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| Keywords:         | tonsils, adenoids, nasopharyngeal tract, WUPyV |
Table I. Virological analysis of polyomavirus in lymphoid and nasopharyngeal samples.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>No.</th>
<th>Polyomavirus detected</th>
<th>Respiratory virus detected</th>
<th>Co-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WUPyV</td>
<td>KIPyV</td>
<td>BKV</td>
</tr>
<tr>
<td>Adenoids</td>
<td>83</td>
<td>23 (27.7%)</td>
<td>-</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>Tonsils</td>
<td>50</td>
<td>6 (12%)</td>
<td>-</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>PBMCs</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>183</td>
<td>29 (15.8%)</td>
<td>-</td>
<td>4 (2.2%)</td>
</tr>
</tbody>
</table>

*10 FLU-2 Adeno-7 RSV

(A) The distribution of WUPyV, KIPyV, BKV, and JCV among secondary lymphoid tissues from immunocompetent children. (B) Detection of WUPyV, KIPyV, influenza virus, RSV, and adenovirus in nasopharyngeal secretions from children with influenza-like infection. FLU, influenza virus; ADENO, adenovirus; RV, respiratory virus; NP, nasopharyngeal.
Table II. Nucleotide variation in the VP2 gene of WUPyV (in bold) in comparison with some GenBank registered sequences (nucleotide position is deduced by alignment with the prototype).

<table>
<thead>
<tr>
<th>Strain (GenBank Acc. No.)</th>
<th>VP2 gene - nucleotide position</th>
<th>Country/year</th>
</tr>
</thead>
<tbody>
<tr>
<td>WU09L (n=27)</td>
<td>A G G C G C G Italy/2010</td>
<td></td>
</tr>
<tr>
<td>WU34L (n=3)</td>
<td>A C C G C G A Italy/2010</td>
<td></td>
</tr>
<tr>
<td>WU35L (n=1)</td>
<td>A S S S S S R Italy/2010</td>
<td></td>
</tr>
<tr>
<td>WU45L (n=2)</td>
<td>A G G C G C A Italy/2010</td>
<td></td>
</tr>
<tr>
<td>WU61L (n=1)</td>
<td>G G C G C C A Italy/2010</td>
<td></td>
</tr>
<tr>
<td>GQ988386_WU709</td>
<td>G G C G C C A China/2008</td>
<td></td>
</tr>
<tr>
<td>EF444550_WU_S1</td>
<td>G G C G C C G USA/2003</td>
<td></td>
</tr>
<tr>
<td>EU711058_WU</td>
<td>G G C G C G A Germany/2007</td>
<td></td>
</tr>
<tr>
<td>FJ464552WUV-RM3</td>
<td>G G C G C G A Italy/2009</td>
<td></td>
</tr>
</tbody>
</table>
Secondary lymphoid tissue as an important site for WU polyomavirus infection in immunocompetent children

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Key words: adenoids, nasopharyngeal tract, tonsils

Running title: Lymphoid tissue and WUPyV in children
Abstract

The polyomaviruses KI and WU (KIPyV and WUPyV) have been identified in respiratory specimens from children with acute respiratory infections, which suggests the respiratory tract as a possible site of infection. However, the persistence of infection in the lymphoid system is unknown. Fresh samples (n=211) of tonsils, adenoids, and peripheral blood mononuclear cells (PBMCs) from 83 immunocompetent children (mean age 4.8 years) were tested for amplification of the KIPyV VP1 and WUPyV VP2 genes. The known BK and JC polyomaviruses and the lymphotropic human herpesvirus HHV-6 were also investigated by quantitative real-time PCR and direct sequencing. In addition, 98 nasopharyngeal swabs collected from children (mean age 6.2 years) affected by seasonal influenza-like illness were tested. Of the lymphoid tissues, 34.9% were positive for WUPyV, 4.8% for BK virus, and 33.8% for HHV-6. KIPyV and JC virus were not detected in these specimens. None of the polyomaviruses were detected in PBMCs. Among the nasopharyngeal samples, the prevalence of WUPyV was 27.5%, although 70% of the positive samples were co-infected with at least one of the following respiratory viruses: influenza virus, adenovirus, and respiratory syncytial virus. Phylogenetic analysis revealed high sequence homology (99%) between lymphoid- and nasopharynx-derived WUPyV strains. These results suggest that the tonsils and adenoids of immunocompetent children are a reservoir for WUPyV infection; probably due to the respiratory route of transmission. In addition, the prevalence of WUPyV was high among the children, and the virus was identified more frequently in older children than during the first years of life.
INTRODUCTION

The recently identified distinct polyomaviruses KI and WU (KIPyV and WUPyV) were identified first in young children with acute respiratory infections, which suggests that these viruses establish infections in the respiratory tract, either transiently or persistently, and are reactivated as a consequence of respiratory disorders [Allander et al., 2007; Gaynor et al., 2007]. Co-infection with one or more respiratory viruses is a common finding in individuals who are positive for KIPyV or WUPyV. However, in a small number of susceptible children, KIPyV or WUPyV was the only virus detected, which suggests that these viruses might cause respiratory disease independently. [Foulongne et al., 2008; Abed et al., 2008]. Nevertheless, unlike in the case of other human and simian polyomaviruses, the aetiopathogenicity of WUPyV and KIPyV remains speculative [Norja et al., 2007].

