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Association of autism with polyomavirus infection in postmortem brains

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Autism is a highly heritable behavioral disorder. Yet, two decades of genetic investigation have unveiled extremely few cases that can be solely explained on the basis of *de novo* mutations or cytogenetic abnormalities. Vertical viral transmission represents a nongenetic mechanism of disease compatible with high parent-to-offspring transmission and with low rates of disease-specific genetic abnormalities. Vertically transmitted viruses should be found more frequently in the affected tissues of autistic individuals compared to controls. Our initial step was thus to assess by nested polymerase chain reaction (PCR) and DNA sequence analysis the presence of cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV1), herpes simplex virus type 2 (HSV2), human herpes virus 6 (HHV6), BK virus (BKV), JC virus (JCV), and simian virus 40 (SV40) in genomic DNA extracted from postmortem temporocortical tissue (Brodmann areas 41/42) belonging to 15 autistic patients and 13 controls. BKV, JCV, and SV40 combined are significantly more frequent among autistic patients compared to controls (67% versus 23%, respectively; $P < .05$). The majority of positives yielded archetypal sequences, whereas six patients and two controls unveiled single–base pair changes in two or more sequenced clones. No association is present with the remaining viruses, which are found in relatively few individuals ($N \leq 3$). Also polyviral infections tend to occur more frequently in the brains of autistic patients compared to controls (40% versus 7.7%, respectively; $P = .08$). Follow-up studies exploring vertical viral transmission as a possible pathogenetic mechanism in autistic disorder should focus on, but not be limited to, the role of polyomaviruses. *Journal of NeuroVirology* (2010) **16,** 141–149.

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Introduction

Autism is a severe neurodevelopmental disorder characterized by social and communication deficits, accompanied by repetitive and stereotyped behaviors, with onset prior to 3 years of age (American Psychiatric Association, 1994). The disease is associated with epilepsy and mental retardation in up to 30% and 80% of cases, respectively (Tuchman and Rapin, 2002; Fombonne, 1999). Epidemiological studies report an incidence of about 20 to 60 in 10,000 newborns (Fombonne, 1999; Rutter, 2005). Autism represents a dimensional continuum ("autism spectrum"), more than a categorical diagnosis: patients display variable degrees of severity, categorically defined into autistic disorder, Asperger's disorder, and pervasive developmental disorder not otherwise specified (American Psychiatric Association, 1994), whereas nonautistic family members often times show psychopathological traits intermediate between those of their autistic siblings

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and the general population (Piven *et al*, 1997). Neuroanatomical and neuroimaging studies have shown microscopic abnormalities in the central nervous system (CNS) of autistic individuals, stemming from deranged neurodevelopmental processes occurring very early in pregnancy, possibly even as early as embryonic days 20 to 25 (Bauman and Kemper, 2005; Miller *et al*, 2005). Approximately 10% of patients suffer from "syndromic" autism, i.e., secondary to a known genetic disorder, such as large chromosomal rearrangements (e.g., duplication of 15q), fragile X syndrome, tuberous sclerosis, and neurofibromatosis, or to identified prenatal teratological agents, such as rubella infection, or exposure to thalidomide and valproic acid (Chess *et al*, 1978; Christianson, 1994; Yamashita *et al*, 2003; Miller *et al*, 2005; Persico and Bourgeron, 2006). In the remaining 90% of patients, nonsyndromic autism has been conclusively identified by family and twin studies as the most "heritable" neuropsychiatric disorder, with concordance rates of 82% to 92% in monozygotic twins versus 1% to 10% in dizygotic twins, sibling recurrence risk at 3% to 6% (compared to 0.2% to 0.6% in the general population), and heritability estimates above 90% (Persico and Bourgeron, 2006). Initial segregation studies supported the involvement of three to four major genes in a large number of patients (Pickles *et al*, 1995). However, an unexpected level of complexity has subsequently emerged: the number of individuals whose autism can be reliable traced back to *de novo* mutations or to submicroscopic genomic rearrangements is extremely small, whereas the vast majority of gene variants associated with autism either confer vulnerability or protection, but do not directly cause the disease (Persico and Bourgeron, 2006; Lintas and Persico, 2009).

