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HIGH PREVALENCE OF THE SIMULTANEOUS EXCRETION OF POLYOMAVIRUSES JC AND BK IN THE URINE OF HIV-INFECTED PATIENTS WITHOUT NEUROLOGICAL SYMPTOMS IN SÃO PAULO, BRAZIL

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SUMMARY

Objective: To evaluate the prevalence of the urinary excretion of BKV and JCV in HIV-infected patients without neurological symptoms. **Methods:** Urine samples from HIV-infected patients without neurological symptoms were tested for JC virus and BK virus by PCR. Samples were screened for the presence of polyomavirus with sets of primers complementary to the early region of JCV and BKV genome (AgT). The presence of JC virus or BK virus were confirmed by two other PCR assays using sets of primers complementary to the VP1 gene of each virus. Analysis of the data was performed by the Kruskal-Wallis test for numerical data and Pearson or Yates for categorical variables. **Results:** A total of 75 patients were included in the study. The overall prevalence of polyomavirus DNA urinary shedding was 67/75 (89.3%). Only BKV DNA was detected in 14/75 (18.7%) urine samples, and only JCV DNA was detected in 11/75 (14.7%) samples. Both BKV and JCV DNA were present in 42/75 (56.0%) samples. **Conclusion:** In this study we found high rates of excretion of JCV, BKV, and simultaneous excretion in HIV+ patients. Also these results differ from the others available on the literature.

KEYWORDS: JC virus DNA; BK virus DNA; Simultaneous excretion; HIV-infected.

INTRODUCTION

JC virus (JCV) and BK virus (BKV) are ubiquitous in the human population[9,29]. Serum-prevalence studies have shown that approximately 70 to 80% of healthy individuals have antibodies against BKV and JCV[3,11,19,20]**.** In immunocompromised individuals, JCV is associated with Progressive Multifocal Leukoencephalopathy (PML), an often fatal disease of the central nervous system[5,13], and with other neurological diseases (granule cell neuronopathy, encephalopathy and meningitis)[39]. In addition, BKV is associated with hemorrhagic cystitis in hematopoietic stem cell transplantation (HSCT) recipients and interstitial nephropathy in kidney transplant recipients and has recently also been shown to be associated with neurologic complications[26,41]. After primary infection, both viruses establish a persistent infection in the kidneys and may be excreted in the urine[15,22].

The prolonged immunosuppression associated with AIDS contributes to the high prevalence of reactivation of these viruses. There are few reports on the prevalence of the urinary excretion of JCV and BKV and the occurrence of simultaneous excretion in HIV-infected patients[7,34].

Also, BKV and JCV have been gathering attention from scientists from different areas of the medicine due to its ability to infect and cause disease in patients with different kind of immunosuppression and patients treated with monoclonal antibody based therapies[30].

The aim of this study was to evaluate the prevalence of the urinary excretion of BKV and JCV in HIV-infected patients without neurological symptoms.

METHODS

Patients

Study and urine samples: Urine samples were collected from 75 HIV-infected patients without any neurologic symptoms or a history of PML who were undergoing regular follow-up at the Outpatient Care Unit of the Instituto de Infectologia Emilio Ribas (IIER), São Paulo, Brazil. The study was approved by the Ethic Research Committee of the IIER. All patients signed an informed consent form.

Sample preparation: BKV and JCV DNA were extracted from 200 µL urine samples using resin columns (QIAamp® DNA Blood Mini Kit, Qiagen, Germany) according to the manufacturer's instructions.

JCV and BKV DNA detection: All samples were initially screened

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using PCR according to protocol described by ARTHUR *et al.*[4] and standardized by FINK *et al.*[18]. This PCR uses primers complementary to the early region of the polyomavirus genome that is shared by JCV and BKV (T antigen) (Table 1).

