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Prevalence Study of Human Polyomaviruses

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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 29th of October 2024 at 13:00.

LIST OF ABBREVIATIONS

BKPyV	BK polyomairus
HPyV6	Human polyomavirus 6
HPyV7	Human polyomavirus 7
JCPyV	JC polyomavirus
KIPyV	Karolinska Institutet polyomavirus
LTag	large tumor antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
NCCR	non-coding control region
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sTag	small tumor antigen
TSPyV	Trichodysplasia spinulosa-associated polyomavirus
VLP	virus like protein
VP1	viral protein 1
WUPyV	Washington University polyomavirus

INTRODUCTION

The development of nucleic acid amplification methods and sequencing techniques has opened up a new era of virological research. In addition and complementary to classical virological methods, the identification of unknown pathogens underlying various infectious diseases has become faster. At the same time, genome hunting is in many cases sequence analyses, or metagenomic analyses, performed on random samples without clinical symptoms using high-throughput sequencing techniques. As a result, the number of newly described virus species has increased dramatically in the 2000s.

Since 2007, the genome sequence of 16 new human polyomavirus species in the family *Polyomaviridae* has been described, in addition to several animal viruses, but the virions have not been isolated [1]. In some cases, disease was targeted, and subsequent research confirmed the role of viruses in disease, but the clinical significance, if any, of most viruses has remained unexplored. The mode of transmission and the targets for replication are not known, and it is unknown whether these viruses cause any disease, and if yes, what the symptoms are [2].

To answer the essential questions, studies in several directions are needed. Seroprevalence studies can answer questions about how prevalent a particular virus is in the population, in different age groups, when the primary infection is most likely to occur, and who might be susceptible to the infection. The data may indicate the mode of transmission but may also reveal geographical variations. These studies are challenging not only to measure antibodies, which requires the collection of blood samples from a large number of different age groups, but also to develop methods and even to produce the necessary antigens, which are not commercially available. From DNA prevalence studies, we can infer the mode of transmission, the site of entry, the dissemination within the body, the possible latency, the mode of excretion from the body, and the site of viral replication. In fact, in the case of appropriate patient data, the disease that may have been caused. These require the collection of various clinical samples, nucleic acid isolation, and PCR methods.

LITERATURE REVIEW

Human polyomaviruses

Members of the *Polyomaviridae* family are non-enveloped viruses with a diameter of 40–45 nm and a circular, double-stranded DNA genome. The icosahedral capsid is composed of 72 capsomeres, each pentamer containing five VP1 (viral protein) proteins and one VP2, and in most species VP3. The capsomeres are linked to each other via the C-terminal part of the VP1 protein. Packaging of the viral genome inside the virions is supported by host cell-derived histone proteins (H2A, H2B, H3, and H4) [2].

The only protein of the virion that comes into contact with the outside world is the VP1 protein, which binds to a receptor on the surface of the susceptible cell; the VP1 is the main antigen of the virus. Recombinant VP1 proteins, produced and purified in a prokaryotic or eukaryotic protein expression system, are able to assemble on their own – without viral DNA, VP2 or VP3 proteins – into capsomeres that form an intact capsid under appropriate conditions. This structure is called a VLP (virus-like particle) and can be used for seroprevalence studies [2, 3].

The genome of polyomaviruses is functionally divided into three parts: the early, the late, and the non-coding control region (NCCR), which contains the replication origins, enhancers, promoters, and transcription start codons. A specific characteristic of polyomaviruses is that both strands of the genome are coding, with the NCCR allowing bidirectional transcription [3]. In one strand, the early region in one direction encodes small (sTag=small tumor antigen) and large tumor antigens (LTag=large tumor antigen) with transforming activities that regulate replication and gene expression. The late region on the other strand, in the opposite direction, also encodes structural proteins VP1, VP2, and VP3, microRNAs, agnoproteins in some viruses, which are required for virion assembly [2].

The first two human pathogenic polyomaviruses were described in 1971: the BK polyomavirus (BKPyV) from the urine of a kidney transplant patient, and the JC polyomavirus (JCPyV) from brain tissue of a patient with progressive multifocal leukoencephalopathy [4, 5]. Their importance was discovered after almost a decade, and it is now known that they infect >80% of the population [6]. They develop a lifelong latency after primary infection, and reactivation can cause severe, even fatal, symptoms in immunosuppressed individuals [7-10].

In 2007, the genome sequences of KI (KIPyV) and WU polyomaviruses (WUPyV) were described from nasopharyngeal samples of children with acute respiratory illness [11, 12].