The widespread nature of WUPyV and KIPyV infection throughout the human population has been confirmed by extensive molecular investigations and supported by recent serological surveys. A pattern of infection that is similar to that of the well-known JC and BK polyomaviruses (JCV and BKV) has emerged, which suggests primary exposure to the virus during childhood, after which infection is sustained by close inter-human contacts [Abedi Kiasari et al., 2008; Kean et al., 2009].

The results of the intensive search for these novel viruses in clinical specimens to determine basic aspects of their natural history, including the sites of viral persistence and the potential routes of spread, have been inconclusive [Bialasiewicz et al., 2009]. Although both
viruses have been found sporadically in faecal specimens from patients with acute nonbacterial gastroenteritis and in some hospitalized infants [Wattier et al., 2008; Ren et al., 2009], WUPyV and KIPyV have been detected more frequently in nasopharyngeal secretions [Abed et al., 2007; Feng et al., 2008; Foulongne et al., 2008; Neske et al., 2008]. Recently, DNA sequences from KIPyV have been detected in lung and paranasal biopsies from cancer patients, which suggests a possible tropism of the virus for these tissues [Babakir-Mina et al., 2009]. In a subsequent study, a role for the lymphoid system has been suggested by the recovery of WUPyV and KIPyV DNA from a large series of archived paraffin-embedded tonsils from adult patients with a wide spectrum of benign and malignant conditions [Babakir-Mina et al., 2009]. These data support the hypothesis that, in analogy with BKV and JCV, these novel polyomaviruses can infect and establish a persistent infection in tissues that are rich in B cells [Patel et al., 2008].

The study was designed to evaluate the role of secondary lymphoid tissues, apart from the respiratory tract mucosa, as sites of persistence for KIPyV and WUPyV. The frequency of infection by WUPyV, KIPyV, JCV, and BKV was investigated in fresh tonsils, adenoids and peripheral blood mononuclear cells (PBMCs) from a selected cohort of immunocompetent children. For comparison, a series of nasopharyngeal specimens from children with comparable demographic characteristics, which were collected during the surveillance of seasonal influenza, were also tested.

MATERIALS AND METHODS

Patients and the Collection and Processing of Samples
The study involved a total of 309 selected clinical samples that had been submitted to the Diagnostic Virology Laboratory of the Burlo Garofolo Children’s Hospital (Trieste, Italy). Specimens of tonsils (n=50), adenoids (n=83), and peripheral blood lymphocytes (n=78) were collected between April and September 2005 from 83 children with tonsillar and/or adenoidal benign lymphoid hyperplasia. In addition, 98 nasopharyngeal samples were collected during November 2007 and March 2008 from children with influenza-like illness. To assess the demographic and clinical factors that might be associated with polyomavirus infection, approval was obtained from the Institutional Scientific Board of Burlo Garofalo Children’s Hospital to conduct a review of medical records to select children who met the following general criteria: Caucasian origin; living in the urban area of North–eastern Italy; age 1–10 years; no history of blood transfusion; no hospitalization within the previous 2 years; no systemic medication for a chronic condition; normal haematological data, admission and discharge diagnosis. Additional criteria for children who were undergoing elective tonsillectomy and/or adenoidectomy were: no respiratory tract disease, fever or acute infectious processes diagnosed at the time of tissue sampling.

Aliquots of each specimen were stored at –80°C in viral transport medium (veal infusion broth in distilled water plus gentamicin and amphotericin) until molecular testing was complete. Strict measures to avoid cross-contamination were maintained throughout the study, including the use of separate rooms for the processing and molecular analysis of the samples.

