PUBBLICAZIONE 3

Nello studio precedente non si sono avute evidenze di trasmissione materno-fetale dei PVs umani presi in esame (BKV e JCV) durante la fase terminale di gravidanza, inoltre l'infezione/riattivazione di tali virus sembra essere rara, almeno nel periodo gestazionale investigato.

Lo scopo del presente lavoro è verificare ed approfondire la frequenza di infezione/riattivazione dei PVs umani BKV e JCV e l'eventuale trasmissione verticale dell'infezione stessa estendendo l'analisi al primo, secondo e terzo trimestre di gravidanza e al primo mese di vita dei rispettivi neonati. Lo studio è stato condotto su una popolazione di 19 donne con gravidanza fisiologica e patologica arruolate presso la I Divisione di Ostetricia e Ginecologia dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità"di Novara, e sui rispettivi neonati seguiti dal reparto di Pediatria dell'Azienda Ospedaliero-Universitaria "Maggiore della Carità"di Novara. Durante questo studio sono stati raccolti e analizzati mediante metodiche molecolari sia qualitative che quantitative (nested-PCR e quantitative-PCR) per la ricerca di genoma virale: A) 57 campioni di urina e 57 campioni di siero provenienti dalle donne in gravidanza (19 campioni di urina e siero per ogni trimestre analizzato), B) 19 campioni di siero fetale provenienti dal cordone ombelicale prelevati al momento del parto, e C) 38 campioni di siero, 38 campioni di secrezioni delle prime vie aeree e 26 campioni di urina appartenti ai neonati, raccolti durante le visite ambulatoriali di routine ad una settimana e aun mese di vita. Inoltre, in collaborazione con il Laboratorio di Microbiologia del Dipartimento di Pediatria della "Johns Hopkins" University School of Medicine di Baltimora, sono stati condotti studi immunologici su tutti i campioni di siero, per la determinazione di immunoglobuline dirette contro specifici antigeni virali mediante metodiche di diagnostica immunologica (VLP-ELISA).

I risultati di tale studio ci consentono di affermare che: 1) la trasmissione verticale dell'infezione da BKV e JCV non rappresenta una via prioritaria nella popolazione umana; 2) nelle donne in gravidanza sussiste una elevata frequenza di IgG anti-JCV e/o -BKV, a dimostrazione di una elevate diffusione della infezione da questi virus nella popolazione generale; 3) la riattivazione della infezione -evidenziata dalla identificazione di IgA e IgG specifiche anti- JCV e -BKV- è un evento relativamente frequente, ma che sembra essere limitato al solo compartimento uro-genitale, senza coinvolgimento sistemico; 4) l'infezione primaria non si manifesta frequentemente, mai nella nostra casistica e solo occasionalmente in altre casisitiche più numerose; 5) la trasmissione degli anticorpi madre-figlio avviene in modo passivo, ciò è confermato dai livelli decrescenti dei titoli anticorpali ad un mese di vita del neonato.

VERTICAL TRANSMISSION SEEMS NOT A FREQUENT MODE OF SPREAD FOR JC AND BK POLYOMAVIRUSES IN HUMANS.

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INTRODUCTION

The human polyomaviruses (PVs) BK Virus (BKV) and JC Virus (JCV), are widely diffuse in the worldwide[1-4]. Since their discovery in 1971[5,6] the pathogenic role of BKV and JCV in immune deficiency conditions has been well documented: JCV causes progressive multifocal leucoencephalopathy in AIDS patients, whereas BKV is the etiologic agent of the so called polyomavirus-associated nephropathy, that occurs in renal transplanted patients[7-10]. The primary infection by JCV and BKV usually occurs during childhood with only slight respiratory symptoms[11] thereafter, they persist latently in several organs, but mainly in urogenital system[12], brain[13] and also in circulating leucocytes[14]. Reactivation of both viruses is common and it is frequently associated with asymptomatic viruria[15-17]. While the natural history of infection is well established, the way of transmission of both BKV and JCV is still not known with certainty. Several hypothesis have been proposed, such as respiratory[18,19], oral-fecal[20], or urinary route of transmission[2,12,21]. Moreover, also on the base of the high frequency of PV infection in childhood, a few authors have investigated the possibility of a vertical way of transmission[16,23-28], already demonstrated for other animal homologue polyomaviruses, as the murine (MuPV)[29] and Simian polyomavirus (SV-40)[30], but with conflicting results.

In a previous paper[31], we did not find any evidence of trans-placental transmission of JCV and BKV in a population of 300 unselected pregnant women and their offspring. However, the study was only partially conclusive due to the evaluation was performed during the 3rd trimester of pregnancy only, by the search for viral genome in maternal urine, blood and in umbilical cord blood by qualitative molecular methods. Moreover, in that paper, serologic investigation of both PVs in mothers and newborns was not performed.

To better evaluate the possibility of vertical transmission (including trans-placental and/or maternal-fetal ways) of JCV and BKV, the present study was extended over the 1st, 2nd and 3rd trimester of pregnancy in 19 unselected pregnant women. Moreover, their newborns were evaluated during the first month of life. All the samples were investigated by means of qualitative and quantitative molecular techniques in order to search for BKV and JCV genome. In addition serological data were obtained by means of VLP-ELISA test.

MATERIAL AND METHODS

Case studies

The study was performed on pregnant women and their respective newborns followed at the Obstetrics and Gynecology Unit of Azienda Ospedaliero-univeristaria Maggiore della Carità (Novara, Italy) between May 2008 and Agust 2009.

The criteria of selection included: 1) Peripheral blood (PB) and urine samples taken at routine follow-up in each one gestational trimester for the mothers and PB samples at one week and one month of life for the babies; 2) Umbilical cord blood samples taken immediately after deliveries, and 3) Nasopharyngeal secretion samples taken at one week and one month of life for the babies. Moreover, when possible, urine samples were additionally taken from the babies at one week and one month of life.

Using these strict criteria, 19 cases were included in the study.

Informed consent was obtained from each patient and the study design was approved by the medical ethics committee of the Azienda Ospedaliero-universitaria Maggiore della Carità.

A total of 57 urine and PB samples from mothers, 19 umbilical cord blood samples, 38 pediatric PB samples, 38 nasopharyngeal secretion samples and 26 pediatric urine samples were collected.

Urine samples were concentrated by means of centrifugation at 700 g for 10 minutes. Maternal urine samples were divided in two aliquots: one was submitted to cytological analysis, the other was stored at -20°C for molecular analysis. Conversely, all the urine samples from newborns, were used for molecular analysis due to scarcity of material.

All blood samples (PB and UCB) were centrifuged at 700 g/min for 10 min, after which the serum was separated and stored in 1.5 mL tubes at -20°C. The sera subdivided in two aliquots and used both for molecular and serological analysis.

Nasopharyngeal secretions were collected from all the newborns by means of Dacron fiber tip swab. and immediately resuspended in 400µL of PBS and submitted to DNA extraction for molecular analysis.

Cytological analysis

Five mL of the urine pellets, obtained from the first centrifugation, were then cytocentrifuged at 250 g/min for 10 min onto two slides, which were both fixed in 95% ethanol, stained with Papanicolau stain, and cytologically examined in order to identify the presence of decoy cells (DCs) indicating active PV replication[32].

DNA extraction and multiplex nested- PCR for LT amplification

DNA was extracted from urine, serum and nasopharyngeal samples using commercial columns (Nucleospin virus, Macherey-Nagel, Germany) with a silica matrix and high DNA binding capacity.