Table 1
Primers used for PCR

Primer	Viral Region	Sequence	Reference
PEP1	AgT	AGTCTTTAGGGTCTTCTAC	(4)
PEP2	AgT	GGTGCCACCTATGGAACAG	(4)
JLP-15	VP1 (VJC)	ACAGTGTGGCCAGAATTCACTACC	(1)
JLP-16	VP1 (VJC)	TAAAGCCTCCCCCCCAACAGAAA	(1)
BKV1	VP1 (VBK)	GAAGTTCTAGAAGTTAAAACTGGG	(25, 31)
BKV2	VP1 (VBK)	GTGGAAATTACTGCCTTGAATAGG	(25, 31)

The target sequence was amplified in a reaction with a final volume of 50 µL containing 10 µL of template, 10X PCR Buffer minus Mg, 200 µM dNTPs, 0.5 µM of each primer, 1.5 mM $MgCl_2$, and 2.5 U/reaction Taq polymerase.

The reaction was submitted to an amplification of 40 cycles on a Perkin Elmer PTC 200 thermocycler as follows: 94 ºC for 1.5 minutes, 55 ºC for 1.5 minutes and 72 ºC for two minutes. An initial DNA denaturation step for 10 minutes and a final extension step for seven minutes at 72 ºC were also included. The length of the obtained fragment from the amplified samples was 173 bp.

All positive samples for AgT by PCR were submitted to two other PCR assays using sets of primers complementary to the genes that express the capsid protein, VP1, which differ between JCV and BKV, allowing the discrimination between these two polyomavirus.

For JCV, we adopted the protocol described by AGOSTINI *et al.*[1] with some modifications. The cycle conditions were as follows: 95 ºC for five minutes for initial DNA denaturation followed by 50 cycles of 95 ºC for one minute, 63 ºC for 1.5 minutes, and 72 ºC for one minute and then 72 ºC for 10 minutes for a final extension step. The length of the obtained fragment from the amplified samples was 215 bp.

For BKV, we adopted the protocol described by KRUMBHOLZ *et al.*[31] with some modifications. The cycle conditions were as follows: 94 ºC for five minutes for initial DNA denaturation followed by 45 cycles of 95 ºC for one minute, 63 ºC for 1.5 minutes, and 72 ºC for one minute and then 72 ºC for 10 minutes for a final extension step. The length of the obtained fragment from the amplified samples was 353 bp.

PCR products were submitted to gel electrophoresis in a 1.5% agarose gel stained with ethidium bromide, and visualized after UV light exposition.

The original protocol was modified by adding glycerol (57%), an adjuvant to improve the amplification of regions rich in G-C[12], and Cresol red (2.5 µg/µL) to dye the PCR product on the agarose gel[24]. For all PCR, positive and negative controls were added. The negative control consisted of the PCR mixture containing water instead of DNA template and to avoid contamination, the risks were eliminated by separating work areas for mixture preparation and PCR reactions.

The sets of primers used for PCR are presented in Table 1.

Statistical analysis: Analysis of the data was performed by the Kruskal-Wallis test with *post hoc* Dunn's test for numerical data and Pearson or Yates corrected Chi-squared for categorical variables. Values of $p < 0.05$ were considered significant. SPSS 10.0 (IBM, Chicago, IL, USA) and GraphPad software (La Jolla, CA, USA) were used.

RESULTS

A total of 75 patients consented to participate in the study. The median age was 43 years old (range: 24 to 69 years old). Of these 75 patients, 49 (65.3%) were men, and 26 (34.7%) were women. The T cell CD4+ count was determined for 42/75 patients and ranged from 12 to 1,179 cells/mm³ (median, 250 cells/mm³).

The overall prevalence of polyomavirus DNA urinary shedding was 67/75 (89.3%). Only BKV DNA was detected in 14/75 (18.7%) urine samples, and only JCV DNA was detected in 11/75 (14.7%) samples. Both BKV and JCV DNA were present in 42/75 (56.0%) samples. No association was observed between the frequency of JCV and BKV virus shedding and the degree of immunodeficiency ($p = 0.250$).

Twenty-two women (22/26, 84.6%) and 45 men (45/49, 91.8%) presented viral excretion ($p = 0.568$). A higher percentage of positive samples was found among males than among females; however, this difference was not statistically significant (Table 2).