Their nucleic acid has been detected in upper respiratory tract secretions in several studies, which has led to the hypothesis of their pathogenic role in respiratory diseases, either as single or co-infectious viruses, but this has not yet been proven [13-16]. Merkel cell polyomavirus (MCPyV) was described in 2008 from a very rare, highly aggressive skin tumor, Merkel cell carcinoma (MCC) [17]. MCPyV is an etiological agent in the development of MCC; depending on the geographical region, it is found in nearly 80% of tumors, clonally integrated into the host cell genome [18].

In 2010, human polyomavirus 6 (HPyV6) and human polyomavirus 7 were discovered in skin samples from healthy people [19]. These two viruses are possibly associated with epidermal hyperplasia in transplanted patients with pruritic, brownish, scaly skin lesions [20, 21]. Also in 2010, trichodysplasia spinulosa-associated polyomavirus (TSPyV) was described from spicular growths on the face of a heart transplant patient [22]. The virus is named after the underlying disease of the patient at the time of sampling, trichodysplasia spinulosa, which is exclusively found in immunosuppressed patients and is characterized by spicular skin growths in different parts of the face, alopecia of the affected areas, and a distorted appearance due to skin thickening [23].

KI polyomavirus és WU polyomavirus

Despite the fact that seroprevalence and DNA prevalence studies were started immediately after the discovery of KI and WU polyomaviruses, their clinical significance is still unclear. The 55–96% KIPyV and 69–99% WUPyV seropositivity in adults from different regions of the world suggest that the viruses are widespread, although there is considerable variation [24-29]. Based on the transmission rate detected among children by some research groups, which is similar to that of adults, a significant proportion of primary infections most likely occurs in early childhood.

The mode of transmission, the site of entry, and the site of replication are unknown, and it is still unknown whether the virus remains in the body as a latent infection after primary infection. Transmission via respiratory secretions, oral entry, and even fecal-oral transmission are possible. As both KIPyV and WUPyV have been detected in nasopharyngeal specimens from patients with respiratory symptoms, most of the studies has focused on respiratory specimens.

KIPyV DNA was detected in 0–34.4% of upper respiratory tract samples from healthy individuals or from individuals with respiratory symptoms [13, 14, 30], while WUPyV DNA

was detected in 0–33.3% of the samples [15, 16, 31]. The viruses were rarely or not detected in respiratory specimens from immunocompetent adults, while in samples from children, KIPyV and WUPyV DNA prevalence was 0.1–10.2% and 0.4–27.5%, respectively [13, 14, 32]. Immunosuppressed patients had upper respiratory specimens with KIPyV prevalence of 0.8–34.4% and WUPyV prevalence of 0.8–25.8% [30, 33-35]. Their presence has been confirmed even in the absence of respiratory symptoms, whereas co-infections were either present or not tested in most cases with usually mild symptoms [14, 32, 34, 36-39]. Viruses have also been detected in lower respiratory tract specimens and even in lung tissue [13, 15, 16, 31, 40-42]. In some case reports, the presence of viral antigen suggesting viral replication and a relatively high copy number have been detected in samples [13, 15, 16, 40], suggesting that KIPyV and WUPyV possibly replicate in the airways. This is strengthened by the observation that in follow-up studies, viruses could be detected in samples from the respiratory tract days or weeks later [43-45].

KIPyV DNA has also been detected in urine, feces and blood, lymphoid tissue, adenoids, tonsils, brain tissue, and benign skin tumors [30, 38, 46-48], while WUPyV nucleic acid has been detected in urine, feces, brain and lymphoid tissue, and blood [43, 45, 49-52]. Although their DNA has been detected in fecal samples from patients with gastrointestinal symptoms, their pathogenic role is questionable due to the co-detection of other pathogens [11, 13, 48]. The proven ability to cause viremia and viruria raises a number of hypotheses. It is possible that viruses may be disseminated from the site(s) of entry by bloodstream within the body, in which secondary lymphoid tissue of the respiratory tract may play a role. The viruses may persist in the cells of the kidneys, urinary tract, and lymphoid tissues, even develop latency, and then reactivate under immunosuppression, being shed via the respiratory tract, urine, and feces, even asymptomatically. No Hungarian seroprevalence data are available for either virus. Our research team has previously published prevalence data from respiratory tract samples. The results were consistent with international data. They showed a higher prevalence of KIPyV and WUPyV DNA in respiratory samples from immunosuppressed individuals (14.3% and 9.1%); the viruses were detected in samples from children (1.7% and 5%), but not in samples from healthy adults [43, 46, 53, 54].

