To assess the stability of polyomavirus antibodies in serial samples over time and the incidence and age-specific prevalence of polyomavirus infections, we established enzyme immunoassays (EIAs) using purified yeast-expressed virus-like particles (VLPs) containing the VP1 major capsid proteins of JC virus (JCV) and the AS and SB strains of BK virus (BKV). A random subsample of 150 Finnish women who had serum samples taken during the first trimester of pregnancy and had a second pregnancy during a 5 year follow-up period was selected, grouped by age of first pregnancy. The polyomavirus antibody levels were similar in samples taken during the first and second pregnancies (correlation coefficient 0.93 for BKV SB and 0.94 for JCV). Analysis of serum samples from 290 Swedish children aged 1–13 years, grouped by age in 2 year intervals, demonstrated that BKV seropositivity increased rapidly with increasing age of the children, reaching 98 % seroprevalence at 7–9 years of age, followed by a minor decrease. JCV seroprevalence increased only slowly with increasing age and reaching 72 % positivity among mothers > 25 years of age. The age-specific seroprevalence of the human polyomaviruses measured using this VLP-based EIA was similar to previous serosurveys by other methods. The stability of the antibodies over time indicates that polyomavirus seropositivity is a valid marker of cumulative virus exposure, and polyoma VLP-based EIAs may therefore be useful for epidemiological studies of these viruses.

**INTRODUCTION**

Human polyomaviruses are endemic and infect a large proportion of mankind worldwide. Primary infections with the human polyomaviruses BK virus (BKV) and JC virus (JCV) occur during childhood and are largely asymptomatic (Dorries, 1998). Following primary infection, both BKV and JCV persist as latent infections in the kidneys and B lymphocytes (Shah, 1996).

Under conditions of severe immunosuppression such as leukaemia, AIDS and organ transplantation both viruses may be reactivated and cause disease. Reactivation of BKV is mainly related to urinary tract diseases such as haemorrhagic cystitis, ureteric stenosis, glomerulonephritis and graft nephropathy (Barbanti-Brodano et al., 1998; Limaye et al., 2001). JCV can be reactivated and induces the CNS disease progressive multifocal leukoencephalopathy (Hou & Major, 2000).

The human polyomaviruses have in vitro transforming abilities, similar to the mouse polyomavirus and the monkey polyomavirus simian virus 40 (SV40) (Barbanti-Brodano et al., 1998). In recent years a possible association of polyomaviruses with human cancer has been reported. JCV has been detected in certain brain tumours, in particular oligoastrocytoma. SV40 was present in polio vaccines produced in rhesus monkey kidney cells during the 1950s and has been detected in several human tumours, including choroid plexus tumours and ependymomas (Del Valle et al., 2001; Rencic et al., 1996; Shah, 1996). BKV has also been detected in a variety of tumours, including neuroblastoma (Flagstad et al., 1999). These reports have created a renewed interest in the epidemiology of these infections (Barbanti-Brodano et al., 1998).

Haemagglutination inhibition (HI) has been the standard method for measurement of antibody titres to BKV and JCV (Hamilton et al., 2000). About 70–90 % of healthy adults are seropositive by HI (Shah, 1996). Seroconversion for BK infection occurs in early childhood and JC seroconversion occurs in late childhood. A study from the USA reported antibodies to BKV in 50 % of children aged 3–4 years and almost all children were seropositive by 10 years of age.
Antibodies to JCV had been acquired by 50% of children aged 10–14 years and about 75% of adults (Shah, 1996).

Four antigenic variants of BKV have been described: the BKV prototype, BKV AS, BKV SB and BKV IV (Jin et al., 1993a). Each strain has been characterized by nucleotide sequencing of the VP1 region, which encodes the major capsid protein of BKV. Specific variations correlate with serological typing by HI. These BKV strains were isolated from urine specimens from several patient groups (Jin et al., 1993b).

In recent years viral serology based on virus-like particles (VLPs) in enzyme immunonoassays (EIAs) has become widely used (Dillner, 1999). EIAs using specific antigens of BKV and JCV have shown a greater sensitivity and precision compared with HI (Hamilton et al., 2000). The objective of the present study was to establish a modern seroepidemiological technology based on yeast-expressed VLPs of JCV, the two major BKV strains, AS and SB, and SV40 (Hale et al., 2002), to investigate the stability of polyomavirus antibodies in serial samples over time and to investigate the incidence and age-specific prevalence of these virus infections.

