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BK VIRUS GENOME IS DETECTABLE IN AUTOPSY SPECIMENS FROM ABORTED FOETUSES



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Keywords:	polyomavirus, vertical transmission, viral capsid protein, transcriptional control region

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Case No.	Mother	Foetus					Placenta		
	Age ^o	Week	Weight [^]	Sex	Malformations	Histology	Dimension	Weight [^]	Histology
1	39	16 th	126	M	Spina bifida	Mielomeningocele	8x7x1	90	Acute corionamniosis
2	31	28 th	380	F	---	---	14x12x1.5	222	---
3	30	17 th	ND	M	---	Intraalveolar meconium and squamous cells	N/A	185	---
4	24	15 th	68	M	---	---	8x4x1	78	Acute corionamniosis
5	35	25 th	520	F	Multiple malformations	Intraalveolar meconium and squamous cells	12.5x10x1.3	136	---
6	35	20 th	364	F	Down's syndrome	---	9.5x8.5x3	118	Acute corionamniosis
7	28	18 th	298	F	Beckwith-Wiedemann's syndrome	---	10x9.5x2	94	---
8	40	19 th	175	M	---	---	14.5x8x2.5	175	Acute corionamniosis funisitis
9	29	17 th	270	M	---	Foetal bronchopneumonia	N/A	180	Acute corionamniosis funisitis
10	36	17 th	ND	M	Down's syndrome	---	10x3	206	---

N/A= not available. ^o Years; [^] grams

Table I:

Main demographic and pathologic data of pregnant women, fetuses and foetal adnexa

For Peer Review

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Case No.	Organs	VP1	TCR
2	Brain	POS	neg
	Heart	POS	neg
	Lung	POS	POS
4	Lung	Neg	POS
5	Liver	Neg	POS
	Heart	POS	POS
6	Heart	POS	Neg
	Thymus	POS	Neg
	Brain	Neg	POS
	Kidney	POS	Neg
	Lung	POS	Neg
7	Lung	Neg	POS
	Heart	POS	Neg
	Liver	POS	POS
	Kidney	POS	Neg
9	Kidney	POS	Neg
	Brain	Neg	POS
10	Kidney	Neg	POS
	Liver	Neg	POS
	Heart	POS	neg
	Lung	POS	neg
	Thymus	POS	neg

VP1= Viral Protein 1

TCR: Transcription Control Region

Table II

Distribution of BKV genomic sequences, in foetal organs identified by amplification of VP1 and TCR regions.

Case No.	Organs	VP1•	TCR♦
2	Brain	BKV type I MM	neg
	Heart	BKV type I MM	neg
	Lung	BKV type I MM	BKV WWT G→A (S5); G→A (S18)
4	Lung	neg	BKV WW C→T(P31); G→A (S18)
5	Liver	neg	BKV WW C→T (P31); G→A (S18)
	Heart	BKV type I MM	BKV WW C→T (P31)
6	Heart	BKV type I MM	neg
	Thymus	BKV type I MM	neg
	Brain	neg	BKV WW C→T (P31); G→A (S18)
	Kidney	BKV type I MM	neg
	Lung	BKV type I MM	neg
7	Lung	neg	BKV WWT G→A (S5); G→A (S18)
	Heart	BKV type IV MG G→A (1792)	neg
	Liver	BKV type IV (IV)	BKV 128-I
	Kidney	BKV type I MM A→G (1801)	neg
9	Kidney	BKV type I GS C→A (1794)	neg
	Brain	neg	BKV WWT G→A (S67)
10	Kidney	neg	BKV 128-I Δ (P32-P41)
	Liver	neg	BKV WWT G→A (S18)
	Heart	BKV type I MM	neg
	Lung	BKV type I MM	neg
	Thymus	BKV type I MM	neg

- Genotype and subtype according to Jin et al., 1993;
♦ Genotype and subtype according to Negrini et al., 1991.
→, substitutions;
Δ, deletion.

