Seroprevalence of human polyomavirus 9 and cross-reactivity to African green monkey-derived lymphotropic polyomavirus

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Human polyomavirus 9 (HPyV9) was discovered recently in immunocompromised patients and shown to be genetically closely related to B-lymphotropic polyomavirus (LPyV). No serological data are available for HPyV9, but human antibodies against LPyV have been reported previously. To investigate the seroepidemiology of HPyV9 and the sero-cross-reactivity between HPyV9 and LPyV, a capsomer-based IgG ELISA was established using the major capsid protein VP1 of HPyV9 and LPyV. VP1 of an avian polyomavirus was used as control. For HPyV9, a seroprevalence of 47% was determined in healthy adults and adolescents (n = 328) and 20% in a group of children (n = 101). In both groups, the seroreactivities for LPyV were less frequent and the ELISA titres of LPyV were lower. Of the HPyV9-reactive sera, 47% reacted also with LPyV, and the titres for both PyVs correlated. Sera from African green monkeys, the natural hosts of LPyV, reacted also with both HPyV9 and LPyV, but here the HPyV9 titres were lower. This potential sero-cross-reactivity between HPyV9 and LPyV was confirmed by competition assays, and it was hypothesized that the reactivity of human sera against LPyV may generally be due to cross-reactivity between HPyV9 and LPyV. The HPyV9 seroprevalence of liver transplant recipients and patients with neurological dysfunctions did not differ from that of age-matched controls, but a significantly higher seroprevalence was determined in renal and haematopoietic stem-cell transplant recipients, indicating that certain immunocompromised patient groups may be at a higher risk for primary infection with or for reactivation of HPyV9.

INTRODUCTION

Human polyomavirus 9 (HPyV9) is the most recently identified of the nine human polyomaviruses (PyVs) reported to date, and was first detected in a renal transplant patient (Scuda et al., 2011). Later, the same virus was also found in human skin (Sauvage et al., 2011). PyVs are small, non-enveloped, circular dsDNA viruses. Primary infection with BK virus (BKV) and JC virus (JCV) occurs in childhood and is usually asymptomatic (Moen & Johannessen, 2008). Subsequently, these viruses establish a latent infection. Reactivation can occur in immunocompromised patients and cause serious disease, such as BKV-associated nephropathy or haemorrhagic cystitis (Gardner et al., 1971; Jiang et al., 2009), JCV-associated progressive multifocal leukoencephalopathy (Hou & Major, 2000; Jiang et al., 2009; Padgett et al., 1971) and trichodysplasia spinulosa caused by trichodysplasia spinulosa-associated PyV (Matthews et al., 2011; van der Meijden et al., 2010). PyVs have been shown to transform cells in vitro and to be tumorigenic in small laboratory rodents (Chen et al., 1989; Eddy et al., 1962; Gross, 1953; Stewart, 1953). BKV and JCV have been implicated aetiologically in a number of human cancers, but this issue is still controversial (Abend et al., 2009; Maginnis & Atwood, 2009; zur Hausen, 2008). Merkel cell PyV (MCPyV) plays a causative role in Merkel cell carcinoma, a rare but aggressive skin cancer (Becker et al., 2009; Feng et al., 2008).