METHODS

Study population. A total of 590 serum samples were analysed for antibodies to polyomaviruses. Serum samples from a consecutive series of 1031 serum samples from children aged between 0 and 13 years were originally obtained from the Department of Clinical Virology, Karolinska Hospital, Sweden, and have been characterized in a previous study of the seroepidemiology of papillomavirus infection (af Geijersstam et al., 1999). The children were grouped in age groups of 2 year intervals and a random subsample of 290 sera was selected. In this study, 50 serum samples each were obtained from the groups of children aged 1-1–3, 3-1–5, 5-1–7, 7-1–9 and 9-1–11 years of age and 40 serum samples were obtained from children aged 11-1–13 years.

A total of 1656 serum samples originated from the Finnish Maternity Cohort (FMC) of the National Public Health Institute, Finland. The samples were randomly chosen from the FMC serum bank collected in the population-based serologic screening programme for congenital infections in the first trimester of pregnancy (af Geijersstam et al., 1998; Kibur et al., 2000). In this study, a subsample of 300 serum samples taken from 150 women during their first and second pregnancies was collected. The women were grouped by age at first pregnancy and had a second pregnancy during a 5-year follow-up period. In this study, there were 50 women each aged 14–19, 20–25 and 26–31 years at their first pregnancy.

Polyoma VLPs. VLPs are based on the major capsid protein, VP1. The VP1 coding sequences were incorporated into the yeast expression vector pFX7 under the control of the galactose-inducible promoter (Gedvilaitė et al., 2000; Sasnauskas et al., 1999). The pFX7-derived expression plasmids carrying the VP1 genes were transformed into the yeast Saccharomyces cerevisiae for cultivation. Polyoma VLPs from JCV, from the two antigenic variants of BKV (strains AS and SB) and from SV40 were produced in S. cerevisiae cells. Yeast transformation, cultivation and recombinant protein purification were carried out as described previously (Gedvilaitė et al., 2000; Sasnauskas et al., 1999).

Following disruption of yeast cells, lysate was centrifuged at 3000 g for 10 min at 4°C. Supernatants were then loaded on to a chilled 30% sucrose cushion and ultracentrifuged at 100 000 g for 3 h at 4°C. Resulting pellets were resuspended in 4 ml chilled disruption buffer and loaded on to CsCl gradients ranging from 1.2-3 to 1.38 g ml−1 and centrifuged at 100 000 g for 48 h. Fractions of 1 ml were collected and subjected to SDS-PAGE analysis. Fractions containing protein corresponding to a molecular mass of ~40–45 kDa were pooled, diluted with 1-31 g CsCl ml−1 and recentrifuged on a second CsCl gradient. Fractions were again collected and those containing VP1 were pooled and dialysed against PBS and analysed by SDS-PAGE, Western blot, electron microscopy (Fig. 1) and HI.

Serological analysis. An EIA based on purified VLPs was used to detect the presence of specific antibodies in human sera. The optimum concentration of polyomavirus VLPs and the serum dilutions used were determined by titration using the positive and negative controls. For all viruses, purified VLPs were added to the wells at a concentration of 6·25 ng per well in ice-cold PBS (pH 7·2). Half-area Costar 3690 EIA plates were incubated overnight at 4°C. After washing with 0·1% PBS/Tween, a blocking buffer consisting of 10% horse serum in PBS (HS-PBS) was added and incubated for 1 h at room temperature. The serum samples were diluted 1 : 40 in HS-PBS and incubated for 2 h at room temperature. The plates were washed five times with 150 µl PBS/Tween and an anti-human IgG mouse monoclonal antibody (Eurodiagnostica) diluted 1 : 800 was added and incubated for 2 h at room temperature. The plates were washed five times with 150 µl PBS/Tween and peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology) diluted 1 : 2000 was added and incubated for 10 min at room temperature. Peroxidase substrate (ABTS) was added and incubated for 30 min at room temperature and the absorbance measured at 415 nm.

