Age-Specific Seroprevalences of Merkel Cell Polyomavirus, Human Polyomaviruses 6, 7, and 9, and Trichodysplasia Spinulosa-Associated Polyomavirus

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Six new human polyomaviruses have been identified since 2008 (Merkel cell polyomavirus [MCPyV], human polyomavirus 6 [HPyV6], HPyV7, HPyV9, trichodysplasia spinulosa polyomavirus [TSPyV], and Malawi polyomavirus [MWPyV]). The presence of specific antibodies against MCPyV, HPyV6, HPyV7, HPyV9, and TSPyV in 828 Italian subjects aged 1 to 100 years was investigated by virus-like particle-based enzyme-linked immunosorbent assays (ELISAs). The findings indicate that all of these new polyomaviruses circulate widely in humans, with seroprevalences in adulthood ranging from 39.4% for HPyV9 to 87.1% for MCPyV, and that primary exposure is most intense in childhood, with the exception of HPyV7 and HPyV9, for which the seroprevalence increased throughout life. The proportion of subjects with high antibody titers was found to increase with age for MCPyV and to decrease with age for TSPyV.

Six new human polyomaviruses have been identified since 2008, including the Merkel cell polyomavirus (MCPyV), associated with Merkel cell carcinoma (1); human polyomaviruses 6, 7, and 9 (HPyV6, HPyV7, and HPyV9) (2-4), not associated with any human disease; trichodysplasia spinulosa polyomavirus (TSPyV), detected in skin lesions of patients with a rare skin disease, trichodysplasia spinulosa (5, 6); and the recently discovered Malawi polyomavirus (MWPyV), isolated from stools of a healthy child (7).

Polyomaviruses are small naked DNA viruses with a capsid composed of three proteins, VP1, VP2, and VP3. The VP1 proteins of these polyomaviruses have the capacity to self-assemble into virus-like particles (VLPs) when expressed in eukaryotic systems, allowing the development of assays to detect specific antibodies and to evaluate the seroprevalence of such infections. Little is known about the natural history of these new polyomaviruses in humans (8, 9). However, serological studies have shown that a large proportion of adults have been exposed to these viruses. The age-specific seroprevalences also indicate widespread exposure early in life to MCPyV (10-13) and TSPyV (14, 15). Assays using VLPs or GST-VP1 seem to be type specific, since no evidence of cross-reactivity has been reported between MCPyV and TSPyV (14, 15), BK polyomavirus (BKPyV) and TSPyV (15), MCPyV, BKPyV, and JC polyomavirus (JCPyV) (10-13), or MCPyV, HPyV6, and HPyV7 (3).

The aim of this study was to investigate and compare age-specific seroprevalences of 5 new human polyomaviruses. We showed that MCPyV and TSPyV are the most prevalent of these new polyomaviruses and that the differences in seroprevalence among polyomaviruses are suggestive of differences in modes of transmission and/or in the rate of persistence of the infection.

MATERIALS AND METHODS

Subjects and samples. Serum samples were collected from 828 individuals from 2010 to 2012. Participants ranged in age from 1 to 100 years and included 350 males and 478 females. Subjects aged 18 to 65 years were healthy blood donors, and sera from subjects aged 1 to 17 years and those aged 66 to 100 years were obtained from discarded clinical laboratory samples, after routine analyses. The hospital records indicated that these samples were from subjects without a history of immuno-suppression/ depression, organ transplantation, immunosuppressive drug treatment, or HIV infection. The County Ethics Committee of Ferrara, Italy, approved the project. Consent from participants was not requested for polyomavirus testing, and samples were therefore deidentified and analyzed anonymously, with indication of age and gender only. All serum samples were stored at -20°C until tested.

Production of VLPs. Production of HPyV9 and MCPyV VLPs in insect cells has been described previously (12, 16), and VLPs were also generated for HPyV6, HPyV7, and TSPyV. Briefly, VP1 proteins from HPyV6 and HPyV7 were PCR amplified from the p6VP1 and p7VP1 plasmids, respectively (3). The TSPyV VP1 coding sequence was obtained by total synthesis with a codon usage-adapted sequence for expression in Spodoptera frugiperda cells (Genscript, Piscataway, NJ) (sequences were based on those under GenBank accession no. HQ696595 and NC014361.1, respectively). After sequence verification, the different VP1 genes were cloned under the control of the polyhedrin promoter of the pFastBac Dual plasmid and further used to generate recombinant baculoviruses, using the Bac-to-Bac system (Invitrogen, FisherScientific, Illkirch, France). HiFive cells maintained in Grace medium (Invitrogen) were infected with the different recombinant baculoviruses for production of the 5 polyomavirus VLPs. VLPs were purified by ultracentrifugation (18 h at 30,000 rpm in a Beckman SW 32 rotor) in a CsCl gradient. The fraction with a density of 1.272 was diluted in phosphate-buffered saline (PBS) and submitted to ultracentrifugation (3 h at 32,000 rpm in a
Beckman SW 32 rotor). The pellet was resuspended in PBS and observed with a JEOL 1011 electron microscope (12, 16) (Fig. 1).

