

# Identification of WU polyomavirus from pediatric patients with acute respiratory infections in Beijing, China

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**Abstract** A novel polyomavirus (WU virus) has been identified in pediatric patients with acute respiratory tract infections (ARI), but its role as a respiratory pathogen has not yet been demonstrated. To investigate if WU virus is related to acute respiratory infections in infants and children in Beijing, specimens collected from 674 pediatric patients with ARI from April 2007 to May 2008 and from 202 children without ARI were used for this investigation. Common respiratory viruses were tested by virus isolation and/or antigen detection by indirect immunofluorescent assay followed by RT-PCR or PCR for other viruses associated with respiratory infections in specimens collected from patients with ARI before WU virus DNA was detected. WU virus DNA was detected by initial screening and secondary confirmation PCR for all specimens. The region encoding the VP2 gene of the virus was amplified from 17 WU-virus-positive clinical specimens, and sequence analysis was performed. Thirty-eight of 674 (5.6%) specimens from patients with ARI and 3 of 202 (1.5%) specimens from children without ARI yielded PCR products with the predicted molecular weight, using either screening or confirmation primer sets, indicating that these specimens were WU virus positive. However, more than 60% of the 38 WU-virus-positive specimens from patients with ARI were also positive for one or more respiratory viruses. The nucleotide and deduced amino acid sequences of the region encoding the VP2 gene from 17 Beijing WU viruses shared high homology (>98.5%) with sequences from GenBank and among themselves. The data indicated

that WU virus in Beijing occurred 3.7 times more frequently in pediatric patients with ARI than in those without ARI ( $p < 0.05$ ).

## Introduction

In May 2007, Gaynor and coworkers reported the identification of a novel virus in respiratory secretions from human patients with symptoms of acute respiratory tract infection. The virus was initially detected in a nasopharyngeal aspirate collected from a 3-year-old Australian child diagnosed with pneumonia. Although its multiple DNA fragments shared limited homology with known polyomavirus, the genomic characteristics of these fragments were similar to those of members of the family *Polyomaviridae*, and the virus was therefore referred to as WU polyomavirus (WU virus) [1]. Since then, the detection and molecular characterization of WU virus in clinical respiratory specimens from patients with ARI have been reported in several articles [2–6], but the role that the virus plays as a respiratory pathogen remains to be determined [7].

The family *Polyomaviridae* is a family of small, non-enveloped viruses with icosahedral capsids. Among the 14 different polyomaviruses that have been described, BK virus (BKV) and JC virus (JCV) are the only two known to productively infect and cause disease in humans [8, 9]. BKV has been reported to cause nephropathy, hemorrhagic and non-hemorrhagic cystitis in immunosuppressed patients, and JCV has been linked to progressive multifocal leukoencephalopathy in immunocompromised patients [10]. The mode of transmission for BKV and JCV has not yet been well defined. However, some of the evidence has suggested that respiratory transmission occurs.

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Sero-antibodies for both viruses are prevalent, and seroconversion occurs in childhood, as indicated by seropositive rates for BKV reaching 90% in children aged 5–9 years and for JCV, reaching 50–60% after the age of 10 years. Potential alternative modes of transmission for these viruses include urino-oral, transplacental transmission, and transmission by blood transfusion, semen and organ transplantation. Primary infections with BKV and JCV are typically subclinical or linked to mild respiratory illness and are followed by viral dissemination to the sites of lifelong persistent infection [11].

In addition to WU polyomavirus, another new human polyomavirus, K1, was reported recently. The K1 virus was identified in nasopharyngeal aspirates and feces from patients with respiratory tract infections in Sweden [12]. The finding of these new polyomaviruses rekindled interest in members of the *Polyomaviridae* and their relationship to human diseases. The purpose of this study was to find out if WU virus is associated with acute respiratory infections in pediatric patients in Beijing, China.

## Materials and methods

### Patients and specimens

Specimens were collected from 674 pediatric patients who visited the affiliated Children's Hospital to Capital Institute of Pediatrics for ARI during April 2007 to May 2008. These included 318 throat swabs (TS) from outpatients and 356 nasopharyngeal aspirates (NPA) from hospitalized patients. The ages of these patients ranged from 11 days to 16 years; 434 were males and 240 were females.