Fig. 1. Negative-stain electron microscopy of BKV AS VLPs expressed in yeast (S. cerevisiae) cells. Bar, 100 nm.
The mouse monoclonal antibody NCL-JCBK, which reacts with both JC and BK polyomaviruses, was purchased from ImmunKemi (Knowles et al., 1991). The antibody was used as positive control, diluted 1:10,000. Human reference sera from three renal transplant recipients, who tested positive for BKV DNA in urine by PCR, were used as positive controls. The sera were used at a dilution twofold lower than the endpoint titre: 1:10,240, 1:640 and 1:40,960, respectively. The reference sera were provided by the Swedish Institute for Infectious Disease Control. A pool of sera from healthy blood donors obtained from the Blood Donor Centre (Karolinska Hospital, Sweden) was also used as a positive control serum, diluted 1:40. One of the serum samples obtained from the Department of Clinical Virology (Karolinska Hospital, Sweden) from a child aged 1 year and 9 months was used as negative control serum.

For definition of cut-off values, the mean value and standard deviation (SD) were calculated from the log-transformed absorbance values in the group of children between 1-1 and 3 years of age and the cut-off values defined as the mean value plus 1 SD of the log absorbance values among the 1-1-3-year-old children. Zero absorbance values were set to half of the lowest detectable absorbance before log transformation.

**RESULTS**

The antibody titres against BKV AS and SB were strongly correlated with each other, with the titres and seroprevalences generally being slightly higher for the BKV SB strain (Fig. 2a; Fig. 3; Table 1). Seropositivity for AS with concomitant seronegativity for SB was uncommon and found in less than 5% of samples (Table 1; compare seroprevalences of total BKV with those of SB or AS).

![Fig. 2](http://vir.sgmjournals.org) 1501

**Fig. 2.** (a) Scatter plot of antibody reactivity to BKV AS and BKV SB ($r=0.76$) in children aged 1–13. (b) Scatter plot of antibody reactivity between JCV and BKV SB ($r=0.09$).

![Fig. 3](http://vir.sgmjournals.org) 1501

**Fig. 3.** Prevalence of antibodies to the polyomaviruses BKV SB, BKV AS and JCV in children and pregnant women.
The correlation coefficients between JCV antibody titres and antibody titres against both strains of BKV were very low ($r=0.14$ for JC vs AS and $r=0.09$ for JC vs SB), indicating little or no cross-reactivity between the viruses (Fig. 2b). The same sera that were used in Fig. 2 were also assayed for antibody reactivity against SV40. Antibodies reactive with SV40 VLPs were found in 20–60% of human sera, with the age distribution closely following the AS seroprevalence distribution among age groups (data not shown). The SV40 antibody titres were strongly correlated to the BKV titres, in particular to the AS titres ($r=0.70$). There were also significant correlations between SV40 antibody levels and SB antibody levels ($r=0.56$), as well as with JCV antibody levels ($r=0.30$). SV40 antibody testing by this assay was therefore not considered to be specific and was discontinued.

The BKV and JCV seropositivities were quite stable over time in the 5-year follow-up study of pregnant women (Fig. 4a, b). For both BKV SB and JCV, there were no cases of seroconversion on follow-up and no cases of seroreversion (loss of seropositivity). The correlation between the antibody titres in the first and second pregnancies was very high: the correlation coefficient was $0.93$ for SB and $0.94$ for JC (Fig. 4a, b).

Repeat testing of the sera from the pregnant women several months apart found that interassay variability was of the same magnitude as the biological variability over time (coefficient of variation (CV) for repeat testing of same samples: $21.5\%$ for SB and $25.1\%$ for JC; CV for variability between analysis of samples taken at first pregnancy compared with analysis of samples taken at second pregnancy: $19.9\%$ for SB and $27.1\%$ for JC). Most of the variability in percentage of absorbance readings was seen for either very low absorbance readings close to zero or very high absorbance readings. In no case did the interassay variability result in a ‘seroconversion’.