Table III: BKV genotypes, subtypes (VP1), archetypes (TCR) and genomic mutations identified in foetal organs.

BK VIRUS SEQUENCES IN SPECIMENS FROM ABORTED FETUSES

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43 **Shortened title:** BKV sequences in tissues of aborted fetuses

ABSTRACT

Given the conflicting results of the few published studies, the aim of this retrospective molecular-based study of 10 aborted fetuses that underwent complete autopsy and 10 placentas was carried out to determine whether BK polyomavirus (BKV) can be transmitted transplacentally. The interruption of pregnancy was due to a miscarriage (five cases) or a prenatal diagnosis of severe intrauterine malformations (five cases). Samples from the brain, heart, lung, thymus, liver and kidney were taken from each fetus, and two samples were obtained from all of the placentas. The presence of BKV was investigated by means of PCR using primers specific for the transcription control region (TCR) and viral capsidic protein 1 (VP1) and DNA extracted from formalin-fixed, paraffin-embedded tissue. BKV genome was detected in 22 of 60 samples (36.6%) from seven fetuses (70%), regardless of the cause of abortion: VP1 was amplified in 12 samples (54%), TCR in seven (32%), and both in three (14%). VP1 was also detected in one placental sample. BKV sequences were most frequently detected in heart and lung (five cases), but sequence analyses of TCR and VP1 revealed a high degree of genomic variability among the samples taken from different organs and the placenta. These results indicate that BKV can cross the placenta during pregnancy and become latent in fetal organs other than the kidney and brain (previously considered the main targets of BKV latency). This may happen in early pregnancy and does not seem to be associated with an increased risk of abortion.

Key words: Polyomavirus; vertical transmission; viral capsid protein; transcriptional control region.

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INTRODUCTION

BK virus (BKV) is a human polyomavirus (PYV) that causes BKV-associated nephropathy in renal transplant patients [Randhawa et al., 1999].

The primary infection is usually asymptomatic and occurs during childhood; after which BKV persists latently in the body and can reactivate during major or minor immune suppressive conditions [Nebuloni et al., 1999]. Little is known about its mechanisms of infection and latency, or its target organs, but it has been claimed that BKV spreads mainly as a result of respiratory transmission. However, although this way of transmission has been supported by epidemiological studies showing a high prevalence of seroconversion to BKV infection (in as many as 80% of the human population), no laboratories have yet isolated BKV in samples cultured from respiratory secretions [Shah, 2000]. On the other hand, molecular investigations have detected BKV genome in kidney and brain, and a few authors have reported the less frequent involvement of other organs and tissues, including liver, lung, bone, genital organs and tonsils [Reploeg et al., 2001].

Some investigators have suggested that the mother-fetus pathway may be an alternative or complementary route of BKV transmission [Taguchi et al., 1975; Gibson et al., 1981; Kalvatchev et al. 2008], although this has been denied by others [Chang et al., 1996; Coleman et al., 1980]. One previous study of 300 pregnant women did not find any evidence of the transplacental transmission of BKV [Boldorini et al., 2008]. However, all of the newborns were alive and well, and there were no abortions, whereas another study found BKV genome in 12 out of 15 aborted fetuses, which suggests that transplacental BKV passage may actually be frequent [Pietropaolo et al., 1998].

The aim of this study was to search for BKV DNA sequences in various organs sampled from 10 aborted fetuses with the dual objective of: a) exploring the possibility of transplacental infection by BKV; and b) identifying any site or tissue prevalence of BKV latency.

MATERIAL AND METHODS

Samples

This retrospective study involved ten fetuses selected from files of the Pathology Unit of Ospedale Maggiore that underwent complete autopsies between 2004 and 2008.