The discrepancy between heritability estimates and genetic findings in autism and other disorders has been primarily interpreted in terms of genetic heterogeneity, polygenic modes of transmission, incomplete penetrance, phenocopies, and gene-gene and gene-environment interactions (Maher, 2008). An alternative explanation is represented by vertical viral transmission occurring during early pregnancy or even mediated by parental gametes, as well-documented for several viruses, including human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) (Englert *et al*, 2004). Using this "trojan horse" mechanism, viral infections can mimic complex genetic transmission modes, since disease incidence and severity will depend upon viral strain and titre, genetic makeup of host and virus, maternal immune response during pregnancy, and environmental and hormonal factors (van den Pol, 2006).

In this study, we address a fundamental prerequisite of the "vertical viral transmission" hypothesis, namely that the genome of vertically transmitted viruses involved in the disease should be detected significantly more often in the affected tissues of patients compared to controls. We have thus assessed the presence of several neurotropic viruses (JC virus [JCV], BK virus [BKV], simian virus 40 [SV40], cytomegalovirus [CMV], Epstein-Barr virus [EBV], herpes simplex virus 1 [HSV1], herpes simplex virus 2 [HSV2], and human herpes virus 6 [HHV6]) in postmortem brains of autistic patients and controls. Using nested polymerase chain reaction (PCR) followed by DNA sequence analysis, we describe a significant association between neocortical polyomavirus infection and autism.

Results

Viral genomes were detected in 12/15 (80%) autistic patients versus 8/13 (62%) controls (odds ratio [OR] = 2.50, 95% confidence interval [CI] = 0.46–13.52; $P = .40$, n.s.). Polyomaviruses represent the only viral species significantly more present in postmortem brains of autistic patients compared to controls (Table 1). BKV, JCV, and SV40, either singly or in combination, were detected in 10/15 (67%) patients versus 3/13 (23%) controls (OR = 6.67, 95% CI = 1.24–35.71; $P = .029$). All other viruses were found in ≤3 patients and/or controls, with no difference in incidence (Table 1). Polyviral infections were also detected more often among autistic patients compared to controls (6/15 = 40% versus 1/13 = 7.7%, respectively), although this difference did not reach statistical significance (OR = 8.00, 95% CI = 0.81–78.74; $P = .084$). Viral titres were estimated at or below 1 copy/60 ng of genomic DNA, corresponding to 10^{-3} to 10^{-4} genome equivalents per cell, but this estimate is likely spurious due to the low yield of viral genomes following DNA extraction from postmortem tissue (see Discussion). Positives amplified in either one or two PCR reactions out of five trials per virus; only subject UMB4671, a 4-year-old autistic girl, was consistently positive for HHV6 in all five trials, probably due to a viral insertion into her genomic DNA. The majority of positives yielded archetypal sequences, whereas six patients and two controls unveiled single base pair changes in two or more sequenced clones (Table 2).

Discussion

The present study reports enhanced frequencies of polyomavirus infection in postmortem neocortical tissues of autistic patients. Instead, CMV, EBV, HSV1, HSV2, and HHV6 are found less frequently and do not appear associated with the disease. Also frequent is the coinfection of the same autistic brain by more than one viral family or species (for example, BKV + JCV in three patients, and CMV + HHV6 in two patients) (Table 1). Although not reaching

Table 1 Presence and cumulative frequency of viral genomes in postmortem neocortical tissue of 13 controls (C), 1 unaffected sibling (S), and 15 autistic patients (A)