Another set of throat swabs was collected from December 2008 to March 2009 from 202 infants and children who visited the affiliated Children's Hospital to Capital Institute of Pediatrics for a regular health check-up, and it was confirmed that these children had no symptoms of respiratory infection in the preceding 10 days when informed consent was obtained from their parents. The ages of these children ranged from 1 month to 14 years; 118 were males and 84 were females.

The original study was approved by the Ethics Committee of Capital Institute of Pediatrics.

### Screening for respiratory viruses other than WU virus

Upon arrival, respiratory specimens from 674 patients with ARI were processed routinely and then centrifuged at 2,500 rpm for 10 min. The supernatant from NPA or TS was used to inoculate MDCK cells for the isolation of influenza virus (Inf) A and B, Hep-2 and Vero cells for

isolation of respiratory syncytial virus (RSV) and adenovirus (ADV), and LLC-MK2 cells for isolation of parainfluenza virus (PIV) types 1–3 [13]. The pellets from NPS were resuspended in several drops of sterilized PBS and spotted onto an acetone-cleaned slide. Then, anti-ADV, Inf A and B, PIV 1–3, and RSV monoclonal antibodies were used for specific viral antigen identification by indirect immunofluorescence assay (IFA) (Chemicon, Inc.) followed by addition of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG, which would bind to the antigen–antibody complex.

In addition to virus isolation and antigen detection, DNA and RNA were extracted from 150  $\mu$ l of NPA or TS specimens from patients with ARI using Trizol (Invitrogen, Inc.) and suspended in 25  $\mu$ l of 8 mM NaOH or 20  $\mu$ l DEPC-treated water for DNA or RNA, respectively, according to the manufacturers' instructions. Polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) were employed for detection of human bocavirus (HBoV), human metapneumovirus (hMPV), rhinovirus (Rh), PIV 1–4, enterovirus (EV), and human coronavirus (hCoV)-NL63, hCoV-OC43, and hCoV-229E, as described previously [14–19].

Samples from the 202 children without ARI were first processed for WU virus DNA detection, and then samples that were WU virus positive were tested for Inf A and B, RSV, ADV and PIV 1–3 by virus isolation, and for hMPV, Rh, PIV 1–4, EV, hCoV-NL63, hCoV-OC43, hCoV-229E, and HBoV by RT-PCR or PCR as described above.

After the procedures for virus isolation, antigen detection and RNA/DNA extraction were completed, the remaining specimens were kept frozen at  $-70^{\circ}\text{C}$  for further analysis.

### Identification of WU virus from specimens

PCR was performed for initial screening for WU virus with all specimens, using primer pair AG0044 (5'-TGTTACA AATAGCTGCAGGTCAA-3') and AG0045 (5'-GCTGCA TAATGGGGAGTACC-3'), which were able to amplify a 250-bp fragment from the VP2-encoding region of the virus. Then, a second PCR was performed with primers AG0048 (5'-TGTTTTTCAAGTATGTTGCATAA-3') and AG0049 (5'-CACCCAAAAGACACTTAAAAGAAA-3') to amplify a 244-bp fragment from the 3' end of the large T antigen (LTag)-encoding region for confirmation, using sequences of published primer sets in the literature [1]. All of these PCR products were analyzed by 2% agarose gel electrophoresis. To confirm the result obtained by PCR, three of the amplicons that were picked out from among the WU-virus-positive samples from patients with ARI, obtained by PCR with LTag-region primers, and one amplicon from among the WU virus-positive samples from

children without ARI, obtained with VP2-region primers, were subsequently sequenced.

The  $\chi^2$  test was employed to evaluate differences in WU-virus-positive rates in the corresponding age groups between children with and without ARI.

To avoid contamination, all PCR procedures, including specimen processing, RNA and DNA extraction, PCR and RT-PCR amplification, and product analysis were performed in different laboratory rooms.

#### Amplification of the VP2-encoding region of WU virus

The gene fragment for the VP2-encoding region of WU virus was amplified by nested-PCR from 17 WU-virus-positive specimens from patients with ARI with following primers based on the published genomic sequences of the WU virus BO (GenBank accession number EF444549): VP2-forward (5'-GTATTGGTGCTACCGTCTCG-3') and VP2-reverse (5'-CTTCAGCAGTTTTAAGTGGG-3') for the first PCR, expected product size, 1,409 bp; VP2-sense (5'-GCCGACAGCCGTTGGATATA-3') and VP2-antisense (5'-AATAGACCAATAATGTGGGG-3') for the nested PCR, expected product size, 1,248 bp. The PCR cycles consisted of an initial heating step at 95°C for 5 min, 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min, and a final extension at 72°C for 10 min.

