KI Polyomavirus Sequences in Respiratory Specimens from Bulgarian Children

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ABSTRACT
KI Polyomavirus (KI PyV) has been recently discovered in the respiratory specimens of children and HIV-positive patients. The etiological role of KI PyV in respiratory disease has not been proved yet, but as a member of the polyomavirus family, the virus has oncogenic potential and is probably involved in development of human disease, especially in individuals with immunodeficiency. The published prevalence of KI PyV among children with respiratory diseases ranges from 1 to 4.8%, but in HIV-positive patients it is much higher.
The aim of the study was to evaluate the prevalence of KI PyV in Bulgarian children with respiratory diseases. Specimens were collected from 123 children at an age from 1 to 16 years divided in two groups: first group of 86 children with respiratory diseases; and a control group of 37 healthy children. Samples were tested using a nested polymerase chain reaction (PCR) method. All patients that were positive for KI PyV were also tested by ELISA for IgM antibodies against other respiratory viruses.

This is the first report concerning the detection of KI PyV in Bulgaria. The prevalence of KI PyV in the group with the respiratory diseases and the control group of children was established as 3 out of 86 and 1 out of 37, respectively. The mean prevalence was 3.25%. A high rate of co-infection with other respiratory viruses (Influenza virus, RS virus) was observed in the patients positive for KI PyV. In one patient KI PyV was determined as a sole etiologic pathogen, but other respiratory viruses such as Metapneumovirus, Corona virus, Bocavirus, etc. were not investigated.

Although the prevalence of KI PyV among Bulgarian children with respiratory disorders according to studied subgroups was with frequency between 3.33% and 3.84%, with a mean of 3.49%, an evident role of KI PyV in the causation of respiratory diseases has not been established.

Keywords: KI polyomavirus, PCR, sequencing

Introduction
All nine Human Polyomaviruses that have been discovered in the world to date are widespread in the human population. They are small double stranded DNA-viruses that possess oncogenic potential. The human Polyomavirus hominis 1 (BK virus) and Polyomavirus hominis 2 (JC virus) have been known since 1971, but all following viruses have been described after 2007. The third polyomavirus was named KI after the abbreviation of Karolinska Institutet in Stockholm, Sweden (2), where it was discovered in 2007. Like the other polyomaviruses KI polyomavirus (KI PyV) is a small sized 45 nm, uncoated DNA virus with high resistance to organic solvent and thermal factors. The viral capsid is built up of 3 specific proteins: VP1, VP2 and VP3, and the viral core contains the genetic material (14).

KI PyV is widespread around the world and has been reported in Australia, North America, Africa and Europe, infecting a large number of birds, rodents and primates. Serological investigations show a high prevalence of antibodies in humans similarly to the other polyomaviruses: 55% according to Kean et al. (15); 55.3%, to Neske et al. (17); and 66.3%, to Nguyen et al. (18). The main susceptible groups are children aged 3 years or younger (13), immunocompromised and transplanted patients at a wide age range (16). There is still scarce information about the tropism of KI PyV. To date KI PyV has been isolated from respiratory secretions and stool (1) and from sewage (8), showing that the most probable routes of infection are respiratory and alimentary. In contrast to other polyomaviruses (BK and JC), KI has not been detected in blood and urine, nor has been isolated from brain tumours of immunocompetent patients (12). However, in HIV-positive patients Barzon et al. (4) reported isolation of KI PyV from brains in 25% of the patients with Progressive Multifocal Leucoencephalopathy (PML), and in 30% of HIV-positive patients without PML. The viruses were located in all investigated regions of the nervous system. However, presence of KI PyV has not been registered in HIV-negative patients.