Polyomaviruses associated with skin disease

Based on international seroprevalence studies, 42–88% of adults are seropositive for MCPyV, 52–93% for HPyV6, 55–80.3% for HPyV7, and 63–80% for TSPyV [55-59]. These

data suggest that the viruses are widespread in the population. However, it is noteworthy how large the range of the adult seroprevalence data was published by different research groups.

Based on DNA prevalence surveys from around the world, MCPyV has been detected in 11%, HPyV6 in 12–30%, HPyV7 in 9.7–12.4%, and TSPyV in 0–1.7% of healthy skin tissue samples [19, 60-64]. Viral shedding from the skin surface has been confirmed for HPyV6 and HPyV7 [19, 21, 61]. With age, the prevalence of HPyV6 and HPyV7 increased along with the amount of virions, with enhanced viral replication and shedding observed in older individuals. Although the target cells of HPyV6 and HPyV7 are not known, the role of epidermal cells, keratinocytes in the pathogenesis of persistent viral infection has been suggested by some studies [18, 20, 65].

The presence of the viruses have also been tested in various skin and non-skin tumors. In different skin tumors, the prevalence of MCPyV was 4–21%, HPyV6 3–42.3%, HPyV7 0.4–4.3%, and TSPyV 0.8–1.7% [62, 64, 66-71]. Their possible role in carcinogenesis, mainly in skin tumors, was suggested but has not been confirmed, except the role of MCPyV in MCC. The DNA of the viruses has been detected in respiratory tract samples, blood, feces, and urine, as well as in various tissue samples. The mode of transmission of the viruses is not known. The fact that they have been detected in skin samples from healthy individuals and confirmed to be shed asymptomaticly suggests a direct skin-to-skin transmission. The low viral DNA load in non-tumor tissues and the high rate of transmission suggest that they are likely to establish a latent infection in the body. However, in addition to these, transmission via urine and respiratory secretions cannot be ruled out due to the proven viruria and the presence of the viruses in respiratory secretions.

No seroprevalence data have been published for any of the viruses from Hungary, and we have Hungarian DNA prevalence studies only with MCPyV. As the tumor is rare, a small number of cases with MCC have been reported. Two studies confirmed the presence of MCPyV DNA in the majority (77%) of tumors [72, 73], while in one study only 37.5% of tumors were found to be viral DNA positive [74]. No cases of HPyV6, HPyV7, or TSPyV related disease have been reported from Hungary.

AIMS

The aims of our research were the following:

I. One of the aims of our work was to study the seroprevalence of KIPyV, WUPyV, MCPyV, HPyV6, HPyV7 and TSPyV in immunocompetent children and adults, for which we planned the following studies:
   - Viral VP1 protein production in a bacterial protein expression system.
   - Detection of antiviral IgG antibodies in human serum samples using a self developed ELISA method.
   - Evaluation of results, comparison with already published data.

II. Our second aim was to study the prevalence of KIPyV and WUPyV DNA in respiratory samples:
   - We planned to test for the presence of viral DNA in nasopharyngeal samples from children and adults using real-time PCR.
   - We planned to investigate the presence of WUPyV and KIPyV in respiratory specimens collected during the COVID-19 pandemic, also to study whether they are co-infected with SARS-CoV-2.

MATERIALS AND METHODS

Seroprevalence studies

Samples

For our seroprevalence studies, serum samples were selected between 2016 and 2021 from among the samples received for diagnostic studies at the University of Debrecen, Medical Microbiology. We studied the seroprevalence of KIPyV using 692 serum samples and WUPyV using 705 serum samples. Seroprevalence studies of MCPyV, HPyV6, HPyV7 and TSPyV were performed using the same cohort of 552–552 sera. Samples were divided into age groups. Detailed data are shown in **Tables 1** and **2**.

Table 1: *Data of the samples used for KIPyV and WUPyV seroprevalence study*

	KIPyV			WUPyV		
Age groups	**Number of samples**	**Age in Years, Min-Max (Median)**	**Female/Male**	**Number of samples**	**Age in Years, Min-Max (Median)**	**Female/Male**
0,7–1 years	20	0.7–1.9 (1.3)	7/13	22	0.7–1.9 (1.4)	8/14
2–5 years	82	2.1–5.9 (3.5)	34/48	88	2.1–5.9 (3.4)	36/52
6–9 years	67	6.1–9.9 (7.6)	33/34	68	6.1–9.9 (7.5)	33/35
10–13 years	67	10.1–13.9 (11.8)	34/33	96	10.1–13.9 (11.9)	52/44
14–20 years	105	14–20.9 (16)	59/46	114	14–20.9 (16)	66/48
21–39 years	101	21–39.6 (31)	48/53	92	21–39.6 (31.6)	42/50
40–59 years	125	40–59.5 (50)	64/61	116	40–59.5 (50)	59/57
>60 years	125	60–92 (72)	64/61	109	60–92 (70.1)	57/52
Total	**692**	**0.7–92 (21.9)**	**343/349**	**705**	**0.7–92 (16.8)**	**353/352**
Adults	**367**	**18–92 (50.2)**	**187/180**	**332**	**18–92 (50.1)**	**168/164**
Children (<18 years)	**325**	**0,7–17.9 (9.6)**	**156/169**	**373**	**0.7–17.9 (7.9)**	**185/188**