The median and interquartile ranges for age were not statistically different among the following groups: JCV (43 ± 12, ranging from 30 to 54), BKV (39.5 ± 14.5, 24-53), simultaneous excretion (44.5 ± 12.75, 29-69), positive (43 ± 13, 24-64), negative (44 ± 11.5, 34-54) and total (44 ± 11.5, 34-54). These comparisons were made using the Kruskal-Wallis test ($p = 0.203$) with *post hoc* Dunn's multiple comparison test ($p > 0.05$ for all groups) (Fig. 1).

Table 2
Sex and positivity for polyomavirus BKV and JCV

Sex (n)	BKV+ (%)	JCV+ (%)	Simultaneous excretion	Negative	*p* value
Male (49)	9 (18.3)	8 (16.3)	28 (57.0)	4 (8.1)	
Female (26)	5 (19.2)	3 (11.5)	14 (53.8)	4 (15.4)	0.768
Total (75)	14 (18.7)	11 (14.7)	42 (56.0)	8 (10.7)	

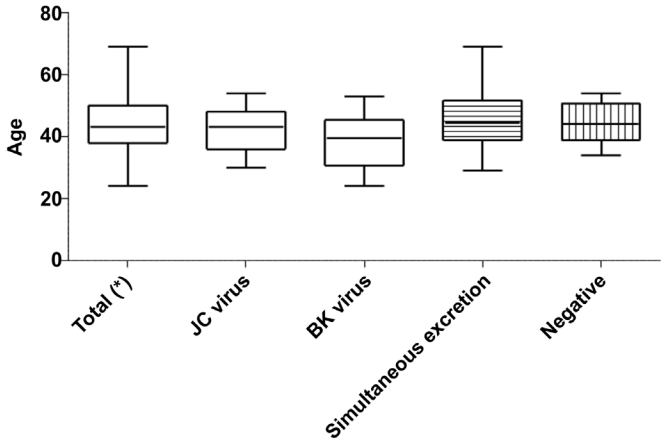

Fig. 1 - Box plots of the age ranges of patients excreting polyomaviruses. (*) Total included positive (JC, BK and simultaneous) and negative samples.

We stratified the individuals by age and analyzed the number of positive cases for BKV and JCV. All subsets included a high number of positive individuals, and no significant difference in the proportion of positive individuals was observed between subsets (Table 3).

Table 3
Detection of JCV and BKV DNA and simultaneous excretion in HIV+ patients stratified by age

Age (n)	JC (%)	BK (%)	Simultaneous(%)	Negative (%)
20-29 (3)	0	1 (7.1)	1 (2.4)	0
30-39 (22)	4 (36.4)	5 (35.7)	10 (24)	3 (37.5)
40-49 (31)	5 (45.4)	6 (42.9)	18 (42.6)	3 (37.5)
50-59 (18)	2 (18.2)	2 (14.3)	12 (28.6)	2 (25)
>59 (1)	0	0	1 (2.4)	0
Total (75)	11/75 (14.6)	14/75(18.7)	42/75(56)	8/75(10.6)
p value (Pearson χ^2)	0.888	0.212	0.511	0.949

There was also no statistically significant difference in the medians and interquartile ranges of the T cell CD4+ count among all of the groups: JCV (250 ± 287.5, ranging from 12 to 550), BKV (339 ± 377, 160-700), simultaneous excretion (200 ± 416, 46-1,179), positive (220 ± 404.5, 12-1,179), negative (539 ± 600.5, 72-1,016); $p = 0.602$ by the Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test ($p > 0.05$ for all groups) (Fig. 2).

DISCUSSION

We observed that 42/75 (56.0%) of the HIV-infected patients were excreting both JCV and BKV. There are few reports describing the simultaneous excretion of JCV and BKV in this patient population. BEHZAD-BEHBAHANI *et al.,* reported a prevalence of simultaneous excretion of JCV and BKV of 5.9% in HIV+ patients[7]. MARKOWITZ *et al.*, observed concomitant excretion of JCV and BKV in four out of 122 (3.3%) HIV-infected patients[34]. KNOWLES *et al*., also found low prevalence of the excretion of both viruses among HIV-infected patients (11/81, 13.2%)[28].