#### Sequence analysis of the VP2-encoding region

The amplified VP2-encoding regions were sequenced (by Invitrogen Inc.), and the complete sequences were aligned using the MegAlign program of DNASTar software. Reference sequences used for phylogenetic analysis of the VP2 genes of WU virus from specimens from this study were obtained from GenBank (WU virus: NC\_009539; WU virus BO: EF444549; WU virus S1, S2, S3, S4, S5: EF444550, EF444551, EF444552, EF444553, EF444554; WU virus CLEF: EU296475).

## Results

### WU virus DNA detected from specimens from children with ARI

Of the 674 specimens collected from patients with ARI and tested for WU virus, 38 (5.6%) yielded a PCR product with the predicted molecular weight, using both primers targeting VP2 for screening and primers targeting the LTag for confirmation, suggested that these specimens were WU virus positive. Furthermore, the PCR products for 3 of these 38 positive specimens with the expected molecular weight for the LTag-encoding region were sequenced, and

these sequences were compared to that of the WU virus prototype BO reported by Gaynor and colleges and the WU virus CLEF reported from China. It was shown that these three amplicons shared high nucleotide sequence homology with the WU viruses BO and CLEF (96.6 and 98%) and among themselves (97.1–99%), indicating that these specimens were truly WU virus positive. Among the 38 WU-virus-positive specimens, no other viral pathogen was detected in 15 specimens (39.5%, 15/38). In the remaining 23 WU virus-positive specimens (60.5%), 13 contained one other virus (4: EV, 3: RSV, 1: OC43, 1: Rh, 1: HBoV, 1: PIV1, 1: PIV2 and 1: PIV3), nine contained two viruses (4: EV and HBoV, 2: EV and PIV1, 1: EV and PIV4, 1: EV and Rh, 1: PIV1 and HBoV), and 1 contained three viruses (Rh, HBoV and PIV3).

The clinical diagnosis for these WU-virus-positive patients, listed in Table 1, indicated that WU virus was detected most frequently from patients with pneumonia (7.5%, 19/254), followed by patients with bronchitis (4.9%, 2/41), upper respiratory infections (URI) (4.9%, 15/307) and bronchiolitis (2.9%, 1/34). No WU virus was detected in patients with asthma and unexplained fever. WU virus was detected in one of the 24 patients diagnosed as “other”, complicated with respiratory infection.

Comparison of symptoms for the patients from whose specimens only WU virus was detected with those of patients for whom other viruses were detected in addition to WU virus indicated that the differences between these two groups were not statistically significant (data not shown).

The age profile of WU-virus-positive patients indicated that WU virus was detected in patients in all age groups from newborn to 9 years old. The highest frequency of WU-virus-positive results was in the group younger than 1 month (12.5%), followed by children who were 6 years (11.6%) and 3 years of age (7.4%). No WU virus was found in children aged 9–16 years (Table 2).

During the study period of over 1 year, from April 2007 to May 2008, a peak was observed in October 2007, and

**Table 1** Clinical associations of WU virus infections

Clinical diagnosis	No. tested	No. WU virus positive (%)
URI	307	15 (4.9)
Bronchitis	41	2 (4.9)
Pneumonia	254	19 (7.5)
Bronchiolitis	34	1 (2.9)
Unexplained fever	4	0
Asthma	10	0
Other	24	1 (4.2)
Total	674	38 (5.6)

**Table 2** Comparison of WU virus positive rates between the corresponding age groups for children with and without ARI

Age	Specimens from children with ARI % Positive (no. positive/no. tested)	Specimens from children without ARI % Positive (no. positive/no. tested)
<1 m	12.5 (5/40)	0 (0/0)
1–3 m	3.9 (3/77)	0 (0/2)
4–6 m	3.9 (2/51)	0 (0/17)
7–11 m	4.5 (4/89)	0 (0/23)
1 y	3.0 (3/100)	0 (0/43)
2 y	6.7 (6/90)	0 (0/16)
3 y	7.4 (6/81)	5.9 (1/17)
4 y	6.3 (4/64)	0 (0/23)
6 y	11.6 (5/43)	2.9 (1/35)
9–16 y	0 (0/39)	3.8 (1/26)
Total	5.6 (38/674)*	1.5 (3/202)*

*m* month/months, *y*: year/years

\*  $0.05 > P > 0.01$  by  $\chi^2$  test ( $\chi^2 = 6.00$ )

other smaller peaks were found in June 2007 and April 2008 (Fig. 1).

