,70] However, two other studies, similar to ours, did not detect the viral DNA.[32,39] MWPyV DNA was detected in one middle ear discharge and

2.7% of throat secretions taken during tonsillectomy. MWPyV DNA was detected in 4.9% of nasopharyngeal samples collected during the SARS-CoV-2 pandemic. MWPyV prevalence was significantly (p<0.0001) higher in children (38/360) than in adults (17/770). In addition, a significant age difference between MWPyV positive (0.8–38 years, median: 3.6 years) and negative individuals (1–92 years, median: 32.2 years) was also detected (p=0.0001; Mann-Whitney U test), with positive samples identified in younger individuals. No sex differences were detected.

We also tested for the co-infection of SARS-CoV-2 and MWPyV in nasopharyngeal samples collected during the COVID-19 pandemic. MWPyV DNA positivity was significantly more frequent among SARS-CoV-2 RNA negative patients (41/565) than among SARS-CoV-2 RNA positive patients (14/565) in the overall cohort (7.3% vs. 2.5%; p=0.0003) and among adults (17/385 vs. 0/385; p<0.0001), Among children, we also detected MWPyV DNA more frequently in SARS-CoV-2 RNA negative respiratory specimens (24/180) compared to SARS-CoV-2 RNA positive (14/180), but the difference was not statistically significant (p=0.122). Since we also tested for the presence of HPyV9 in 919 specimens, we also analysed for dual polyomavirus infection. In six samples, both HPyV9 and MWPyV DNA were detected (6/919; 0.65%), in 10.9% (6/55) of MWPyV positive samples both polyomaviruses were detected. Of the samples, four were from children (2.1–5,3 yrs; median: 2.3 yrs) and two were from adults (30.1 and 30.6 yrs). One child (2.4 yrs) also had SARS-CoV-2 infection at the time of sampling, the respiratory samples of the others were SARS-CoV-2 RNA negative.

The pathogenesis of MWPyV, mode of transmission, portal of entry, site of replication remain unknown, nor do we know whether latent infection may occur in the body. In addition to faecal-oral transmission, respiratory transmission and oral invasion of the virus are also suspected. Our results support this hypothesis. Interestingly, we also detected MWPyV DNA in a middle ear fluid, suggesting that the virus may be transmitted to the middle ear. Further studies and analysis of a larger number of samples would be needed to clarify this.

MWPyV sequence analysis results

The entire NCCR region was amplified and sequenced from 21 MWPyV positive samples. Four novel point mutations were described, and a 13 base pair deletion in one isolate. Although the biological significance of the MWPyV NCCR sequence variants is not yet clear, it is hypothesized that these mutations may affect transcription factor binding sites and the function of promoters and enhancers.[22] Haplotype network analysis was used to test whether NCCR sequence variances were correlated with the sample types from which they were isolated or the geographical region from which they originated. However, haplotypes did not show any correlation with either the sample types or the geographical region, and haplotypes were not clustered based on these.

STLPyV DNA prevalence

STLPyV DNA was detected in 3.4% of the throat swab samples. In addition, we detected the virus in a middle ear discharge (6.7%) and in an adenoid tissue (1%). Tese two patients, the throat swab sample taken at the same time was also positive for STLPyV DNA. All polyomavirus positive samples were from children. STLPyV was not detected in tonsils. 1.4%

of nasopharyngeal samples collected during the COVID-19 pandemic were found to be STLPyV DNA positive. STLPyV positivity was significantly higher (p<0.0001) in respiratory samples from children (13/360, 3.6%) than adults (3/770, 0.4%). A significant age difference was detected (p=0.0001) between STLPyV negative (1.3-28.5 years, median: 4.5 years) and STLPyV positive (0.8-92 years, median: 30.7 years) individuals, with viral DNA present in samples from younger individuals, mainly children. No sex differences were detected. STLPyV positivity rate was significantly (p=0.0039) higher in SARS-CoV-2 RNA negative samples than in SARS-CoV-2 RNA positive samples. In fact, the same significant difference was observed among children (11/385 vs. 2/358; p=0.01). The three STLPyV positive adult samples were from SARS-CoV-2 RNA negative individuals. MWPyV and STLPyV co-infection was detected in seven samples (7/1130; 0.6%), four samples from children and three from adults. MWPyV DNA was also present in 43.8% of STLPyV DNA positive samples, while SARS-CoV-2 co-infection was present in only 1.3% of samples (2/16). Only one sample (1/16; 6.3%) had all three viruses detected. One child (3.2 years) had both HPyV9 and STLPyV DNA in a SARS-CoV-2 RNA negative respiratory specimen.

Our results suggest the respiratory transmission of STLPyV and even the possibility of viral invasion into the middle ear.

STLPyV sequence analysis results

In our work, two complete STLPyV genomes were sequenced. We then performed phylogenetic analysis together with additional sequences from GenBank. We found that, based on both the phylogenetic tree and the network analysis, the sequences were divided into two main branches. However, no geographical separation was confirmed as samples from the same region were found in both branches. At present, too few sequences are available to draw definitive conclusions, so further sequence data from different geographical regions is needed.

