



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Merkel cell polyomavirus DNA in tumor-free tonsillar tissues and upper respiratory tract samples: Implications for respiratory transmission and latency

Kalle Kantola^{a,*}, Mohammadreza Sadeghi^{a,1}, Anne Lahtinen^a, Minna Koskenvuo^c, Leena-Maija Aaltonen^d, Merja Möttönen^e, Jaana Rahiala^f, Ulla Saarinen-Pihkala^f, Pekka Riikonen^g, Tuomas Jartti^h, Olli Ruuskanen^h, Maria Söderlund-Venermo^a, Klaus Hedman^{a,b}

^a Department of Virology, Haartman Institute, University of Helsinki, P.O. Box 21, FIN-00014, Helsinki, Finland

^b Helsinki University Central Hospital Laboratory Division, Haartmaninkatu 3, FIN-00290, Helsinki Finland

^c Department of Pediatrics, Stanford University, Palo Alto, CA 94305-5164, USA

^d Department of Otorhinolaryngology-Head and Neck Surgery, Helsinki University Central Hospital, Haartmaninkatu 4 E, FIN-00290, Finland

^e Department of Pediatrics and Adolescence, Oulu University Hospital, P.O. Box 23, FIN-90029, Oulu, Finland

^f Hospital for Children and Adolescents, Helsinki University Hospital, Stenbäckinkatu 11, FIN-00290, Helsinki, Finland

^g Department of Pediatrics, Kuopio University Hospital, P.O. Box 1777, FIN-70211, Kuopio, Finland

^h Department of Pediatrics, Turku University Hospital, Kiinamylynkatu 4-6, FIN-20520 Turku, Finland

ARTICLE INFO

Article history:

Received 20 March 2009

Accepted 20 April 2009

Keywords:

MCPyV
KIPyV
WUPyV
Tonsil
PCR

ABSTRACT

Background: Merkel cell polyomavirus (MCPyV) was discovered recently. It is considered a potential causative agent of Merkel cell carcinoma, a life-threatening skin cancer.

Objectives: To study the prevalence of MCPyV in a large number of clinical samples of various types. Most of the samples were examined also for the other newly found polyomaviruses KI (KIPyV) and WU (WUPyV).
Study design: Altogether 1390 samples from immunocompetent or immunocompromised patients, including (i) tonsillar tissues and sera from tonsillectomy patients; (ii) nasopharyngeal aspirates (NPAs) and sera from wheezing children and (iii) nasal swabs, sera and stools from febrile leukemic children were studied for MCPyV. The tonsils, nasal swabs and stools were also studied for KIPyV and WUPyV.

Results: MCPyV DNA was detected in 14 samples altogether; 8 of 229 (3.5%) tonsillar tissues, 3 of 140 (2.1%) NPAs, 2 of 106 (1.9%) nasal swabs and 1 of 840 (0.1%) sera. WUPyV and KIPyV were detected in 5 (2.2%) and 0 tonsils, 1 (0.9%) and 4 (3.8%) nasal swabs and 0 and 2 (2.7%) fecal samples, respectively. The patients carrying in tonsils MCPyV were of significantly higher age (median 42 years) than those carrying WUPyV (4 years, $p < 0.001$).

Conclusions: MCPyV DNA occurs in tonsils more frequently in adults than in children. By contrast, WUPyV DNA is found preferentially in children. MCPyV occurs also in nasal swabs and NPAs, in a frequency similar to that of KIPyV and WUPyV. The tonsil may be an initial site of WUPyV infection and a site of MCPyV persistence.

© 2009 Elsevier B.V. All rights reserved.

Abbreviations: MCPyV, Merkel cell polyomavirus; KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; NPA, nasopharyngeal aspirate; MCC, Merkel cell carcinoma; HBoV, human bocavirus; RSV, respiratory syncytial virus.

* Corresponding author at: Department of Virology, Haartman Institute, University of Helsinki, P.O. Box 21, FIN-00014 Helsinki, Finland.
Tel.: +358 9 19126502/50 3059817 (mobile); fax: +358 9 19126491.