DNA was extracted using a commercial kit (QIAamp Tissue Kit; Qiagen, Mannheim, Germany) and used immediately for PCR analysis. The Rnase P gene (Roche Applied Science, Mannheim, Germany) was chosen as the cellular target to test the integrity of the DNA sample.
Molecular Evaluation

Ten microlitres of each DNA sample were used as a template in a conventional PCR amplification, with primers that targeted the WUPyV VP2 and KIPyV VP1 genes. The conditions for PCR amplification and the primer sequences have been described previously [Allander et al., 2007; Gaynor et al., 2007]. The WUPyV VP2 and KIPyV VP1 products were 250 bp and 207 bp, respectively. Sequences of WUPyV (AP-p002 and AP-p003) and KIPyV (pUC18-KIPyV) that had been cloned in plasmid vectors were used as positive controls. JCV and BKV were detected by real-time PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Milan, Italy) as described previously [McNees et al., 2005]. In brief, 10 µl of DNA sample and 10 µl of each specific standard scale dilution were added in duplicate to a final volume of 50 µl of reaction mix, which contained 900 µM primers against sequences in the conserved N-terminal region of the large T-Ag gene, 100 µM TaqMan FAM (6-carboxy-fluorescein)-MGB probe, and 25 µl of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Milan, Italy). JCV-Mad1 and BKV-Dunlop-1 plasmid vectors were included separately as positive controls. Reactions without DNA template were used as negative controls. Each clinical sample was tested twice. In addition, the genomic DNA of the reference lymphotropic human herpesvirus (HHV)-6 was investigated in the same series, as described previously [Allard et al., 1990].

PCR products of the expected sizes (207 bp for WUPyV and 250 bp for KIPyV), as determined by visualization on 0.8% acrylamide gels stained with ethidium bromide, were sequenced bidirectionally (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Milan, Italy) with an ABI PRISM 310 sequencer, in accordance with the manufacturer’s guidelines. Contiguous sequences were assembled using the Sequencer
software version 4.6 (Gene Codes Corp, Ann Arbor, MI, USA), and aligned with the polyomavirus sequences deposited in GenBank using the Blast algorithm.

Nasopharyngeal secretions were also evaluated for the presence of respiratory viruses, including adenovirus, respiratory syncytial virus (RSV), and influenza virus, by standardized molecular tests [Allard et al., 1990; Zhang and Evans, 1991; Valle et al., 2006]. For the analysis of RSV and influenza virus, RNA was purified with an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). To detect RSV, 10 µl of RNA were amplified by real-time PCR using specific primers and probes (RSVA and RSVB) for the F protein, whereas, for influenza virus, 10 µl of the extracted RNA were amplified using specific primer sets for the type A and B matrix protein (MP) regions and for the 1 HA and 3 HA genes.

**Statistical Analysis**

Data are presented as numbers and proportions tabulated in contingency tables. The $\chi^2$ test was used to evaluate the difference between proportions. $P<0.05$ was considered to be significant.

**RESULTS**

Table I shows the rates of detection for the pathogens in lymphoid and nasopharyngeal samples from the enrolled children. RNaseP gene sequences were amplified successfully in all 309 samples tested.

Amplicons of the expected size that were shown to correspond unequivocally to WUPyV sequences by DNA sequence determination were detected in 29/83 (34.9%) of the children who underwent tonsillectomy or adenoidectomy. The frequency of infection with WUPyV was similar to that found for the reference lymphotropic virus HHV-6 in the same series [27/83 (33.8%),
The mean age of the children infected with WUPyV was 4.0 years (range, 2–6 years), showing a prevalence of infection of 51.7% (15/29). Conversely, BKV was detected rarely (4/83; \(P=0.001\) vs. HHV-6) and no sequences from JCV or KIPyV were identified.

WUPyV was detected more frequently in samples of adenoids (23/83, 27.7%) than in tonsils (6/50, 12%) \((P<0.001)\). WuPyV was not detected in both the adenoids and the tonsils in any of the WUPyV-positive subjects. BKV was found only in 1/83 of the samples of adenoids and 3/50 of the tonsil samples.

WUPyV was the only one of the newly discovered polyomaviruses that was found in nasopharyngeal samples from the children with respiratory symptoms; WUPyV was identified in 27/98 of these samples (27.5%). The mean age of the children who excreted WUPyV was 3.8 years (range, 1–10 years). Of the positive samples, 19/27 (70%) were co-infected with at least one of the respiratory viruses considered in this study. Conversely, BKV and JCV sequences were not detected in the nasopharyngeal samples and none of the polyomaviruses were found in PBMC samples.

To assess the sequence variation in WUPyV among the 29 lymphoid tissue samples that were positive for this virus, the sequences obtained for the 250-bp region of the VP2 gene were compared with all available WUPyV VP2 sequences deposited in GenBank (Table II). The new isolates clustered into five groups, designated WU9L, WU34L, WU35L, WU45L, and WU61L, on the basis of nucleotide sequence. Phylogenetic analysis revealed a high level of identity (>99%) among the 29 viral strains. Of these, 23/29 (79.3%) showed 100% homology with WU9L, and the nucleotide sequences differed from each other on average of 0.1%.

Direct sequence analysis was possible for five of the 27 WUPyV-positive nasopharyngeal specimens. These were designated WU16NP (three strains), WU237NP (one strain), and
WU32NP (one strain), and showed a high degree of homology with the most representative lymphoid-derived strains (WU9L).