BKV seropositivity increased rapidly with increasing age of the children, reaching $98\%$ seroprevalence at 7–9 years of

### Table 1. Seroprevalence of polyomavirus infection in children aged 1.1–13 years

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Total BKV (AS and/or SB)</th>
<th>BKV AS</th>
<th>BKV SB</th>
<th>JCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1–3 years</td>
<td>10/50 (20%)</td>
<td>8/50 (16%)</td>
<td>10/50 (20%)</td>
<td>8/50 (16%)</td>
</tr>
<tr>
<td>&gt;3–5 years*</td>
<td>31/49 (63%)</td>
<td>24/49 (49%)</td>
<td>29/49 (59%)</td>
<td>13/49 (27%)</td>
</tr>
<tr>
<td>&gt;5–7 years</td>
<td>40/50 (80%)</td>
<td>36/50 (72%)</td>
<td>40/50 (80%)</td>
<td>11/50 (22%)</td>
</tr>
<tr>
<td>&gt;7–9 years</td>
<td>49/50 (98%)</td>
<td>44/50 (88%)</td>
<td>49/50 (98%)</td>
<td>21/50 (42%)</td>
</tr>
<tr>
<td>&gt;9–11 years*</td>
<td>45/49 (92%)</td>
<td>34/49 (69%)</td>
<td>42/49 (86%)</td>
<td>25/49 (51%)</td>
</tr>
<tr>
<td>&gt;11–13 years</td>
<td>31/40 (78%)</td>
<td>22/40 (55%)</td>
<td>31/40 (78%)</td>
<td>13/40 (33%)</td>
</tr>
</tbody>
</table>

*One serum sample was excluded due to reactivity with blank ELISA plates.

![Fig. 4.](image-url)
age, followed by a tendency for a minor decrease (Fig. 3; Table 1). However, among mothers > 25 years of age, there was a high BK seroprevalence of > 96% (Fig. 3). In contrast, the JCV seroprevalences increased more slowly with increasing age and reached the highest seroprevalence (72%) among the mothers > 25 years of age (Table 1; Fig. 3).

**DISCUSSION**

The present study has established a VLP-based EIA for human polyomaviruses. The fact that antibody titres were generally stable over time during a 5 year follow-up indicates that polyomavirus VLP antibodies are a valid marker of polyomavirus cumulative incidences over substantial time spans, thus enabling a meaningful interpretation of seroprevalence data. The fact that the age-specific seroprevalence was similar to that reported in other serosurveys also suggests the validity of the VLP EIA (Dorries, 1998; Shah, 1996). The absence of seroconversions, in spite of the fact that we studied a sizeable cohort of women followed for up to 5 years, indicates that the incidence of new infections with these viruses is a rare event.

The titres against the two antigenic variants of BKV (strains AS and SB) were strongly correlated to each other, but the titres were generally higher for the BKV SB strain. Differences in the DNA sequences encoding the VP1 gene with these viruses is a rare event.

Up to 5 years, indicates that the incidence of new infections with these viruses is a rare event.

The serological response to papillomaviruses.

The abundant presence of antibodies reactive with SV40 VLPs in human sera might be due to cross-reactivity with human polyomaviruses, as suggested by the close correlation of BKV AS and SV40 VLP antibody data. To enable serological studies of possible SV40 infections in humans, further work will be required to establish serological assays for SV40 that lack cross-reactivity with human polyomaviruses.

Recombinant DNA technology has been applied to express polyomavirus structural protein genes, both in prokaryotic and eukaryotic systems. The polyoma VLPs used in this study were based on the major capsid protein, VP1, and produced in yeast cells from *S. cerevisiae* (Gedvilaite et al., 2000; Sasnauskas et al., 1999). VLPs of JCV have also been expressed in *Escherichia coli* and polyomavirus VP1 will also assemble into capsid-like particles in the nucleus of insect cells when expressed in the baculovirus system (An et al., 2000; Ou et al., 1999).

Yeast-derived recombinant VLPs offer many advantages over other expression systems in terms of protein yield, cost and ease of protein expression (Hale et al., 2002; Sasnauskas et al., 1999). In this study, we found such VLPs to be highly serologically reactive, with an optimum amount of VLPs as low as 6 ng per well. The yeast VLP-based assays are therefore likely to be useful in determining past exposure to BKV and JCV in epidemiological studies and for serological diagnosis in patients at risk of polyomavirus-associated diseases or showing early signs of complications due to polyomavirus reactivation.

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