The polyomaviruses are object of great scientific and medical interest because of their high oncogenic potential and possible association with human malignancies. The causal role of KI PyV for the development of respiratory diseases is slightly possible and the majority of studies so far, have not included specimens from asymptomatic patients. Studies that included such a control group, detected viral sequences in asymptomatic patients with a similar prevalence. Linking the infection with
KI PyV and respiratory diseases is further complicated by the observed high rates of co-infection with other respiratory viruses (1, 2, 7, 11, 17). Despite the lack of evidence that KI PyV causes human disease, additional research is warranted to investigate this possibility, especially when considering the similarities of KI PyV to BK and JC in terms of nucleotide sequence and potential route of infection.

Although the etiologic role of KI PyV for the development of human diseases has not been proved yet, the virus belongs to the polyomavirus family, might possess oncogenic potential and probably is involved in human disease, especially in individuals with immunodeficiency (4).

The first retrospective studies of respiratory specimens in Sweden and Australia showed prevalence of KI PyV in 1% (11) and 2.6% (6) respectively. Further studies conducted in Canada and South Korea have shown similar frequencies, but in France (10) the reported prevalence of KI PyV is higher – 4.8%.

This study aimed to examine Bulgarian children with respiratory diseases and to determine the prevalence of KI PyV among them.

Materials and Methods

Study groups

PCR screening for KI PyV was performed on nasopharyngeal specimens collected between December 2009 and January 2011. The tested specimens were collected from 123 children at an age ranging from 1 to 16 years, divided in two groups. The first group included 86 children with respiratory diseases: bronchiolitis, atypical pneumonia, asthmatic bronchitis; and the second, control, group consisted of 37 healthy children. The group of children with respiratory diseases was also divided in two subgroups: one of 26 randomly selected children at an age ranging from 1 to 16 years (hospitalized in the Pediatric Clinic at the Medical University – Sofia, and non-hospitalized children) and a second subgroup of purpose that included 60 children, at an age ranging from 9 months to 5 years, all hospitalized in the University Pediatric Clinic.

Sample preparation and PCR conditions

DNA was extracted from fresh frozen samples by commercially available kit: DNA-Sorb A (Sacace Biotechnologies, Italy).

All 123 samples were screened with a nested PCR assay targeting the VP1 gene. The primers were designed by Invitrogen (USA). PCR was performed with the protocol published by Allander et al. (2) on a Mastercycler personal (Eppendorf, Germany) thermal cycling machine.

Five microliters of extracted DNA were used as a template in the first round of PCR and 2 μl from the amplified DNA, for the second-round nested PCR. The reaction mix for both the first and second-round PCR also included PCR Super Mix (22 mM Tris-HCl /pH 8.4/, 55 mM KCl, 1.65 mM MgCl2, 0.22 mM of each dNTP, 2.0 U of Taq DNA polymerase), and 20 pmol of each primer (1.8 μl - POLVP1-39F; 1.9 μl - POLVP1-324R) to a final reaction volume of 50 μl. The primers for the first PCR were: POLVP1-39F (AAG GCC AAG AAG TCA AGT TC) and POLVP1-363R (ACA CTC ACT AAC TTG ATT TGG). In the second PCR the primers were: POLVP1-118F (GTA CCA CTG TCA GAA GAA AC) and POLVP1-324R (TTT TGC CAGGCT GTA ACA TAC). The cycling conditions for the first and second-round PCR reactions were: 10 min at 94 °C followed by 35 cycles of amplification (94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min) and terminal extension for 10 min at 72 °C. The products were visualized on a 2% agarose gel stained with 10 mg/ml ethidium bromide after horizontal electrophoresis (Thermo EC Classic TM CSSU78). The size of the amplified specific product after the first PCR was 325 bp and after the second-round nested reaction it was 207 bp (Fig. 1).

Fig. 1. Amplification product obtained after the second nested PCR round. Lane 1: DNA marker with a step of 100 bp; lane 2: negative control; lanes 3, 4, 5 and 7 to 12: negative specimens; lane 6: a positive specimen.