E-mail addresses: kalle.kantola@helsinki.fi (K. Kantola), sadeghi355@yahoo.com (M. Sadeghi), annelaht@mappi.helsinki.fi (A. Lahtinen), minnak@stanford.edu (M. Koskenvuo), leena-maija.aaltonen@hus.fi (L.-M. Aaltonen), merja.mottonen@ppshp.fi (M. Möttönen), jrahiala@uku.fi (J. Rahiala), Ulla.Pihkala@hus.fi (U. Saarinen-Pihkala), pekka.riikonen@kuh.fi (P. Riikonen), tuomas.jartti@utu.fi (T. Jartti), olli.ruuskanen@tyks.fi (O. Ruuskanen), maria.soderlund-venermo@helsinki.fi (M. Söderlund-Venermo), klaus.hedman@helsinki.fi (K. Hedman).

¹ These authors contributed equally to this study.

1. Background

The Merkel cell polyomavirus (MCPyV) is a human polyomavirus identified recently in Merkel cell carcinoma (MCC) tissue by digital transcriptome subtraction.¹ A subsequent study confirmed that the virus occurs in ~80% of MCC tissues.^{1,2} The clonal integration of the viral DNA in the human genome in MCC tissues together with its relatively low incidence in skin tissues of healthy controls (~16%) suggested that the virus might be pathogenetically associated with MCC. The potential role of MCPyV in MCC and in other forms of human cancer is yet to be clarified.

2. Objectives

Our study objective was to determine the occurrence of MCPyV in a large number of samples of many different types among

immunocompetent and immunosuppressed individuals. For comparison, we studied the samples also for the two other newly found polyomaviruses KIPyV and WUPyV.

3. Study design

The clinical material comprised nasal swabs, sera and fecal samples from 51 febrile children with acute lymphoblastic leukemia undergoing anticancer treatment (group 1)³; nasopharyngeal aspirates (NPAs) and paired sera from 140 and 248 wheezing children, respectively (group 2)^{3–6}; and matched pairs of tonsillar tissue and sera from 229 asymptomatic children or adults (group 3)⁷ (Fig. 1). The children of groups 1 and 2 have been studied extensively for 14 and 16 respiratory viruses, respectively, including adenovirus; coronaviruses 229E, OC43; rhinoviruses; enteroviruses; human bocavirus (HBoV); influenza A and B viruses; human metapneumovirus and parainfluenza viruses 1–3. The tonsillar tissues were obtained during tonsillectomy due to chronic tonsillitis or tonsillar hypertrophy. Total nucleic acids were extracted from the sera, tonsillar tissues, nasal swabs and NPAs with the DNA Mini kit (Qiagen, Crawley, UK), and from the fecal samples with QIAamp QIAGEN DNA Stool kit (Qiagen) according to the manufacturer's instructions. The tonsillar tissues and fecal samples were examined individually whereas the sera, NPAs and nasal swabs were initially studied in pools of five, followed by individual examination of positive pools. A negative control of molecular biology grade water was extracted and included in the PCR between every 10 samples.

For MCPyV detection, nested PCR was performed using as outer primers the previously described LT3 primers¹ and as inner primers a pair hybridizing to conserved regions within the

LT3 region (LT3iF 5'-TGACGTGGGAGAGTGTTTTGG-3'; LT3iR 5'-GAGGAAGGAAGTAGGAGTCTAGAAAAG-3'). The 25- μ l amplification reaction consisted of 1 \times GeneAmp PCR buffer I (Applied Biosystems, Warrington, UK), each dNTP at 0.2 mM, 20 pmol each primer, 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 μ l of sample. To increase the robustness of fecal PCRs, BSA (New England Biolabs, Ipswich, USA) included at a final concentration of 0.1 μ g/ μ l. The cycling conditions for the first and the second PCRs were 5 min at 95 °C, followed by 40 cycles of amplification (95 °C for 17 s, 58 °C for 20 s and 72 °C for 20 s). All samples positive for MCPyV were reanalyzed with the LT1/M1 nested PCR primer set as described² and sequenced. Samples positive with the LT3 nested primer set but negative with the LT1/M1 primer pair were analyzed with a third (seminested) PCR. Primers LT3sF (5'-CTAAGTGGCCTGTATTAGCTGTAAG-3') and LT3 antisense were used for the first-round PCR whereas the internal primers LT3sF and LT3iR were used for the second-round PCR. The mastermix and cycling conditions were as for the other two PCRs. WUPyV and KIPyV detection was performed by using primer set A as described.⁸ All MCPyV and KIPyV/WUPyV PCR products of correct size were sequenced for confirmation of specificity. For use as positive controls and to determine assay sensitivities by limiting dilution analysis, plasmids containing the VP2 gene of WUPyV (EU693907) and KIPyV (EU358767) and the LT3¹ amplification product of MCPyV (EU375803) were constructed; the amplification products of the VP2 genes were cloned into pCR8/GW/TOPO (Invitrogen; Carlsbad, CA, USA) whereas the MCPyV LT3 region was synthesized and cloned into pGOv4 by Gene Oracle, Inc. (San Leandro, USA). In the MCPyV and KIPyV/WUPyV assays, plasmid controls with 30 and 5 copies/reaction were reproducibly positive, respectively. Of note, the sensitivities were unaffected by the inclusion of genomic human DNA from cultured 293T cells at 100 ng per reaction (4 ng/ μ l). In non-nested format with 40 PCR cycles the LT3 primers had a sensitivity 1 log lower than that of the nested assay both in the presence and absence of human genomic DNA.

