Originally published as:

Trusch, F., Klein, M., Finsterbusch, T., Kühn, J., Hofmann, J., Ehlers, B.
Seroprevalence of human polyomavirus 9 and cross-reactivity to African green monkey-derived lymphotropic polyomavirus
(2012) Journal of General Virology, 93 (4), pp. 698-705.

DOI: 10.1099/vir.0.039156-0

Seroprevalence of the human polyomavirus 9 (HPyV9) and cross-reactivity to the African green monkey-derived lymphotropic polyomavirus (LPyV)

Franziska Trusch (1), Marcus Klein (1), Tim Finsterbusch (1), Joachim Kühn (2), Jörg Hofmann (3)[#], Bernhard Ehlers (1)[#*]

(1) Division of Viral Infections, Robert Koch-Institute, Berlin, Germany.
(2) Institute of Medical Microbiology, Clinical Virology, University Hospital Muenster, Muenster, Germany
(3) Institute of Virology, Charité - Universitätsmedizin Berlin, Berlin, Germany

[#] These authors contributed equally to the work

* Corresponding author:
Dr. B. Ehlers
Division of Viral Infections
Robert Koch Institute
Nordufer 20
13353 Berlin
email: EhlersB@rki.de
phone: ++4930187542347
fax: ++4930187542598

Running title: Seroprevalence of human polyomavirus 9

Key words: Polyomavirus, Polyomaviridae, HPyV9, LPyV, human, seroprevalence, antibody

Abstract

Human polyomavirus 9 (HPyV9) was recently discovered in immunocompromised patients and shown to be genetically closely related to the 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-crossreactivity between HPyV9 and LPyV, a capsomer-based IgG ELISA was established using the major capsid proteins 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 pediatric group of children (n=101). In both groups, the seroreactivities for LPyV were less frequent and the ELISA titers of LPyV were lower. Of the HPyV9-reactive sera, 47% reacted also with LPyV, and the titers 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 titers were lower. This potential sero-crossreactivity between HPyV9 and LPyV was confirmed by competition assays and it is hypothesized that the reactivity of human sera against LPyV may be generally due to crossreactivity 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 hematopoietic stem cell transplant recipients indicating that certain immunocompromised patient groups may be at a higher risk for primary infection with or reactivation of HPyV9.

Introduction

The human polyomavirus 9 (HPyV9) is the most recently identified among the nine human polyomaviruses (PyVs) known 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 double-stranded DNA viruses. Primary infection with BK virus (BKV) and JC virus (JCV) occurs in childhood and is usually asymptomatic (Moens & 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 hemorrhagic 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 TSV-associated *Trichodysplasia spinulosa* (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 etiologically implicated in a number of human cancers, but this issue is still controversial (Abend *et al.*, 2009; Maginnis & Atwood, 2009; zur Hausen, 2008). The Merkel cell polyomavirus (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 sites of persistence. Until today, no commercial sero-assays for the detection of human PyVs are available. Therefore, different assay formats have been set up in the past 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. The serologically best-studied polyomaviruses are BKV and JCV. Infection with BKV occurs generally earlier in childhood than with JCV, 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; Touze *et al.*, 2010; Viscidi *et al.*, 2011), PyVs discovered in respiratory tract specimens (KIV and WUV) (Kean *et al.*, 2009; Neske *et al.*, 2010; Nguyen *et al.*, 2009), and PyVs with skin tropism (HPyV6 and HPyV7) (Schowalter *et al.*, 2010).

For HPyV9, no seroepidemiological studies are available to date. However, HPyV9 is closely related (genome identity: 76%) to the B-lymphotropic polyomavirus (LPyV; also named African green monkey PyV) (Scuda *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 that is closely related to LPyV and induces crossreactive antibodies (Brade *et al.*, 1981; Kean *et al.*, 2009; Takemoto & Segawa, 1983). The aim of the present study was therefore to study the sero-crossreacitvity between HPyV9 and LPyV and to determine the seroprevalence of HPyV9 in children and adults using an ELISA. In addition, we analysed sera of several patient groups. Based on the fact that PyVs are frequently reactivated in immunocompromised transplant recipients, sera of kidney, liver and hematopoietic stem cell transplant recipients were tested. Taking into account that JCV has tropism for the central nervous system and that evidence for the presence of BKV, KIV and WUV in the central nervous system is accumulating (Lopes da Silva, 2011; Barzon *et al.*, 2009; White *et al.*, 2005), we also analysed patients with neurological dysfunctions.

Results

Seroprevalence of HPyV9 and crossreactivity to LPyV

A capsomer-based ELISA was established and used for the detection of HPyV9-VP1 and LPyV-VP1 antibodies. To ensure that the final OD_{450} values for HPyV9-VP1 and LPyV-VP1 were not in part derived from antibodies to VP1 epitopes conserved among the PyVs or resulted from antibodies non-specific for PyVs, the reactivity of the sera to the VP1 of an avian PyV (Budgerigar fledging polyomavirus [BFDPyV]) was measured (Mean OD_{450}=0.06), and the values obtained for each serum subtracted from the ODs measured for VP1 of HPyV9 and LPyV. Using this approach, a pediatric 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 (Figure 1A). Of the 429 sera, 22% revealed reactivity to HPyV9-VP1 only (n=92, OD_{450} 0.08 to 1.0) and 19% exerted reactivity to both HPyV9-VP1 (n=82, OD_{450} 0.08 to 3.2) and LPyV-VP1 (OD_{450} 0.09-3.0), but only 2% had reactivity to LPyV-VP1 only (n=8, OD_{450} 0.09 to 0.8). Of the co-reactive sera, 91% revealed a higher reactivity to HPyV9 than to LPyV (Figure 1B). The HPyV9 and LPyV antibody titers were correlated (correlation coefficient: 0.65) (Figure 1B), indicating a possible sero-crossreactivity between HPyV9 and LPyV.