Sequencing analysis

An amplified PCR product positive for KI PyV was submitted to sequencing analysis in order to verify its specificity and to be further used as a positive control. Amplicons were sequenced by the GeXP system for gene analysis (Beckman Coulter, Switzerland). The amplification product was first analyzed by agarose electrophoresis and then purified using the GeneJet PCR Purification Kit (Beckman Coulter, Cat. No. K0701).

Sequencing PCR of the purified fragment was performed using the kit for rapid sequencing of Beckman Coulter (Cat. No. BC608120) with the following conditions: initial denaturation at 95 °C for 5 min, and 30 cycles of 96 °C for 20 sec, 50 °C for 20 sec, 60 °C for 4 min. The reaction was performed in duplicates with each primer – POLVP3 or POLVP4.

Other serological tests

Patients that were found positive for KI PyV were also tested with ELISA for IgM antibodies against the following acute respiratory infectious pathogens: adenovirus (NovaLisa commercial kit, NovaTec Immundiagnostica, Germany, Cat. No. ADVM0010); Influenza A and B (NovaLisa commercial kit, NovaTec Immundiagnostica, Germany, Cat. No. INFMO290), RS virus (commercial kit Euroimmun, Germany, Cat. No. EI 2670-9601 M), Parainfluenza virus (NovaLisa commercial kit, NovaTec Immundiagnostica, Germany, Cat. No. PAIM0360), Mycoplasma pneumoniae (NovaLisa commercial kit, NovaTec Immundiagnostica, Germany, Cat. No. MYCM0350), and
*Chlamydia pneumoniae* (NovaLisa commercial kit, NovaTec Immundiagnostica, Germany, Cat. No. CHLM0510); all according to the manufacturers’ protocols.

**Results and Discussion**

The three detected KI PyV positive specimens from all 86 investigated children with respiratory diseases are shown in Table 1. In subgroup I of the initially screened children the KI PyV positive sample accounted for 3.84% (1/26). One specimen positive for KI PyV was detected after screening of nasopharyngeal specimens collected from 26 randomly selected children. The amplification product positive for KI PyV was presented with a band lighting at 207 bp after agarose gel electrophoresis (Fig. 1).

In order to verify the specificity of the detected product it was sequenced in the system for genetic analysis GeXP of Beckman Coulter. The obtained sequence of 207 bp using the primer POLVP4 was verified and presented as follows: AGC CAG TAA TGT GAA ATA CAA CAG CTG CTG AGG ATG GGC GTG AGC CCA CCC CTC ATT ACT GTG CAA TTA GCT CTG CCA TTC ATG ACA AGG AAA GCG GTT CAA GTA TCA AAG TGT AAG AAA CTC CAG ATG CTG ACA CAA CTG TAT GTT ACA GCC TGG CAG AAA TTG CTG CCC CTG ATA TAC CAA ATC AAG TTA GTG AGT GTA AGG CC. It was compared and initially analyzed by means of BLAST (at http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a 97% similarity with KI PyV viral strains was documented.

The performed sequencing analysis revealed that the positive amplification product was indicative of a KI PyV and was further used as positive amplification control. The KI PyV sequence was submitted and registered in GenBank, Bethesda, Maryland, USA under the accession number JN582333 from 12.08.2011. This is the first report concerning detection of KI PyV in Bulgaria.

Results and Discussion

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Number of children</th>
<th>Number of KI PyV positive samples</th>
<th>% (+) of all</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>26</td>
<td>1</td>
<td>3.84%</td>
</tr>
<tr>
<td>II</td>
<td>60</td>
<td>2</td>
<td>3.33%</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>3</td>
<td>3.49%</td>
</tr>
</tbody>
</table>

A similar low rate, 3.33% (2/60), was also observed in subgroup II, where two out of 60 examined children were determined as positive (Table 1). Samples with a positive amplification product for KI PyV, marked with bands lighting at 207 bp, are shown in Fig. 2. The mean rate for the whole group of 86 investigated children was 3.49% (3/86).