In order to amplify the LT regions, a multiplex nested-PCR (n-PCR) was performed directly on the urine samples (as proposed by Agostini et al.33) and on the DNA extracted from all serum and oro-pharyngeal samples using PM1+ and PM1- as outer primers, and PM2- (common to all PVs), JC+ (an amplified fragment of 189 bp), BK+ (an amplified fragment of 353 bp) and SV40+ (an amplified fragment of 135 bp) as inner primers, all simultaneously mixed in a single reaction, in order to distinguish the different members of the Polyomavirus genus (**Table 1**). The samples were amplified in a total volume of 25 µL containing 1.25U GoTaq® Flexl DNA polymerase (Promega, Madison, WI, USA) in the presence of 1x Green GoTaq® Flexl buffer (Promega, Madison, WI, USA), 4 mM $MgCl_2$ (2 mM for the inner PCR), 0,2 pmol/µL of each primer (Roche Diagnostics, Milan, Italy), 0.2 mM dNTPs (Promega, Madison, WI, USA), and 10 µL serum and salivary DNA, 2.5 µL urine, and 1 µL of template in the inner PCR reactions using a Eppendorf Mastercycler gradient PCR System. The procedure involved denaturation at 95°C for 5 min, followed by 40 cycles (35 cycles for the inner PCR) of denaturation at 95°C for 40 s, annealing at 61°C (55°C for the inner PCR) for 40 s, and extension at 72°C for 40 s. The cycles were terminated with a final extension at 72°C for 5 min. DEPC-treated DNase/RNase-free water (Bioline, London, UK) was used as the negative control; the positive controls were DNA extracted from PML brain tissue (for JCV), from renal tissue from a subject with histologically proven BKV nephropathy (for BKV) and from an SVG cell line culture (for SV40). The sensitivity of the multiplex nPCR was estimated by amplifying serial dilutions of a JCV-positive sample (data not shown).

Quantitative Real Time-PCR for BKV and JCV

The viral load of samples that tested positive for the PVs genome amplification by means of multiplex n-PCR, was determined by quantitative real-time PCR assay (q-PCR). Specific separated reactions targeting a conserved region of LT gene were settled for BKV and JCV (Primers and probes sequences used were shown in **Table 2**). Both reactions were performed in a final volume of 25 µL containing a 1x Taqman Universal PCR Master Mix (Applied Biosystem, Foster City, CA, USA), 900 nM of each primer, 200 nM probe and 5 µL of extracted nucleic acid. Thermal cycling was carried out on a 7500 Fast Real Time-PCR System (Applied Biosystem, Foster City, CA, USA) according to the following steps: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, at the end of which fluorescence was read.

Each sample was analyzed in triplicate, and each run contained a negative control (DEPC-treated DNase/RNase-free water (Bioline, London, UK)). Standard curves for quantification of BKV and JCV were constructed using serial dilutions of plasmids containing the whole BKV genome (1,5x105 copie/µL (Tebu-bio, Columbia, MD, USA)) and JCV genome (1,1x105 copie/µL (Tebu-bio, Columbia, MD, USA). The detection limit for this assay was determined to be 5 copies/reaction. Data were expressed as logarithm 10 copies of viral DNA per microliter of sample.

Serological analysis

BKV-specific antibodies will be measured in serum samples using a virus like particle (VLP)-based ELISA assay. Briefly, 96-well Maxisorb microtiter plates (Nunc) are coated with purified VLP protein (20 ng /well) followed by treatment with a polyvinyl alcohol containing blocking solution. Serum samples (diluted 1:100 for detection of IgA and IgM and 1:200 for detection of IgG), are allowed to react on the antigen coated plates, and antigen bound immunoglobulin is detected with peroxidase conjugated antibodies to human IgG, IgA, or IgM (Southern BioTech, Birmingham, AL).

The IgA and IgM measurements are performed after an initial blocking step with 15% goat anti-human IgG (Sigma-Aldrich, St. Louis, MO). Color development is initiated by addition of substrate, and optical density (OD) is measured in an automated microtiter plate reader. Positive and negative control sera, sensitivity controls, and reproducibility controls are included in each run. Runs where replicate serum values fall outside the expected coefficient of variation are repeated. Results are recorded both as an OD value, and as a categorical variable (seropositive or seronegative) based on cut off points determined from the distribution of reactivity of samples from young children after excluding outliers.

Statistical analysis

Statistical analysis was performed using XLSTAT 09 software program. Results were presented as geometrical mean±standard deviation, or as median with range, as appropriate. Fisher exact test was run to determine statistical significance as appropriate and statistical results with a P value less than 0,05 were considered significant (95% confidence interval).

RESULTS

Clinical Data

The clinical data of the 19 women included in the study are shown in **Table 3**.

Mean age was 28,5±1,146 years (median: 29 years; range: 20 – 36 years) at the first control (1st trimester of pregnancy). All but two pregnancy were physiologic; the case 8 developed a steatohepatitis during the pregnancy whereas the case 17 received renal transplant and took immunosuppressive drugs during all the pregnancy .

Gestational age at deliveries ranged from 37th to 41st weeks (median: 40th weeks); 13 deliveries (68,4%) were eutocics, whereas six distocics (31,58%), the latter were resolved by cesarean deliveries (five cases) and vacuum extraction (one case).

The babies were nine females and ten males, the average weight at birth was 3396±1,153 gr (median: 3370 gr; range: 2780-4330 gr); all but one had natural feeding.

All the babies were well, four of them (cases 3, 7, 9 and 13) complained jaundice that quickly resolved spontaneously.

Cytological analysis

No decoy cells were identified by cytological examination of the 57 urine samples from pregnant women, despite a careful search. Cytologic analysis of the urine samples from the babies was not performed due to the paucity of samples.

Molecular Analysis

Multiplex n-PCR

Genome of PVs was detected in 20 of 57 (35,1%) urine samples, from 8 pregnant women (42,1%). One of these had only one positive sample, two had two positive samples and in five cases all the samples were PV positive. Notably, in all the cases, the type of PV was stable in the samples taken at different time of pregnancy. On respect to the type of PV isolated, JCV genome was identified in four cases (21%), BKV in three (16%) , and the co-infection BKV- JCV in one case only (**Figure 1**).

The **Figure 2** shows the frequency of excretion and type of PV identified in urine samples from the eight PVs positive pregnant women**.** The overall percentage of urine samples that tested positive for PV genome did not change significantly over the three controls. However, the analysis of the single cases, disclosed a variability of genome detection during the three trimester of pregnancy. In detail, BKV positivity was identified in three cases (1, 2, 7): the case 1 over the three controls, in the case 2 during 2nd and 3rd control and in the case 7 in 1st control only. JCV positivity

was identified in four cases (3, 4, 9, 18): cases 3, 4 and 9 over the three controls, and case 18 during the 1st and the 3rd control. One case (13) was positive for both BKV and JCV in all the controls (**Table 4**).

Multiplex n-PCR performed on PB samples of pregnant women and on all the samples from newborns (UBC, PB, nasopharyngeal secretion and urine samples) was negative in every cases.