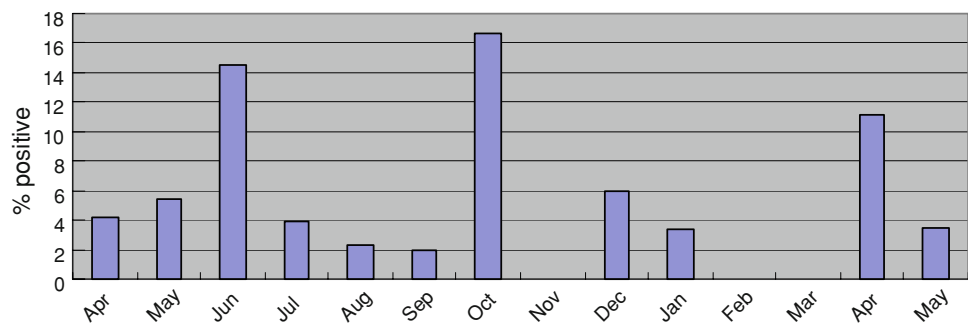
WU virus DNA detected from specimens from children without ARI

Out of the 202 specimens collected from children who visited the hospital for a health check-up, three (1.5%, 3/202) were WU-virus-DNA positive, including one from a 3-year-old boy, one from a 7-year-old girl, and another one from a 9-year-old boy, as shown by PCR with primers targeting VP2 for screening or PCR with primers targeting the LTAg for confirmation, suggesting that these specimens were WU virus positive. The result was then reconfirmed by sequence analysis for one of these three amplicons using VP2-region primers. No other virus was detected in these three specimens, either by virus isolation or by RT-PCR and PCR.

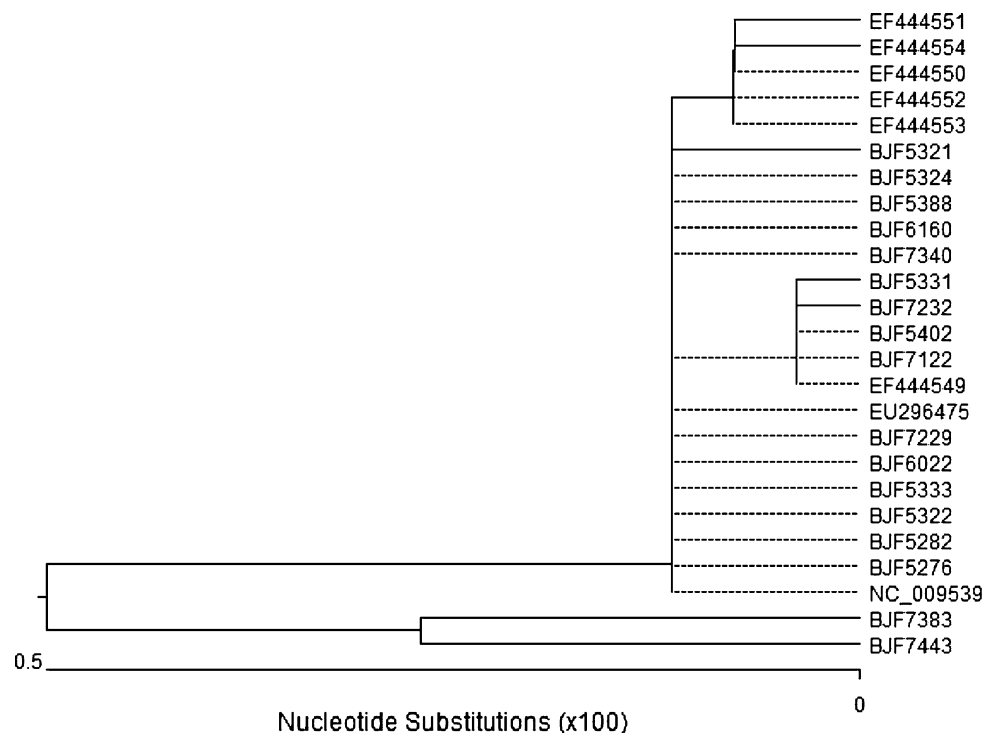
The overall positive rate of WU virus detection in specimens collected from children with ARI was higher (about 3.7 times) than that in specimens from children without ARI ( $P$  value of  $0.05 > P > 0.01$  by  $\chi^2$  test), and this was statistically significant, whereas no significant difference was found in the corresponding age groups (Table 2).

