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ORIGINAL ARTICLE ***Early pregnancy***

Footprints of BK and JC polyomaviruses in specimens from females affected by spontaneous abortion

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STUDY QUESTION: Are JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) infections associated with spontaneous abortion (SA)?

SUMMARY ANSWER: There is no association of JCPyV or BKPyV with SA.

WHAT IS KNOWN ALREADY: A large number of risk factors have been associated with SA. The role of polyomaviruses, including JCPyV and BKPyV, in SA remains to be clarified.

STUDY DESIGN, SIZE, DURATION: This is a case–control study including women affected by spontaneous abortion (SA, $n = 100$, the cases) and women who underwent voluntary interruption of pregnancy (VI, $n = 100$, the controls).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Viral DNAs were investigated by qualitative PCR and quantitative droplet-digital PCR (ddPCR) in matched chorionic villi tissues and peripheral blood mononuclear cells (PBMCs) from SA ($n = 100$) and VI ($n = 100$). Indirect ELISAs with mimotopes/synthetic peptides corresponding to JCPyV and BKPyV viral capsid protein 1 epitopes were then employed to investigate specific IgG antibodies against JCPyV and BKPyV in human sera from SA ($n = 80$) and VI ($n = 80$) cohorts.

MAIN RESULTS AND THE ROLE OF CHANCE: JCPyV DNA was detected in 51% and 61% of SA and VI samples, respectively, with a mean viral DNA load of 7.92 copy/10^4 cells in SA and 5.91 copy/10^4 cells in VI ($P > 0.05$); BKPyV DNA was detected in 11% and 12% of SA and VI specimens, respectively, with a mean viral DNA load of 2.7 copy/10^4 cells in SA and 3.08 copy/10^4 cells in VI ($P > 0.05$). JCPyV was more prevalent than BKPyV in both SA and VI specimens ($P < 0.0001$). In PBMCs from the SA and VI cohorts, JCPyV DNA was detected with a prevalence of 8% and 12%, respectively, with a mean viral DNA load of 2.29 copy/10^4 cells in SA and 1.88 copy/10^4 cells in VI ($P > 0.05$). The overall prevalence of serum IgG antibodies against JCPyV detected by indirect ELISAs was 52.5% and 48.7% in SA and VI groups, respectively, whereas BKPyV-positive sera were found in 80% SA and 78.7% VI samples.

LIMITATIONS, REASONS FOR CAUTION: This study did not investigate the presence of viral mRNA and/or proteins, which are indicative of an active viral infection, and these might be taken into consideration in future studies.

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WIDER IMPLICATIONS OF THE FINDINGS: JCPyV and BKPyV DNA sequences were detected and quantitatively analyzed for the first time by PCR/ddPCR in chorionic villi tissues and PBMCs from SA and VI specimens. Moreover specific immunological approaches detected serum IgG against JCPyV/BKPyV. Statistical analyses, however, do not indicate an association between these polyomaviruses and SA.

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Key words: polyomavirus / BKPyV / JCPyV / pregnancy / miscarriage / PCR / droplet-digital PCR / viral DNA load / ELISA

Introduction

Spontaneous abortion (SA) is considered the most common adverse pregnancy outcome, with an estimated occurrence rate of about 15–20% of all pregnancies. This phenomenon affects ~70% of pregnancies during the first trimester (12 weeks), whereas 30% of late miscarriages occur during the 13th–22nd gestation weeks (Cardenas *et al.*, 2010; Giakoumelou *et al.*, 2016). A large number of risk factors, such as age, stress, obesity, ethnic origin and male factors as well as smoking and alcohol consumption, have been associated with SA (Coste *et al.*, 1991; De la Rochebrochard and Thonneau, 2002; Sopori, 2002; Lashen *et al.*, 2004; Maconochie *et al.*, 2007; Rotondo *et al.*, 2012). Furthermore, other factors including anatomical, psychological and genetic alterations, other than immunological and/or hormonal causes, are significant in abortive events (Rai and Regan, 2006; Cardenas *et al.*, 2010; Mor and Cardenas, 2010; Suzumori and Sugiura-Ogasawara, 2010).