Table 2: *Sample data for seroprevalence study of MCPyV, HPyV6, HPyV7 and TSPyV*

Age groups	**Number of samples**	**Age in Years, Min-Max (Median)**	**Female/Male**
<6 years	38	0.8–5.9 (2.8)	15/23
6–9 years	36	6.1–9.9 (7.6)	19/17
10–13 years	45	10.1–13.9 (11.7)	22/23
14–20 years	87	14–20 (16)	47/40
21–39 years	114	21–39.5 (30.8)	57/57
40–59 years	128	40–59.5 (50)	66/62
>60 years	104	60–85 (69)	57/47
Total	**552**	**0.8–85 (33)**	**283/269**
Adults	**359**	**18–85 (47.3)**	**187/172**
Children (<18 years)	**193**	**0.8–17.9 (11.7)**	**96/97**

Antigen production, protein expression, protein purification

Antigens for ELISA assays were produced using a bacterial protein expression system. For this purpose, the gene encoding the VP1 capsid protein was codon-optimized and synthesized based on reference sequences, except KIPyV sequence which was not codon-optimized. The insert was cloned into a pTriEx™-4 Neo vector, amplified in the *Escherichia coli* XL-1 Blue strain, and finally verified by sequencing. Expression of VP1 proteins was performed in *Escherichia coli* Origami™ B(DE3)pLacI, except for the VP1 protein KIPyV, which was produced in *Escherichia coli* Rosetta-gami™ B(DE3)pLacI. Protein expression was induced. VP1 proteins were purified under denaturing conditions by affinity chromatography (Protino NI-TED Packed Columns) using the 6xHis tag. The proteins were then dialyzed using a Slide-A-Lyzer Dialysis cassette, concentrated on an Amicon column if necessary, and stored frozen until use. The quality of the purified VP1 proteins was assessed by Coomassie Brilliant Blue staining and Western blot, while BCA assay was used for quantitative analysis.

ELISA

For the detection of IgG antibodies against VP1 proteins, an indirect, colorimetric ELISA method was developed. We optimized all steps of the immunoassay method and performed our assays. Optical density (OD) value was measured at 450 nm using a MultiSkan Sky Microplate spectrophotometer, and the results were corrected by the values measured at 620 nm. Our ELISA measurements were performed in duplicate for all serum samples. OD values were sorted in increasing order and then fitted with a trend line. The obtained function was used to calculate an inflection point, which is the cut-off value for the ELISA. Samples with OD >cut-off+10% were considered seropositive.

DNA prevalence studies

Samples

To study the DNA prevalence of KI and WU polyomaviruses, nucleic acids from nasopharyngeal samples sent to Medical Microbiology for SARS-CoV-2 PCR from August 2020 to April 2021 and in October 2021 were selected. A total of 1030 samples were collected from 680 SARS-CoV-2 RNA positive and 350 SARS-CoV-2 negative patients. The age range of the patients was 0–94.2 years (median: 38.1 years). Nucleic acid isolation was performed using MagNA Pure 96 DNA and Viral NA Small Volume Kittel (Roche) or

Chemagic Viral DNA/RNA 300 Kit H96 (PerkinElmer) Kit according to the manufacturer's instructions.

Real-time PCR

A quantitative, real-time PCR method with TaqMan assay was used to detect the DNA of KI and WU polyomaviruses. Primers and probes designed for the VP2-3 region of KIPyV and the VP1 region of WUPyV were used [75]. PCR was performed in 50 µL final volume as follows: 10 µL template nucleic acid, 500-500 nM forward and reverse primers, 200 nM probe, 25 µL 2X TaqMan Universal PCR Master Mix. The PCR reaction was performed in a QuantStudio 5 Real-Time PCR instrument with the following protocols: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. The detection of the fluorescent signal was performed at 60 °C.

Statistical analysis

For statistical tests, Mann-Whitney U test, Fisher's exact test, $\chi 2$ trend test, and Spearman rank correlation analysis were used using GraphPad Prism 9.4.0 software. Differences were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

For all the viruses studied, the purified VP1 proteins produced were considered suitable for ELISA.