Sequence analysis of the VP2-encoding region

Among the sequences of the 17 amplicons that were amplified from specimens from ARI patients and sequenced in the VP2 coding region, 5 (ID no. BJF5276, BJF5282, BJF5322, BJF5331 and BJF5333) were submitted to GenBank and were given the accession numbers EU693903, EU693904, EU693905, EU693906 and EU693907, respectively. All of the VP2-coding-region sequences, named BJF5321, BJF5324, BJF5388, BJF6160, BJF7340, BJF5331, BJF7232, BJF5402, BJF7122, BJF7229, BJF6022, BJF5333, BJF5322, BJF5282, BJF5276, BJF7383, and BJF7443, were 1,248 bp in length, encoding a protein of 415 aa. The nucleotide and deduced amino acid sequences of the VP2 regions shared high homology (>98.5%) with the sequences obtained from GenBank, and among themselves as well. Phylogenetic analysis of the nucleotide sequences showed that 15 out of 17 Beijing WU viruses clustered more closely with NC\_009539, EU296475 and EF444549 in the phylogenetic tree than BJF7383 and BJF7443 (Fig. 2). The clinical data indicated that both BJF7383 and BJF7443 were collected from patients diagnosed with pneumonia in May 2008. While no other virus was detected with BJF7383, PIV3 was detected in same specimen with BJF7443.

**Fig. 1** Seasonality of WU virus: proportion detected in each calendar month over a 1-year period

**Fig. 2** Phylogenetic analysis of WU viruses BJF5321, BJF5324, BJF5388, BJF6160, BJF7340, BJF5331, BJF7232, BJF5402, BJF7122, BJF7229, BJF6022, BJF5333, BJF5322, BJF5282, BJF5276, BJF7383, and BJF7443 from Beijing, and WU virus: NC\_009539; WU virus B0: EF444549; WU virus S1, S2, S3, S4, S5: EF444550, EF444551, EF444552, EF444553, EF444554; and WU virus CLEF: EU296475 based on nucleotide sequences of the VP2-encoding region



## Discussion

The discovery of new polyomaviruses has attracted interest in the *Polyomaviridae* and their relationship to human disease. In this study, 674 specimens collected from pediatric patients with ARI in Beijing were tested for WU virus in addition to common respiratory viruses and other newly identified viruses associated with acute respiratory infections. Thirty-eight (5.6%) out of these 674 specimens yielded PCR products with the predicted molecular weight, using either screening or confirmation primer sets for WU virus, suggesting the presence of this recently identified WU virus in the respiratory tracts of infants and young children with ARI in Beijing, China. Although WU virus was also identified from specimens collected from children without symptoms of ARI, indicating that some WU virus infections might be asymptomatic, WU virus occurred 3.7 times more frequently in pediatric patients with ARI than in those without ARI ( $p < 0.05$ ) in this study, suggested that WU virus is a kind of pathogen of ARI in Beijing children.

Of the 38 WU-virus-positive specimens from infants and children with ARI, more than one viral pathogen was detected in 23 (60.5%) of them, suggesting that coinfection with WU virus and other viruses is common. However, in terms of clinical manifestations, no statistically significant differences could be seen between the co-infected patients and those with WU virus only.

It was found in this study that WU virus was present not only in patients with acute lower respiratory tract infections, such as pneumonia and bronchiolitis, but also in patients with upper respiratory infections, and WU virus was detected in children of almost every age group up to 8 years.

In agreement with earlier findings [2–4], the data from this study on the phylogenetic analysis of the nucleotide sequences of VP2 genes indicate that this sequence is highly conserved. However, slight differences were found in sequences from two specimens collected from patients with pneumonia during same period, so further investigation is needed to learn more about this new polyomavirus.

In conclusion, WU virus was detected not only in pediatric patients with either acute lower or upper respiratory tract infections but also in children without ARI in Beijing, China. Further investigations are underway to determine the etiological role and clinical significance of this virus.

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