The impact of pathogenic infections in pregnant woman is not completely elucidated (Donders *et al.*, 2000; Contini *et al.*, 2018). Many reports pointed out that several infectious agents might have a role during pregnancy-related problems, including SA. Indeed, nearly 15% and 66% of early and late SAs, respectively, are associated with potential pathogenic infections (Donders *et al.*, 2000; Srinivas *et al.*, 2006; Baud *et al.*, 2008).

An association of viral infections with increased risk of pregnancy loss has been published (Giakoumelou *et al.*, 2016). Different viruses, such as human cytomegalovirus, dengue virus, herpes simplex virus, adenovirus and adeno-associated virus are able to infect the placenta, the trophoblast and/or the cytotrophoblasts (MacCalman *et al.*, 1996; Burton and Watson, 1997; Fisher *et al.*, 2000; De Freitas *et al.*, 2018).

The potential involvement of JC polyomavirus (JCPyV) in SA is of interest. It has been reported that ~50% of healthy subjects carry serum IgG antibodies against JCPyV, indicating that this polyomavirus is fairly ubiquitous in humans (Bononi *et al.*, 2018). Nevertheless, after the primary and asymptomatic infection, this viral agent remains lifelong in the host in a latent state. The reactivation of JCPyV may occur in healthy adults and immune-compromised/suppressed patients. Notably, two previous investigations from our laboratory showed that DNA belonging to JCPyV is present in human sperm fluids (Rotondo *et al.*, 2017a) and was associated with male infertility (Comar *et al.*, 2012). Additionally, JCPyV DNA sequences have been detected in urine samples of pregnant women (Arthur and Shah, 1989; Flaegstad *et al.*, 1991). These data may suggest that the JCPyV could be sexually transmitted and potentially able to infect the uterus/embryo. Thus, JCPyV infection could be a potential risk factor for the pregnancy and embryo development.

Little is known about the potential role of BK polyomavirus (BKPyV) on adverse pregnancy outcomes. The few data available are discordant. BKPyV is common in kidney transplant recipients and, world-wide, the infection rate is in the range of 65–90% in the general population (Comar *et al.*, 2004; Pietrobon *et al.*, 2017). BKPyV infection is usually latent and asymptomatic in the general population with the exception of immune-compromised/suppressed patients (Pietrobon *et al.*, 2017). The presence of BKPyV DNA sequences was previously associated with a negative pregnancy outcome, suggesting a possible role of this human polyomavirus (HPyV) in SA (Pietropaolo *et al.*, 1998; Boldorini *et al.*, 2011).

The aim of this investigation was to verify the association between JCPyV/BKPyV infections and SA. To this end, qualitative PCR was used to analyze DNAs from aborted chorionic villi tissue samples and peripheral blood mononuclear cells (PBMCs) collected from females affected by SA (the cases) and females undergoing voluntary interruption of pregnancy (VI: the control group). Viral DNA sequences were then quantified using specific quantitative droplet digital PCR (ddPCR) in chorionic villi specimens and PBMCs from the SA and VI groups. In addition, the prevalence of serum IgG antibodies against JCPyV and BKPyV was investigated employing indirect ELISAs with specific synthetic peptides as mimotopes of the JCPyV and BKPyV viral capsid protein 1 (VP1).

Materials and Methods

Human subjects

In this study, two different cohorts of pregnant women were enrolled, i.e. females with SA ($n = 100$, the cases), and females undergoing VI ($n = 100$, the controls). In SA and VI groups, the exclusion criteria were: patients found to be positive for infectious agents/diseases, such as HIV, hepatitis B virus, hepatitis C virus, syphilis, during the year before the sample collection; the presence of congenital or acquired immune deficiency syndrome/diseases, or immune-suppressive therapies during the year before the sample collection; and well known causes of spontaneous abortions, such as genetics, severe uterine or hormonal dysregulation, and use of teratogenic drugs. The inclusion criteria were as follows: patient's age in the range 18–42 years; gestational age within the first 12 weeks; and the cohort of VI pregnancy was composed of females according to the Italian law, named Bill 194, Article 6, and Comma B. Clinical samples were collected by Dr. Roberta Capucci and Dr. Alice Poggi, Obstetrics and Gynecology Clinic, University Hospital of Ferrara (Contini *et al.*, 2018).