Viral Load

The median peaks and range of detection for JCV and BKV in urine of pregnant women were calculated during each trimester of pregnancy (**Figure 3**). The median peak of JCV was 3,5 $\log_{10}$ copies/μL (range: 1,5-4,8 $\log_{10}$ copies/μL), in the 1st trimester, significantly decreased in the 2nd (1,7 $\log_{10}$ copies/μL (range: 0-3,7 $\log_{10}$ copies/μL), and raised in the 3rd trimester (3,73 $\log_{10}$ copies/μL (range: 2,2-4,6 $\log_{10}$ copies/μL). Conversely, the median peak of BKV constantly increased during the pregnancy: it was 1,7 $\log_{10}$ copies/μL (range: 0-2,8 $\log_{10}$ copies/μL), in the 1st trimester, 2,3 $\log_{10}$ copies/μL (range: 0-3,8 $\log_{10}$ copies/μL), in the 2nd and 3,5 $\log_{10}$ copies/μL (range: 0-4,7 $\log_{10}$ copies/μL) in the 3rd trimester.

The **Figure 4** shows the pattern of BKV and JCV viral load in urine over the three trimester of pregnancy in each pregnant women.

The JCV viral load decreased in four cases in the 2nd trimester and tested negative in one case (**fig 4a**) but interestingly, it raised again in the last control in all the cases.

Conversely, the pattern of BKV viral load (**Figure 4B**) was more unpredictable. Viral load of cases 1 and 13 slightly increased over the 1st and 2nd control but more significantly in the 3rd trimester; the case 7 tested positive only in the 1st control, whereas the case 2, was negative in the 1st trimester, and positive in the 2nd and 3rd trimester.

Serological Analysis

Data from VLP-ELISA test in serum samples of pregnant women are summarized in **Figure 5**. The overall prevalence of anti-PV IgG antibodies was 89% (17/19); in detail, 9 cases (47%) had anti-BKV IgG, one case (5%) anti-JCV IgG and 7 cases (37%) had anti- BKV and JCV IgG,. For practical purpose, the latter were considered once BKV-specific IgG and once JCV-specific IgG.

The seroreactivity to JCV was stable over the time during the three controls in all the cases, but the OD values of anti-JCV IgG decreased during the three trimesters (**Figure 6A**); indeed, the median OD values for anti-JCV IgG were: 1,674 OD (range: 0,646-2,0665 OD) in the 1st trimester, 1,467 OD (range: 0,731-2,229 OD) in the 2nd , and 1,012 OD (range: 0,433 - 2,049 OD) in the 3rd

trimester. Of note, anti-JCV antibodies IgM and IgA were also detected in case 13 over the three controls, whereas they were found only in 2nd and 3rd trimester in the case 3.

The seroreactivity was also stable for BKV over the time, in all but two cases (case 8 and 4), where anti-BKV IgG were detected only in the 2nd and 3rd trimester respectively. The median OD values for anti-BKV IgG (**Figure 6B**) increased during the 2nd control from 0,8465 OD (range: 0-2,51OD) at the 1st control, to 1,33025 OD (range: 0-2,45 OD), and afterward decreased to1,0645 OD (range: 0,1815- 2,0545 OD) at the 3rd control. Interestingly, anti-BKV IgA were also detected in 5 cases over the three controls, one of these tested positive also for anti-BKV IgM; in another case anti-BKV IgM was detected in the 3rd control (case 4).

The seroreactivity to BKV and/or JCV obtained from serum samples of newborns was specular to that found in the mothers. The median OD values for anti-JCV and anti- BKV IgG decreased during the first month of life but without a total vanishing. Median OD levels were: a) anti-JCV IgG: 0,7765 OD (range: 0,4955- 2,017 OD) at delivery; 0,76075 OD (range: 0,1715-1,9525 OD) at one week of life and 0,482 OD (range: 0,2395-1,267OD) at one month; b) anti-BKV IgG: 1,6725 OD (range: 0,5515-2,255OD) at delivery; 1,7255 OD (range: 0,337-2,2295 OD) at one week of life and 1,04 OD (range: 0,1635- 1,919 OD) at one month.

The case 4 showed a particular serological condition. Seroreactivity to both anti -BKV IgM and IgG was found in the 3rd trimester of pregnancy, whereas the serum sample of the newborn started to be seroreactive from one week, and maintained positivity in one month control.

JCV and BKV viruria in seroreactive pregnant women

The frequency of viruria in the groups of JCV and BKV seroreactive pregnant women was different. As shown in the **figure 7**, 62.5% (5 cases of 8)of pregnant women JCV seroreactive showed viruria, in comparison with only 25% (4 cases of 12)of the BKV seroreactive group; however the difference did not reach a statistical significance (P=0,0994).

The **figure 8**, compares the median OD value of anti-JCV and BKV IgG in pregnant women with and without viruria. In the JCV pregnant women the median OD value was higher (1,656; range: 0,809-2,229) for the group with viruria than in the group without viruria (1,232; range: 0,433- 1,809); conversely the median anti-BKV OD values in pregnant women with viruria was lower (0,886; range: 0,485-1,967) as compared to the group without viruria (1,153; range: 0,1815-2,51).

Viral load of JCV and BKV in urine were compared with OD values of anti-JCV and BKV IgG in serum samples over the three controls during pregnancy. As shown in the **figure 9A** either JCV viral load in urine and OD values of anti-JCV IgG decreased between 1st and 2nd control,

whereas an increase of viral load and decrease of anti-JCV IgG was found between 2nd and 3rd trimester of pregnancy. The pattern of BKV viral load in urine and OD values of anti-BKV IgG had a similar trend; the **figure 9B** shows a constant increase of both values over the three controls.

TABLES

TABLE 1: Multiplex n-PCR primers

REGION	NAME	POSITION	SEQUENCE
OUTERN	PM1+	*4022-4045	5’-TCT TCT GGR YTA AAR TCA TGC TCC-3’
	PM1-	*4550-4572	5’-TTW TAG RTK CCA ACC TAT GGA AC-3’
INNER	PM2-	*4388-4411	5’-GGT AGA AGA CCC YAA RGA CTT TCC-3’
	JC+	°4086-4108	5’-ATA TTA TGA CCC CCA AAA CCA TG-3’
	SV+	**4291-4314	5’-ATA ATT TTC TTG TAT AGC AGT GCA-3’
	BK+	*4059-4085	5’-GAA TGC TTT CTT CTA TAG TAT GGT ATG-3’

Wobble position IUB code: Y=C/T; R=A/G; W=A/T; K=G/T;
* BKV Dunlop strain GenBank Acession Number: V01108;
°JCV Complete genome GenBank Acession Number: J02226;
** SV40 complete genome GenBank Acession Number: J02400.

TABLE 2: Q-PCR primers and probes

PV	NAME	POSITION	SEQUENCE
BKV	BKV for	*4986-5012	5’-ATT CAT TCT CTT CAT TTT ATC CTC GTC-3’
	BKV rev	*5078-5049	5’-AAT CTT CCC TTA ATG AGA AAA GCT TAT TTA -3’
	BKV probe	*5016-5046	5’-FAM-CCC TTT GTC AGG GTG AAA TTC CTT ACA CTT C-TAMRA-3’
JCV	JCV for	°4299-4321	5’-GAG TGT TGG GAT CCT GTG TTT TC -3’
	JCV rev	°4352-4375	5’-GAG AAG TGG GAT GAA GAC CTG TTT-3’
	JCV probe	°4323-4350	5’-FAM- TCA TCA CTG GCA AAC ATT TCT TCA TGG C-TAMRA -3’

* BKV Dunlop strain GenBank Acession Number: V01108;
°JCV Complete genome GenBank Acession Number: J02226.