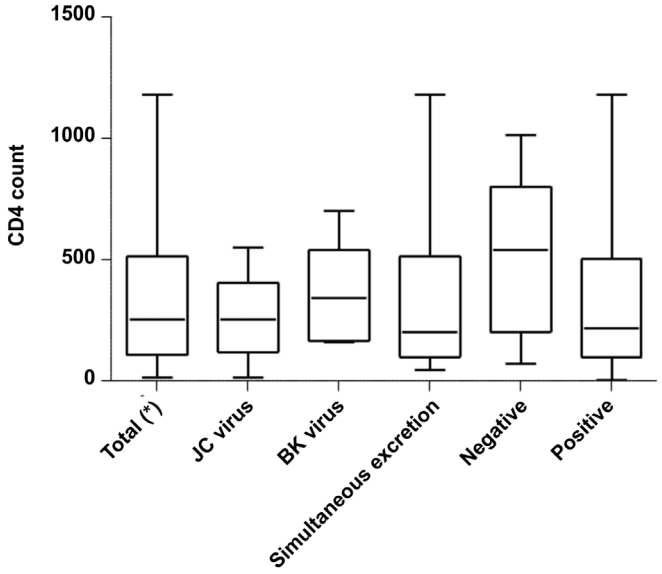

Fig. 2 - Box plots of the T cell CD4+ counts of patients excreting polyomaviruses. (*) Total included positive and negative excretion.

In our study the prevalence of BKV in patients who were shedding only BKV is similar to that found by MARKOWITZ *et al*.[34], and BEHZAD-BEHBAHANI *et al.*[7], ranging from 14% to 20%. However, we must consider those patients with simultaneous excretion, which makes the frequency of BKV urinary shedding much higher, reaching 74.6%. Also, a higher rate BKV shedding compared to JCV was reported in patients with other immunosuppressive conditions[16].

Moreover, it is known that HIV can transactivate many viruses, among which the polyomavirus JC[14]. There are no data on the dispersion of JC virus and BK in our country, our results could reflect a high circulation of both viruses in the general population and its reactivation in the presence of immunodeficiency frames.

BKV has been shown to have the ability to cause encephalitis[26,41] and hemorrhagic cystitis[6] in AIDS patients, as well as urinary system diseases in HSCT and kidney transplant recipients.

PML, formerly a very rare disease[10], is now one of the most frequent opportunistic diseases among AIDS patients[2,8,23], and even after the introduction of Highly Active Anti-Retroviral Therapy (HAART), the incidence of PML did not decrease like that of other opportunistic infections[2].

Including the patients who were excreting both viruses, 70% of patients of this study were JCV positive. This JCV prevalence is higher than that found in other similar studies[7,21]. The high rate of JCV urinary shedding could indicate a high exposure to the virus among this population.

We did not find any association between JCV and BKV urine excretion and the degree of immunodeficiency, as measured by the T CD4+ cell count. This finding has also been reported in previous studies. BEHZAD-BEHBAHANI *et al.* reported no statistically significant difference in the prevalence of JCV and BKV viruria between HIV+ patients with T cell CD4+ counts lower and higher than 200 cells/mm^3[7], and MATOS *et al.*,

did not find any association between JCV shedding and the degree of immunodeficiency[35]. MARKOWITZ *et al*., also did not find any increase in the frequency of JCV in HIV-infected patients with different degrees of immunodeficiency but did find a correlation between BKV shedding and the degree of immunodeficiency: 8% of patients with T cell CD4+ counts higher than 500 cells/mm³ shed BKV and 37.5% of patients with T cell CD4+ counts lower than 200 cells/mm³ shed BKV[34]. The same finding was reported by KNOWLES *et al.,* 1999, who found BKV DNA in only 9/40 (22.5%) patients with T cell CD4+ counts higher than 200 cells/mm³ and in 21/40 (51.2%) patients with T cell CD4+ counts lower than 200 cells/mm³ [(28)]. The prevalence of JCV urine shedding was not significantly associated with the degree of immunodeficiency.