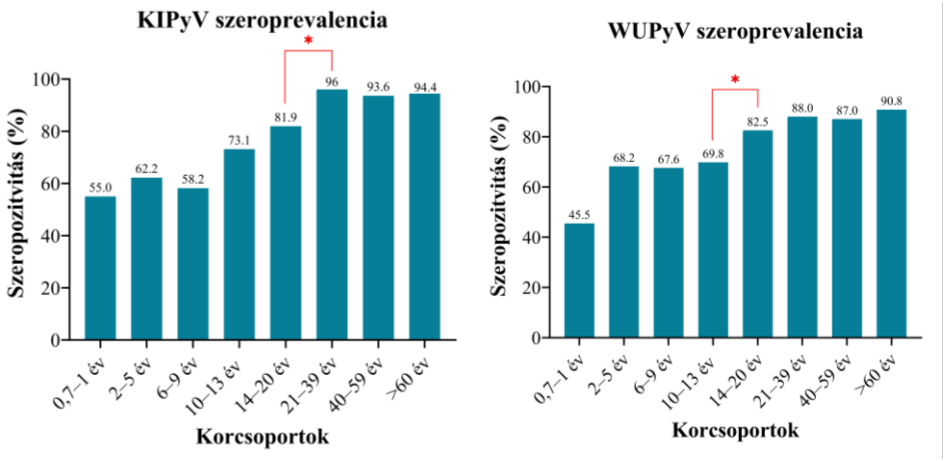
KIPyV and WUPyV belong to the same genus, with 65% identical VP1 protein sequences [76]. MCPyV and TSPyV, also belonging to the same genus, have 57% identical VP1 protein sequences [77]. HPyV6 and HPyV7 are also closely related, with 69% identical VP1 protein sequences [65]. It is suggested that antibodies against VP1 may cross-react, with results being questionable if serological tests are not specific enough. The samples used for the serological testing of KIPyV and WUPyV were partially overlapping, so the OD values of the samples tested in both assays were analyzed for correlation. The MCPyV, HPyV6, HPyV7 and TSPyV ELISA tests were performed with the same samples, so the measured OD values were also subjected to correlation analysis. The correlation analysis showed a significant but moderate correlation (r=0.335, $p<0.0001$) between the OD values of KIPyV and WUPyV. A similar moderate, significant correlation (r=0.326, $p<0.0001$) was also observed between the OD values of HPyV6 and HPyV7. For MCPyV and TSPyV, the correlation was low but significant (r=0.277, $p<0.0001$). For polyomaviruses of different genus, the measured seroreactivity values did not show any correlation. Other research groups have published significant but weak correlations [78-81]. Possible cross-reactivity was tested with some selected samples. Serum samples were pre-incubated with 1000 ng of homologous or heterologous protein, and ELISA was performed. After preincubation with homologous proteins, sera became seronegative, whereas heterologous proteins had no such effect. All these results indicate that our ELISA assays were sufficiently specific for the viruses tested.

KI és WU polyomavirus seroprevalence studies

A statistically confirmed increase in KIPyV seroreactivity, a significant difference in OD values, was observed between 10–13 and 14–20 ($p=0.01$) and between 14–20 and 21–39 years age groups ($p=0.005$). Seroprevalence, the proportion of seropositive samples in each age group, is shown in **Figure 1**. Our measurements showed a KIPyV seropositivity rate of 82.1% in the total population, 68.9% in children (<18 years) and 93.7% in adults (>18 years). The age of seropositives was significantly higher in the overall population ($p=0.0001$) as well

as in children (p=0.0002) compared to seronegatives. A significant proportion of primary infections occurred in early childhood, with a 55% transmission rate detected in children under two years of age. The seropositive rate significantly increased with age, as confirmed by the $\chi 2$ trend test (p<0.0001). A significant increase in seropositivity (p=0.015) was observed between the age groups 14–20 and 21–39 years, which is also consistent with the seroreactivity results. The highest seropositivity of 96% was detected in the 21–39 age group. The proportion of antibody-positives was only slightly, but not significantly lower among older people. All these results are in line with international publications [26, 29, 58, 82]. Based on our results, KIPyV is an ubiquitous virus in Hungary.