TABLE 3: Clinical data

CASE	MOTHERS DATA			NEWBORNS DATA			
	AGE	PATOLOGY	DELIVERY	GA/SEX	WEIGHT	PATOLOGY	FEEDING
1	31	none	CD	41^{+1}/F	4050 gr	no one	maternal
2	36	none	ED	ND/M	3500 gr	no one	maternal
3	32	none	CD	37/F	3430 gr	jaundice	maternal
4	25	none	ED	41/M	4060 gr	no one	maternal
5	28	none	ED	40/M	3480 gr	no one	maternal
6	34	none	ED	40/F	3130 gr	no one	maternal
7	29	none	ED	ND/F	3370 gr	jaundice	maternal
8	29	steatohepatitis	CD	37/F	2840 gr	no one	maternal
9	27	none	ED	39/M	3130 gr	jaundice	maternal
10	30	none	ED	40^{+3}/M	3080 gr	no one	maternal
11	28	none	VE	41/F	2800 gr	no one	maternal
12	31	none	ED	41/M	3970 gr	no one	maternal
13	27	none	ED	40/M	4300 gr	jaundice	maternal
14	27	none	ED	40/F	3050 gr	no one	maternal
15	20	none	ED	40/F	3270 gr	no one	maternal
16	31	none	ED	40/M	3540 gr	no one	maternal
17	32	renal transplant recipient	CD	38/F	2780 gr	no one	maternal
18	25	none	CD	40/M	4330 gr	no one	maternal
19	24	none	ED	40/M	3040 gr	no one	maternal

ND= Not Determined; ED= Eutocic Delivery; CD= Cesarean Delivery; VE= Vacuum Extraction; GA= Gestational Age (weeks+ days); M= Male; F=Female.

TABLE 4: Results of multiplex n-PCR on the urine samples of the eight pregnant women tested positives for PVs genome.

CASE	1st TRIMESTER	2nd TRIMESTER	3rd TRIMESTER
1	BKV	BKV	BKV
2	NEG	BKV	BKV
3	JCV	JCV	JCV
4	JCV	JCV	JCV
7	BKV	NEG	NEG
9	JCV	JCV	JCV
13	BKV/JCV	BKV/JCV	BKV/JCV
18	JCV	NEG	JCV

FIGURES

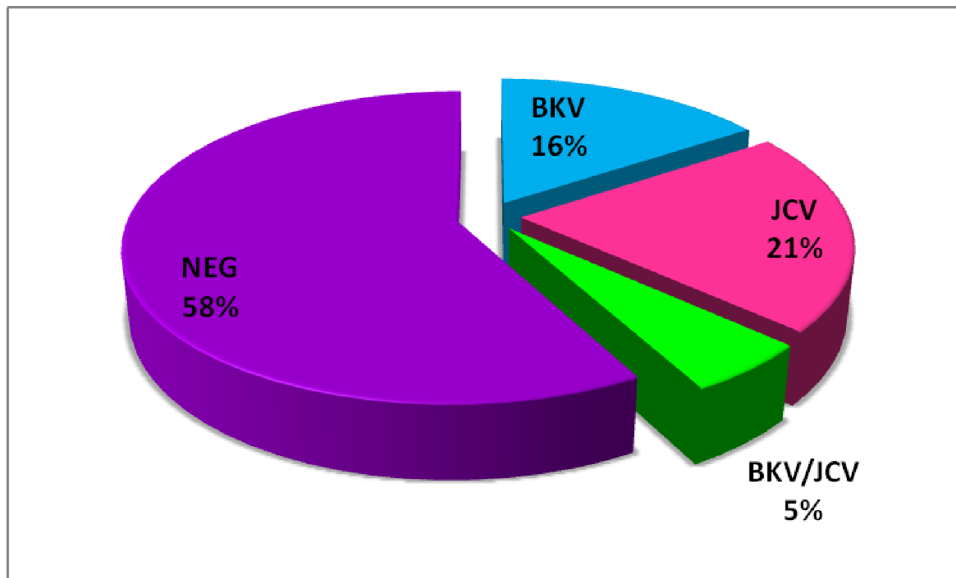


Figure 1: Distribution of PVs urinary excretion among women during pregnancy.

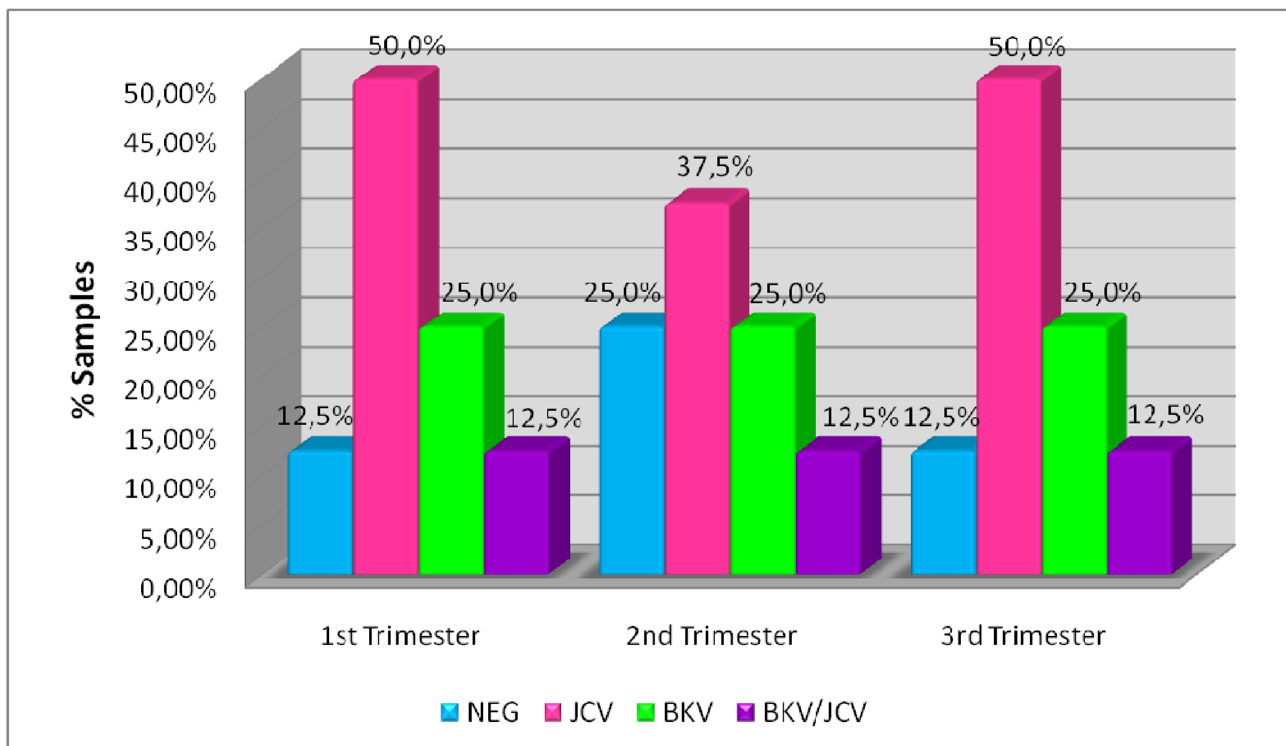


Figure 2: Frequency of excretion and type of PV identified in urine samples from the eight pregnant women that tested positive for PV genome by multiplex n-PCR in the 1st, 2nd and 3rd trimester of pregnancy.