Because of (i) these observations, (ii) the previously reported presence of LPyV antibodies in human sera (Brade *et al.*, 1981; 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 crossreactivity between HPyV9 and LPyV was further analysed. For this purpose, 10 human sera, reactive for both HPyV9-VP1 and LPyV-VP1, were compared with 10 sera of AGMs, the natural hosts of LPyV, for their reactivities against HPyV9-VP1 and LPyV-VP1. Six of the AGM sera co-reacted with HPyV9 and LPyV (the other 4 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 (examples shown in Figures 2A and 2B). These data further indicated sero-crossreactivity between HPyV9 and LPyV, and competition assays were carried out for confirmation. By pre-incubating HPyV9-reactive human sera with up to 5 µg/ml of soluble HPyV9-VP1, the anti-HPyV9-VP1 ELISA reactivity was reduced to approximately 20%. The reactivity was also reduced by pre-incubation with LPyV-VP1, but only to approximately 85% (example in Figure 2C). Conversely, the anti-LPyV-VP1 ELISA reactivity of AGM sera was reduced to approximately 30% by pre-incubating the AGM sera with up to 5 µg/ml of soluble LPyV-VP1. With HPyV9-VP1, the reactivity was only reduced to approximately 80% (example in Figure 2D). Pre-incubation of human and AGM sera with up to 5 µg/ml of soluble BKV-VP1 had no reducing effect (data not shown).

Seroprevalence of HPyV9 in age groups

The seroprevalence of HPyV9 was 13% in children of age 2-5 and rose to 38% in the group of age 11-20. In young adults of age 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 of age >60. 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 (Figure 3). In neither age group, a noteworthy difference in HPyV9 seroprevalence between male and female adults was observed (data not shown).

Seroprevalence of HPyV9 in patient panels

Sera from kidney (n=100), hematopoietic 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 to age-matched controls. A significantly elevated HPyV9 seroprevalence was seen in the groups of kidney and hematopoietic stem cell transplant recipients. The liver transplant recipients and the patients with neurological dysfunctions did not show significant differences to the controls (Figure 4A). The means of netto absorbances were significantly elevated in all four patient groups (Figure 4B).

HPyV9 infection of the index patient

HPyV9 had been discovered in an immunocompromised patient 837 days after kidney/pancreas transplantation (Scuda *et al.*, 2011). Sera taken at day 837 and at different time points thereafter were tested here for HPyV9 IgM antibodies, IgG antibodies and 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 was measured. During the following 2 weeks, IgM increased and, with delay and more slowly, also IgG. From day 852, the IgG titer further increased and remained constant after day 1093 at OD_{450} 2.8 for approximately 1.5 years, while the IgM titer decreased. The IgG avidity index (AI) rose from AI=0.35 at day 839 to AI=0.70, 0.99 and 0.97 at days 852, 1093 and 1552, respectively (Figure 5, lower part).

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 (Figure 5, upper part). 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 have determined the seroprevalence of the recently identified HPyV9 with an ELISA using VP1 capsomers as antigen. Beside hemagglutinin inhibition test (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 successfully used in several studies on polyomavirus 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 do not contain specific antibodies against BFDPyV and therefore used the reactivities against BFDPyV-VP1 as a measure for either unspecific 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 pediatric and a healthy adult population were used to determine the age of primary infection with HPyV9 and overall prevalence. The results indicate that infection with HPyV9 occurs in children and young adults and that healthy adults are frequently infected, similar to other human polyomaviruses (Carter *et al.*, 2009; Kean *et al.*, 2009). Seroprevalence of HPyV9 has its maximum (53%) in early adulthood (age 21-30) and slightly declines towards older age (Figure 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 titers were observed. This is in line with earlier observations on BKV in kidney transplant recipients (Bodaghi *et al.*, 2009; Brade *et al.*, 1981; 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. The IgM, IgG and PCR data, shown in Figure 5, indicate that a primary HPyV9 infection had likely occurred around day 837 after kidney/pancreas transplantation.

We also observed higher levels of seroprevalence and IgG titers in patients undergoing hematopoietic stem cell transplantation which might be a consequence of the passive transfer of immunoglobulins from blood donors seropositive for HPyV9. While the application of blood products in the group of our kidney transplant recipients was rather rare, almost all stem cell transplant recipients received multiple blood donations during the hospitalisation period. Furthermore, the administration of polyvalent immunoglobulins to 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 approximately 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.*, 1981; 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 crossreacting 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; Delbue *et al.*, 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 on the nucleic acid and protein level closely related to LPyV and therefore is the likely candidate for the previously postulated LPyV-like unknown human PyV. HPyV9 was identified by PCR in serum, plasma and urine of immunocompromised subjects (Scuda *et al.*, 2011), later also in human skin (Sauvage *et al.*, 2011).

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

Based on (i) the correlation of HPyV9 and LPyV antibody titers (Figure 1B), (ii) the near complete absence of sera specifically reacting with LPyV only (Figure 1B) and (iii) the reciprocal reactivities of human and AGM sera with HPyV9 and LPyV, respectively (Figure 2A,B), our study clearly indicates that HPyV9 and LPyV serologically crossreact. Taken together we put forward the hypothesis that the reactivity of human sera against LPyV may be generally due to crossreactivity between HPyV9 and LPyV. Whether LPyV is infectious for humans remains to be clarified. Furthermore, it can be generally concluded that both nucleic acid-based and antibody-based detection methods are necessary to prove the infection with a certain polyomavirus.