ID	Status	BKV	JCV	SV40	CMV	EBV	HSV1	HSV2	HHV6	Any virus[a]	Any polyomavirus[b]
UMB1541	C				+					+	0
UMB1377	C									0	0
UMB1407	C									0	0
UMB1185	C		+							+	+
UMB1860	C									0	0
B6221	C								+	+	0
B6207	C		+							+	+
B4211	C									0	0
B5873	C		+							+	+
B3829	C				+					+	0
B5251	C									0	0
UMB1500	C							+		+	0
UMB1706	C				+			+		++	0
Frequency in controls		0 (0%)	3 (23%)	0 (0%)	3 (23%)	0 (0%)	0 (0%)	2 (15%)	1 (8%)	8 (62%)	**3 (23%)**
B5321	S					+				+	0
UMB1174	A			+						+	+
B5342	A									0	0
B5569	A									0	0
UMB4721	A			+						+	+
B6337	A				+				+	++	0
B6294	A		+		+				+	+++	+
B5173	A	+	+							++	++
B5000	A						+			+	0
B5144	A									0	0
UMB144	A	+								+	+
UMB1349	A		+							+	+
UMB4671	A		+						+	++	+
B5666	A	+	+							++	++
UMB1182	A				+					+	0
UMB1638	A	+	+							++	++
Frequency in autistics		4 (27%)	6 (40%)	2 (13%)	3 (20%)	0 (0%)	1 (7%)	0 (0%)	3 (20%)	12 (80%)[c]	**10 (67%)**[d]

Note. Significant differences between autistic patients and controls are in bold. [a]Number of controls or autistic patients positive to at least one viral species. [b]Number of controls or autistic patients positive to at least one polyoma species. [c]Autistic patients versus controls, $P = .40$. [d]Autistic patients versus controls, $P < .05$.

statistical significance, this trend is interesting in light of the synergistic action previously demonstrated between different viral species, such as HIV and JCV, when coinfecting the centralnervous system (CNS) of the same host (Gorrill *et al*, 2006)

Our experimental approach appears both sensitive and reliable. Interestingly, rare cases of viral genome integration in humans have been reported for HHV6 (Clark *et al*, 2006), the only virus consistently detected in one individual in each of five separate PCR trials. The probability of false-positives and false-negatives was minimized by preventing cross-contaminations, sequencing several clones from PCR products, and performing on different days five experiments per virus, each involving patients and controls altogether. At least one sample scored positive for each viral species (Table 1), and 22/40 (55%) nested-PCR experiments yielded at least one positive sample. Viral titers appear low, but this estimate is spuriously decreased by DNA extraction procedures from frozen brain tissue, which yield mean recovery rates for microorganism genomes blunted by as much as 100-fold in controlled experimental settings (Little *et al*, 2009). Hence, our results are potentially compatible with viral titers in live tissue possibly as high as 1 copy of viral DNA every 10 to 100 cells.

Several human studies have addressed possible viral contributions to autism (Libbey *et al*, 2005). Conclusive evidence has been provided only for prenatal rubella infection (Chess *et al*, 1978). Suggestive evidence comes from a number of positive clinical studies and case reports describing the onset of autism following infection with HSV, CMV, and poxvirus, whereas at least three reports were negative (for review, see Libbey *et al*, 2005). These studies report either direct searches of viral genomes in biomaterials such as blood and urines, or indirect measures of immune activation. The present report for the first time addresses the presence of neurotropic viruses directly in postmortem brain, undoubtedly the organ most affected in autistic disorder. Here 67% of autistic patients are positive for a polyomavirus infection, whereas our 23% positives among controls are perfectly superimposable to

Table 2 Single–base pair substitutions and related amino acid changes, if present, relative to Genbank reference sequences[a]