Ethical approval

Written informed consents were obtained from all women included in the study at the time of the hospital admission. The study was approved by the county ethics committee of Ferrara, Italy.

Samples

Chorionic villi tissue specimens from embryos (n = 200), the corresponding PBMCs (n = 200) and serum samples (n = 160) from females were from our sample collection, as published previously (Contini *et al.*, 2018). Blood and chorionic villi samples were collected within 12 h after the abortion. All chorionic villi samples were obtained by expert gynecologists using standard procedure. Medical termination of pregnancy within the 12th week was induced by vaginal prostaglandin administration. After spontaneous expulsion of the product of conception, chorionic villi were sampled using disposable Safetouch system collection bottles. They were suspended in saline and placed at +4° until sample processing, up to 12 h later Chorionic villi were manually selected and cleaned from the surrounding tissues with sterilized scissors and scalpels, in safety conditions under a biohazard hood. DNA extraction from samples was then carried out, within minutes. Viral DNA analyses were performed 3 weeks after the sample collection. Peripheral blood samples were collected soon after the abortion (within 12 h). In order to separate serum and PBMCs, blood samples were immediately centrifuged. Sera from 80 (out of 100) available samples from SA and VI females were analyzed for the presence of JCPyV antibodies. Serological assays were performed within 3 weeks after the sample collection.

DNA and sera isolation

DNA was purified from chorionic villi as previously reported (Kooper *et al.*, 2009; Contini *et al.*, 2018). Briefly, chorionic tissue (~25 mg each specimen) was incubated overnight with proteinase K at 56°C to allow tissue digestion. Then, DNA was extracted using a QIAmp DNA Blood and Tissue Extraction Kit (Qiagen, Milan, Italy), as reported (Rotondo *et al.*, 2016, 2018a). PBMCs and sera were isolated by density gradient using Histopaque-1077 (Sigma-Aldrich, Milan, Italy). Sera were stored at −80°C until the time of the analysis. DNA from PBMCs was isolated and analyzed by PCR as reported (Rotondo *et al.*, 2017b; 2018b). DNA was stored at −80°C until the time of the analysis.

Viral DNA detection

In the first phase of our analysis, the presence of viral DNA in SA and VI chorionic villi and PBMCs was assessed by two different qualitative techniques. Specifically, DNA sequences belonging to JCPyV were investigated by PCR, whereas BKPyV DNA was investigated by nested-PCR (Rotondo *et al.*, 2017a). A positive control, consisting of the recombinant plasmid vector pMITCR1A containing the complete JCPyV genome (NC_001699.1) was employed. BKPyV DNA was investigated by nested-PCR using two different primer sets targeting the Tag region (De Mattei *et al.*, 1994; 1995), (Table I). The recombinant plasmid vector pBR322_BKPyV containing the complete BKPyV genome (AB301099.1) was employed. Qualitative techniques were carried out with 500 ng human genomic DNA (Barrandon and Green, 1987; Martini *et al.*, 2004; Rotondo *et al.*, 2015). Multiple negative controls, represented by two DNA extraction controls containing salmon sperm DNA and distilled water, and an additional technical PCR negative control (sterile water), were included in each PCR reaction. Samples were analyzed three times, without different results. PCR amplicons were analyzed by electrophoresis on a 2% agarose gel.