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. Pediatric samples (n=101; range: <1month to 11 years; median 6 years) were randomly selected from a larger panel of serum samples collected for routine virus diagnostics at the university hospital of Munster, Germany. Sera from kidney (n=100; range: 5–77 years; median 52 years), hematopoietic 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, Berlin, Germany. Approval of the local Ethics Committee was obtained. AGM sera were collected from 10 animals housed at the Paul-Ehrlich-Institute (Langen, Germany).

Expression and purification of recombinant proteins

The sequences of the major capsid proteins VP1 of HPyV9, LPyV, BKV and BFDPyV (Genbank accession numbers: HQ696595, M30540, NC001538, AB453159) were codon-optimized, commercially synthesized (MrGene GmbH, Regensburg, Germany) and inserted into a pTriEx-1.1 plasmid modified to generate VP1 constructs tagged with 6xhistidine at the C-terminus. For VP1 expression, the recombinant vectors were transformed in *E. coli* Rosetta(DE3)pLacI™ cells (Novagen, San Diego, USA). After induction of expression insoluble recombinant proteins were obtained in inclusion bodies and purified with BugBuster Protein Extraction Reagent (Novagen) after lysis of cells and inclusion bodies with 1000 U Lysozym (Novagen). Separation of VP1 from other *E. coli* proteins was done under denaturing conditions with 8M urea that was finally removed by dialysis. Purity of proteins was analysed with SDS–Page and Western Blot using an anti-His monoclonal antibody (Sigma-Aldrich, St. Louis, USA). Protein concentration was determined by measuring with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Additionally, the assembly of expressed VP1 to capsomers was confirmed by electron microscopy (data not shown).

ELISA and statistical analysis

An ELISA was developed by coating F96 polysorp micro well™ Plates (Nunc, Thermo Scientific, Roskilde, Denmark) with purified VP1 (50 ng per well) in PBS (pH 7.2) for 1 h at 37 °C. Plates were washed 3x with 800 µl PBS / 0.05 % Tween (PBS-T). To inhibit non-specific binding 200 µl blocking buffer (PBS-T with 5 % casein) per well was added 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 3x with 800 µl PBS-T and a HRPO-conjugated, secondary rabbit anti-human IgG antibody (Dianova, Hamburg, Germany) diluted 1:10,000, was added for detection of IgG antibody. A POD-conjugated, secondary sheep anti-human IgM antibody (Seramun, Heidesee, Germany) diluted 1:20,000, was added for

detection of IgM antibody. After an additional washing step (3x with 800 µl PBS-T) peroxidase substrate TMB (tetramethylbenzidene, Taastrup, Denmark) was added for 10 min at room temperature in the dark. The reactions were stopped with 2N H_2SO_4. Optical density (OD_{450}) was measured on a microplate spectrometer (BMG Labtech, Offenburg, Germany) at λ=450 nm. All blank wells had adsorbance values < 0.1. The optimal concentration of the antigen used to coat the microtiter plates and the optimal dilution of sera and conjugate was determined by checkerboard titration.

The data were analysed with the X^2-test to estimate significance of differences among independent groups of individuals. Correlation analysis between HPyV9 and LPyV reactivities was performed with the Spearman rank correlation test.

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

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

Cut-off value

The COV 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 ODs measured in VP1-coated wells. The COV defining a positive serologic response was defined as the mean of all negative ODs plus standard deviation (COV_{HPyV9}: OD_{450} 0.08; COV_{LPyV}: OD_{450} 0.09).

DNA extraction and PCR.

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

Acknowledgements

The excellent technical assistance of Severine Lepek, Gabriele Kerger, Sonja Liebmann, Nezlisah Yasmum, and Cornelia Walter as well as the supply with African green monkey sera by Stephen Norley is gratefully acknowledged.

Figure legends

Figure 1 Reactivity of human sera to VP1 of HPyV9 and LPyV. (A) The percentage of seroreactivity of pediatric sera (n=101; age: <1month to 11 years) and sera of healthy adults and adolescents (n=328; age: 16 – 72 years) against VP1 of HPyV9 (black bars) and LPyV (grey bars) in capsomer-based ELISA is shown. (B) Correlation of antibody reactivity against VP1 of HPyV9 and LPyV (correlation coefficient = 0.65) in the 429 sera analysed. Cut-off values for the detection of HPyV9 (0.08, vertical line) and LPyV (0.091, horizontal line) are indicated by dashed lines. Upper left area shows LPyV reactivity only (2%), upper right area HPyV9 and LPyV co-reactivity (19%), bottom left area seronegative samples (57%) and bottom right area HPyV9 reactivity only (22%). Magnified and highlighted by grey color is the part of (B) which includes only sera of OD_{450} <0.5.

Figure 2 Crossreactivity of HPyV9 and LPyV antibodies. The seroreactivity of human sera (A) and AGM sera (B) to HPyV9-VP1 (black bars) and LPyV-VP1 (grey bars) was measured with ELISA. The influence of pre-incubation of a human serum (C) and an AGM serum (D) with 0.1-5µg/ml soluble VP1 of HPyV9 (closed squares) or LPyV (closed circles) 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 0.1-5µg/ml of BKV-VP1. This antigen was used as negative control and defined as 100% (dashed lines).

Figure 3 HPyV9 und LPyV seroreactivities in age groups. For HPyV9 (black bars) and LPyV (grey bars), percentages of VP1-specific IgG reactivities of sera from pediatric individuals and healthy adults and adolescents stratified by age are shown. In the youngest group, sera of toddlers under one year were omitted because of the likely presence of maternal antibodies.

Figure 4 HPyV9 seroprevalence in patients. (A) Percentages of IgG reactivities, specific for HPyV9-VP1, of sera from kidney-transplant recipients (KTx), hematopoietic stem cell transplant recipients (HSCTx), liver transplant recipients (LTx) and patients with neurological symptoms (NS) in comparison to age-matched healthy control groups (AC). * = p> 0.05 and ** = p> 0.01, as calculated by X^2-test. (B) Means of HPyV9 IgG reactivities in each patient group compared to those of AC.

