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Detection of WU polyomavirus DNA by real-time PCR in nasopharyngeal aspirates, serum, and stool samples

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Abstract
Background: The human WU polyomavirus (WUPyV) has been recently described as a novel virus in respiratory tract samples.
Objective: To investigate the viral load of WUPyV in nasopharyngeal aspirates (NPAs), stool, and serum samples of pediatric patients with acute respiratory tract diseases.
Study design: We established a real-time PCR for WUPyV DNA and tested NPA obtained between 2002 and 2007 from pediatric in-patients with acute respiratory tract diseases. In addition, 14 stool and 14 serum samples of children with WUPyV DNA positive NPA were analysed.
Results: WUPyV DNA was found in 5.2% of 1232 NPA. The median viral load in the NPA was 950 copies/ml (maximum 3.4E10 copies/ml). The WUPyV load in NPA was neither associated with the coinfection status nor with the clinical diagnoses. WUPyV DNA was found in 3 of 14 serum samples and in 2 of 14 stool samples. The WUPyV load in NPA tended to be higher in viremic children.
Conclusion: WUPyV DNA was found in NPA, serum, and stool of hospitalised children with acute respiratory tract diseases. Further studies are necessary to determine whether WUPyV is a human pathogen.

1. Introduction
Respiratory tract infections are a leading cause of human morbidity and are mainly caused by viruses. Molecular methods have enabled the detection of a number of unknown viruses in respiratory tract samples such as several coronaviruses as well as the human bocavirus (hBoV).1–3 Most recently, two novel viruses of the family polyomaviridae were detected in respiratory samples and named KI polyomavirus4 and WU polyomavirus (WUPyV).5 Two human polyomaviruses were known before, the JC and BK polyomavirus. These viruses cause persisting infections, which are usually asymptomatic in immunocompetent individuals but may lead to severe disease under immunosuppression.6

In the first description of WUPyV, DNA was detected in 37 (3.0%) of 1245 samples from Australian children with acute respiratory tract infection and in 6 of 890 respiratory samples obtained from adults and children in the United States.5 Most of the WUPyV DNA positive patients (86%) were younger than 4 years of age. Strikingly, coinfections were detected in 72% of the patients. Meanwhile, similar findings of WUPyV detection have been reported from South Korea, Canada, Scotland, the United States, and Germany with prevalence rates ranging from 1.6% to 7%.7–11 Because WUPyV DNA was also detected in samples of a control group without respiratory symptoms, the clinical relevance of positive WUPyV DNA results in respiratory samples has been questioned.11 Rather, positive WUPyV DNA results in the respiratory tract may constitute an incidental detection of a persistent virus. However, further investigations are necessary to clarify this issue.

The initial studies on WUPyV infections have mostly used conventional single-round PCR for WUPyV detection. In order to facilitate WUPyV DNA analysis and to quantify WUPyV DNA in clinical samples, we designed a real-time PCR assay. Quantification of WUPyV DNA may be important with respect to the pathogenic relevance of positive WUPyV DNA results, as has been suggested for hBoV.12 In the present study, we describe the evaluation of the WUPyV real-time PCR and its application for the detection of WUPyV DNA in nasopharyngeal aspirates (NPAs). In addition, we studied stool and serum samples of children with WUPyV DNA positive NPA.

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2. Materials and methods

2.1. Samples

The specimens tested for WUPyV infection consisted of unselected stored NPA that were received from January 2002 through September 2005 and from January 2007 through July 2007 from the Children’s Hospital of the University of Würzburg for screening of respiratory viruses. The aspirates were collected using a catheter tube and an aspiration trap. After suction the tube was rinsed with sterile saline. All samples had been tested for the presence of antigens of adenovirus, influenza A and B virus, parainfluenza viruses 1–3, and respiratory syncytial virus (RSV) by immunofluorescence assay and for the presence of hBoV DNA as described previously. In addition, archived stool and serum samples of children with positive WUPyV DNA in NPA were tested if they were obtained no longer than 14 days before or after the NPA. All samples had been stored at −20 °C or below until DNA extraction. The study was approved by the ethics committee of the medical faculty of the University of Würzburg.

2.2. Real-time WUPyV PCR

DNA was extracted from 200 μl of the NPA and serum samples using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) and from 140 μl of stool suspensions using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), which is known to purify both RNA and DNA. Both kits were used according to the instructions of the manufacturers. The elution volume of the extractions was 50 μl in case of the NPA and serum samples and 60 μl for the stool suspensions. Less than the stated starting material was used for the extraction procedures in exceptional cases, if the volume of the archived samples was otherwise insufficient. One negative control was extracted and amplified for every five patient samples. Extracted nucleic acids were stored at −70 °C until PCR testing.

Primers and probe for the real-time PCR were selected by Primer Express 2.0 software (Applied Biosystems, Darmstadt, Germany) from the C-terminal region of the large T-antigen that was used for a qualitative WUPyV PCR previously. Sequencing of 62 WUPyV positive samples had revealed high sequence conservation in this region. Blasting of primers and probe against GenBank to exclude unspecific binding did not reveal any significant homologies with other organisms. The real-time PCR was carried out in a final volume of 20 μl consisting of 5 μl of extracted DNA, primers WU2958s (CCTGTTAGTATTTTCACCTCATGTA) and WU2865a (TGTCAGCAATTCGATAGGGCTATATAT) at a final concentration of 400 nM, the probe WU2925s-TM (6FAM-AAACTTGTAATGGAAGAAGCTTAGACA-TAMRA) at a final concentration of 100 nM, and 1 μl Quantitect probe master mix (Qiagen). Amplification was performed on an ABI7500 real-time PCR system (Applied Biosystems, Darmstadt, Germany). The cycling conditions were 50 cycles with 30 s at 95 °C and 60 s at 60 °C after a preheating step of 15 min at 95 °C.