Figure 1: *KIPyV and WUPyV seroprevalence by age group*



A significant increase in WUPyV seroreactivity was revealed between 0.7–1 and 2–5 years (p=0.03) and between 14–20 and 21–39 years (p=0.004). Antibodies against WUPyV were detected in 79.1% of the total population, 89.2% of adults (>18 years) and 70.2% of children (<18 years) were infected (**Figure 1**). The age of seronegative individuals was significantly lower in the total study population (p=0.02) and in children (p=0.004). The increase in seropositivity rate with age was also confirmed by the $\chi 2$ trend test for WUPyV (p<0.0001). Our seroprevalence results are in line with those published by others [24, 25, 76, 83]. Our results suggest that the majority of primary infections occured in childhood, with as many as 45% of those younger than 2 years being seropositive and as many as 68% of 6-year-olds. Our results showed a significant increase in seropositivity (p=0.03) in adolescence and young adulthood. We observed a slight increase in seropositivity among adults, with the highest antibody-positivity rate (90.8%) detected among the elderly. Our results confirm that WUPyV is ubiquitous in Hungary, with the majority of the population being infected during childhood [29, 56, 82, 84].

KI and WU polyomavirus prevalence in airway samples

KIPyV DNA was detected in the respiratory specimens of 2 SARS-CoV-2 infected (2/680; 0.29%) and 3 SARS-CoV-2 RNA negative patients (3/350; 0.86%), KIPyV prevalence in the studied cohort was 0.49% (5/1030). However, all KIPyV positive samples were from adults, so the adult prevalence was 0.74% (5/680). Nucleic acid of WUPyV was detected in a sample from a SARS-CoV-2 RNA positive adult and in a sample from a SARS-CoV-2 RNA negative child (1/680; 0.15% and 1/350; 0.29%), resulting in a prevalence of 0.19% (2/1030) of WUPyV DNA detected in the chort. The results from the adult samples did not differ significantly from those measured in the respiratory tract samples of immunocompetent adults by other research groups [43, 46, 53, 85, 86]. However, contrary to our expectations, no KIPyV was detected in the respiratory tract samples of children, while WUPyV was detected in only one sample. This is remarkably different from the pre-pandemic prevalence of 5% WUPyV and 1.7% KIPyV derived from respiratory samples from children [46]. The association between SARS-CoV-2 and the two polyomaviruses has been previously investigated by an Italian research team. DNA prevalence of 24.1% for KIPyV and 4.5% for WUPyV in SARS-CoV-2 RNA positive airway samples from adult patients was published, whereas neither virus was detected in SARS-CoV-2 RNA negative samples [32]. Also compared to previous DNA prevalence results from a study of adult respiratory tract samples, a remarkably high positivity polyomavirus positivity rate was observed [13, 85-87].

The question arises why KIPyV was not detected in samples from children, why WUPyV was detected in only one case, and why our data differ from those published by the Italian research group. The explanation could be a methodological difference, but we used the same PCR method with many similar methodological steps. Although different sample types were analysed (oropharyngeal and nasopharyngeal samples), both sample types are equally suitable for testing based on previous publications [13, 46, 85, 86, 88-90]. Immunosuppression may result in a higher prevalence of KIPyV and WUPyV. In the case of the Italian study, the immunological status of the study participants is not fully known, and we also had no complete information about the immune status of the individuals tested. It is important to note that the Italian team collected the samples at the beginning of the pandemic, when the restrictive measures just started in Italy (between March 2020 and May 2020). In contrast, we

collected the samples August–October 2020 and October 2021, after and in between long periods of school and day care closures, lockdown, measures that significantly restricted social contacts,. International publications proved that the introduction of restriction measures reduced the spread of many respiratory infections [91, 92]. Since one potential pathway of transmission for KIPyV and WUPyV is by airborne route, it is very likely that our lower prevalence data is due to these events.