PyV serology has been used as an indicator for PyV infection because of the absence of overt symptoms during primary infection and insufficient knowledge of the sites of persistence. To date, no commercial seroassays for the detection of human PyVs are available. Therefore, different assay formats have been set up by a number of laboratories using a wide variety of antigen preparations including cultured viruses, virus-like particles (VLPs) formed by
the major structural protein VP1 or PyV capsomers. Serologically, the best-studied PyVs are BKV and JCV. Infection with BKV generally occurs earlier in childhood than JCV infection, and the prevalence in healthy adults is around 50–96 % for BKV and 50–70 % for JCV (Antonsson et al., 2010; Bodaghi et al., 2009; Carter et al., 2009; Egli et al., 2009; Kean et al., 2009; Viscidi & Clayman, 2006). High seroprevalences have also been determined for MCPyV (Tolstov et al., 2009; Touzé et al., 2010; Viscidi et al., 2011), PyVs discovered in respiratory tract specimens (KI virus and WU virus) (Kean et al., 2011; Takemoto & Segawa, 1983; zur Hausen & Gissmann, 1979). It has been reported that up to 30% of adult humans have antibodies against LPyV, and it has been speculated that either LPyV is infectious for humans or an unknown human PyV exists against LPyV, and it has been speculated that either LPyV is infectious for humans or an unknown human PyV exists that is closely related to LPyV (Scuda et al., 2011; Takemoto & Segawa, 1983; Segawa, 1983; zur Hausen & Gissmann, 1979). It has been reported that up to 30% of adult humans have antibodies against LPyV, and it has been speculated that either LPyV is infectious for humans or an unknown human PyV exists that is closely related to LPyV and induces cross-reactive antibodies (Brade et al., 1980; Kean et al., 2009; Takemoto & Segawa, 1983). The aim of the present study, therefore, was to study the sero-cross-reactivity between HPyV9 and LPyV and to determine the seroprevalence of HPyV9 in children and adults using an ELISA. In addition, we analysed serum samples from several patient groups. Based on the fact that PyVs are frequently reactivated in immunocompromised transplant recipients, sera from kidney, liver and haematopoietic stem-cell transplant recipients were tested. Taking into account the fact that JCV has tropism for the central nervous system and that evidence for the presence of BKV, KI virus and WU virus in the central nervous system is accumulating (Barzon et al., 2009; Lopes da Silva, 2011; White et al., 2005), we also analysed patients with neurological dysfunctions.

RESULTS

Seroprevalence of HPyV9 and cross-reactivity to LPyV

A capsomer-based ELISA was established and used for the detection of HPyV9 and LPyV VP1 antibodies by measuring A450. To ensure that the final A450 values for HPyV9 and LPyV VP1 were not derived in part from antibodies to VP1 epitopes conserved among the PyVs or resulting from antibodies non-specific for PyVs, the reactivity of the sera to the VP1 of an avian PyV, budgerigar fledging PyV (BFDPyV), was measured (mean A450 = 0.06), and the values obtained for each serum sample subtracted from the A450 values measured for VP1 of HPyV9 and LPyV. Using this approach, a paediatric population of 101 subjects and 328 healthy adults and adolescents were tested. HPyV9 seroprevalences of 20% (20/101 children) and 47% (154/328 adults and adolescents) were determined. For LPyV, reactivities of 6% (6/101 children) and 26% (84/328 adults and adolescents) were obtained (Fig. 1a). Of the 429 sera, 21% revealed reactivity to HPyV9 VP1 only (n = 92, A450 0.08–1.0) and 19% exerted reactivity to both HPyV9 and LPyV VP1 (n = 82, A450 0.08–3.2 and 0.09–3.0, respectively), but only 2% had reactivity to LPyV VP1 only (n = 8, A450 0.09–0.8). Of the cross-reactive sera, 91% revealed a higher reactivity to HPyV9 than to LPyV (Fig. 1b). The HPyV9 and LPyV antibody titres were correlated (correlation coefficient 0.65) (Fig. 1b), indicating a possible sero-cross-reactivity between HPyV9 and LPyV.