**DISCUSSION**

The presence of polyomaviruses WUPyV and KIPyV in respiratory tract specimens from children with acute respiratory infection suggests that the respiratory tract is a site of viral persistence. Although not proven, the notion that the respiratory tract, apart from the mucosa, is a site of persistent infection seems to be supported by the recent recovery of WUPyV and KIPyV sequences from archival tonsillar transformed tissues [Babakir-Mina et al., 2009].

To clarify whether secondary lymphoid tissues act as sites of viral persistence, fresh specimens of tonsils, adenoids, and PBMCs from a selected group of asymptomatic immunocompetent children were screened. At the same time, to evaluate the prevalence of infection with these polyomaviruses within the same geographic area, an independent series of nasopharyngeal swabs from children diagnosed with influenza-like illness was investigated. This survey demonstrated that WUPyV was the only one of the newly discovered polyomaviruses that could be detected in the tonsils (12%) or adenoids (27.7%) of this healthy paediatric population. The overall prevalence rate of WUPyV (34.9%) was similar to that of the lyphotropic reference virus HHV-6, which was detected in 33.8% of children. These findings emphasized the unexpectedly high prevalence of WUPyV in the secondary lymphoid system, which suggested the possible involvement of these tissues as a site for WUPyV persistence.

To the best of our knowledge, this is the first report demonstrating that the secondary lymphoid organs of immunocompetent children play an important role in WUPyV infection; a role that is favoured possibly by the respiratory route of transmission of the virus. The validity of
these findings was supported by the timing of collection of the lymphoid samples. The samples were collected during a period that was unrelated to the season of respiratory epidemic diseases, and processed for virological evaluation before the influenza-like cases were enrolled.

In the study described above, the frequency of WUPyV in lymphoid tissues was significantly higher than that reported for other human polyomaviruses [Patel et al., 2008]. Among the well-known human polyomaviruses, only BKV was recovered (in 4.8% of the children tested). This reinforced the notion that, with the exception of WUPyV, the presence of polyomaviruses in tonsils and adenoids was probably transient, and that different routes of viral transmission were involved [Comar et al., 2010]. These data were consistent with a high frequency of recovery of WUPyV (27.5%) from the nasopharyngeal secretions of children affected by influenza-like illness, which were collected during a recent influenza season. Nevertheless, the low level of sequence variation that was observed between lymphoid and nasopharynx-derived strains was consistent with a high level of conservation among the WUPyV strains circulating in this population.

In this study, the frequency of detection of WUPyV in children with influenza-like illness differed (27.5% vs 1.4%) from that established by Debiaggi et al [2010]. In addition, the previous study showed that primary infection occurred during the first months of life (1–24 months) suggesting that WU/KIPyVs have a limited circulation in Italy and a low pathogenic potential in young children [Debiaggi et al., 2010]. In contrast, in the present study, WUPyV was found frequently in older children (mean age 3.8 years), as shown recently for KIPyV [Wattier et al., 2010].
In agreement with previous studies, neither KIPyV nor WUPyV DNA was detected in peripheral lymphocytes, which indicated the absence of any systemic involvement by PBMCs in healthy subjects, as observed in general for other members of the polyomavirus family.

JCV and KIPyV sequences were not detected in the tonsils or adenoids of the children enrolled in this study. Although the lack of JCV was not unexpected in young children, the absence of KIPyV was a rather surprising finding that was in contrast with previous data that demonstrated the presence of a subclade of KIPyV in the tonsils of Italian adults [Babakir-Mina et al., 2009]. Although the absence of KIPyV in lymphoid specimens suggests that host susceptibility for KIPyV differs from that for WUPyV, as for JCV and BKV, these conflicting results were resolved when the presence of KIPyV was investigated in the nasopharyngeal secretions of immunocompromised subjects. In this North–Eastern Italy area, although infection with KIPyV seemed to be a rare event in immunocompetent children, this polyomavirus was detected in 0.5% of hospitalized children aged 15–36 months (data not shown).

In summary, the high prevalence of WUPyV in tonsils and adenoids from immunocompetent children and the high degree of homology of these lymphoid-derived strains with the most representative nasopharynx-derived strains suggests that lymphoid tissues serve as an important site of viral persistence. This finding provides support for the hypothesis that WUPyV is the only known human polyomavirus that has a predilection for respiratory transmission among children. Nevertheless, the number of patients with respiratory symptoms who were positive for WUPyV and/or KIPyV was low, and there was no strong evidence for an aetiological role of these viruses in respiratory disease. Furthermore, the frequent co-detection of other known respiratory pathogens and the lack of control groups make it difficult to establish a
causal link with clinical symptoms. It might be that KIPyV and WUPyV could play a role in respiratory disease in at least a subset of immunocompromised patients.

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