Detection of anti-VP1 antibodies. Microplates (Maxisorp; Nunc) were coated overnight at 4°C with MCPyV, HPyV6, HPyV7, HPyV9, or TSPyV VLPs (100 ng/well in PBS) as previously described (16). Briefly, sera were diluted 1:100, and peroxidase-conjugated anti-human IgG (Southern Biotech, Clinisciences, Nanterre, France) diluted 1:20,000 was used to detect human IgG binding. Histograms of optical density (OD) values for 1- to 10-year-old children (data not shown) revealed a bimodal age distribution of seroreactivity. The cutoff point for seropositivity was defined as the mean of the lower distribution plus 2 standard deviations and was equal to 0.194, 0.169, 0.188, 0.185, and 0.192 for MCPyV, HPyV6, HPyV7, HPyV9, and TSPyV, respectively.

Samples were considered to have high levels of antibodies when the OD value was greater than the median for the seropositive samples, i.e., 2.431 for MCPyV and 1.102 for TSPyV.

MCPyV and TSPyV antibody titers were determined for 48 children aged 1 to 9 years and 48 adults aged more than 60 years. Sera were randomly selected from the subjects who were positive for both anti-MCPyV and anti-TSPyV. Sera were serially diluted 2-fold from 1:100 to 1:204,800, and the endpoint antibody titer was determined as the last dilution that yielded a positive result. Antibody titers were considered to be high if they were in the fourth quartile for all the samples tested for each virus. A high anti-MCPyV titer was >3,200, and a high anti-TSPyV titer was >1,600.

Statistical methods. Logistic regression with adjustment for age and gender was performed using XLStat software (Addinsoft, France). Age- and gender-adjusted odds ratio estimates (OR*) with 95% confidence intervals (95% CI) were obtained to assess the magnitude and statistical significance of the associations between high levels of antibodies, gender,
and age. Correlation analysis of polyomavirus seroreactivities was performed with the Spearman rank correlation test.

RESULTS

Age-specific seroprevalences for the five polyomaviruses investigated are shown in Fig. 2. For MCPyV, the seroprevalence increased with age, from 41.7% in children aged 1 to 4 years to 87.6% in those aged 15 to 19 years. MCPyV seroprevalence was relatively stable in adulthood (79.0% to 96.2%). High levels of infection were also observed for TSPyV and HPyV6. TSPyV antibodies were detected in 31.3% of infants aged 1 to 4 years, and the level increased to 75.3% for children aged 15 to 19 years. There was a slight increase in seroprevalence with age in adulthood, increasing from 64.4% of 30- to 39-year-olds to 91.1% of those aged 80 years and older. Similarly, HPyV6 antibodies were detected in 37.5% of 1- to 4-year-old children and 61.8% of 15- to 19-year-old children, and the seroprevalence increased slightly in adulthood, from 67.1% for 30- to 39-year-old adults to 98.2% for those aged 80 years and older. The seroprevalences of HPyV7 were lower than those shown above, with only 10.4% of 1- to 4-year-old children and 36.0% of 15- to 19-year-olds being seropositive. In adulthood ($\geq$ 20 years), there was a clear increase in seroprevalence with age (44.9% to 85.7%). The lowest seroprevalence observed was for HPyV9, since only 10.4% of 1- to 4-year-olds and 33.7% of 15- to 19-year-old children were seropositive. As for HPyV7, the HPyV9 seroprevalence increased regularly with age, reaching only 41.0% for 60- to 69-year-olds and 69.6% for those aged 80 years and older. In adulthood ($\geq$ 20 years), seroprevalences for MCPyV, HPyV6, HPyV7, HPyV9, and TSPyV were 87.0%, 83.4%, 63.6%, 39.4%, and 76.4%, respectively. Correlation analysis of OD values for all polyomaviruses investigated (Table 1) showed no correlation between these polyomaviruses, with the exception of HPyV6 and HPyV7, for which a Spearman coefficient of 0.433 ($P < 10^{-4}$) was found (Table 1).