Because of (i) these observations, (ii) the previously reported presence of LPyV antibodies in human sera (Brade et al., 1980; Kean et al., 2009) and (iii) the fact that the genomes and encoded proteins of HPyV9 and LPyV are remarkably similar (genome identity 76%; VP1 amino acid identity 87%) (Scuda et al., 2011), the potential cross-reactivity of HPyV9 and LPyV was analysed further. For this purpose, ten human sera, reactive for both HPyV9 and LPyV VP1, were compared with ten sera of African green monkeys (AGMs), the natural host of LPyV, for their reactivity against HPyV9 and LPyV VP1. Six of the AGM sera co-reacted with HPyV9 and LPyV, whilst the other four were negative for both antigens. Importantly, in contrast to the human sera, the reactivity of the positive AGM sera was always higher to LPyV than to HPyV9 (Fig. 2a, b). These data further indicated sero-cross-reactivity between HPyV9 and LPyV, and competition assays were carried out for confirmation. By pre-incubating HPyV9-reactive human sera with up to 5 μg soluble HPyV9 VP1 ml−1, the anti-HPyV9 VP1 ELISA reactivity was reduced to ~20%. The reactivity was also reduced by pre-incubation with LPyV VP1, but only to ~85% (Fig. 2c). Conversely, the anti-LPyV VP1 ELISA reactivity of AGM sera was reduced to ~30% by pre-incubating the AGM sera with up to 5 μg soluble LPyV VP1 ml−1 (Fig. 2d). With HPyV9 VP1, the reactivity was only reduced to ~80%. Pre-incubation of human and AGM sera with up to 5 μg soluble BKV VP1 ml−1 had no reducing effect (data not shown).

Seroprevalence of HPyV9 in age groups

The seroprevalence of HPyV9 was 13% in children aged 2–5 years and rose to 38% in the group aged 11–20. In young adults aged 21–30, a maximum prevalence of 53% was measured. In the older age groups, a steady decline was observed, resulting in a 35% prevalence in subjects >60 years. The age distribution of LPyV reactivity closely followed the distribution of HPyV9 reactivity, and the number of LPyV-positive sera was smaller in each age group (Fig. 3). In neither age group was a noteworthy difference in HPyV9 seroprevalence between male and female adults observed (data not shown).

Seroprevalence of HPyV9 in patient panels

Sera from kidney (n = 100), haematopoietic stem-cell (n = 50) and liver (n = 50) transplant recipients, as well as...
sera from patients with neurological dysfunctions (n=50), were analysed in the HPyV9 ELISA and compared with age-matched controls. A significantly elevated HPyV9 seroprevalence was seen in the groups of kidney and haematopoietic stem-cell transplant recipients, whereas the liver transplant recipients and the patients with neurological dysfunctions did not show significant differences from the controls (Fig. 4a). The means of the net absorbances were significantly elevated in all four patient groups (Fig. 4b).

**HPyV9 infection of the index patient**

HPyV9 was originally discovered in an immunocompromised patient 837 days after a kidney/pancreas transplantation (Scuda et al., 2011). Sera taken at day 837 and at different time points thereafter were tested for HPyV9 IgM antibodies, IgG antibodies and the avidity of IgG antibodies (sera from earlier time points were not available for antibody testing). At day 837 after transplantation, only a weak IgM absorbance value was measured. Over the following 2 weeks, IgM levels increased and, with a delay and more slowly, so did IgG levels. From day 852, the IgG titre increased further and remained constant after day 1093 at an \( A_{450} \) value of 2.8 for approximately 1.5 years, whilst the IgM titre decreased. The IgG avidity index (AI) rose from 0.35 on day 839 to 0.70, 0.99 and 0.97 on days 852, 1093 and 1552, respectively (Fig. 5, bottom panel).

To detect the genome of HPyV9, DNA samples extracted from the patient sera were analysed with HPyV9-specific nested PCR. PCR was positive for HPyV9 with sera taken at days 837 and 839 after transplantation. Other sera taken at earlier or later time points were PCR negative (Fig. 5, upper panel). Additional analysis of the samples with generic PyV PCR (Scuda et al., 2011) did not reveal the presence of LPyV or human PyVs other than HPyV9.