Figure 5 Identification of a primary HPyV9 infection in a kidney/pancreas-transplant recipient. In the upper part of the figure, positive (+) and negative (Ø) results of HPyV9-specific PCR with serum samples are shown. In the bottom part of the figure, HPyV9-specific IgG reactivities (closed circles) and IgM reactivities (open squares) are shown. Avidity of IgG antibodies is indicated as avidity index (AI, max. 1.0).

References

Abend, J. R., Jiang, M. & Imperiale, M. J. (2009). BK virus and human cancer: innocent until proven guilty. *Seminars in cancer biology* **19**, 252-260.

Antonsson, A., Green, A. C., Mallitt, K. A., O'Rourke, P. K., Pawlita, M., Waterboer, T. & Neale, R. E. (2010). Prevalence and stability of antibodies to the BK and JC polyomaviruses: a long-term longitudinal study of Australians. *J Gen Virol* **91**, 1849-1853.

Barzon, L., Squarzon, L., Militello, V., Trevisan, M., Porzionato, A., Macchi, V., De Caro, R. & Palu, G. (2009). WU and KI polyomaviruses in the brains of HIV-positive patients with and without progressive multifocal leukoencephalopathy. *J Infect Dis* **200**, 1755-1758.

Becker, J. C., Schrama, D. & Houben, R. (2009). Merkel cell carcinoma. *Cell Mol Life Sci* **66**, 1-8.

Bodaghi, S., Comoli, P., Bosch, R., Azzi, A., Gosert, R., Leuenberger, D., Ginevri, F. & Hirsch, H. H. (2009). Antibody responses to recombinant polyomavirus BK large T and VP1 proteins in young kidney transplant patients. *J Clin Microbiol* **47**, 2577-2585.

Brade, L., Muller-Lantzsch, N. & zur Hausen, H. (1981). B-lymphotropic papovavirus and possibility of infections in humans. *J Med Virol* **6**, 301-308.

Carter, J. J., Paulson, K. G., Wipf, G. C., Miranda, D., Madeleine, M. M., Johnson, L. G., Lemos, B. D., Lee, S., Warcola, A. H., Iyer, J. G., Nghiem, P. & Galloway, D. A. (2009). Association of Merkel cell polyomavirus-specific antibodies with Merkel cell carcinoma. *J Natl Cancer Inst* **101**, 1510-1522.

Chen, J. D., Neilson, K. & Van Dyke, T. (1989). Lymphotropic papovavirus early region is specifically regulated transgenic mice and efficiently induces neoplasia. *J Virol* **63**, 2204-2214.

Costa, C., Bergallo, M., Terlizzi, M. E., Cavallo, G. P., Cavalla, P. & Cavallo, R. (2011). Lack of detection of lymphotropic polyomavirus DNA in different clinical specimens. *J Clin Virol* **51**, 148-149.

Delbue, S., Tremolada, S., Branchetti, E., Elia, F., Gualco, E., Marchioni, E., Maserati, R. & Ferrante, P. (2008). First identification and molecular characterization of lymphotropic polyomavirus in peripheral blood from patients with leukoencephalopathies. *J Clin Microbiol* **46**, 2461-2462.

Delbue, S., Tremolada, S., Elia, F., Carloni, C., Amico, S., Tavazzi, E., Marchioni, E., Novati, S., Maserati, R. & Ferrante, P. (2010). Lymphotropic polyomavirus is detected in peripheral blood from immunocompromised and healthy subjects. *J Clin Virol* **47**, 156-160.

Eddy, B. E., Borman, G. S., Grubbs, G. E. & Young, R. D. (1962). Identification of the oncogenic substance in rhesus monkey kidney cell culture as simian virus 40. *Virology* **17**, 65-75.

Egli, A., Infanti, L., Dumoulin, A., Buser, A., Samaridis, J., Stebler, C., Gosert, R. & Hirsch, H. H. (2009). Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis* **199**, 837-846.

Faust, H., Pastrana, D. V., Buck, C. B., Dillner, J. & Ekstrom, J. (2011). Antibodies to Merkel cell polyomavirus correlate to presence of viral DNA in the skin. *J Infect Dis* **203**, 1096-1100.

Feng, H., Shuda, M., Chang, Y. & Moore, P. S. (2008). Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* **319**, 1096-1100.

Focosi, D., Maggi, F., Andreoli, E., Lanini, L., Ceccherini-Nelli, L. & Petrini, M. (2009). Polyomaviruses other than JCV are not detected in progressive multifocal leukoencephalopathy. *J Clin Virol* **45**, 161-162.

Gardner, S. D., Field, A. M., Coleman, D. V. & Hulme, B. (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet* **1**, 1253-1257.

Gross, L. (1953). A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proc Soc Exp Biol Med* **83**, 414-421.

Hou, J. & Major, E. O. (2000). Progressive multifocal leukoencephalopathy: JC virus induced demyelination in the immune compromised host. *J Neurovirol* **6 Suppl 2**, S98-S100.

Jiang, M., Abend, J. R., Johnson, S. F. & Imperiale, M. J. (2009). The role of polyomaviruses in human disease. *Virology* **384**, 266-273.

Kean, J. M., Rao, S., Wang, M. & Garcea, R. L. (2009). Seroepidemiology of human polyomaviruses. *PLoS pathogens* **5**, e1000363.

Knowles, W. A., Pipkin, P., Andrews, N., Vyse, A., Minor, P., Brown, D. W. & Miller, E. (2003). Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. *J Med Virol* **71**, 115-123.

Lopes da Silva, R. (2011). Polyoma BK virus: an emerging opportunistic infectious agent of the human central nervous system. *The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases* **15**, 276-284.