Three NPAs testing positive for MCPyV DNA were selected for DNase treatment to determine the encapsidation status of the viral DNA. The NPAs were centrifuged at 16,000 \times g for 10 min whereafter both the supernatants and the pellets (after resuspension and sonication) were diluted 1:1 with molecular biology grade water and treated with 100 units of DNase I (Roche, Mannheim, Germany) or mock treated with water. After 2 h at 37 °C, the enzyme was inactivated with 8 mM EDTA and heating at 70 °C for 10 min. DNA was isolated with DNA Mini kit (Qiagen).

4. Results

The three novel polyomaviruses, MCPyV, KIPyV and WUPyV, were detected in the nasal swabs of the leukemic children and NPAs of the wheezing children at frequencies of 0.9–3.8% (Fig. 1). Sera from the children with MCPyV DNA in NPA were PCR-negative. Two of the 75 fecal samples (2.7%) tested positive for KIPyV DNA whereas all tested negative for MCPyV and WUPyV. Of the 840 sera studied for MCPyV, only one from a leukemic child was PCR-positive. The WUPyV/KIPyV screening of NPAs and sera from the wheezing children had been done in a prior study (manuscript in submission). For MCPyV the highest frequency of viral DNA detection in any sample type was in the tonsillar tissues; in this group of 229 subjects the average ages of the patients that were PCR-positive for MCPyV or WUPyV were 39.4 years (median 42; range 7–70) and 4.8 years (median 4; range 2–8), respectively, whereas the average ages of the patients PCR-negative for the two viruses were 22 years (median 20; range 2–72) and 23 years (median 21; range 2–72), respectively. The ages of the patients with MCPyV or WUPyV DNA in tonsils were ranked⁹ and compared with the unequal variance *t*-test, yielding a statistically significant difference of $p < 0.001$ (Fig. 2). Also the differ-

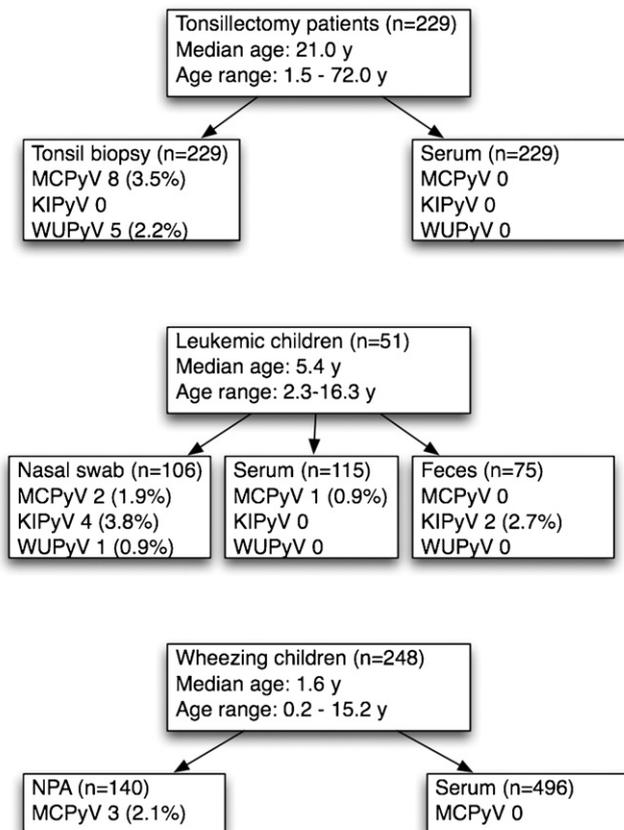


Fig. 1. Patient and sample groups. Data are numbers (%) of PCR-positive samples. Only samples testing positive for MCPyV with at least two different PCRs and subsequent sequencing are included.