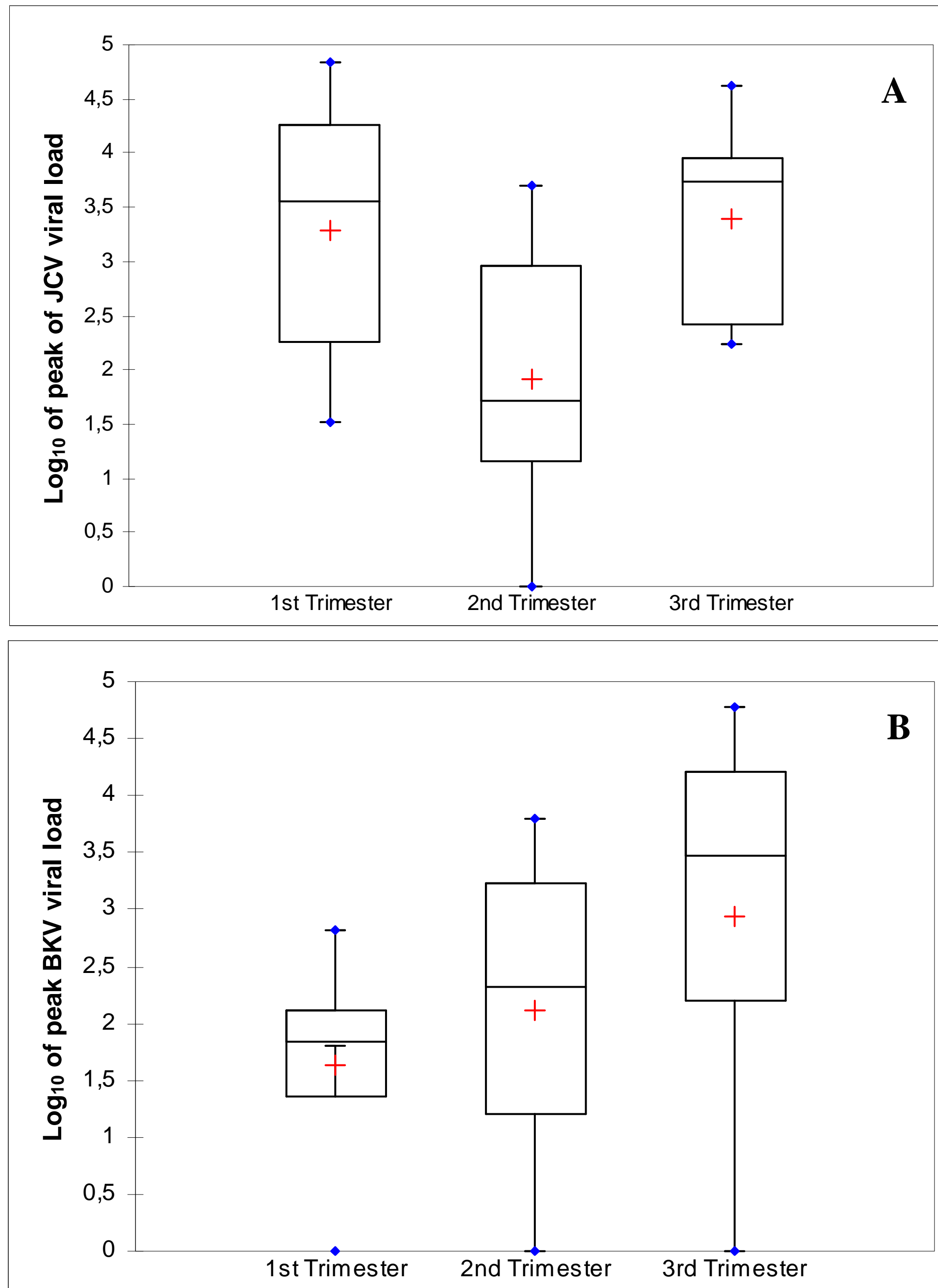


Figure 3: A) Box plots of $\log_{10}$ peak JCV viral load in pregnant women during the three gestational period. **B**) Box plots of $\log_{10}$ peak JCV viral load in pregnant women during the three gestational period. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.

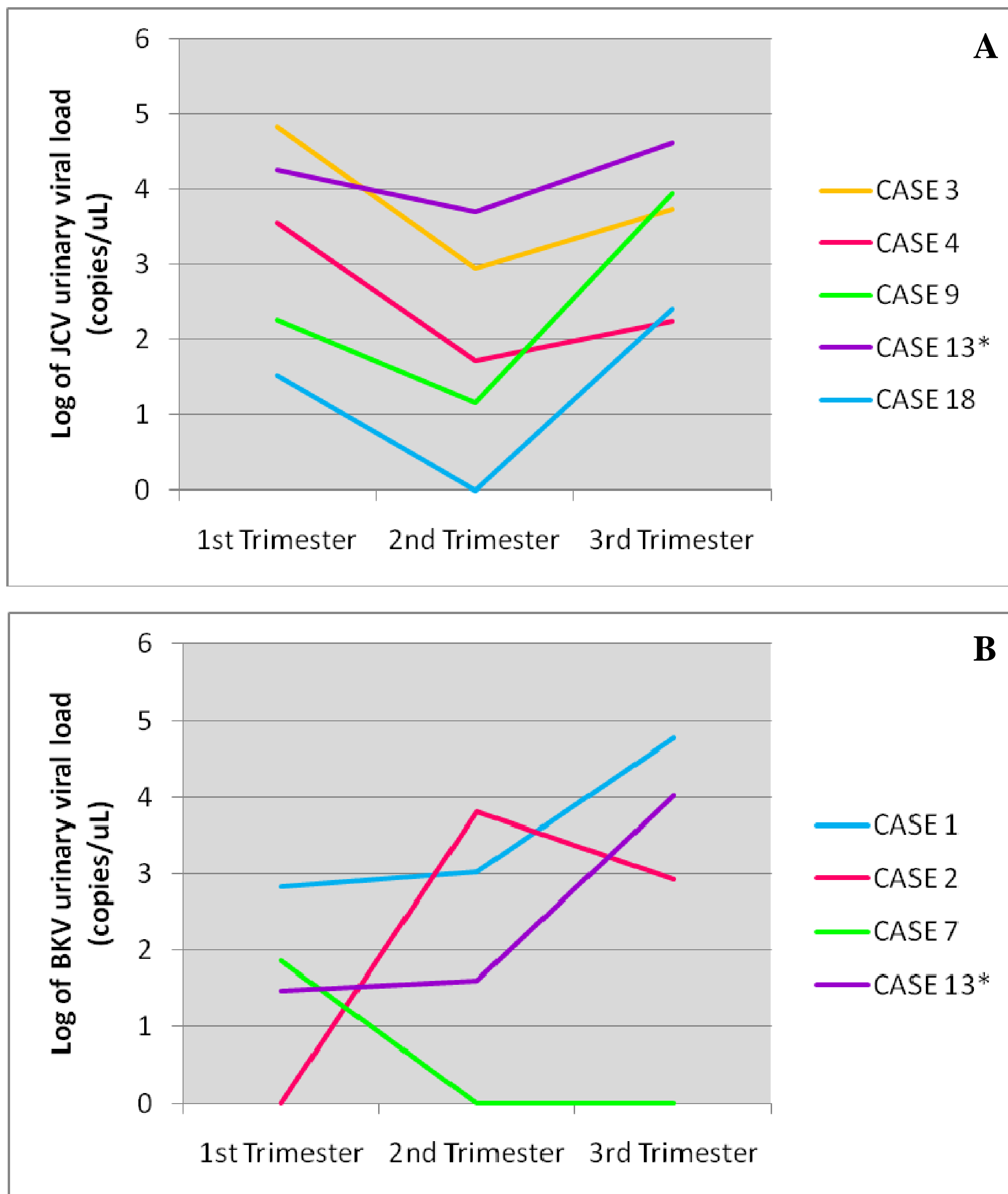


Figure 4: A) Pattern of JCV viral loads in pregnant women during the three trimesters of gestation. **B**) Pattern of BKV viral loads in pregnant women during the three trimesters of gestation. Data are shown as $\log_{10}$PVs copies/µL. No viruria is represented using a value of 1 PVs copy/µL of urine.