**DISCUSSION**

We determined the seroprevalence of the recently identified HPyV9 with an ELISA using VP1 capsomers as antigen. In addition to haemagglutinin inhibition tests (Bodaghi et al., 2009; Knowles et al., 2003) and VLP-based assays (Egli et al., 2009; Faust et al., 2011), capsomer-based ELISA formats have been used successfully in several studies on PyV serology (Carter et al., 2009; Kean et al., 2009; Schowalter et al., 2010; van der Meijden et al., 2011). In the present study, we also tested the reactivity of all sera against the VP1 of BFDPyV. We presumed that the analysed human sera did not contain specific antibodies against BFDPyV and therefore we used the reactivities against BFDPyV VP1 as a measure of either non-specific binding to VP1 proteins or reactions against common PyV epitopes. By subtraction of the BFDPyV reactivities from the HPyV9 reactivities, we enhanced the specificity of the...
ELISA for HPyV9 antibodies. A similar approach was carried out previously using murine PyV as the control virus for evaluating the reactivity of human sera against MCPyV, HPyV6 and HPyV7 (Schowalter et al., 2010).

A paediatric and a healthy adult population were used to determine the age of primary infection with HPyV9 and its overall prevalence. The results indicated that infection with HPyV9 occurs in children and young adults and that healthy adults are frequently infected, similar to other human PyVs (Carter et al., 2009; Kean et al., 2009). The seroprevalence of HPyV9 reached its maximum (53%) in early adulthood (age 21–30) and declined slightly towards older age (Fig. 3), resembling that of BKV (Egli et al., 2009; Kean et al., 2009; Knowles et al., 2003). This age distribution suggests that re-exposure to or reactivation of persisting HPyV9 may not occur frequently in immunocompetent, healthy adults. However, in immunocompromised patients undergoing kidney transplantation, significantly higher levels of seroprevalence and IgG titres were observed. This is in line with earlier observations on BKV in kidney-transplant recipients (Bodaghi et al., 2009;)

Fig. 2. Cross-reactivity of HPyV9 and LPyV antibodies. (a, b) The seroreactivity of human sera (a) and AGM sera (b) to HPyV9 VP1 (filled bars) and LPyV VP1 (shaded bars) was measured by ELISA. (c, d) The influence of pre-incubation of a human serum (c) and an AGM serum (d) with soluble HPyV9 VP1 (●) or LPyV VP1 (■) (0.1–5 μg ml⁻¹) as competing antigens before ELISA with HPyV9 VP1 (c) and LPyV VP1 (d) as bound antigen is shown. The values were normalized to those obtained with 0.1–5 μg BKV VP1 ml⁻¹. This antigen was used as a negative control and was defined as 100% (dashed lines).

Fig. 3. HPyV9 and LPyV seroreactivities in different age groups. For HPyV9 (filled bars) and LPyV (shaded bars), the percentages of VP1-specific IgG reactivities of sera from paediatric individuals and healthy adults and adolescents stratified by age are shown. In the youngest group, sera of toddlers <1 year of age were omitted because of the probable presence of maternal antibodies.
Brade et al., 1980; Egli et al., 2009; van der Meijden et al., 2011), and may indicate that these patients have an elevated risk of primary infection or reactivation of persistent HPyV9. One example is the patient in whom HPyV9 was first identified. IgM, IgG and PCR data (Fig. 5) indicated that a primary HPyV9 infection had probably occurred around day 837 after kidney/pancreas transplantation.

We also observed higher levels of seroprevalence and IgG titres in patients undergoing haematopoietic stem-cell transplantation, which might be a consequence of the passive transfer of immunoglobulins from blood donors seropositive for HPyV9. Whilst the application of blood products in the group of our kidney-transplant recipients was rather rare, almost all stem-cell transplant recipients received a number of blood donations during the hospitalization period. Furthermore, the administration of polyvalent immunoglobulins to a few transplanted patients may have contributed to a higher HPyV9 seroprevalence in this patient group. However, HPyV9 reactivation or infection may have played an additional role.