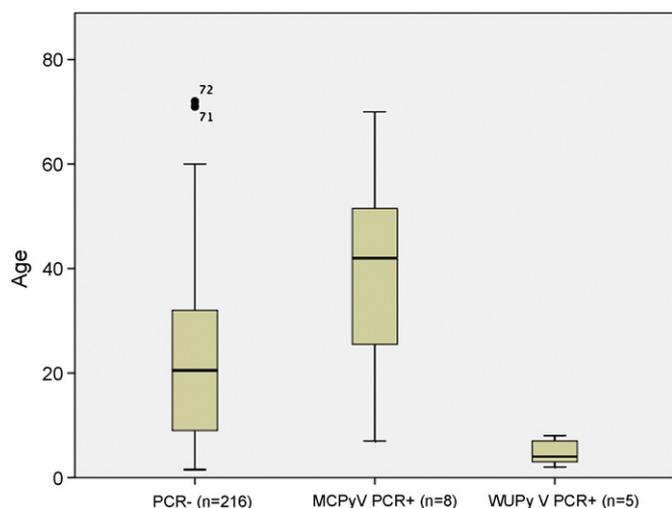


Fig. 2. A box plot illustration of the ages of tonsillectomy patients with or without MCPyV DNA in tonsil tissue. Each box corresponds to the interquartile range, with the upper boundary of the box representing the 75th percentile and the lower boundary representing the 25th percentile. The two outliers are shown with black circles, and the horizontal line indicates the median value. Excluding the outliers, the T-bars that extend from the boxes show the maximum and minimum values. Of note, only one of the subjects positive for MCPyV was <21 years of age (a 7-year-old child with hyperplastic palatine tonsils).

ence in the median ages of the patients with and without MCPyV ($p=0.032$) or WUPyV ($p<0.001$) in tonsil was statistically significant. Serum samples from the tonsillectomy patients collected at the time of operation were PCR-negative for the three viruses. Most of the tonsillar MCPyV findings were from patients with chronic tonsillitis or tonsillar hyperplasia, 5/108 (4.9%) and 3/42 (7.1%), respectively. WUPyV occurred in patients with hyperplastic palatine or adenoid tonsillitis (2 of 25, 8%), chronic tonsillitis (2 of 108, 1.9%), and chronic pansinusitis (1 of 2, 50%).

All three wheezing children with MCPyV DNA in NPA (aged 1.7, 1.2 and 4.2 years) showed co-infections with other respiratory viruses (HBoV, rhinovirus and enterovirus along with respiratory syncytial virus (RSV)). Of the two leukemic children with MCPyV DNA in NPA (aged 7 and 4 years), one tested positive for influenza A and RSV antigens whereas the other remained negative in tests for 12 respiratory viruses. In addition to fever, both had cough and rhinitis.

Among the 16 samples that, in our entire study, were positive for MCPyV by using the LT3 nested PCR, 13 tested positive also with the LT1/M1 nested PCR. Of the three LT3 nested PCR-positive samples that were negative with the LT1/M1 nested PCR, one tested positive with the LT3 seminested PCR whereas the other two remained negative. All PCR products were sequenced for confirmation of specificity. Throughout our study, only samples testing MCPyV positive with two different PCRs were considered MCPyV positive and are shown in Fig. 1. Although not shown, the lower number of LT1/M1 positive samples was probably due to the lower sensitivity of the assay.²

Three of our MCPyV-positive NPAs were treated (before DNA isolation) with DNase I. Two were reproducibly resistant to the enzyme with no change in the level of viral DNA. One sample was in three repeated assays fully susceptible to DNase I (PCR-negative versus PCR-positive), suggesting that the viral DNA occurred in nonencapsidated form.