Viral DNA load quantification

JCPyV and BKPyV DNA loads in SA and VI chorionic villi and PBMC samples were analyzed using a specific ddPCR assay. This quantitative method of viral DNA detection was performed using the QX200™ Droplet Digital™ PCR-System (Bio-Rad, Segrate, Milan, Italy) to determine the copy number of viral DNA (Mazzoni *et al.*, 2017a). In our experiments, this ddPCR method does not need an internal-positive control because it detects, in a specific manner, viral DNA sequences. In our experience, ddPCR allows us to reveal down to 1 viral DNA molecule, as an absolute detection per reaction (Mazzoni *et al.*, 2017a), whereas with the traditional quantitative PCR the limit of detection is in the range of 5–10 viral DNA molecules (Pancaldi *et al.*, 2011). The ddPCR reaction mixture consists of 11 µl of a 2× ddPCR super mix (Bio-Rad, Segrate, Milan, Italy), 1 µl of the target primers/probes set (900 nM primers/250 nM probe), and 10 µl of DNA (~100 ng per reaction). The cellular eukaryotic translation initiation factor 2C1 gene, located at chromosome 1p34.3, was used to determine the human cell equivalents of each sample. The viral DNA load values were reported as viral copies per 10^4 human cell equivalents (copy/10^4 cells). Samples and controls were analyzed in triplicate and by three different operators in three experiments. Negative controls (DNA extraction controls) and an additional control containing distilled water were included in each ddPCR reaction.

Indirect ELISA

An indirect ELISA was developed and standardized to detect specific antibodies against JCPyV and BKPyV VP1 in human sera using JCPyV VP1 K and VP1 N, and BKPyV VP1 L and VP1 M, mimotopes (Pietrobon *et al.*, 2017; Bononi *et al.*, 2018). The amino acid sequences of the two JCPyV VP1s are: VP1 K: NH-KSISISDTFESDSPNRD-COOH; VP1 N: NH2-LMNVHSNGQATHDNGAGK-COOH. The amino acid sequences of the two BKPyV VP1s are: VP1 L: NH2-LKLSAENDFSSDSPERK–COOH; VP1 M: NH2–MLNLHAGSQKVHEHGGGK–COOH (Tognon *et al.*, 2015; Mazzoni *et al.*, 2014a; 2014b; 2016; 2017b). The human peptide hNPS, amino acid sequence NH2-SFRNGVGTGMKKTSFQRAKS-COOH, was employed as a negative control (Guerrini *et al.*, 2010). Peptides, synthesized by standard procedures, were purchased from UFPeptides s.r.l. (Ferrara, Italy).

The different steps of the indirect ELISA tests were carried out as published previously (Pietrobon *et al.*, 2017; Bononi *et al.*, 2018). Briefly, plates were coated with 5 µg of the selected peptide for each well and diluted with Coating Buffer (Candor Bioscience, Germany) at 4°C for 16 h. Peptide blocking was performed with 200 µl/well of the Blocking Solution (Candor Bioscience, Germany) at 37°C for 90 min. Each sample was analyzed three times in duplicate wells. Color intensity in wells was determined by optical density (OD) where the immune-complexes formed. Cut-off values were determined for each assay using an OD reading of the three negative control sera that were added three times to the standard deviation (+3 SD) (Pietrobon *et al.*, 2017; Bononi *et al.*, 2018).

Statistical analysis

The prevalence of viral DNAs in SA and VI chorionic tissues and PBMCs was statistically analyzed using the chi-square trend test. The viral DNA load and ELISA OD values were analyzed with D'Agostino-Pearson test for normality and means were compared using the non-parametric Mann–Whitney-*U* test. Fisher's exact test was used to compare the prevalence of antibodies anti-JCPyV and BKPyV in different groups. Statistical analyses were carried out using Graph PadPrism version 5.0 for Windows (GraphPad, La Jolla, CA, USA). *P*-values <0.05 were considered statistically significant.

Results

Qualitative PCR of the viral DNA

DNAs from chorionic villi and PBMC specimens of VI and SA affected females (Table II) were investigated by PCR techniques for the presence of JCPyV and BKPyV DNAs. JCPyV DNA was detected by

Table I Primer sets used in PCR techniques to investigate and quantify JC and BK polyomavirus DNA sequences in human chorionic villi tissue specimens and peripheral blood mononuclear cells.