Maginnis, M. S. & Atwood, W. J. (2009). JC virus: an oncogenic virus in animals and humans? *Seminars in cancer biology* **19**, 261-269.

Matthews, M. R., Wang, R. C., Reddick, R. L., Saldivar, V. A. & Browning, J. C. (2011). Viral-associated trichodysplasia spinulosa: a case with electron microscopic and molecular detection of the trichodysplasia spinulosa-associated human polyomavirus. *J Cutan Pathol* **38**, 420-431.

Moens, U. & Johannessen, M. (2008). Human polyomaviruses and cancer: expanding repertoire. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* **6**, 704-708.

Neske, F., Prifert, C., Scheiner, B., Ewald, M., Schubert, J., Opitz, A. & Weissbrich, B. (2010). High prevalence of antibodies against polyomavirus WU, polyomavirus KI, and human bocavirus in German blood donors. *BMC Infect Dis* **10**, 215.

Nguyen, N. L., Le, B. M. & Wang, D. (2009). Serologic evidence of frequent human infection with WU and KI polyomaviruses. *Emerg Infect Dis* **15**, 1199-1205.

Padgett, B. L., Walker, D. L., ZuRhein, G. M., Eckroade, R. J. & Dessel, B. H. (1971). Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. *Lancet* **1**, 1257-1260.

Pastrana, D. V., Tolstov, Y. L., Becker, J. C., Moore, P. S., Chang, Y. & Buck, C. B. (2009). Quantitation of human seroresponsiveness to Merkel cell polyomavirus. *PLoS pathogens* **5**, e1000578.

Sauvage, V., Foulongne, V., Cheval, J., Ar Gouilh, M., Pariente, K., Dereure, O., Manuguerra, J. C., Richardson, J., Lecuit, M., Burguiere, A., Caro, V. & Eloit, M. (2011). Human polyomavirus related to african green monkey lymphotropic polyomavirus. *Emerg Infect Dis* **17**, 1364-1370.

Schowalter, R. M., Pastrana, D. V., Pumphrey, K. A., Moyer, A. L. & Buck, C. B. (2010). Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe* **7**, 509-515.

Scuda, N., Hofmann, J., Calvignac-Spencer, S., Ruprecht, K., Liman, P., Kuhn, J., Hengel, H. & Ehlers, B. (2011). A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus. *J Virol* **85**, 4586-4590.

Stewart, H. L. (1953). Pulmonary tumors in animals with particular reference to mice. *Acta Unio Int Contra Cancrum* **9**, 512-528.

Takemoto, K. K. & Segawa, K. (1983). A new monkey lymphotropic papovavirus: characterization of the virus and evidence of a related virus in humans. *Prog Clin Biol Res* **105**, 87-96.

Tolstov, Y. L., Pastrana, D. V., Feng, H., Becker, J. C., Jenkins, F. J., Moschos, S., Chang, Y., Buck, C. B. & Moore, P. S. (2009). Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. *Int J Cancer* **125**, 1250-1256.

Touze, A., Gaitan, J., Arnold, F., Cazal, R., Fleury, M. J., Combelas, N., Sizaret, P. Y., Guyetant, S., Maruani, A., Baay, M., Tognon, M. & Coursaget, P. (2010). Generation of Merkel cell

polyomavirus (MCV)-like particles and their application to detection of MCV antibodies. *J Clin Microbiol* **48**, 1767-1770.

van der Meijden, E., Janssens, R. W., Lauber, C., Bouwes Bavinck, J. N., Gorbalenya, A. E. & Feltkamp, M. C. (2010). Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. *PLoS pathogens* **6**, e1001024.

van der Meijden, E., Kazem, S., Burgers, M. M., Janssens, R., Bouwes Bavinck, J. N., de Melker, H. & Feltkamp, M. C. (2011). Seroprevalence of Trichodysplasia Spinulosa-associated Polyomavirus. *Emerg Infect Dis* **17**, 1355-1363.

Viscidi, R. P. & Clayman, B. (2006). Serological cross reactivity between polyomavirus capsids. *Adv Exp Med Biol* **577**, 73-84.

Viscidi, R. P., Rollison, D. E., Sondak, V. K., Silver, B., Messina, J. L., Giuliano, A. R., Fulp, W., Ajidahun, A. & Rivanera, D. (2011). Age specific-seroprevalence to Merkel cell polyomavirus, BK virus and JC virus. *Clin Vaccine Immunol*.

White, M. K., Gordon, J., Reiss, K., Del Valle, L., Croul, S., Giordano, A., Darbinyan, A. & Khalili, K. (2005). Human polyomaviruses and brain tumors. *Brain research Brain research reviews* **50**, 69-85.

zur Hausen, H. (2008). Novel human polyomaviruses--re-emergence of a well known virus family as possible human carcinogens. *Int J Cancer* **123**, 247-250.

zur Hausen, H. & Gissmann, L. (1979). Lymphotropic papovaviruses isolated from African green monkey and human cells. *Med Microbiol Immunol* **167**, 137-153.