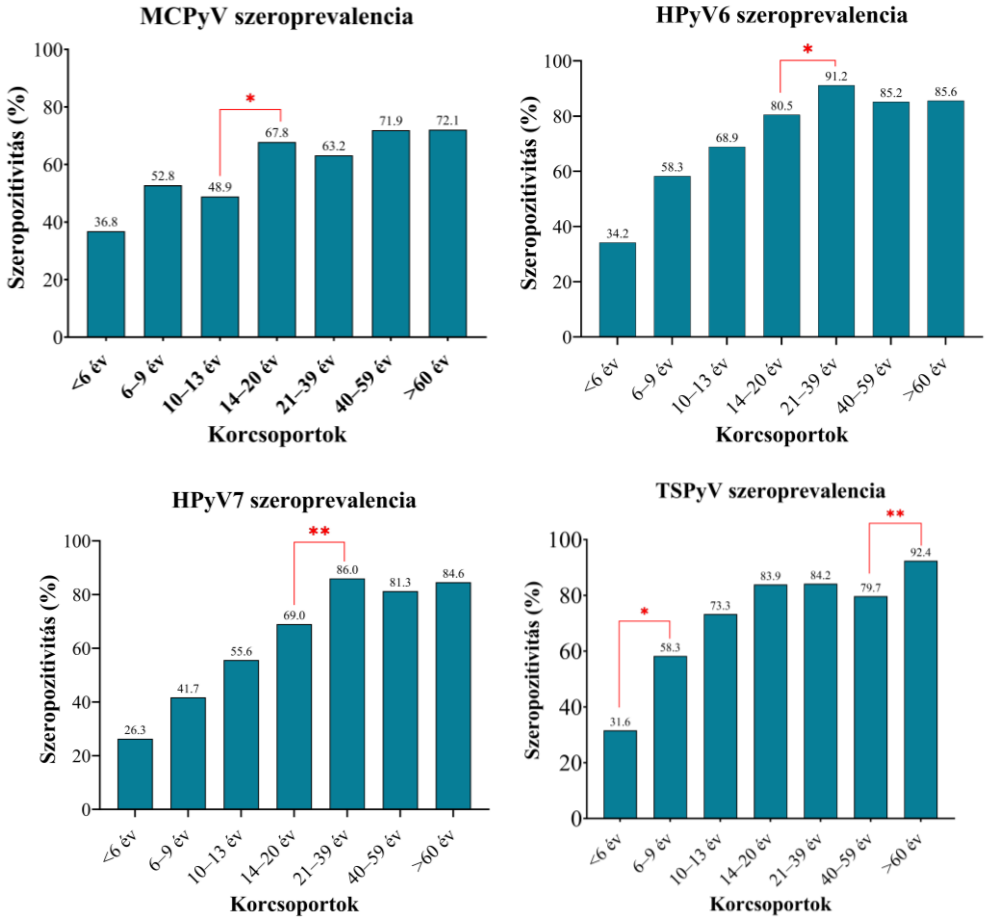
Seroprevalence analysis of polyomaviruses associated with skin disease

No statistically significant difference in OD values was observed between age groups when testing seroreactivity against MCPyV antigen. For HPyV6, a statistically significant increase in seroreactivity was observed in the age groups 6–9 years (p=0.0359) and 21–39 years (p=0.0192), whereas for HPyV7 a significant increase in serorectivity was detected in the age groups 14–20 and 21–39 years (p=0.021 and p=0.003, respectively). A significant increase in OD values measured by TSPyV ELISA was observed between the two youngest (p=0.009) and the two oldest age groups (p=0.029).

In the total study group, the MCPyV infection rate was 63.9%, while the seroprevalence in the adult population was 69.3%. Higher seroprevalence were observed for HPyV6, HPyV7 and TSPyV, with 79.2%, 72.5% and 78.4% seropositivity rates, respectively. For these viruses, the adult seropositivity was above 80%. Although females and males were represented proportionally in the overall cohort, our results showed that significantly more women were infected with MCPyV, HPyV6 and HPyV7 compared to men (p=0.01, p=0.046, p=0.017, respectively). Among adults, no sex difference was found, but among children, significantly more girls were seropositive for HPyV6 and HPyV7 than boys (p=0.0036, p=0.0062). Similar data have not been published by others. There is currently no explanation for the sex differences, and further studies with larger sample sizes and a better understanding of the mode of transmission would be needed.

Seropositivity increased with age for all four viruses, as confirmed by the $\chi 2$ trend test (MCPyV: p=0.0004, HPyV6, HPyV7, TSPyV: p<0.0001) (**Figure 2**).

Figure 2: *Seroprevalence of polyomaviruses associated with skin disease*



For all four viruses, in line with international reports, the proportion of seropositives at young ages was already relatively high [24, 29, 79, 83, 93-96]. Among children under 6 years of age, seropositivity ranged from 26.3 to 36.8%, depending on the virus. A significant increases in the seroprevalence was observed for MCPyV between 10–13 and 14–20 years age groups (p=0.0396) and for HPyV6 and HPyV7 between 14–20 and 21–39 years age groups (p=0.0396, p=0.0052). Consistent with the results of TSPyV seroreactivity, we detected a significant increase in the proportion of seropositives between the two youngest (p=0.0346) and the two oldest (p=0.0084) age groups.