![Fig. 2](image-url)  
**Fig. 2.** Amplification products obtained from 60 children with respiratory diseases after the second nested PCR round. Lane 1: DNA marker with a step of 100 bp; lane 2: negative control; lanes 3, 4, 5 and 7 to 10: negative samples; lanes 6 and 11: positive samples; lane 12: a positive control (sequence JN582333). The control specimen was tested for a possible co-infection with the other described respiratory pathogens and was not found positive. However, the available diagnostic kits for respiratory pathogens did not include several more rare respiratory viruses such as coronavirus, metapneumovirus, human bocavirus, etc. Although it is possible that KI PyV may

**TABLE 2**

Summarized results concerning the investigation of the three KI PyV-positive specimen for co-infection with other respiratory pathogens

<table>
<thead>
<tr>
<th>KI PyV (+) PCR children</th>
<th>Influenza A virus</th>
<th>Adenovirus</th>
<th>RS virus</th>
<th>Parainfluenza virus</th>
<th>Mycoplasma pneumoniae</th>
<th>Chlamydia pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>№1 (of 26)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>№2 (of 60)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>№3 (of 60)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- negative result; + positive result

**TABLE 3**

Summarized results for the prevalence of KI PyV determined by PCR in the investigated 123 children

<table>
<thead>
<tr>
<th>Number of tested children</th>
<th>Children with respiratory diseases</th>
<th>Healthy Children</th>
<th>Total/ Mean KI PyV rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>3 out of 86 (3.49%)</td>
<td>1 out of 37 (2.7%)</td>
<td>4 out of 123 (3.25%)</td>
</tr>
</tbody>
</table>
be the causative pathogen of a respiratory disease, still other microbial agents, including not yet identified ones, should be taken into consideration.

The other two of the three KI positive patients from the second group with 60 children were at the same time determined as positive for Influenza A virus and RS virus, respectively, both with positive titers of specific IgM antibodies. The observed high rate of co-infection is similar to the rate reported by other authors (1, 2, 7, 11, 17). The results obtained from the investigation of all KI PyV-positive children’s sera for acute infection with other respiratory pathogens are shown in Table 2.

After the PCR screening of the group with healthy children aged from 1 to 16 years, it was found that one specimen was positive for KI PyV, probably indicating an asymptomatic infection. The positivity rate for KI PyV among the tested healthy children (1 out of 37) was very similar to the rate determined for the children with respiratory diseases (3 out of 86). The summarized results from the PCR investigation for KI PyV of all 123 children: 86 children with respiratory diseases and 37 healthy children, are shown in Table 3.

According to our results, the prevalence of KI PyV among children with respiratory diseases is 3.49% (3/86); among healthy children, 2.7% (1/37); and a mean of 3.25% (4/123), which is similar to the results of data previously published by other authors (6, 9, 10, 11). Within the group of children with respiratory diseases, the obtained rate of KI PyV in the randomized group (1 out of 26, 3.84%) was similar to the rate of KI PyV in the purpose screening group (2 out of 60, 3.3%), with no significant difference compared to the rate of KI PyV in healthy children (1 out of 37, 2.7%). The rate of KI PyV probably depends on the current spread of the virus among the studied groups, rather than on the kind of investigated contingent (children with respiratory diseases or healthy children).

Conclusions
The prevalence of KI PyV among Bulgarian children with respiratory disorders was shown to be between 3.33% and 3.84%, according to the studied contingents and the current spread of the virus. Our results are similar to the previously published data (between 1 and 4.8%) (3, 4, 5, 6).

The role of KI PyV as an evident etiological agent for development of respiratory diseases has not been established. Moreover, a high rate of co-infection (2 out of 3), and detection of KI PyV in healthy people, in accordance with the published data (1, 2, 3, 5, 6), necessitates further research in order to gain better knowledge concerning KI polyomavirus.

REFERENCES