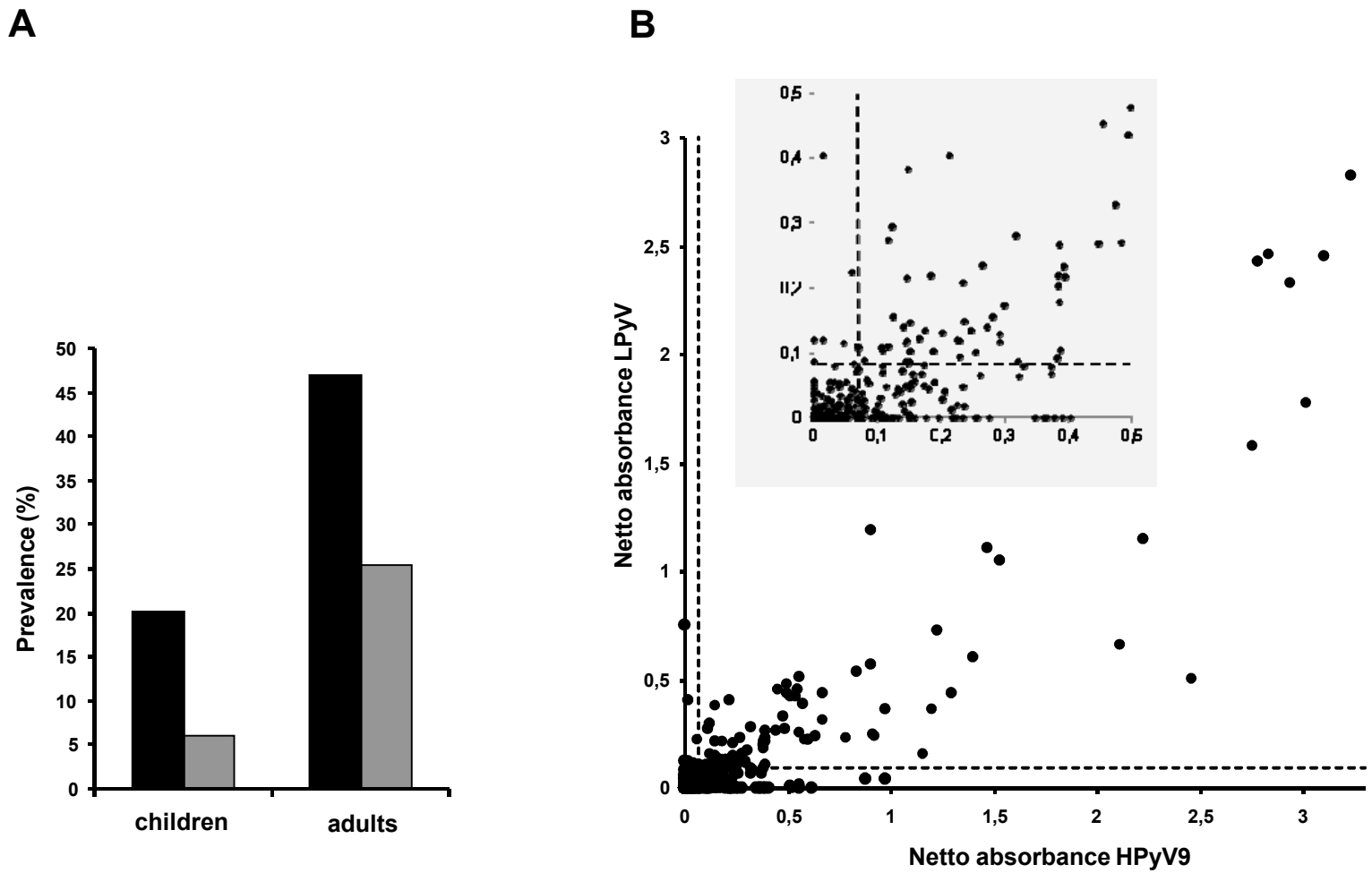
A
B
Prevalence (%)
50
45
40
35
30
25
20
15
10
5
0
children
adults
Netto absorbance LPyV
Netto absorbance HPyV9
3
2,5
2
1,5
1
0,5
0
0,1
0,2
0,3
0,4
0,5

Figure 1

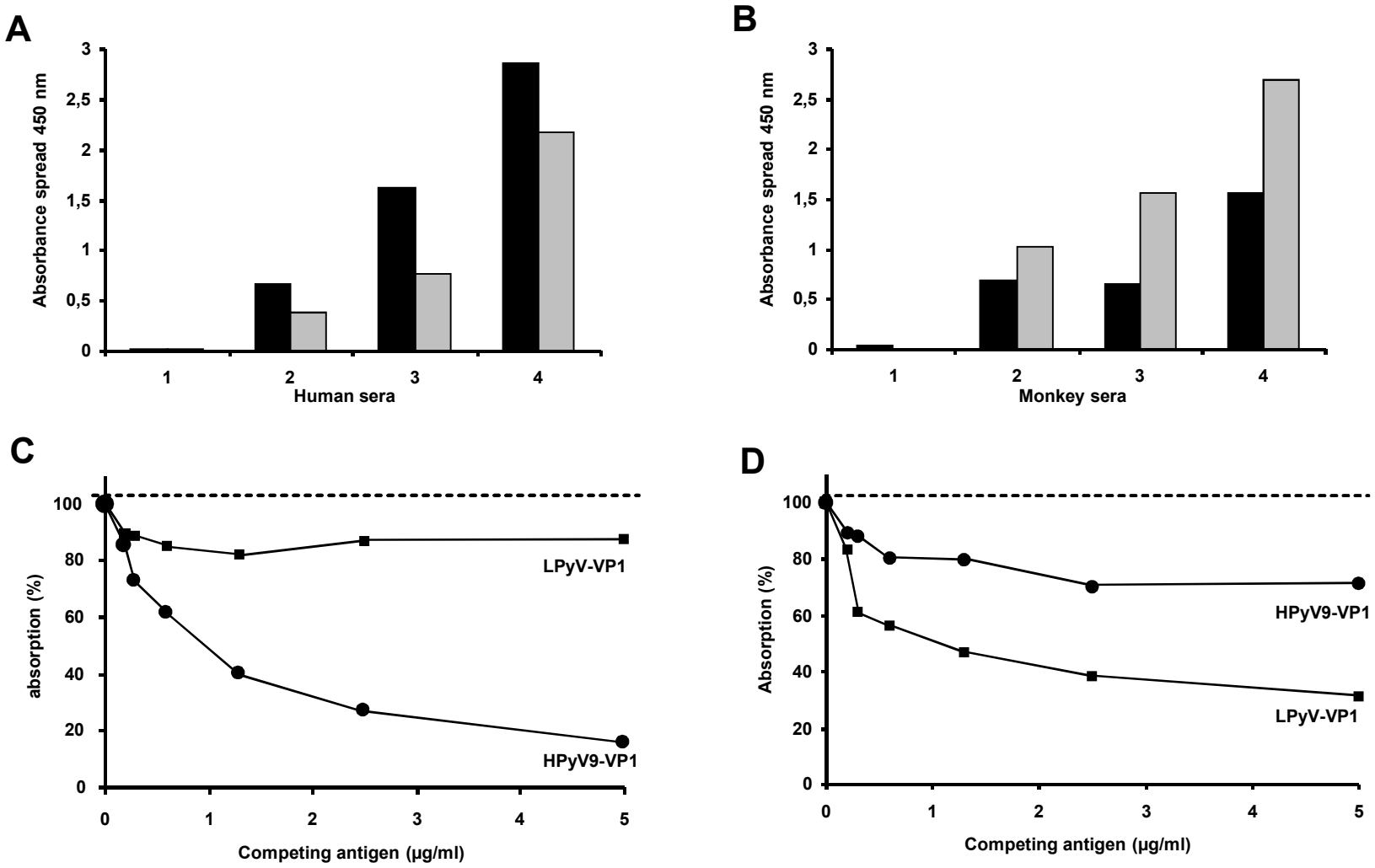
A
Absorbance spread 450 nm
3
2,5
2
1,5
1
0,5
0
1
2
3
4
Human sera
B
Absorbance spread 450 nm
3
2,5
2
1,5
1
0,5
0
1
2
3
4
Monkey sera
C
absorption (%)
100
80
60
40
20
0
LPyV-VP1
HPyV9-VP1
0
1
2
3
4
5
Competing antigen (µg/ml)
D
Absorption (%)
100
80
60
40
20
0
HPyV9-VP1
LPyV-VP1
0
1
2
3
4
5
Competing antigen (µg/ml)