For all four viruses, our results are consistent with the seroprevalence published by other research groups. However, it is interesting to note that the data published from different regions of the world differ by up to 30–50% [29, 79, 80, 93, 97]. This may be explained by variation in the immunoserological method used, but may also be due to geographical differences in virus distribution. However, there may be geographically distinct genetic variants of the viruses which may even differ in antigenicity. To investigate this, we compared our results with those of all publications from which we could extract data for

statistical analysis comparable with our own results. Based on the literature data, 42–88% of adults are seropositive, and our own data of 69.3% is acceptable. Our data are identical or very similar to those from the Czech Republic, Italy, Iran, Cameroon and Australia [24, 79, 98-101]. Significant differences were found when analyzing data from Japan, China, the United States, the Netherlands and Spain [6, 25, 29, 56, 84, 102-106]. Similar results were found for studies using very different methods, while significant differences were also revelaed using almost identical methodologies. This suggests that methodological differences are probably not behind the differences in seroprevalnce data, but rather that there may be actual geographical differences.. This is confirmed by some genome sequence analyses that have suggested a geographical separation of genotypes, but further studies are needed to confirm this [107, 108]. Our adult seroprevalence of HPyV6 and HPyV7 (87.7% and 83.8%, respectively) also matches well with the international data [24, 29, 56, 58, 79, 80, 83, 93]. The comparison of the literature data to the data from this project showed a trend of increasing seropositivity with age, with similarities observed in the antibody positivity rates measured in each age group. However, a significant difference was also observed in some cases. Similarly to those mentioned for MCPyV, in addition to methodological and antigenic differences, there may also be genetic differences in the viruses, which may be geographically distinct. Our own TSPyV seroprevalence of 85% in adults was slightly higher than the 63–80% published by others [25, 29, 79, 80, 109, 110]. However, in our comparisons, the trend of seropositivity with age and the rate of transmission were similar to those reported by other research groups, with significant differences only compared to two studies [29, 56, 79, 80, 94, 111]. For all four viruses, transmission by skin-to-skin direct contact is possible, but we cannot exclude the transmission via respiratory secretions either.

SUMMARY

In the present study, we examined the prevalence of six polyomaviruses from the family *Polyomaviridae*. For our seroprevalence studies of KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, and TSPyV, the capsid antigen (VP1 protein) of all six viruses, was successfully produced in bacterial protein expression system. We developed and optimized an indirect, colorimetric ELISA method for the detection of IgG antibodies against VP1 using the antigens for all six viruses. The assays were performed using large numbers of serum samples from immunocompetent children and adults, and Hungarian seroprevalence was measured and analysed in different age groups. We found that all polyomaviruses studied are ubiquitous in Hungary, which is supported by the adult seroprevalence data. According to our results, 93.7% of adults are seropositive for KIPyV, 89.2% for WUPyV, 69.3% for MCPyV, 87.7% for HPyV6, 83.8% for HPyV7 and 85% for TSPyV. Antibody positivity rates increased with age for all six viruses, with primary infections occurring mostly in childhood, and at very young ages (<6 years) for KIPyV and WUPyV. There was little change in seropositivty rates after young adulthood. Higher seroprevalence rates of MCPyV, HPyV6 and HPyV7 were observed among females. For TSPyV, we observed a significant increase in seropositvity rates in the older age group, above 60 years. By comparing our data with those of international publications, we found that our seroprevalence data is similar to or in agreement with those measured by others. The relatively large differences in the published seropositivity rates are probably not due to differences in serological methods or in the production of antigens.

During the SARS-CoV-2 pandemic, the prevalence of KIPyV and WUPyV DNA in respiratory samples was investigated for possible co-infection. No association between polyomaviruses and SARS-CoV-2 infection was detected. The prevalence of both viruses was much lower than expected, with no KIPyV DNA detected in samples from children and WUPyV DNA detected in only one sample. We hypothesized that the spread of both viruses may have been inhibited by lockdown measures.

NEW FINDINGS

- ✓ We produced antigens of six polyomaviruses suitable for immunoassays, and designed and optimized an ELISA method for seroprevalence studies.
- ✓ We investigated the seroprevalence of KIPyV, WUPyV, MCPyV, HPyV6, HPyV7 and TSPyV polyomaviruses in different age groups in Hungary.
- ✓ We have found that the seroprevalence of KIPyV, WUPyV, MCPyV, HPyV6, HPyV7 and TSPyV increased with age, and these viruses are ubiquitous in Hungary. Primary infections mostly occur in childhood.
- ✓ Comparative analysis has confirmed the hypothesis that MCPyV seroprevalence, or even the virus itself, shows geographical variation.
- ✓ No association was found between KIPyV, WUPyV and SARS-CoV-2 infection. We hypothesize that restrictive measures inhibited the spread of both polyomaviruses.

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   Microorganisms. 10 (4), 1-12, 2022.
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3. Katona, M., **Jeles, K.**, Takács, P., Csoma, E.: DNA and seroprevalence study of MW and STL polyomaviruses.
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