A plasmid containing the PCR product obtained with the primers AG0048 and AG0049 cloned into the vector pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) was used as positive control and for the standard curve. Viral loads were calculated from the threshold cycle (Ct) values of the individual samples with respect to the standard curve. General laboratory procedures to prevent PCR contamination were strictly adhered to.

2.3. Statistical analysis

Statistical analysis was carried out by using GraphPad Prism Version 3.0c for Mac (GraphPad Software, San Diego, USA) and SPSS Version 15 for windows (SPSS, Chicago, USA).

3. Results

3.1. Validation of the quantitative real-time PCR

In order to validate the quantitative real-time PCR, the linearity of the assay, the lower limit of detection, the intra-assay and interassay variation, and the specificity were investigated. The amplification curve was linear over the range from 5 to at least 5 × 10^6 copies/reaction. For standard curves of 28 independent runs, the mean value of the slope was −3.53 with a standard deviation of 0.1. The mean of the coefficient of correlation was 0.992 with a standard deviation of 0.014. The lower limit of detection was determined by probit analysis based on a total of 24 replicates in three independent runs of a twofold dilution series. A concentration of 7.1 copies/reaction (95% confidence interval: 4.8–20.4 copies/reaction) was detectable with 95% probability (data not shown). This corresponds to 360 copies/ml of starting material (serum or NPA). The reliability of the real-time PCR was assessed by analyzing the intra-assay and interassay variation. The intra-assay coefficient of variation of the Ct values for three replicates was 1.32% or lower for standard concentrations of 5–5 × 10^3 copies/reaction (data not shown). The interassay coefficient of variation of a run control (nominal concentration, 5 × 10^5), which was regularly included in each run, was 15.1% (data not shown). To test the primers and probe for unspecific binding, samples known to be positive for JC, BK, and KI polyomavirus, hBoV DNA and, adenovirus DNA were tested in the WUPyV real-time PCR assay. All samples were negative in the WUPyV assay.

3.2. Quantitative detection of WUPyV DNA in NPA

For clinical evaluation of the WUPyV real-time PCR, 1232 samples from 1134 children that had previously been tested by conventional qualitative WUPyV PCR were retested with the real-time assay. After analysis of discrepant results between the qualitative and the real-time PCR assay by repeat-testing and by additional PCR assays in different genome regions, two samples remained that were positive only in the real-time assay (110 and 140 copies/ml) and one sample that was positive only in the qualitative assay. Overall, the frequency of WUPyV DNA detection in our study population was 5.2% with the real-time PCR assay. The median viral load of all WUPyV positive NPA was 9.5 × 10^2 copies/ml. The maximum value was 3.4 × 10^3 copies/ml. Twenty-two (34.4%) of the positive samples had viral loads below the 95% limit of detection (360 copies/ml). A comparison of WUPyV loads with respect to the clinical diagnosis did not reveal any significant differences (p = 0.98 by Kruskal–Wallis test; Fig. 1). In 32 (50%) of the WUPyV positive NPA, evidence of infection with other respiratory viruses was detected. The median WUPyV loads of the
Mann–Whitney test) or positive stool samples (1.7 × 10^9 copies/ml) were found in a small proportion (4.7%) of the WUPyV DNA positive NPA, the majority of these samples (51.6%) contained only low viral loads of less than 1000 copies/ml. Although the significance of viral load results in NPA is limited to some extent because of technical variations in the acquisition procedure (e.g. the volume of the NPA on arrival in the lab ranged approximately from 1 to 10 ml), the observed viral loads in patient samples differed by a factor of 10^8, a range much larger than could be accounted for by variation in NPA volumes obtained. In analogy to hBoV, the observed WUPyV load distribution is compatible with primary WUPyV viremia associated with high viral loads in few of the children and incidental detection of low copy numbers of persistent virus in most of the children.

For two of the three children with very high viral load in NPA, serum samples from the same disease episode were available for retrospective testing. WUPyV viremia was found in both of these children. Again in analogy to the findings described for hBoV, this may be an indication of the clinical relevance of WUPyV in a subset of the children. Serological studies to determine the time point of primary WUPyV infection will be necessary to further elucidate this issue.

Based on the hypothesis that high viral loads may have pathogenic relevance, we compared the WUPyV load in NPA according to the clinical diagnosis. Significant differences of the median viral load were not observed between the different disease groups. However, this analysis is limited by the retrospective nature of our study, by the high number of coinfections, and by the fact that clinical information was analyzed only for WUPyV positive patients. Stool samples from 2 of 14 children were positive for WUPyV DNA. This finding may be explained by replication of WUPyV in gastrointestinal epithelium. However, both of the children had high WUPyV loads in NPA, which points to swallowing of WUPyV as an alternative explanation for the presence of WUPyV DNA in stool samples. Whether the WUPyV DNA in stool samples is derived from infectious or from degraded virus particles and if WUPyV may be a cause of gastroenteritis remains to be determined.

In conclusion, we have successfully established and used a real-time PCR assay for the detection of WUPyV DNA. Application of this assay for quantitative analysis has shown that WUPyV DNA can be found in NPA samples at very high titers. In addition to the respiratory tract, WUPyV was also found in serum and stool samples. However, the clinical relevance of WUPyV infections remains uncertain both for the respiratory and for the gastrointestinal tract.

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