* Case co-infected by BKV and JCV.

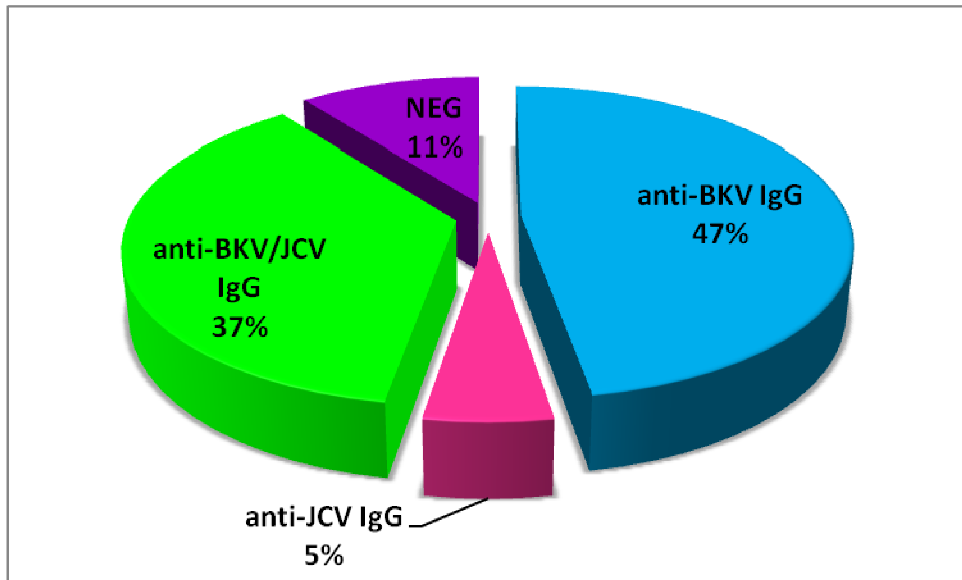


Figure 5: Prevalence of specific anti-BKV and/or anti-BKV IgG among the pregnant women.

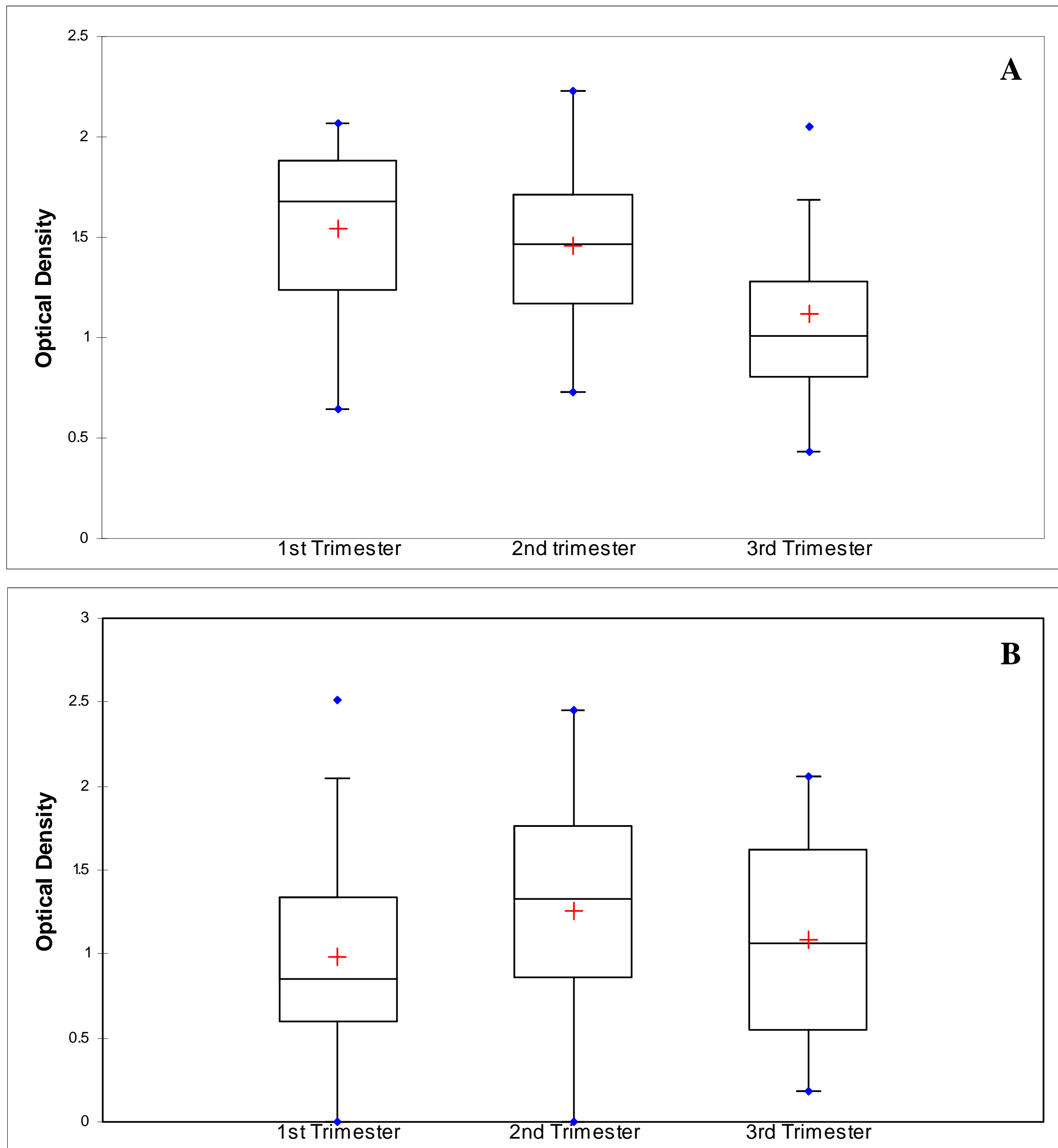


Figure 6: A) Box plots of OD anti-JCV IgG value for pregnant women during the three gestational period. **B**) Box plots of OD anti-BKV IgG value for pregnant women during the three gestational period. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.

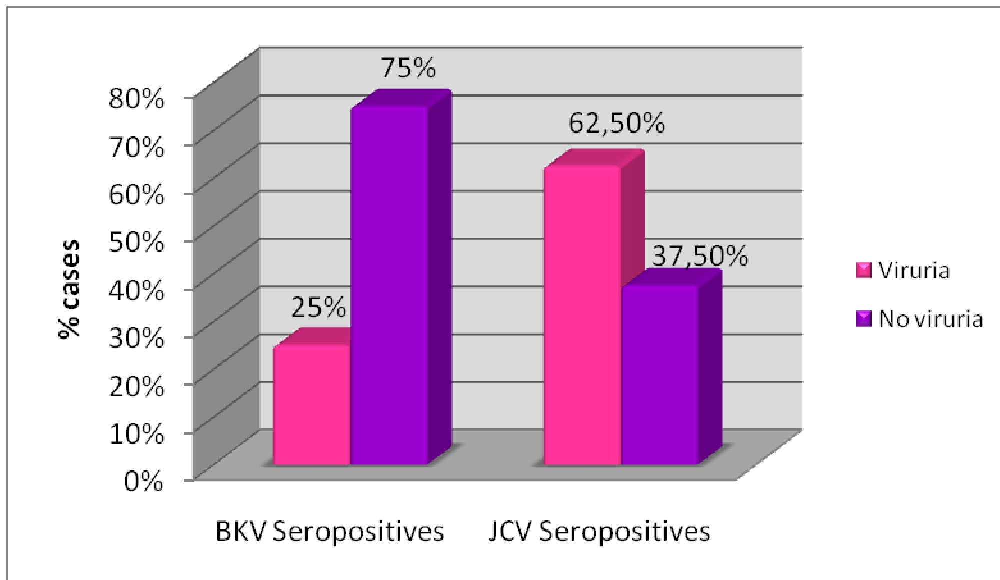


Figure 7: Frequency of BKV and JCV viruria in BKV and/or JCV seropositive women during pregnancy.