The selection criteria included: a) sampling of the main fetal organs, placenta and placental adnexa; b) no evidence of organ maceration (e.g. due to the permanence of the fetus in amniotic fluid after death) as evaluated by means of the light microscopy of hematoxylin and eosin-stained sections; and c) the amplification of the β -actin gene from all of the autopsy tissue samples by means of polymerase chain reaction (PCR).

Histological evaluation

Small autopsy samples were taken from the brain, heart, lung, thymus, liver and kidney of each fetus, fixed in 10% formalin and embedded in paraffin; 4 μ m thick sections were then obtained and stained with hematoxylin and eosin.

Molecular biology studies

DNA extraction

Five 5- μ m-thick sections were cut from the paraffin-embedded fetal and placental tissue samples using a microtome blade that was cleaned with xylene between each block in order to avoid sample cross-contamination, and then placed in a 1.5 mL tube. DNA was extracted using EDTA-SDS/proteinase K followed by phenol chloroform, and resuspended with 30 μ l of DEPC-treated and RNase-free water (Bioline, London, UK).

The amount of extracted DNA was determined by spectrometry, and a standard concentration of 300 ng/ μ L was established.

Polymerase chain reaction assays

PCR of the β -actin gene was used to evaluate the degree of DNA fragmentation in each case, with the gene being amplified in two steps using the primers B1-B2 and AW75-AW76, which respectively amplify a fragment consisting of 586 and 202 base pairs (bp).

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3 Amplification with B1 (5'-CCA AGG CCA ACC GCG AGA AGA TGA C-3') and B2 (5'-AGG
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Amplification with B1 (5'-CCA AGG CCA ACC GCG AGA AGA TGA C-3') and B2 (5'-AGG
GTA CAT GGT GGT GCC GCC AGA C-3') was performed in a total volume of 25 μ L containing
0.05 U BioTaq DNA polymerase (Bioline, London, UK) in the presence of 1x Bioline NH₄ buffer,
1.5 mM MgCl₂ (Bioline, London, UK), 0.4 pmol/ μ L of each primer (Roche Diagnostic, Milan,
Italy), 0.2 mM dNTPs (Bioline, London, UK), and 5 μ L of fetal tissue DNA. The PCR was then
performed using an Eppendorf Mastercycler gradient PCR system (Eppendorf, Hamburg,
Germany). The samples were amplified by means of denaturation at 94°C for 5 min, followed by 35
cycles at 94°C for 30 sec, annealing at 66°C for 30 sec, and extension at 72°C for 45 sec, and
ending with a final extension at 72°C for 7 min.

The subsequent reaction with AW75 (5'-CCT TCC TGG GCA TGG AGT CCT G-3') and AW76
(5'-GGA GCA ATG ATC TTG ATC TTC-3') primers was performed in a total volume of 25 μ L
containing 0.05 U BioTaq DNA polymerase (Bioline, London, UK) in the presence of 1x Bioline
NH₄ buffer, 3 mM MgCl₂ (Bioline, London, UK), 0.2 pmol/ μ L of each primers (Roche Diagnostic,
Milan, Italy), 0.2 mM dNTPs (Bioline, London, UK), and 5 μ L of fetal tissue DNA. The samples
were amplified by denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 90 sec,
annealing at 55°C for 30 sec, and extension at 72°C for 90 sec, and ending with a final extension at
72°C for 7 min.

The BKV viral protein 1 (VP1) region was amplified using VP1F (1681-1700 nucleotide position;
5'-ACT GGG GTA GA TGC TAT TAC-3') and 327-2 (1937-1956 nucleotide position; 5'-TGC
ATG AAG GTT AAG CAT GC-3') as outer primers; and VP1F and VP1R (nucleotide position
1869-1888; 5'-GTA GAT TTC CAC AGG TTA GG-3') as inner primers. The amplified fragments
consisted of 276 bp (outer reaction) and 208 bp (inner reaction). Amplification was performed in a
total volume of 25 μ L containing 0.05 U BioTaq DNA polymerase (Bioline, London, UK) in the
presence of 1x Bioline NH₄ buffer, 1.5 mM MgCl₂ (Bioline, London, UK), 0.6 pmol/ μ L of each
primer (0.4 pmol/ μ L for inner PCR) (Roche Diagnostic, Milan, Italy), and 0.2 mM dNTPs (Bioline,
London, UK). In the first step, 5 μ L of tissue DNA were added to the PCR mixture and, in the