A complete NCCR region was amplified and sequenced from 11 STLPyV positive samples. Three novel variants and three novel point mutations were identified. Based on the results of haplotype network analysis, the STLPyV NCCR sequences were sorted into two groups, but no geographic or sample type clustering was observed for STLPyV.

***In vitro* functional genome analysis of HPyV9**

Functional analysis of HPyV9 promoters in different cell types can help to identify the target cells of the virus. In addition, biological differences in the markedly different sequence variants can be studied. In our *in vitro* experiments, we investigated the promoter activities of two HPyV9 isolates (the reference sequence 9 and UF-1) in different cells. Because of the possible respiratory transmission and replication of the virus, we chose the primary airway epithelial cell, the lung fibroblast cell MRC-5, and the lung epithelial cell line A549 for our studies. Similar experiments were performed with the HEK-293 renal epithelial cell as well as A549, which allowed comparison of results.[71-73] The Caco-2 cell line is a colon epithelial cell line similar to SW480 used by others. The SK-HEP-1 endothelial cell line was chosen because the endothelium of blood vessels may play a key role in the spread of the virus within the body.

Promoter activities of both isolates were strongest in A549 cells and weakest in MRC-5 fibroblasts. Promoter 9_E of the reference sequence showed significantly stronger activity than

9_L in Caco-2, primary airway epithelium, SK-HEP-1 and MRC-5 cells. This was not observed in A549 cells, whereas in HEK-293 cells the activity of 9_L was significantly stronger. The latter was also observed by two other research groups.[71,72] The activity of UF-1 promoters was nearly identical in Caco-2 cells, but significantly stronger UF-1_L activity was measured in A549, HEK-293 and SK-HEP-1 cells. Our results in HEK-293 cells are in agreement with data from two previous publications.[71,72] In airway epithelial and fibroblast cells, the activity of UF-1_L was significantly weaker compared to UF-1_E. UF-1 promoters showed higher activity in all cell types tested, except HEK-293. In our cotransfection experiment, LTAg resulted in a significant increase in the activity of late promoters (9_L and UF-1_L) in all cell types. The results are consistent with the function of LTAg as a protein that promotes viral replication but also transactivates late genes.[74] In A549, HEK-293 and primary cells, luciferase activities were altered to a similar extent for both NCCRs. The strongest effect was measured in SK-HEP-1 cells. In this cell type, UF-1_L activity increased much more in response to LTAg compared to 9_L activity and also compared to that measured in other cells. The activity of the 9_E promoter decreased to a similar extent in all cells in response to LTAg, and the difference was statistically significant in all but the primary cells. The activity of the UF-1_E promoter varied with cell type in response to LTAg, showing no change in A549 and HEK-293 cells, a significant increase in SK-HEP-1 cells and a significant decrease in primary airway epithelial cells. Only two research groups performed similar experiments with A549 and HEK-293 cells. Similar but different results have also been published.[71,72] The latter may be due to methodological differences.

Although low promoter activity was measured in primary airway epithelial cells, the highest value was observed in the A549 cell line. This suggests that HPyV9 may enter and replicate in the respiratory tract. Our results suggest that A549 and HEK-293 cells may be suitable for further investigation of HPyV9 replication.

Summary

During our research, we investigated the seroprevalence of three human polyomaviruses — HPyV9, MWPyV, and STLPyV — using a large number of serum samples collected from immunocompetent children and adults. For this purpose, we produced viral antigens and developed an ELISA method. To study the respiratory transmission and replication of these viruses in the respiratory tract, we performed DNA prevalence analyses using tonsil, adenoid, and lung tissues, as well as respiratory samples. Additionally, we examined the viral regulatory region of HPyV9 and studied promoter activity *in vitro* in various cell types.

Based on our results, HPyV9 and MWPyV seropositivity rates increased with age among children and young adults. Among people over 21 years of age, the seropositivity rate did not change significantly. According to our results, 36.2% of adults (>18 years) were seropositive for HPyV9 and 54% for MWPyV, meaning that a significant proportion of adults are susceptible to both viral infections. STLPyV primary infections occured in early childhood, under 6 years of age. 81.2% of adults were seropositive, the virus is ubiquitous in the population.

Neither polyomavirus was detected in lung tissue. HPyV9 DNA was detected in 1% of tonsils and STLPyV in 1% of adenoids. In the throat swab sample taken during tonsillectomy, HPyV9 was not detected, but MWPyV DNA was present in 2.7% and STLPyV DNA in 3.4% of the samples. Moreover, both viruses were detected in 1-1 middle ear discharge. HPyV9 DNA was detected in 5.2%, MWPyV in 4.9%, and STLPyV in 1.4% of nasopharyngeal samples collected during the COVID-19 pandemic, mainly in SARS-CoV-2 RNA negative samples. HPyV9 prevalence was higher in samples from adults, and MWPyV and STLPyV DNA positivity were higher in children. Our results suggest the possibility of respiratory transmission of all three viruses, the airway and oral cavity may be the portal of entry, with MWPyV and STLPyV even entering the middle ear. We obtained valuable full-genome and NCCR sequences from the samples and performed sequence analyses. Novel MWPyV and STLPyV NCCR variants and mutations were identified.