Virus	ID	Status	SNP position	Amino acid change
BKV	UMB144	A	4417C-T (2/6)	No change
	B5666	A	None	—
	UMB1638	A	4384A-G, 4415G-A (3/3 for both)	No change, S92F
	B5173	A	None	–
JCV	UMB1349	A	None	–
	B5666	A	4293T-C (3/3)	H123R
	UMB1638	A	None	–
	UMB4671	A	None	–
	B6294	A	None	–
	B5173	A	None	–
	B5873	C	4326A-G (3/3)	F112S
	B6207	C	None	–
	UMB1185	C	None	–
SV40	UMB1174	A	None	–
	UMB4721	A	None	–
CMV	B6337	A	None	–
	B6294	A	None	–
	UMB1182	A	None	–
	B3829	C	None	–
	UMB1706	C	None	–
	UMB1541	C	None	–
EBV	B5321	S	None	–
HSV1	B5000	A	65164C-T (7/7)	No change
HSV2	B6337	A	None	–
	UMB1500	C	None	–
	UMB1706	C	None	–
HHV6	UMB4671	A	87138G-A, 87157A-T, 87278A-G (4/4 for all)	–
(B variant)	B6294	A	87278A-G (3/3)	–
	B6221	C	87138G-A, 87157A-T, 87278A-G (4/4 for all)	—

Note. In parentheses, number of clones carrying the substitution/total number of sequenced clones. [a]Genbank reference sequence numbers: BKV, AB301103; JCV, AB195640; SV40, EF579804; CMV, BK000394; EBV, V01555; HSV1, FJ593289; HSV2, EU939311; HHV6 (B variant), AF157706.

previously published data (Elsner and Dörries, 1992). Indeed, a recent magnetic resonance imaging (MRI) study documented for the first time the presence of temporal lobe and/or white matter abnormalities similar to those produced by viral infections, such as congenital CMV, in as many as 36% of autistic children (Boddaert *et al*, 2009). Parent-to-offspring transmission of JCV due to close proximity and possibly mediated by fomites has been conclusively demonstrated (Kitamura *et al*, 1994; Kunitake *et al*, 1995; Zheng *et al*, 2004). This mechanism, which does not truly represent a vertical transmission because the infectious agent can reach the offspring both from the mother and from the father, is unlikely to be involved in autism because neuropathological studies of autistic brains document the derangement of neurodevelopmental processes occurring during early pregnancy (Bauman and Kemper, 2005; Miller *et al*, 2005). Prenatal transplacental or early postnatal viral infections have been the transmission mechanisms generally proposed in the literature (Clark *et al*, 2006). Transplacental passage of polyomaviruses is indeed quite possible, although it has not been conclusively demonstrated (Pietropaolo *et al*, 1998; Kalvatchev *et al*, 2008; Boldorini *et al*, 2008). Vertical viral transmission through parental gametes also appears an intriguing possibility, for the following reasons: (a) polyomaviruses have been detected in sperm cells and fluids (Martini *et al*, 1996; Monini *et al*, 1996; Barbanti-Brodano *et al*, 2004); (b) converging experimental approaches have demonstrated an inappropriate and persistent activation of the innate immune system, even in some of the same brain tissues assessed in the present study (Vargas *et al*, 2005; Garbett *et al*, 2008; Palmieri *et al*, 2010; Lintas *et al*, 2009). This immune imbalance is compatible with the persistence of an unresolved, early-onset viral infection accompanied by autoimmune phenomena; (c) polyomaviruses can cause autoimmune disorders (Rekvig *et al*, 2006), which are especially frequent among first-degree relatives of autistic patients (Comi *et al*, 1999); (d) polyomaviruses can produce genomic instability, which is frequently encountered at the gametic level in parents of autistic children and results in the generation of Copy Number Variants (Marshall *et al*, 2008).