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3 second step, 2.5 μ L of template. The PCR was performed using an Eppendorf Mastercycler gradient
4 PCR system (Eppendorf, Hamburg, Germany). The samples were amplified by denaturation at 95°C
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6 for 5 min, followed by 35 cycles at 95°C for 40 sec, annealing at 55°C for 40 sec (56°C for inner
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8 PCR), and extension at 72°C for 40 sec, and ending with a final extension at 72°C for 5 min.
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12 The BKV transcriptional control region (TCR) was amplified using BRP+ (57-77 nucleotide
13 position; 5'-GAA AAA GCC TCC ACA CCC TTA-3') and BRP2 (349-368 nucleotide position; 5'-
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15 GCC AAG ATT CCT AGG CTC GC-3') as outer primers, and BRP1 (91-111 nucleotide position;
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17 5'-TTG AGA GAA AGG GTG GAG GC-3') with BRP2 as inner primers. The amplified
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19 fragments consisted of 302 bp (outer reaction) and 277 bp (inner reaction). Amplification was
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21 performed in a total volume of 25 μ L containing 0.05 U BioTaq DNA polymerase (Bioline,
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23 London, UK) in the presence of 1x Bioline NH₄ buffer, 1.5 mM MgCl₂ (Bioline, London, UK),
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25 0.6 pmol/ μ L of each primer (Roche Diagnostic, Milan, Italy), and 0.2 mM dNTPs (Bioline, London,
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27 UK). In the first step, 5 μ L of tissue DNA were added to the PCR mixture and, in the second step,
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29 2.5 μ L of template. The PCR was performed using an Eppendorf Mastercycler gradient PCR system
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31 (Eppendorf, Hamburg, Germany). The samples were amplified by denaturation at 95°C for 5 min,
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33 followed by 35 cycles at 95°C for 40 sec, annealing at 55°C for 40 sec (50°C for inner PCR), and
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35 extension at 72°C for 40 sec, and ending with a final extension at 72°C for 5 min.
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43 All of the DNA amplification products were analysed by means of 2% agarose gel electrophoresis,
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45 and visualised using SYBR safe DNA gel stain (Invitrogen Molecular Probes, Oregon, USA). PCR
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47 sensitivity was estimated by amplifying serial dilutions of a BKV-positive sample. Each sample
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49 underwent three PCRs, and the BKV region amplified in two of the three was considered positive
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51 (data not shown).
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53 Direct DNA sequencing

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56 DNA fragments from all of the VP1- and TCR-positive samples were separated by electrophoresis
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58 on 3% agarose gel, and one fragment of each sample (derived from the inner reaction) was excised,
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60 extracted and purified using a commercial kit (PCR clean-up gel extraction, NucleoSpin, Macherey-

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3 Nagel, Duren, Germany). A cycle sequencing PCR reaction was performed as previously described
4 [Boldorini et al., 2009] and, in order to avoid Taq polymerase errors, the sequence in each sample
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6 was analysed twice. The sequences were read manually; when the electropherogram showed two
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8 peaks at the same height in the same nucleotide position, these were considered to be two distinct
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10 sequences. The genotypes and subtypes of the isolated BKV strains were classified on the basis of
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12 the VP1 region sequence, as proposed by Jin *et al.* [Jin et al., 1993], and their archetypes were
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14 classified on the basis of the TCR region sequence, as proposed by Negrini *et al.* [1991].
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19 *Immunohistochemistry*

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22 Immunohistochemistry was performed using a polyclonal antibody against the simian virus SV40
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24 (Lee Biomolecular Research Labs, San Diego, CA; dilution 1:20,000), which cross-reacts with
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26 human BKV virus in all cases that are PCR positive for BKV genome [Boldorini et al., 2005].
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RESULTS

Table I shows the main pathological and demographic characteristics of the pregnant women and fetuses.