Virus	Primes names	Primers sequence (5′→3′)	Amplicon size (bp)	Annealing temp. (°C)	Reference
PCR					
JCPyV	JC_VP1_Seq_F	ACAGTGTGGCCAGAATTCCACTAC	214	63	Rotondo *et al.* (2017a)
	JC_VP1_Seq_R	TAAAGCCTCCCCCCCAACAGAAA			
Nested-PCR					
BKPyV	PCR1-BB4	TTTTGGAACCTGGAGTAGCTCAGA	578	58	*This paper*
	PCR1- BB5	GCTTGACTAAGAAACTGGTGTAGATCAG			
	Nested-BB2	TAGGTGCCAACCTATGGAACAGA	189	58	De Mattei *et al.* (1994); (1995)
	Nested-BB3	GGAAAGTCTTTAGGGTCTTCTACC			
ddPCR					
JCPyV	JCV_AT_F	AGTGTTGGGATCCTGTGTTTTCA	71	60	*This paper*
	JCV_AT_R	GTGGGATGAAGACCTGTTTTGC			
	JCV_AT Probe	FAM-CATCACTGGCAAACAT-NFQ			
BKPyV	BKV_AT_F	AGTGTTGAGAATCTGCTGTTGCT	96	60	*This paper*
	BKV_AT_R	GGAGTTCCTTTAATGAAAAATGGGATGAAG			
	BKV_AT Probe	FAM- CATCACTGGCAAACAT-NFQ			

JCPyV, JC polyomavirus; BKPyV, BK polyomavirus; ddPCR, droplet-digital PCR.

Table II Characteristics of the patients affected by spontaneous abortion and voluntary interruption of the pregnancy.

	Number of patients	Age of the patients (years, mean ± SD)	Number of pregnancies (mean ± SD)	Weeks of gestation (mean ± SD)
SA	100	36 ± 5	2.4 ± 1.6	8.2 ± 1.3
VI	100	32 ± 6	2.5 ± 1.2	10.2 ± 1.5

SA, spontaneous abortion; VI, voluntary interruption.

qualitative PCR in 51% (51/100) of SA chorionic tissue specimens and 61% (61/100) of VI samples (Table III). Nested-PCR investigation showed that BKPyV DNA was present in 11% (11/100) and 12% (12/100) of SA and VI chorionic tissue specimens, respectively (Table III). The difference between SA and VI groups was not statistically significant for both viruses ($P > 0.05$). However, JCPyV DNA sequences were the most prevalent compared to those of BKPyV in both SA and VI groups ($P < 0.0001$). Furthermore, viral co-infections were observed in both SA and VI samples. Specifically, 10% (10/100) of both SA and VI chorionic tissue specimens were simultaneously JCPyV and BKPyV DNA-positive.

In PBMCs, JCPyV DNA was revealed in 8% (8/100) of samples from SA group, whereas it was detected in 12% (12/100) of samples from VI group (Table III). The difference between SA and VI groups was not statistically significant ($P > 0.05$). DNA sequences belonging BKPyV were not detected in PBMCs from either the SA or VI group.

All PBMCs from females tested BKPyV-negative, thus excluding contamination from the mothers' PBMCs to chorionic villi. Similarly, the great majority of JCPyV-positive chorionic villi, 51% from SA and 61%

Table III Prevalence of JCPyV and BKPyV DNA sequences in chorionic villi and peripheral blood mononuclear cell samples from females affected by SA and VI of pregnancy.

Tissue sample	Number of positive samples/ samples analyzed (%)	
	JCPyV	BKPyV
CV from SA	51/100 (51)[a]	11/100 (11)
CV from VI	61/100 (61)[a]	12/100 (12)
	$P = 0.1543$	$P = 0.8246$
PBMCs from SA	8/100 (8)	0/100 (0)
PBMCs from VI	12/100 (12)	0/100 (0)
	$P = 0.4804$	$P = 1.000$

[a]JCPyV DNA sequences were the most prevalent compared to those of BKPyV in both SA and VI groups ($P < 0.0001$).

from VI, do not correspond to JCPyV-positive PBMCs of the paired mothers, being both JCPyV-positive only in two and three samples in the SA and VI groups, respectively.