Figure 2

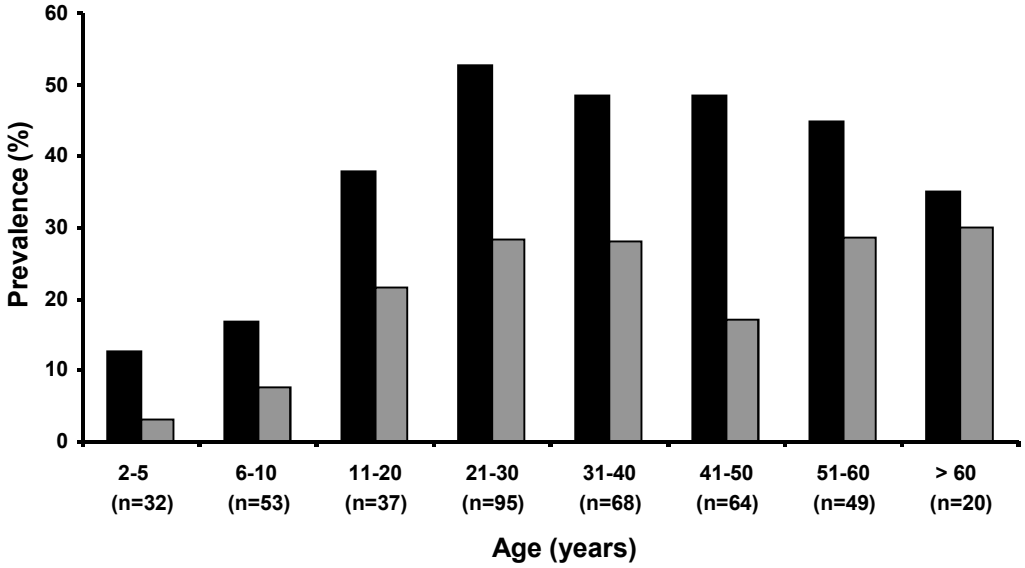
60
50
40
30
20
10
0
Prevalence (%)
2-5 (n=32)
6-10 (n=53)
11-20 (n=37)
21-30 (n=95)
31-40 (n=68)
41-50 (n=64)
51-60 (n=49)
> 60 (n=20)
Age (years)