The interruption of pregnancy was due to a prenatal ultrasound and/or genetic diagnosis of serious intrauterine malformations in five cases: two cases of Down syndrome, and one each of spina bifida, Beckwith-Wiedemann syndrome, and multiple malformations. The other five cases were spontaneous abortions.

The six male and four female fetuses had a median gestational age of 18 weeks (mean 19.6; range 16-28) and weighed 68-520 g (median 284; mean 275.1); placental weight was 78-222 g (median 155.5; mean 148.4). The median age of the mothers was 33 years (mean 32.7; range 24-40).

Histological findings

Non-specific acute chorionamniosis was found in five cases (#1, #4, #6, #8 and #9), associated with acute funisitis in two cases (#8 and #9) and fetal bronchopneumonia in one (#9); the morphology of the fetal organs was normal and their development was in line with their gestational ages. The morphological findings in two cases (#3 and #5) indicated acute intrauterine fetal death, including meconium and squamous cells within the pulmonary alveoli; the histological analyses supported the prenatal diagnoses of spina bifida and Beckwith-Wiedemann syndrome.

Molecular analysis

Amplification of the VP1 and/or TCR regions revealed BKV sequences in 22 of 60 samples (36.6%) obtained from seven fetuses (70%): the VP1 region was amplified in 12 samples (54%), TCR in seven (32%), and both in three (14%). Sequences were detected in only one placental sample by means of the amplification of VP1 region.

Table II shows the organ distribution of BKV genome, which was detected most frequently in the heart and lung (5/7 cases, 70%); in four cases, BKV infection involved three or more organs, and in one case was found only in one lung sample (case #4).

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2
3 Table III shows the genotype, subtype and archetype distribution of BKV, and the genomic
4 variations observed in the VP1 and TCR. In three cases, sequence analysis of the TCR revealed
5 variability in the samples taken from different organs: in one case (#5), a G→A substitution of
6 BKV WW was found in the liver but not in the heart, and two different BKV archetypes (WWT and
7 128-I) were detected in cases #7 and #10. Sequence analysis of VP1 revealed two different
8 genotypes (I and IV) and three subtypes in case #7.
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11 One placenta sample showed the GS subtype, with a single bp substitution (G→C nt 1807);
12 interestingly, the corresponding fetus (#2) had a different subtype (MM).
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15 Immunohistochemistry analysis using the anti-SV40 antibody was negative in all cases.
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DISCUSSION

The transplacental transmission of PYVs after the intraperitoneal inoculation of pregnant hamsters by SV40 and pregnant mice by murine polyoma virus (MuPyV) has been clearly documented in animal models [Patel et al., 2009; Zhang et al., 2005], but human transplacental JCV and BKV transmission is less clear.

A previous study of 300 pregnant women in the third trimester of pregnancy did not reveal any evidence of the transplacental crossing of PYVs in the offspring [Boldorini et al., 2008], but a study of 15 aborted fetuses found viral genome in 12 placenta and brain samples, and nine kidney samples [Pietropaolo et al., 1998]. Since no other similar studies were performed in humans, the present study was designed to evaluate further the presence of BKV in foetal samples and search for viral genome in samples of organs other than brain and kidney, which have previously been considered the target organs of BKV latency.

The main finding was the amplification of BKV TCR and/or VP1 genomic sequences in samples obtained from seven out of 10 fetuses tested by nested PCR. The heart and lung were the organs in which viral genome was most frequently detected; kidney and brain were less frequently positive. In three cases, BKV was detected in samples taken from three or more organs. These findings clearly indicate that BKV can cross the placenta during pregnancy and become latent in organs and tissues other than kidney and brain.