Viral DNA load quantification

The viral DNA load from chorionic tissues and PBMCs was analyzed by the ddPCR method (Table II; Fig. 1). JCPyV and BKPyV DNAs, detected by qualitative PCR assays in chorionic tissues, were confirmed by ddPCR. The mean JCPyV DNA load detected in chorionic tissues was 7.92 copy/10^4 cells (range 0.57–154.64 copy/10^4 cells) in SA samples ($n = 51$) and 5.91 copy/10^4 cells (range 0.63–31.87 copy/10^4 cells) in VI samples ($n = 61$, Fig. 1). The difference of the JCPyV DNA load between the two groups, SA versus VI, is not statistically significant ($P > 0.05$). The mean BKPyV DNA load detected in chorionic tissues was 2.7 copy/10^4 cells (range 0.83–5.11 copy/10^4 cells) in SA samples ($n = 11$) and 3.08 copy/10^4 cells (range 0.69–10.44 copy/10^4 cells) in VI samples ($n = 12$, Fig. 1). The difference of the BKPyV DNA load between the two groups, SA versus VI, is not statistically significant ($P > 0.05$).

JCPyV DNA sequences, detected by qualitative PCR in PBMCs, were confirmed by ddPCR (Fig. 1). The mean JCPyV DNA load observed in PBMCs was 2.29 copy/10^4 cells (range 0.46–10.57 copy/10^4 cells) in SA group ($n = 8$) and 1.88 copy/10^4 cells (range 0.66–5 copy/10^4 cells) in VI group ($n = 12$). The difference in JCPyV DNA loads between groups was not statistically significant ($P > 0.05$). DNA sequences belonging to BKPyV were not detected by ddPCR in both SA and VI groups, confirming the nested-PCR data.

Detection of JCPyV and BKPyV antibodies by indirect ELISA

Human sera, from SA ($n = 80$) and VI ($n = 80$) groups were analyzed for IgG antibodies reacting to JCPyV and BKPyV VP1 epitopes.

The prevalence of JCPyV antibodies was analyzed in SA and VI sera. In serum samples from SA and VI groups the overall prevalence of IgG antibodies against JCPyV, combining the data of the positive sera both for the VP1 K and VP1 N mimotopes, was 52.5% (42/80) and 48.7% (39/80), respectively (Table IV): this difference between groups was not statistically significant ($P > 0.05$). In this investigation, sera were considered JCPyV VP1-positive when reacting with both mimotopes K and N.

The prevalence of BKPyV antibodies was analyzed in sera from SA and VI cohorts. IgG antibodies against BKPyV VP1 mimotopes (L + M) were detected in 80% (64/80) of SA and 78.7% (63/80) of VI samples (Table V): this difference between groups was not statistically significant ($P > 0.05$). Sera were considered BKPyV VP1-positive when reacting with both mimotopes L and M.

Discussion

SA is one of the most important challenges of pregnancy. The different risk factors which account for SA are not completely understood. Pathological agents, including viruses and their related infections, may have a role in SA and preterm delivery (Giakoumelou *et al.*, 2016). This study aimed to verify the association between JCPyV/BKPyV infections and SA.