Figure 3

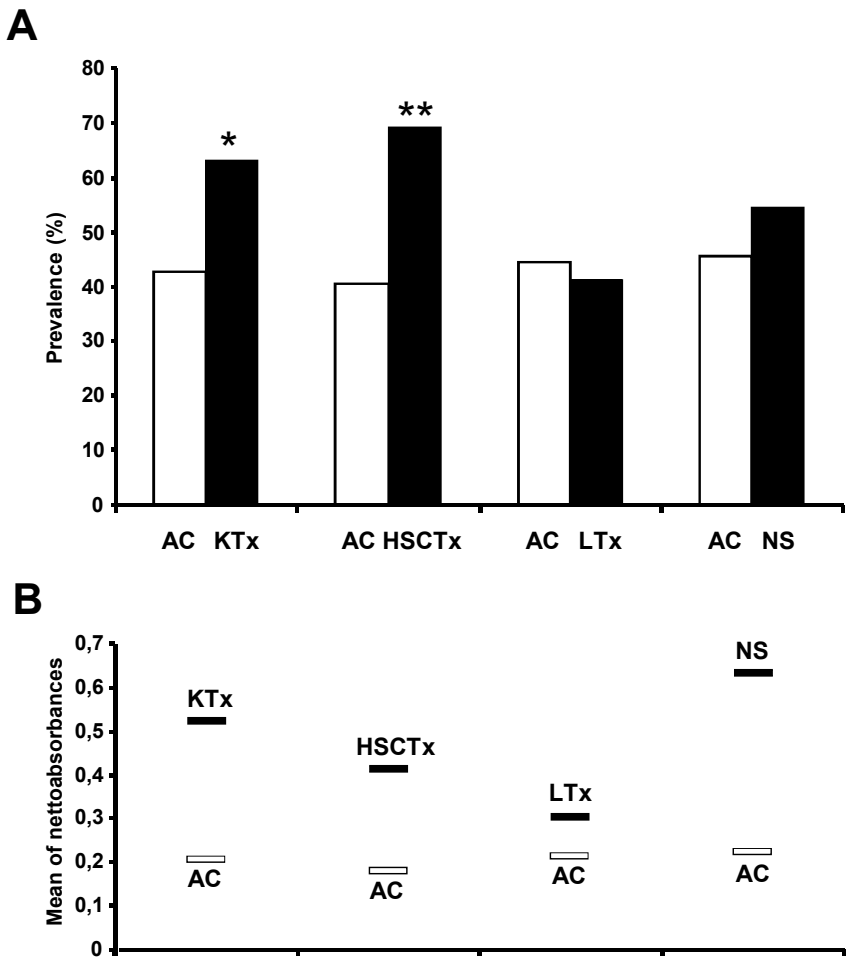
A
Prevalence (%)
80
70
60
50
40
30
20
10
0
*
**
AC KTx
AC HSCTx
AC LTx
AC NS
B
Mean of nettoabsorbances
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
KTx
HSCTx
LTx
NS
AC
AC
AC
AC

Figure 4

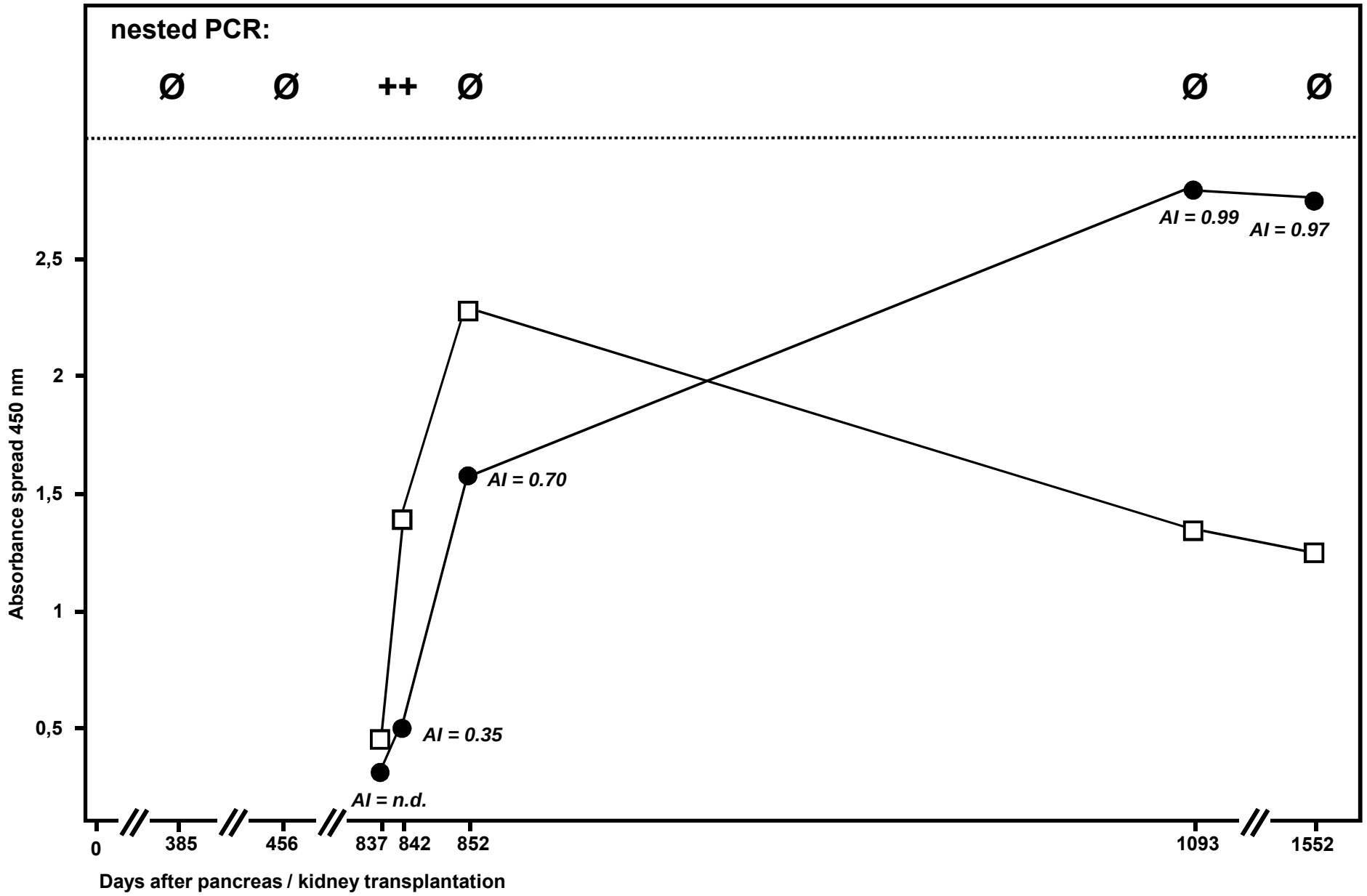
nested PCR:
Ø
Ø
++
Ø
Ø
Ø
AI = 0.99
AI = 0.97
AI = 0.70
AI = 0.35
AI = n.d.
2,5
2
1,5
1
0,5
Absorbance spread 450 nm
0
385
456
837
842
852
1093
1552
Days after pancreas / kidney transplantation

Figure 5