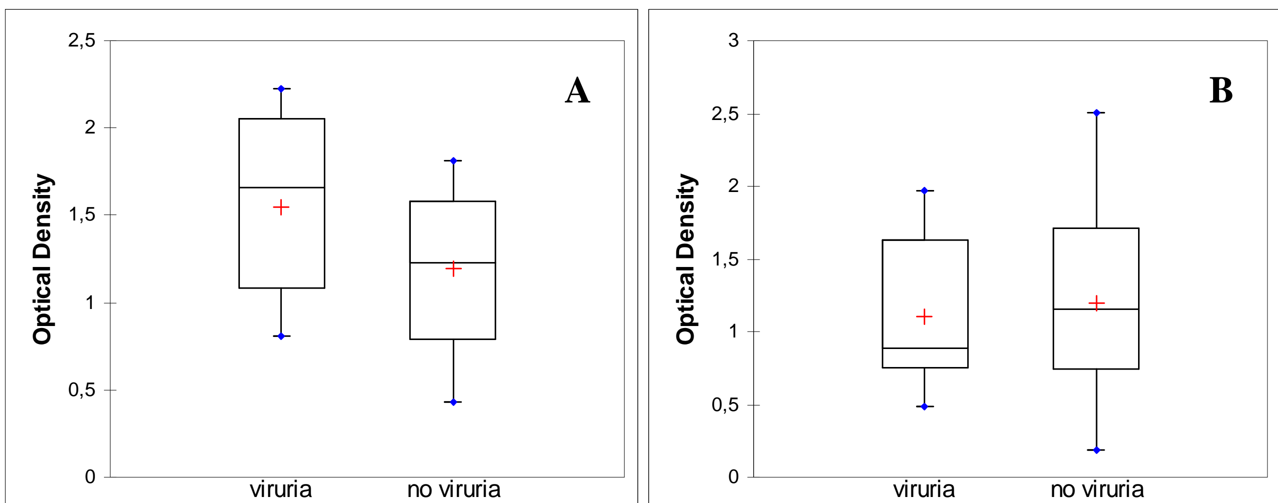
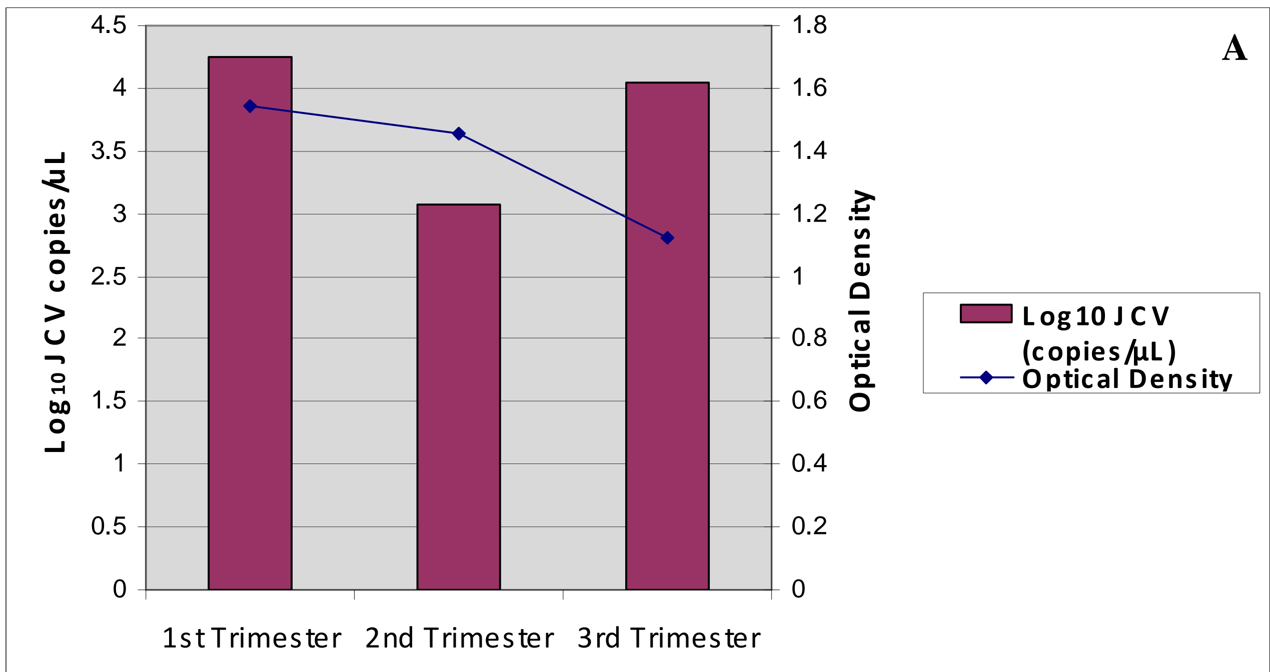


Figure 8: A) Box plots of OD anti-JCV IgG value for pregnant women with and without JCV viruria. **B**) Box plots of OD anti-BKV IgG value for pregnant women women with and without JCV viruria. The five horizontal lines in each box represent the smallest observation, lower quartile, median, upper quartile, and the largest observation.