It has been reported that ~30 % of adult humans have LPyV-neutralizing antibodies (Takemoto & Segawa, 1983). In line with this, an LPyV seroprevalence of 10–18 % was reported using ELISA or reporter-vector assays (Brade et al., 1980; Kean et al., 2009; Pastrana et al., 2009; Viscidi & Clayman, 2006). These observations were taken as evidence that LPyV may be infectious for humans, but it was also speculated that an unknown human PyV closely similar to and cross-reacting with LPyV might exist. Short LPyV-like sequences have been detected by PCR in peripheral blood from immunocompromised and healthy subjects (Delbue et al., 2008, 2010), but in other PCR-based studies no evidence for the presence of LPyV in humans was obtained (Costa et al., 2011; Focosi et al., 2009; Scuda et al., 2011; this study). Importantly, the newly identified HPyV9 is closely related to LPyV at the nucleic acid and protein level and therefore is a likely candidate for the previously postulated unknown LPyV-like human PyV. HPyV9 was identified by PCR in serum, plasma and urine of

Fig. 4. HPyV9 seroprevalence in patients. (a) Percentages IgG reactivity, specific for HPyV9 VP1, of sera from kidney-transplant recipients (KTx), haematopoietic stem-cell transplant recipients (HSCTx), liver transplant recipients (LTx) and patients with neurological symptoms (NS) in comparison with age-matched healthy controls (AC). *P<0.05; **P<0.01, as calculated by χ² test. (b) Means of HPyV9 IgG reactivities in each patient group compared with those of AC.

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Fig. 5. Identification of a primary HPyV9 infection in a kidney/pancreas-transplant recipient. In the upper panel, positive (+) and negative (Ø) results of HPyV9-specific PCR with serum samples are shown. In the bottom panel, HPyV9-specific IgG reactivities (●) and IgM reactivities (□) are shown. The avidity of IgG antibodies is indicated as Al values (maximum value 1.0).
immunocompromised subjects (Scuda et al., 2011), and also later in human skin (Sauvage et al., 2011).

In the serological study presented here, many human sera that were ELISA positive for HPyV9 reacted also with LPyV (n=82), although to a lesser extent. A comparable number of sera (n=92) reacted with HPyV9 only, but 74/92 sera had an A_{450} value of <0.3. Therefore, we believe that, in these 74 sera, the reactivity with LPyV was too low to be measured, i.e. below the cut-off value. Overall, 18/92 HPyV9-positive sera had an A_{450} value between 0.3 and 1. Their sole reactivity with HPyV9 VP1 could be due to the fact that the majority of their reactive antibodies might have specificity for HPyV9 only. Alternatively, the HPyV9-reactive antibodies of these sera may in fact be antibodies against an unknown human PyV that cross-reacts with HPyV9 but not with LPyV.

Based on (i) the correlation of HPyV9 and LPyV antibody titres (Fig. 1b), (ii) the almost complete absence of sera specifically reacting with LPyV only (Fig. 1b) and (iii) the reciprocal reactivities of human and AGM sera with HPyV9 and LPyV, respectively (Fig. 2a, b), our study clearly indicates that HPyV9 and LPyV cross-react serologically. Taken together, we suggest that the reactivity of human sera against LPyV may generally be due to cross-reactivity between HPyV9 and LPyV. Whether LPyV is infectious for humans remains to be clarified. Furthermore, it can be concluded that both nucleic acid-based and antibody-based detection methods are necessary to prove infection with a particular PyV.

**METHODS**

**Collection of human and AGM serum samples.** Human serum samples were collected from healthy adolescents and adults (blood donors) (n=328; age range 16–72 years; median 34.5 years) at the Charité University Hospital, Berlin, Germany. Paediatric samples (n=101; age range <1 month to 11 years; median 6 years) were selected randomly from a larger panel of serum samples collected for routine virus diagnostics at the University Hospital Muenster, Germany; Sera from kidney (n=100; range 5–77 years; median 52 years), haematopoietic stem-cell (n=50; range 1–77 years; median 30 years) and liver (n=50; range 7–78 years; median 57 years) transplant recipients, as well as sera from patients with different neurological symptoms (n=50; range 27–86 years; median 57.5 years) were collected for routine diagnostics at the Charité University Hospital. Approval of the local ethics committee was obtained. AGM sera were collected from ten animals housed at the Paul-Ehrlich-Institute (Langen, Germany).