BKV genome was detected in four of the five cases in which the pregnancy was interrupted as a consequence of chromosomal aberrations (two cases of Down syndromes, one of Beckwith-Widemann disease, and one case of multiple fetal malformations), and in three of the five cases of spontaneous abortion. Although the numbers are small, these findings indicate that BKV infection is not associated with an increased risk of spontaneous abortions, as is also suggested by the finding of BKV genome in placental tissue from six out of 12 women whose pregnancies had a normal outcome [Pietropaolo et al., 1998].

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The main limitations of the study are: 1) the lack of serological and virological data; 2) the placenta and adnexa were negative for BKV genome in all but one case; and 3) the BKV subtype detected in the only positive placenta sample (GS) was different from that detected in the corresponding organ samples (MM).

The first of these limitations is due to the fact that this was a retrospective study of archives samples, and the pregnant women were not pre-selected. Published data indicate that the seroprevalence of anti-BKV IgG in the adult population is about 80% [Shah et al., 2000], and one study of pregnant women found genomic sequences of PYVs in urine and blood samples taken from 6-26% of the cases [Boldorini et al., 2008]; it is therefore reasonable to assume that the figures relating to the women from whom the samples for this study were taken were similar.

The second point can be explained on the basis of the results of a previous retrospective study of autopsied kidneys [Chester et al., 1983], which detected BKV genome only in some of the specimens taken from the same renal tissue, thus suggesting that viral DNA is focally distributed. As the present study examined only two samples of each placenta, the presence of BKV genome in the rest of the tissue cannot be excluded.

Thirdly, sequence analysis of the TCR and VP1 regions of BKV revealed considerable differences in the samples taken from different organs of cases #5, #7 and #10, and between the placenta and fetal samples in case #2. Hypervariability of the TCR region is a typical feature of BKV and JCV and has been related to the ability of these viruses to adapt to different tissues and organs, whereas the detection of more than one BKV strain in samples from cases #7 and #2 may reflect multiple infection by different BKV strains, as found in a study of renal biopsy samples taken from renal transplant patients [Randhawa et al., 1999]. However, as the sequence analysis was made using formalin-fixed samples taken from fetal autopsies, it is also possible that the changes in nucleotide sequences may have been due to technical artefacts.

In the present study, the TCR and VP1 regions were both detectable in the same sample in only three cases; only one of the regions was amplified in most cases. This may have been due to viral

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3 genome fragmentation [Randhawa et al., 2002], a well-known consequence of the formalin fixation
4 of fetal tissues, which need to be kept longer in fixatives than adult tissues. This possibility is
5 supported by the fact that a 202 bp fragment of the human β -actin gene was amplified in all of the
6 samples using the AW75-AW76 primers, but not the larger 586 bp fragment using the B1-B2
7 primers.
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15 The results of the immunohistochemical analysis of all of the samples that were positive for BKV
16 by PCR were negative. This suggests that the infected cells may have had only a few copies of
17 BKV genome, and that these could not be detected by immunohistochemistry that is a relatively
18 low-sensitive technique able to identify viral proteins only when they are present in large amount as
19 during active viral replication.
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27 Finally, since the results obtained in this study showed that transplacental transmission of BKV can
28 occur in humans, an attempt was made to explain the lack of evidence of mother-to-foetal
29 transmission of BKV in our previous study. That study was performed in the latest trimester of
30 pregnancy, by seek the viral genome in blood and urine samples from mothers and umbilical cord
31 blood samples from newborns. Therefore, a transplacental passage of BKV in the first phases of
32 pregnancy and a possible latent infection of fetal organs could be missed; and the lack of serological
33 data precluded evaluation of the possible presence of specific anti-BKV antibodies in the newborns.
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For Peer Review

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