Two significantly different NCCR sequences of HPyV9, the early and late promoter activities were studied using a luciferase reporter vector, ensuring bidirectional expression in respiratory, renal and colon epithelial, and endothelial cell lines, lung fibroblasts, and primary, airway epithelial cells. Promoter activities were the highest in A549 lung epithelial cells, and the lowest in fibroblasts and primary cells. The activity of both promoters of the UF-1 NCCR, which differed significantly from the reference sequence, was found to be stronger in all but one cell. The large T antigen of the virus increased late promoter activities in all cells. Our *in vitro* experiments suggest that the A549 lung epithelial cell line and the HEK-293 renal epithelial cell line may be suitable for HPyV9 viral replication.

New findings

- For seroprevalence assays, we have produced suitable HPyV9, MWPyV and STLPyV VP1 proteins, with which we have designed and optimized a suitable ELISA method.
- We investigated and analysed the seroprevalence of HPyV9, MWPyV and STLPyV in different age groups.
- We found that seropositivity of HPyV9 and MWPyV increased with age until young adulthood, but a significant proportion of adults remained susceptible to infection. Primary STLPyV infections occured in young children, and the majority of adults were seropositive. STLPyV is a ubiquitous virus.
- Our DNA prevalence data support the hypothesis of possible respiratory transmission of the virus, and valuable prevalence and sequence data have been collected and analysed.
- We have established an *in vitro* experimental system that allows functional studies of different NCCR variants of polyomaviruses, including analysis of differences in their biological effects.
- The activity of HPyV9 promoters was investigated in different cell lines and primary cells. We found that the promoters of the UF-1 NCCR haplotype are stronger than those of the reference sequence, LTAg enhances late promoter activities, and A549 airway epithelial cell line and HEK-293 renal epithelial cell line may be suitable for HPyV9 viral replication.

Acknowledgements

I am grateful to my supervisor, Dr. Eszter Csoma, for always being patient and willing to help me. I would also like to thank her for her contribution to my professional development during my PhD years.

I am also grateful to Dr. József Kónya, head of the Department, for the opportunity to carry out my PhD research in the Department of Medical Microbiology.

I would like to thank Professor Lajos Gergely, member of our research group. I also owe special thanks to Dr. Krisztina Jeles for her understanding and joyful support in our work, even in difficult times.

I would also like to thank all the staff of the Department of Medical Microbiology who contributed in any way to our research.

I am very grateful to all those involved in the study, without whom the work would not have been possible.

Finally, with extreme gratitude and love, I thank my Husband who supported me through the difficulties of life and all the other members of my Family for always supporting and encouraging me in the completion of my PhD thesis.

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Registry number: DEENK/592/2024.PL
Subject: PhD Publication List

Candidate: Melinda Katona
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10069547

List of publications related to the dissertation

1. **Katona, M.**, Jeles, K., Takács, P., Csoma, E.: DNA and seroprevalence study of MW and STL polyomaviruses.
   J. Med. Virol. 96 (8), 1-10, 2024.
   DOI: http://dx.doi.org/10.1002/jmv.29860
   IF: 6.8 (2023)
2. **Katona, M.**, Jeles, K., Takács, P., Csoma, E.: Prevalence and in vitro study of human polyomavirus 9.
   Sci. Rep. 14, 1-16, 2024.
   DOI: http://dx.doi.org/10.1038/s41598-024-80806-3
   IF: 3.8 (2023)

List of other publications

3. **Katona, M.**, Jeles, K., Kovács, R. L., Csoma, E.: KI and WU Polyomaviruses: seroprevalence Study and DNA Prevalence in SARS-CoV-2 RNA Positive and Negative Respiratory Samples.
   Microorganisms. 10 (4), 1-12, 2022.
   DOI: http://dx.doi.org/10.3390/microorganisms10040752
   IF: 4.5
4. Jeles, K., **Katona, M.**, Csoma, E.: Seroprevalence of Four Polyomaviruses Linked to Dermatological Diseases: New Findings and a Comprehensive Analysis.
   Viruses-Basel. 14 (10), 1-16, 2022.
   DOI: http://dx.doi.org/10.3390/v14102282
   IF: 4.7
5. Steuer-Hajdu, K., Ványai, B., **Katona, M.**, Szegedi, A.: Az atópiás dermatitisz.
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Pathobiology. 85 (3), 179-185, 2018.
DOI: http://dx.doi.org/10.1159/000481174
IF: 1.831

Total IF of journals (all publications): 21,631
Total IF of journals (publications related to the dissertation): 10,6

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

16 December, 2024

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