Scattergram distributions of OD values for the five polyomaviruses studied indicated that high values were observed for MCPyV and TSPyV but not for HPyV6, -7, and -9 (Fig. 3). High OD values above the median for positive samples were positively associated with age for MCPyV, increasing from 35.03% of

| Virus   | MCPyV RS value$^a$ (P value) | HPyV6 RS value$^a$ (P value) | HPyV7 RS value$^a$ (P value) | HPyV9 RS value$^a$ (P value) | TSPyV RS value$^a$ (P value) |
|---------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| MCPyV   | 1 (0)                        |                              |                              |                              |                              |
| HPyV6   | 0.047 ($<10^{-4}$)           | 1 (0)                        |                              |                              |                              |
| HPyV7   | 0.056 ($<10^{-4}$)           | 0.433 ($<10^{-4}$)           | 1 (0)                        |                              |                              |
| HPyV9   | 0.042 ($<10^{-4}$)           | 0.033 ($<10^{-4}$)           | 0.025 ($<10^{-4}$)           | 1 (0)                        |                              |
| TSPyV   | 0.014 (0.001)                | 0.008 (0.012)                | 0.002 (0.163)                | 0.026 ($<10^{-4}$)           | 1 (0)                        |

$^a$ RS value, Spearman correlation coefficient.

FIG 3 Scattergrams representing the distributions of the 828 human sample reactivities with MCPyV, HPyV6, HPyV7, HPyV9, and TSPyV VLPs (gray bars represent medians).
MCPyV-positive samples for 1- to 9-year-old children to 62.4% of MCPyV-positive samples for those aged 60 years and older (OR* = 2.90; P = 2.5 × 10^-4). In contrast, high enzyme-linked immunosorbent assay (ELISA) levels of reactivity against TSPyV were negatively associated with age, decreasing from 69.8% for 1- to 9-year-old children to 33.7% for older subjects (OR* = 0.22; P ≤ 10^-4) (Table 2).

In order to confirm the high levels of reactivity variations observed throughout the age groups, using single 1/100 dilutions, endpoint dilution titers were determined for a subset of the children (48 children aged 1 to 9 years old) and the oldest adults (48 adults aged more than 60 years). High antibody titers were positively associated with age for MCPyV, increasing from 10.4% of MCPyV-positive samples for 1- to 9-year-old children to 27.1% of MCPyV-positive samples for those aged 60 years and older (OR* = 3.42; P = 0.034). On the other hand, high antibody titers against TSPyV were negatively associated with age, decreasing from 41.7% for 1- to 9-year-old children to 6.3% for older subjects (OR* = 0.09; P = 3.7 × 10^-4) (Table 3). These results confirmed those based on high OD values, using a single dilution.

### DISCUSSION

Antibodies against recently discovered human polyomaviruses were investigated in Italian subjects. Antibodies to MCPyV VP1 were detected in 87% of adults, a proportion similar to the 85% we previously reported for a limited number of blood donors (12) and the 66 to 81% reported for adults in Italy based on a VP1-VLP-based ELISA (13) and slightly higher than the 46 to 64% reported in the United States based on a VP1-GST Luminex-based assay (17), VP1-GST capsomere-based ELISA (10), or VLP-based ELISA (11). Primary exposure to MCPyV probably occurred mainly in early childhood, as there was a seroprevalence of 58% in children less than 10 years of age. This seroprevalence was slightly higher than the 45% reported for children less than 10 years of age in Italy (13) and the 43 to 49% reported for 2- to 15-year-old children in the United States (11) but much higher than the 20 to 30% in the United States reported by Kean et al. (10). In addition, we observed that more than 82% of 10- to 19-year-old subjects had anti-MCPyV antibodies, a proportion higher than the 38 to 60% reported by others for subjects of similar age (10, 13).

We confirmed recently reported findings, obtained using VLP-based ELISA (14) and a VP1-GST Luminex-based assay (15), that TSPyV seroprevalence is high in adults (76%) and comparable to that of MCPyV (87%). However, in contrast to the findings reported by Chen et al. (14), we found that MCPyV seroprevalence was slightly higher than TSPyV seroprevalence in children (Fig. 2). TSPyV seropositivities of 31% among 1- to 4-year-olds and 70% among 5- to 9-year-old children were observed in Italy and were higher than those recently reported by van der Meijden et al. (15) in the Netherlands (41% of 1- to 9-year-old children) and by Chen et al. (14) in Finland (5% of 1- to 4-year-olds and 48% of 6- to 10-year-old children).