**Expression and purification of recombinant proteins.** The sequences of the major capsid protein, VP1, of HPyV9, LPyV, BKV and BFDPyV (GenBank accession nos HQ696595, M30540, NC_001538 and AB435139, respectively) were codon optimized, commercially synthesized (MrGene GmbH) and inserted into a pTriEx-1.1 plasmid modified to generate VP1 constructs tagged with His at the C terminus. For VP1 expression, the recombinant vectors were transformed in *Escherichia coli* Rosetta BL21(DE3)pLacI cells (Novagen). After induction of expression, insoluble recombinant proteins were obtained in inclusion bodies and purified using BugBuster Protein Extraction Reagent (Novagen) after lysis of the cells and inclusion bodies with 1000 U lysozyme (Novagen). Separation of VP1 from other *E. coli* proteins was carried out under denaturing conditions with 8 M urea, which was then removed by dialysis. The purity of the proteins was analysed by SDS-PAGE and Western blotting using an anti-His mAb (Sigma-Aldrich). Protein concentration was determined by using a Pierce BCA Protein Assay kit (Thermo Scientific). Additionally, the assembly of expressed VP1 into capsomers was confirmed by electron microscopy (data not shown).

**ELISA and statistical analysis.** An ELISA was developed by coating F96 PolySorp MicroWell plates (Nunc, Thermo Scientific) with purified VP1 (50 ng per well) in PBS (pH 7.2) for 1 h at 37 °C. Plates were washed three times with 800 µl PBS/0.05% Tween (PBS-T). To inhibit non-specific binding, 200 µl blocking buffer (PBS-T with 5% casein) was added per well for 2 h at 37 °C. Human sera were diluted 1:200 and allowed to react with the antigen-coated wells for 1 h at 37 °C. Plates were washed three times with 800 µl PBS-T and an HRP-conjugated, secondary rabbit anti-human IgG antibody (Dianova) diluted 1:10 000, was added for detection of IgG antibody. An HRP-conjugated, secondary sheep anti-human IgM antibody (Seramun) diluted 1:20 000, was added for detection of IgM antibody. After an additional washing step (three washes with 800 µl PBS-T), the peroxidase substrate tetramethylbenzidine (Kem-En-Tec Diagnostics) was added for 10 min at room temperature in the dark. The reaction was stopped with 1 M H_{2}SO_{4}. A_{450} was measured in a microplate spectrometer (MBG Labtech). All blank wells had A_{450} values of <0.1. The optimal concentration of the antigen used to coat the microtitre plates and the optimal dilution of sera and conjugate were determined by chequerboard titration.

The data were analysed with a χ² test to estimate significance differences among independent groups of individuals. Correlation analysis between HPyV9 and LPyV reactivities was performed with Spearman’s rank correlation test.

For competition assays, serum samples were pre-incubated for 1 h at 37 °C with 0–5 µg VP1 antigen mL⁻¹ before evaluation in the ELISA. For each ELISA plate, a fixed set of sera was used to control for inter-serial variations.

Antibody avidity was measured with a modified ELISA by adding PBS only or 6 M urea in PBS to each well after the serum incubation step. The AI was determined by calculating the ratio of serum incubated with PBS only to serum incubated with 6 M urea.

**Cut-off value.** The cut-off value for the ELISA was determined experimentally. The background reactivities detected in wells without antigen coating and those without both antigen and serum (blanks) were subtracted from the A_{450} values measured in the VP1-coated wells. The cut-off value defining a positive serological response was defined as the mean of all negative A_{450} values plus SD (0.08 for HPyV9 and 0.09 for LPyV).

**DNA extraction and PCR.** DNA extraction from sera and nested PCR with primers specific for HPyV9 VP1 as well as a generic PyV PCR were carried out as described previously (Scuda et al., 2011).

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