The present study has several limitations that must be duly acknowledged. Our sample size is relatively small, a limitation inherent to all studies involving postmortem brains. Other infectious agents not assessed here could play a relevant role in autism. Attempts to replicate our results may encounter difficulties due to low viral titres following DNA extraction from postmortem brain tissue and to the typically patchy distribution of viral infections in the CNS. Finally, our results obviously

Table 3 Brain tissue information for patients and controls

No.	Case no.[a]	Diagnosis	Age (years)[b]	Sex[c]	PMI (h)[d]	Cause of death	Mental retardation	Epilepsy	Other features	Drug therapies at time of death
1	UMB1174	Autism	7	F	14	Sudden death, seizure	Yes	Yes	Coloboma iris, cortical heterotopias, mesial temporal sclerosis, lymphoadenopathy, recurrent infections	Lamictal, Valium[e]
2	UMB4721	Autism	8	M	16	Drowning	Unknown	No	—	None
3	B5144	Autism	20	M	23.7	Trauma	Yes	No	–	None
4	B5173	Autism	30	M	20	Gastrointestinal hemorrage	Yes	Yes	Large ear lobes	Dilantin, Depakote, Tranxene bid, cisapride, clorazepate, folic acid, oxcarbazepine[d]
5	B6294	Autism	16	M	Unknown	Seizure	Unknown	Yes	–	Topamax, Depakote, Allegra, Claritin, NuThera, multivitamin
6	B6337	Autism	22	M	25	Seizure	Yes	Yes	Intestinal lymphoadenopathy, hypertrophic spleen, recurrent otitis	Lamectil, Zonegran, Neurontin, Abilify, flax seed oil, omega-3, multivitamin
7	B5000	Autism	27	M	8.3	Drowning	Yes	No	Septo-optic dysplasia of the septum pellucidum, hypothalamic and pituitary dysfunction	Synthroid, mannitol
8	B5342	Autism	11	F	13	Drowning (seizure?)	Yes	Yes	Recurrent otitis	Topamax, Lamictal, Adderall
9	UMB4671	Autism	4	F	13	Trauma	Yes	No	–	None
10	UMB1349	Autism	5	M	39	Drowning	Yes	No	Hypogonadism and obesity	–
11	UMB5666	Autism	8	M	22	Rhabdomyosarcoma	No info	Yes	Colitis, syndactyly of the fingers and feet	Valproate
12	UMB1182	Autism	9	F	24	Smoke inhalation	Yes	No	–	–
13	UMB1638	Autism	21	F	50	Respiratory failure during seizures	Yes	Yes	Microcephaly, schizophrenia	Zoloft, Zyprexa
14	B5569	PDD-NOS	5	M	25.5	Drowning	No	No	Recurrent otitis, angioedema, food allergies	Prozac, melatonin[d]
15	UMB144	PDD-NOS	10	M	22	Drowning	No info	No info	–	Depakote, imipramine
16	B5321	Unaffected sibling	19	F	12.5	Traffic accident	No	No	–	–
17	UMB1185	Control	4	M	17	Drowning	No	No	–	None
18	UMB1377	Control	6	F	20	Drowning	No	No	–	None
19	UMB1860	Control	8	M	5	Cardiac arrhythmia	No	No	–	None
20	B3829	Control	22	M	12	Central hepatic laceration	No	No	–	None
21	B4211	Control	30	M	23	Cardiac arrhythmia	No	No	–	None
22	B6207	Control	16	M	26	Ischemic heart attack	No	No	–	None
23	B6221	Control	22	M	24	Unknown	No	No	–	None

Table 3 *(Continued)*

No.	Case no.[a]	Diagnosis	Age (years)[b]	Sex[c]	PMI (h)[d]	Cause of death	Mental retardation	Epilepsy	Other features	Drug therapies at time of death
24	B5873	Control	28	M	23	Unknown	No	No	–	–
25	UMB1541	Control	20	F	19	Head injuries	No	No	–	None
26	UMB1407	Control	9	F	20	Asthma	No	No	–	None
27	UMB1706	Control	8	F	20	Rejection of heart transplant	No	No	–	None
28	B5251	Control	19	M	19	Multiple trauma	No	No	–	None
29	UMB1500	Control	6	M	18	Lung infection	No	No	–	None