5. Discussion

In this study MCPyV DNA was detected in clinical samples of many different types: nasal swabs and nasopharyngeal aspirates,

tumor-free tonsillar tissues and sera. Among immunocompetent children, the absence of MCPyV from the 496 serum samples tested is in accordance with our recent study (in submission) in which the two other polyomaviruses KIPyV and WUPyV were absent from all of these sera. Interestingly, our literature survey did not reveal any evidence of occurrence of human polyomavirus DNA in serum or plasma of immunocompetent individuals. On the contrary, blood cells of immunocompetent individuals have been shown to harbor BKV and JCV DNA in several studies. The reported rates of BKV detection in lymphoid cells have been 0%,¹⁰ 26%,¹¹ 53%¹² and 94%¹³ and those of JCV 0%,¹⁰ 1%,¹¹ 10%¹⁴ and 83%.¹³ BKV and JCV DNA have also been reported in the sera or plasma of immunocompromised patients.^{15,16}

Among the three NPAs of wheezing children positive for MCPyV, the invariable occurrence of co-infections (100%) with respiratory viruses may indicate merely an “innocent bystander” role for MCPyV in respiratory disease, as previously suggested for KIPyV and WUPyV.⁸ Further studies are needed to determine whether MCPyV has any contribution to respiratory tract symptoms.

A recent study reported an apparently higher prevalence of KIPyV (8%) than of WUPyV (1%) among respiratory specimens of 200 patients with respiratory symptoms, 89% of whom were immunocompromised.¹⁷ In line with this, we found KIPyV in 3.5% of nasal swabs and 2.7% of fecal samples from the febrile leukemic children whereas WUPyV was found in only 0.9% and 0% of the samples, respectively. Of note, WUPyV has been reported in 2 of 377 (0.5%) fecal samples from immunocompetent children with acute gastroenteritis.¹⁸

Two very recent reports have disclosed the presence of MCPyV in NPA samples of predominantly immunocompetent children (<15 years) with an average genoprevalence of 0.9%.^{19,20} Our study, with even slightly higher MCPyV prevalences of 2% and 3% among immunocompetent and immunocompromised children, respectively, is in line with the other two. Furthermore, we showed widespread occurrence of MCPyV DNA in tonsillar tissue. This may be a general feature of human polyomaviruses, as tonsillar tissues can harbor both JCV²¹ and BKV²² DNA and tonsillar stromal cells have been shown to be susceptible to JCV infection.²³ Blood cell-derived MCPyV positivity of the tonsils could not be completely ruled out as only serum samples (all of which tested negative for MCPyV DNA) obtained at the time of operation were available from our tonsillectomy patients. The potential presence of MCPyV in blood cells of immunocompetent individuals as opposed to serum is an interesting topic for further research.

The age distribution of our MCPyV-positive biopsy donors is in strong agreement with the study of Goh et al.²⁰ showing in NPAs Merkel cell virus DNA more frequently in adults (8.5%) than in young children (0.6%). This is very interesting, as in the NPAs of immunocompetent individuals KIPyV and WUPyV occur more frequently in children than adults.²⁴ In light of the equivalent NPA prevalences of WUPyV and MCPyV among immunocompetent children, the notable age difference in tonsillar occurrence of MCPyV versus WUPyV possibly reflects a major dissimilarity in the life cycles of these two viruses, related with persistence versus primary infection. Indeed, our small-scale DNase data suggest that even among children MCPyV DNA in the upper airways is not necessarily encapsidated among all individuals. On the basis of our results and those of Goh et al.²⁰ showing MCPyV DNA mainly in adults, it is tempting to speculate that the occurrence of MCPyV in the respiratory tract may involve active shedding coupled with respiratory transmission among children, leading to DNA persistence towards adulthood.

Conflict of interest

No conflicts.

Acknowledgments

This study was supported by Helsinki University Central Hospital Research & Education and Research & Development funds, the Sigrid Jusélius Foundation and the Academy of Finland and the Medical Society of Finland (FLS). We wish to thank Drs. Tobias Allander from the Karolinska institute (Sweden) and David Wang from the Washington University (USA) for providing the KIPyV and WUPyV plasmids, respectively.