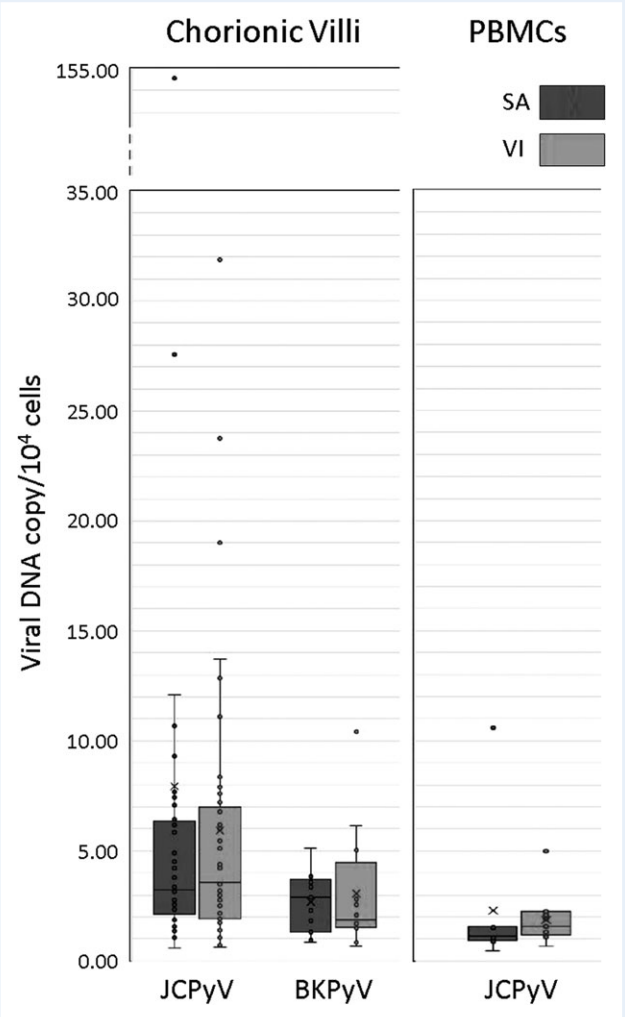

Figure 1 Viral DNA load distribution in chorionic villi specimens and peripheral blood mononuclear cells. Mean viral DNA load (*y*-axis: viral DNA copy/10^4 cells) of: JC polyomavirus (JCPyV) in spontaneous abortion (SA) ($n = 51$) and voluntary interruption of pregnancy (VI) ($n = 61$) chorionic villi specimens, and in SA ($n = 8$) and VI ($n = 12$) peripheral blood mononuclear cells (PBMCs); BK polyomavirus (BKPyV) in SA ($n = 11$) and VI ($n = 12$) chorionic villi specimens. Boxes represent the 25th and 75th percentiles, lines within the boxes represent the median, the X symbol represents the mean viral DNA load, whereas the error bars represent the SD. The difference in viral load between SA and VI groups was not statistically significant ($P > 0.05$). Dark circles represent outlier values.

In this study, for the first time, JCPyV DNA sequences were investigated and quantified in chorionic villi from women affected by SA (cases) and females undergoing VI (controls). JCPyV DNA was detected in chorionic villi of both SA and VI groups, and with a similar prevalence (51% versus 61%, respectively). Quantitative data, obtained for the first time by ddPCR, show a low viral DNA load in both cases and controls. Our results may suggest that JCPyV is not associated with SA. Analysis of PBMCs confirmed our data, showing a low prevalence of JCPyV-positive samples, with few viral DNA copies. These qualitative/quantitative results indicate the absence of a systemic and active infection of this HPyV in women with SA and undergoing VI. To the best of our

Table IV Prevalence of IgG antibodies reacting to JCPyV VP1 mimotopes detected in sera from SA and VI of pregnancy groups.

Patient	Number of Sera	Median age (years) ± SD	Number of positive samples (%)		
			VP1 K	VP1 N	VP1 (K + N)
SA	80	35 ± 4	49 (61.2)[a]	41 (51.2)[a]	42 (52.5)[a]
VI	80	31 ± 5	39 (48.7)	40 (50.0)	39 (48.7)

In SA and VI sera, the prevalence of IgG antibodies against JCPyV VP1 K and VP1 N was as reported above. With the exception of a small number of samples, sera reacted with the VP1 K and N peptides with a similar prevalence. Accordingly, seronegative samples for the JCPyV VP1 K peptide failed to react with JCPyV VP1 N epitopes. Sera were considered JCPyV VP1-positive when reacting with both mimotopes K and N (VP1 K + N).
The prevalence of anti-JCPyV antibodies in SA and VI was statistically analyzed using the chi-square trend test.
[a]SA versus VI, $P > 0.05$.

Table V Prevalence of IgG antibodies reacting to BKPyV VP1 mimotopes detected in sera from SA and VI of pregnancy groups.