[a]Autism Tissue Program identifier. [b]Mean age (± SD) for the autism group = 13.36 ± 8.78, for controls = 15.23 ± 8.89; Student $t = -0.516$, 26 *df*, $P = .610$. [c]M:F ratio = 10:5 for the autism group and 9:4 for controls; chi-squared = 0.021, 1 *df*, $P = .885$. [d]PMI = postmortem interval; mean PMI (± SD) for the autism group = 22.54 ± 10.91, for controls = 18.92 ± 5.46; Student $t = 1.073$, 25 *df*, $P = .293$. [e]Pharmacological therapy from the last available report, dating back to less than a year prior to death.

do not demonstrate vertical viral transmission, as these infections could represent late, bystanding consequences of a malfunctioning immune system. Nonetheless, this study documents for the first time an increased incidence of polyomavirus infections in postmortem brains of autistic patients. These results do spur interest into further investigating the vertical viral transmission hypothesis *in vivo*. The incidence of polyomavirus infections and viral titres measured in peripheral tissues and/or bodily fluids, as well as immune response parameters, could provide valuable indirect information, whereas investigations focused on parental gametes should yield direct evidence. Both approaches are being actively pursued in our laboratory.

Materials and methods

Brain tissue samples

Frozen postmortem brain tissues dissected from the superior temporal gyrus (Brodmann areas 41/42) of 15 autistic patients and 13 normal controls (Table 3), were obtained through the Autism Tissue Program from the NICHD Brain and Tissue Bank (Baltimore, MD) and the Harvard Brain Tissue Resource Center (Belmont, MA). Patients and controls were selected based on age, sex, and postmortem interval (PMI), which are superimposable (Table 3). One unaffected sister of an autistic patient was assessed separately, due to frequent "autism spectrum" traits in first-degree relatives (Piven *et al*, 1997). The superior temporal gyrus was selected, as this neocortical brain region hosts well-documented structural and functional abnormalities in autistic individuals (Zilbovicius *et al*, 2006). These same brain specimens have been the object of several previous reports from our group (Campbell *et al*, 2007; Garbett *et al*, 2008; Palmieri *et al*, 2010; Lintas *et al*, 2009).

Nested PCR, DNA cloning, and sequencing

DNA was recovered by phenol/chloroform extraction and ethanol precipitation, following brain tissue digestion with proteinase K at 55°C overnight. Nested PCR was performed for the following virus species: JCV, BKV, SV40, CMV, EBV, HSV1, HSV2, and HHV6. Each individual was screened for each virus by five independent nested-PCR experiments. Each PCR experiment included all individuals (i.e., patients and controls) and negative controls for the first and second round PCRs; in order to minimize the risk of contaminations, positive controls were PCR-amplified separately and run on the same agarose or acrylamide gel for band size determination. Each individual was recorded as "positive" if at least one PCR experiment yielded an amplified product of appropriate size, later cloned and definitively confirmed by DNA sequencing. PCR cycling conditions were 95°C for 5 min; 35 to 40 cycles at 95°C for 30 s,