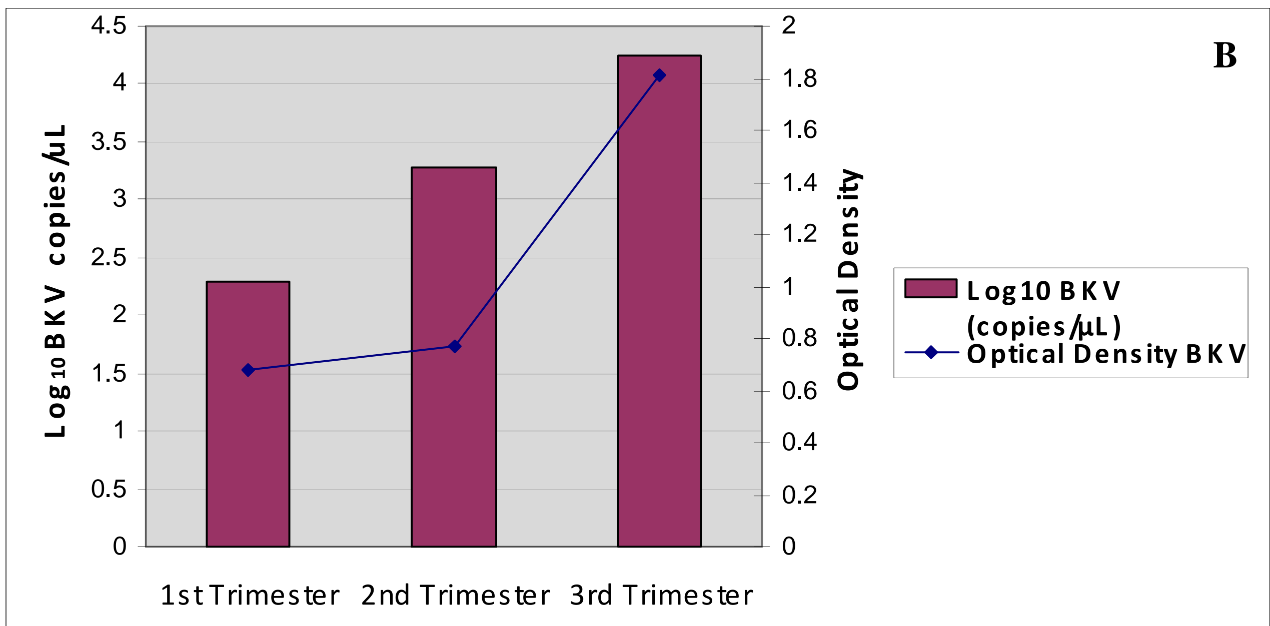


Figure 9: A) Mean JCV viral loads in urine and mean OD anti-JCV IgG values durinf pregnancy in JCV seropositive women. **B**) Mean BKV viral loads in urine and mean OD anti-BKV IgG values durinf pregnancy in BKV seropositive women.

DISCUSSION

Vertical transmission of DNA and RNA virus among humans and animals has been well documented. The modes of viral transmission include passage across the placenta over the pregnancy, during birth-related processes, soon after birth, or also in combined ways. The overall rates of such mode of transmission can be quite different among different virus species: i.e. the rates of vertical transmission of varicella zoster virus and human immunodeficiency virus range from 2 to 20% and from 13 to 30% respectively, in comparison with cytomegalovirus in which vertical transmission has been reported in only 0.2% of the cases[34]. The potential for these modes of transmission for members of the *Polyomaviridae* family is controversial.

In our study, we tried to clarify whether vertical transmission of human Polyomavirus JCV and/or BKV could be a mode of spread of one or both viruses, and, possibly, to indicate its frequency. Therefore, we performed, to the best of our knowledge, the first molecular and serological-based study in a population of pregnant women and their offspring, the last followed up to one month of life.

The main result of this study was that in our population no evidence of vertical transmission could be found. The persistence of BKV and JCV-specific IgG in 89% of maternal sera reflects what it was previously reported in sero-epidemiologic studies of the adult population[1] and by Shah et al.[26] that reported BKV-specific IgG prevalence in 91% of pregnant women.

Seven cases (35%) showed evidence of BKV (70%) and JCV (30%) reactivation, as demonstrated by the acquisition of IgG and/or IgA specific antibodies during pregnancy, and this reactivation persists until delivery in all the cases. This finding is similar to that reported by Gibson et al.[27], but quite different from that of Shah et al.[26], that observed BKV reactivation in 5% of their cases. In that study, however the sera were collected several months distance each one, and a transitory reactivation in between could not be detectable. The significance of this reactivation in pregnancy is controversial. In our cases, median OD levels (data not shown) for both JCV and BKV-specific IgA and IgM were low, near to the cut-off positivity level, therefore, systemic viral reactivation was quite unlikely. McCance and Mims[35], have found in mice infected immediately after birth with murine polyoma virus, that pregnancy reactivates local infection in kidneys but not in other organs or in the fetus. In contrast, primary viral infection acquired during pregnancy could be followed be transplacental passage of murine polyomavirus as reported by McCance and Mims[36] and Zhang et al.[29]. The first hypothesis could not be excluded, although in our study there were no evidence of urinary reactivation of PV, indeed no decoy cells were identified and viral load in urine does not correlate with presence of BKV and JCV specific IgA or IgM.

Finally, the rates of reactivation of BKV were higher than JCV in the present study in contrast with results obtained by Gibson et al.[27].

In our study we found no evidence of primary infection by JCV or BKV but this occurrence is only rarely reported in other studies concerning human PVs in clinical setting[24-26].

Umbilical cord blood samples, blood samples taken at one week and one month of life from the babies clearly indicated that BKV and JCV specific immunity are passively transmitted to the newborn. It was demonstrated by: a) absence of viral genome in all the samples from newborn and babies and b) decrease of median OD value of BKV and JCV specific antibodies from umbilical blood samples to blood samples taken at one month of life where they tend to disappear. These data confirm early studies by Shah et al.[26], Borgatti et al.[24], and Coleman et al.[25], that were performed in largest series of pregnant women.

Molecular analysis identified PV genome in urine samples from less than 50% of pregnant women, whereas blood samples from newborn and babies as well as urine and nasopharyngeal secretion were negatives.

These data confirm what was found in a previous paper[31] where molecular-based study performed on 300 pregnant women yield no evidence of viral genome detection in blood samples from mothers and umbilical cord, but with PV identification in urine.

The comparative analysis between viral load in urine and median OD values for JCV and BKV specific IgG seems evidence differences in viral excretion for JCV and BKV. The BKV viruria increased parallel with IgG antibodies during the pregnancy, whereas JCV viruria was almost stable but median OD level of IgG decreased. These findings, not previously studied in literature in clinical setting, could be related to different viral kinetic between JCV and BKV during pregnancy in analogy to what reported in other more severe immune deficiency conditions, such as renal transplanted or AIDS subjects.

In this paper, sequence analysis of transcriptional control region and viral capsidic protein of BKV and JCV was not carried out. Recently, Patel et al.[30], demonstrate that different strains of SV40 as produced by TCR rearrangements, had different ability to produce transplacental infection However, in a previous study we did not find a significant rearrangements in BKV and JCV isolated from urine samples of pregnant women.

The small number of cases included in our study could be a bias in drawing definite conclusions; however either molecular and serologic data seem denied that vertical transmission could be a frequent way of BKV and JCV spread in human population. Since we have not found primary infection by BKV or JCV we are not able to exclude that this occurrence during pregnancy could be a serious risk of congenital transmission for these viruses.

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CONCLUSIONI

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A conclusione dei nostri studi, condotti con metodiche molecolari e siero-immunologiche, si può affermare che l'infezione da BKV e JCV pur essendo estremamente diffusa nella popolazione generale, viene verosimilmente contratta durante l'infanzia o l'età adulta, mentre l'infezione congenita –sia essa transplacentare, peripartum o perinatale- non risulta dimostrabile e dunque non rappresenta una via prioritaria di diffusione del virus.

L'analisi di sequenza della regione genomica tardiva VP1 di BKV, da noi valutata nella sua totalità in soggetti portatori di trapianto di rene con e senza nefropatia BKV-associata non ha evidenziato mutazioni significativamente correlate con lo sviluppo della patologia renale. Tuttavia sono state identificate mutazioni che sembrano più specificamente presenti nei soggetti con nefropatia BKV-associata. Ciò potrebbe suggerire che l'analisi di sequenza possa risultare uno strumento utile nella identificazione di ceppi virali a maggiore patogenicità. Un recente lavoro (citato come voce bibliografica N°56) ha inoltre suggerito che mutazioni di regioni genomiche virali specifiche di SV40 possano associarsi a maggiore propensione per questo virus ad associarsi a trasmissione verticale in criceti. Ciò ci stimolerà ad ulteriori investigazioni per valutare il significato clinico di varianti virali che occorrono nella popolazione generale.

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