A higher seroprevalence was observed for HPyV6 (83%) than for HPyV7 (64%) in adults, similar to the levels reported by Schowalter et al. (3), who used VLP-based ELISAs (69% for HPyV6 and 35% for HPyV7). HPyV9 seroprevalence slowly increased in age in children, reaching only 34% at 15 to 19 years of age and being similar in children and young adults to levels recently reported in Germany (13% in 2- to 5-year-old children and 38% in 11- to 20-year-olds) by Trusch et al. (18), who used a VP1-GST capsomere-based ELISA. However, our results differed for adulthood, since a regular increase in seroprevalence with age was observed, reaching 70% for subjects aged 80 years and older, whereas Trusch et al. (18) reported a peak seroprevalence of 53% in young adults (21- to 30-year-olds) and then a decrease to 35% for 60-year-olds. The differences in seroprevalences reported for these five polyomaviruses between studies could represent true differences in seroprevalence in different countries but could also reflect differences in study populations, in techniques used for the detection of antibodies, and in cutoff definitions.

In our series, the age distribution of positive samples for HPyV6 was similar to those observed for MCPyV and TSPyV, for which infection in adulthood seems rare. In contrast, the regular increase in seroprevalence with age for HPyV7 and HPyV9 suggests that transmission of these two polyomaviruses occurs
throughout life. In addition, we confirmed that there is no cross-reactivity between these new human polyomaviruses, with the exception of HPyV6 and HPyV7, in contrast to the results reported by Schowalter et al. (3). However, cross-reactivity between polyomaviruses has already been reported for BKPyV and simian virus 40 (SV40) (19, 20) and for HPyV9 and African green monkey-derived lymphotropic polyomavirus (LPyV) (16, 18).

Our study has some limitations because the subjects were either blood donors or nonhospitalized patients. However, the sera investigated had been drawn from subjects without a history of immuno-suppression/depression, organ transplantation, immunosuppressive drug treatment, or HIV infection. It is thus unlikely that the seroprevalences reported were significantly different from those in the general Italian population. In addition, there was some heterogeneity in the different VP1 preparations used, but it is unlikely that this would explain the differences in reactivity observed between the different polyomaviruses. No difference in reactivity against monoclonal antibodies was observed between VLPs and capsomeres for papillomaviruses (21), and this can be expected to be the same for polyomaviruses, since all structures have conformational epitopes at the surface. In addition, the variations in the proportions of capsomeres, intermediate-sized capsids, and full-sized VLPs between the different VP1 preparations are unlikely to explain the differences in reactivity observed between polyomaviruses, since, for example, the proportion of small particles and capsomeres was greater in HPyV6 than in HPyV7 preparations but the HPyV6 seroprevalence was higher than the HPyV7 seroprevalence.

A clear increase in high ELISA OD values with age, confirmed by a larger proportion of high titers, was observed for MCPyV antibodies (Tables 2 and 3), in agreement with the findings of Viscidi et al. (13) suggesting that antibody titers increased with age and with those of Tolstov et al. (11) indicating that in most subjects who seroconverted, MCPyV IgG levels increased with advancing age. A correlation between MCPyV loads and antibody titers has been reported (22, 23), suggesting that life-long persistence of MCPyV infection is correlated with higher antibody levels. The increase in MCPyV antibody levels with age may thus reflect the fact that subjects with high antibody titers had active viral replication associated with VP1 production, a state more frequently observed in older subjects due to waning immunity.

Decreases in the proportions of both high TSPyV ELISA OD values and high TSPyV antibody titers were observed with advancing age (Tables 2 and 3), in agreement with a recent study on BKPyV (13), suggesting that TSPyV did not replicate actively in the majority of adult individuals. The persistence of TSPyV is not known. However, the fact that antibody levels declined with age suggests that in contrast to the case for MCPyV, active replication of this polyomavirus is a rare event in adulthood or TSPyV replication is restricted to an immunoprivileged site. Since trichodysplasia spinulosa is a very rare disease that occurs in immunocompromised patients, it could be speculated that primary infection with TSPyV, which is asymptomatic, becomes latent in most individuals and may be reactivated under conditions of immunosuppression.

In conclusion, our findings emphasize the fact that there are differences between the age-specific seroprevalences of these 5 new human polyomaviruses. Because our study cohort was not population based, the seroprevalences reported do not necessarily indicate precise seroepidemiology. However, our findings demonstrate that these polyomaviruses circulate widely in the Italian population, with primary exposure occurring mainly in early childhood, with the exception of HPyV9. For all of the polyomaviruses investigated, there was a tendency toward an increase in seroprevalence in adulthood with advancing age. In addition, the increase in antibody levels with age observed for MCPyV suggests a reactivation of the virus at older ages, with waning immunity with age potentially being responsible for reactivation of infection. In contrast to the case for MCPyV, the decrease in antibody levels with age observed for TSPyV suggests that persistence and latency in immunocompetent individuals are less frequent with this polyomavirus, thus resulting in lowering of antibody levels. Such differences also suggest that these five new human polyomaviruses may have different modes of transmission and capacities of persistence.

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