Table 4 Primers and PCR conditions used in this study

Virus	Primer name	Primer sequence (5′-3′)	PCR annealing temp	Reference
BKV	BK/JC-F	GAAAGTCTTTAGGGTCTTCTACC	59°C	Tognon *et al*, 2001
	BK/JC-R	TAGGTGCCAACCTATGGAACAGA		Tognon *et al*, 2001
	BK-F1, nesting	GGTGGTGTTGAGTGTTGAGAATC	59°C	—
JCV	BK/JC-F	GAAAGTCTTTAGGGTCTTCTACC	59°C	Tognon *et al*, 2001
	BK/JC-R	TAGGTGCCAACCTATGGAACAGA		Tognon *et al*, 2001
	JC-R1, nesting	GGGAATCCTGGTGGAATACAT	59°C	—
SV40	SV40-F1	GCATGACTCAAAAAACTTAGCAATTCTG	58°C	Martini *et al*, 2002
	SV40-R1	CTTTGGAGGCTTCTGGGATGCAACT		Martini *et al*, 2002
	SV40-F2	GATGGCATTTCTTCTGAGCA	55°C	—
	SV40-R2	CACTTGTGTGGGTTGATTGC		—
CMV	HHV-F1	GTCTTGCGCACCAGATCCAC	60°C	Tafreshi *et al*, 2005
	HHV-R1	GTCGTGTTTGACTTTGCCAGC		Tafreshi *et al*, 2005
	Cito-F2	AAAAAGACAGGCCGTCACGTAGT	63°C	—
	Cito-R2	TCAGGAAGACTATGTAGTGGGAACG		—
EBV	EBV-F1	GGCTGGTGTCACCTGTGTTA	59°C	Telenti *et al*, 1990
	EBV-R1	CCTTAGGAGGAACAAGTCCC		Telenti *et al*, 1990
	EBV-F2	TGGGTCTCAGTGACATGGAA	58°C	—
	EBV-R2	TCCCACCCCAGTAGTTACCA		—
HSV1	HHV-F1	GTCTTGCGCACCAGATCCAC	60°C	Tafreshi *et al*, 2005
	HHV-R1	GTCGTGTTTGACTTTGCCAGC		Tafreshi *et al*, 2005
	Hsv1-F2	GTGTGTAACTCGGTGTACGGGTTC	62°C	—
	Hsv1-R2	CAGCACAAATATGGAGTCCGTGTC		—
HSV2	HHV-F1	GTCTTGCGCACCAGATCCAC	60°C	Tafreshi *et al*, 2005
	HHV-R1	GTCGTGTTTGACTTTGCCAGC		Tafreshi *et al*, 2005
	Hsv2-F2	CCATCAAGGTGGTGTGCAACTC	62°C	—
	Hsv2-R2	GTAGATGATGCGCATGGAGTACG		—
HHV6	HHV6-F1	CGCAGAGACATATCGTTCCGATGG	63°C	Victoria *et al*, 2005
	HHV6-R1	AGAACCGTCGCATCAATTACTCGC		Victoria *et al*, 2005
	HHV6-F2	AATAGGAGCCTTGCTGGTCAGAAC	63°C	Victoria *et al*, 2005
	HHV6-R2	CCTGGACCCCACAAAACCTAACG		Victoria *et al*, 2005

annealing temperature for 30 s, and 72°C for 30 to 40 s according to amplicon length; and 72°C for 10 min. Primers and PCR annealing temperatures are largely from previous studies (Telenti *et al*, 1990; Tognon *et al*, 2001; Martini *et al*, 2002; Tafreshi *et al*, 2005; Victoria *et al*, 2005), as summarized in Table 4. First and second round PCRs were performed in a total volume of 25 μl including 10× AccuSure Buffer (Bioline, London, UK), 2.5 mM AccuSure $MgCl_2$, 0.8 mM dNTPs (TaKaRa, Shiga, Japan), 2.5 units of Taq AccSure DNA polymerase, and 0.4 μM of each primer (Invitrogen, Carlsbad, CA). Approximately 60 ng of genomic DNA was used as a template for the first PCR; 4 μl of a 1:20 dilution of the first PCR product was used as a template for the second PCR. PCR products were analyzed by electrophoresis on a 1% to 2% agarose or acrylamide gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light. The sensitivity of nested PCRs was preliminarily tested using serial 10-fold dilutions of plasmids containing the appropriate viral genomic segment (range $0–10^8$ copies) and found to range between <1 and <10 copies/μl. Appropriately sized PCR products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA), using the TOPO TA reagents according to the manufacturer's protocol. Three to seven clones per individual were sequenced, using a CEQ8000 DNA sequencer (Beckman-Coulter, Fullerton, CA). Single–base pair changes were considered reliable when present in at least two clones.

Statistical analysis

Contrasts were performed using Student *t*, chi-square, or Fisher's exact test, reporting also odds ratio (ORs) and 95% confidence intervals (CIs) wherever appropriate. Two-tailed *P* values were applied.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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