References

- Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 2008;**319**:1096–100.
- Kassem A, Schopflin A, Diaz C, Weyers W, Stickeler E, Werner M, et al. Frequent detection of Merkel cell polyomavirus in human merkel cell carcinomas and identification of a unique deletion in the VP1 gene. *Cancer Res* 2008;**68**:5009.
- Koskenvuo M, Mottonen M, Rahiala J, Saarinen-Pihkala UM, Riikonen P, Waris M, et al. Respiratory viral infections in children with leukaemia. *Pediatr Infect Dis J* 2008;**27**:974–80.
- Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, et al. Human bocavirus and acute wheezing in children. *Clin Infect Dis* 2007;**44**:904–10.
- Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. *Emerg Infect Dis* 2004;**10**:1095–101.
- Kantola K, Hedman L, Allander T, Jartti T, Lehtinen P, Ruuskanen O, et al. Sero-diagnosis of human bocavirus infection. *Clin Infect Dis* 2008;**46**:540–6.
- Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, et al. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. *Proc Natl Acad Sci USA* 2006;**103**:7450–3.
- Norja P, Ubillos I, Templeton K, Simmonds P. No evidence for an association between infections with WU and KI polyomaviruses and respiratory disease. *J Clin Virol* 2007;**40**:307–11.
- Conover WJ, Iman RL. Rank transformations as a bridge between parametric and nonparametric statistics. *Am Stat* 1981;**35**:124–9.
- Sundsford A, Flaegstad T, Flo R, Spein AR, Pedersen M, Permin H, et al. BK and JC viruses in human immunodeficiency virus type 1-infected persons: prevalence, excretion, viremia, and viral regulatory regions. *J Infect Dis* 1994;**169**:485–90.
- Dolei A, Pietropaolo V, Gomes E, Di Taranto C, Ziccheddu M, Spanu MA, et al. Polyomavirus persistence in lymphocytes: prevalence in lymphocytes from blood donors and healthy personnel of a blood transfusion centre. *J Gen Virol* 2000;**81**:1967–73.
- Azzi A, De Santis R, Ciappi S, Leoncini F, Sterrantino G, Marino N, et al. Human polyomaviruses DNA detection in peripheral blood leukocytes from immunocompetent and immunocompromised individuals. *J Neurovirol* 1996;**2**:411–6.
- Dorries K, Vogel E, Gunther S, Czub S. Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals. *Virology* 1994;**198**:59–70.
- Ferrante P, Omodeo-Zorini E, Caldarelli-Stefano R, Mediatì M, Fainardi E, Granieri E, et al. Detection of JC virus DNA in cerebrospinal fluid from multiple sclerosis patients. *Mult Scler* 1998;**4**:49–54.
- Drachenberg CB, Hirsch HH, Papadimitriou JC, Gosert R, Wali RK, Muni-venkatappa R, et al. Polyomavirus BK versus JC replication and nephropathy in renal transplant recipients: a prospective evaluation. *Transplantation* 2007;**84**:323–30.
- Nickeleit V, Klimkait T, Binet IF, Dalquen P, Del Zenero V, Thiel G, et al. Testing for polyomavirus type BK DNA in plasma to identify renal-allograft recipients with viral nephropathy. *N Engl J Med* 2000;**342**:1309–15.
- Mourez T, Bergeron A, Ribaud P, Scieux C, de Latour RP, Tazi A, et al. Polyomaviruses KI and WU in immunocompromised patients with respiratory disease. *Emerg Infect Dis* 2009;**15**:107–9.
- Ren L. WU polyomavirus in fecal specimens of children with acute gastroenteritis, China. *Emerg Infect Dis* 2009;**15**:134–5.
- Bialasiewicz S, Lambert SB, Whitley DM, Nissen MD, Sloots TP. Merkel cell polyomavirus DNA in respiratory specimens from children and adults. *Emerg Infect Dis* 2009;**15**:492–4.
- Goh S, Lindau C, Tiveljung-Lindell A, Allander T. Merkel cell polyomavirus in respiratory tract secretions. *Emerg Infect Dis* 2009;**15**:489–91.
- Monaco MC, Jensen PN, Hou J, Durham LC, Major EO. Detection of JC virus DNA in human tonsil tissue: evidence for site of initial viral infection. *J Virol* 1998;**72**:9918–23.
- Goudsmit J, Wertheim-van Dillen P, van Strien A, van der Noordaa J. The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils. *J Med Virol* 1982;**10**:91–9.
- Monaco MC, Atwood WJ, Gravell M, Tornatore CS, Major EO. JC virus infection of hematopoietic progenitor cells, primary B lymphocytes, and tonsillar stromal cells: implications for viral latency. *J Virol* 1996;**70**:7004–12.
- Ren L, Gonzalez R, Xie Z, Zhang J, Liu C, Li J, et al. WU and KI polyomavirus present in the respiratory tract of children, but not in immunocompetent adults. *J Clin Virol* 2008;**43**:330–3.