Patient	Number of Sera	Median age (years) ± SD	Number of positive samples (%)		
			VP1 L	VP1 M	VP1 (L + M)
SA	80	35 ± 4	70 (87.5)[a]	65 (81.2)[a]	64 (80.0)[a]
VI	80	31 ± 5	61 (76.5)	69 (86.2)	63 (78.7)

IgG antibodies against BKPyV VP1 L and M epitopes were detected in SA and VI groups with the prevalence indicated above. Sera reacted with the BKPyV VP1 L and M peptides with a similar prevalence and the majority of seronegative samples for the BKPyV VP1 L peptide failed to react with BKPyV VP1 M epitopes. Sera were considered BKPyV VP1-positive when reacting with both mimotopes L and M (VP1 L + M).
The prevalence of anti-BKPyV antibodies in SA and VI was statistically analyzed using the chi-square trend test.
[a]SA versus VI, $P > 0.05$.

knowledge, only one study investigated the association of JCPyV with miscarriage. Specifically, the presence of JCPyV DNA was studied in a small sample size ($n = 15$) of placentae derived from SA, however none of the samples tested positive for this polyomavirus (Pietropaolo *et al.*, 1998). This discrepancy could be due to the different age of tissues analyzed, i.e. chorionic villi versus placentae.

In our investigation, SA and VI serum samples were analyzed to detect anti-JCPyV IgG antibodies by indirect ELISAs with specific JCPyV mimotopes from VP1 antigens. The immunological data confirm our molecular results, indicating that both groups were infected with JCPyV and show a high prevalence (52.5–48%) of antibodies against this HPyV.

BKPyV DNA analyzed in chorionic villi from women affected by SA and control women, showed a similar low prevalence (nearly 12%) and viral DNA load in the two groups. Notably, this is the first investigation in which BKPyV DNA has been quantified in chorionic villi by ddPCR assay. Our results suggest that BKPyV may latently infect the female genital tract, without impacting embryo formation. Accordingly, the lack of active BKPyV infection accountable for miscarriage is also demonstrated by the fact that none of the PBMCs from both groups tested positive for this viral DNA. DNA sequences belonging to BKPyV have been previously detected with relatively high prevalence in a small group of in placentae ($n = 15$) from aborted tissues, suggesting that this virus may be transmitted vertically by crossing the placenta and hypothetically infect the fetus (Pietropaolo *et al.*, 1998). Similar data were obtained more recently in another study conducted on aborted fetuses ($n = 10$) derived from both miscarriages and voluntary interruptions (Boldorini *et al.*, 2010).

In the present study, indirect ELISAs using specific BKPyV mimotopes from VP1 antigens were employed for the detection of anti-BKPyV IgG antibodies in human sera from SA and VI groups. Our immunologic data indicate that the majority of SA (80%) and VI (78.7%) sera carry IgG antibodies against BKPyV. The high prevalence of anti-BKPyV IgG antibodies detected in our case and control groups may indicate that this virus is commonly present in humans. Statistical analyses of molecular and immunological data suggest that the BKPyV infection is not associated with SA.

In conclusion, our investigation addressed the role of JCPyV and BKPyV as pathogenic SA risk factors. JCPyV was more prevalent than BKPyV in analyzed specimens. BKPyV genomic sequences were sporadically identified with only a few DNA copies in chorionic villi from both SA and VI groups. The low viral DNA load, observed for the first time by ddPCR, may suggest that both JCPyV and BKPyV are present in chorionic villi in a latent phase. The analysis conducted on PBMCs showed JCPyV-positive samples. Furthermore, our immunological data confirmed that JCPyV and BKPyV antibodies can be detected in a specific manner in pregnant women, with prevalence similar to non-pregnant females. Statistical analyses of our data do not support an association between JCPyV/BKPyV and SA.

Authors' roles

Study concept, design and supervision: F.V. C.C., F.M. and M.T. Sample analysis, interpretation of data and experiments execution: A.T., J.C.R., F.M., M.M., E.M., I.B. Statistical analysis: J.C.R., E.M., A.T., I.B. Drafting

of the manuscript: J.C.R., A.T. Critical revision/discussion of the manuscript for important intellectual content: F.V., C.C., F.M. and M.T. Administrative, technical, or material support: A.T., J.C.R., I.B., M.M.

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Conflict of interest

All authors have no potential conflicts of interest.

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