In the present study, there was no association between JCV and BKV shedding and sex, $p = 0.768$ (Table 2), in contrast to a previous study that reported a substantially higher shedding rate among male individuals[35].

We observed a slightly higher frequency of the urinary shedding of both viruses in older individuals; however, the ages of the groups (JCV, BKV, simultaneous excretion and negative) were not significantly different (Table 2). This increase has been reported for JCV in an immunocompetent population, in which the older the population, the higher the frequency of JCV urinary shedding[36,42]. In an immunocompetent population BK virus did not show an increase in the frequency of shedding with age[36,42].

In immunocompetent populations from Asia and Europe, the prevalence of JCV urinary shedding was considerably higher than BKV[17,27,35,37,40], but in HIV-positive patients, the urinary shedding of these viruses has not been well evaluated. SUNDSFJORD *et al.*, found a higher number of BKV-positive than JCV-positive urine samples for HIV+ patients, but this difference was not significant[38].

Interestingly, we found that the prevalence of urinary shedding of BKV in our population was slightly higher than the JCV urinary shedding prevalence, but this difference was not statistically significant, probably due to the small number of samples after stratification by age and to the large number of positive samples in all age groups ($p = 0.193$).

Although the data found in this study, the following work has some limitations: the HIV, BKV and JCV load were not available. However, other studies found no direct association between HIV viral load and excretion of polyomavirus. MATOS *et al.,* described that in HIV and JCV co-infected individuals, there was no statistical difference between HIV viral load and detection of JCV DNA in the urine. Also similar results were found, by LEDESMA *et al*., regarding BK detection and HIV viral load[32].

Also this study does not have a control group. However a recent work published by MACHADO *et al.*, 2011, found different rates of JCV and BKV urinary shedding in HIV+ children and non-infected children. In this study the BKV frequency was significantly higher in HIV infected patients in comparison of healthy subjects (54.3% vs. 12.5%)[33]. However the age of the participants of this study is very different from ours, it was not possible to compare the two groups. The biological basis and the possible viral interactions associated with the high prevalence of co-infection have not been well described, also little is known about the shedding dynamics of these viruses in these population therefore, further studies should be done. In summary, in our study, we found high rates of urinary shedding of JCV (70.7%) and BKV (74.7%) in this HIV-infected patients group. In addition, we also found high rates of the simultaneous excretion of JCV and BKV (56%) in HIV+ patients, a result that has not been reported before.

RESUMO

Alta prevalência de excreção simultânea de poliomavírus JC e BK na urina de pacientes HIV+ sem sintomas neurológicos em São Paulo, Brasil

Objetivo: Avaliar a prevalência de excreção urinaria de vírus JC (VJC) e vírus BK (VBK) em pacientes HIV+ sem sintomas neurológicos. **Métodos:** Amostras de urina de pacientes HIV+ sem sintomas neurológicos foram testados para a presença de VJC e VBK através da técnica de PCR. As amostras foram triadas para a presença de poliomavírus com par de *primers* complementares a região precoce do genoma do VBK e do VJC (AgT). A presença foi confirmada através de dois outros ensaios de PCR dirigidos a região do gene VP1 de ambos os vírus. A análise estatística foi realizada com auxílio do teste de Kruskal-Wallis para dados numéricos e Pearson ou Yater para variáveis categóricas. **Resultados:** Ao todo foram inclusos no estudo 75 pacientes. A prevalência geral de excreção de poliomavírus na urina foi de 67/75 (89,3%). O DNA do vírus VBK foi detectado em 14/75 (18,7%) das amostras de urina, e o DNA do VJC foi detectado em 11/75 (14,7%) das amostras testadas. Ambos os vírus estavam presentes simultaneamente em 42/75 (56%) das amostras de urina. **Conclusão:** Encontramos, no presente estudo, uma alta taxa de excreção de VJC, VBK e excreção simultânea em pacientes HIV+. Ainda, esses resultados diferem de outros disponíveis na literatura.

COMPETING INTERESTS

The authors declared that there are no competing interests.

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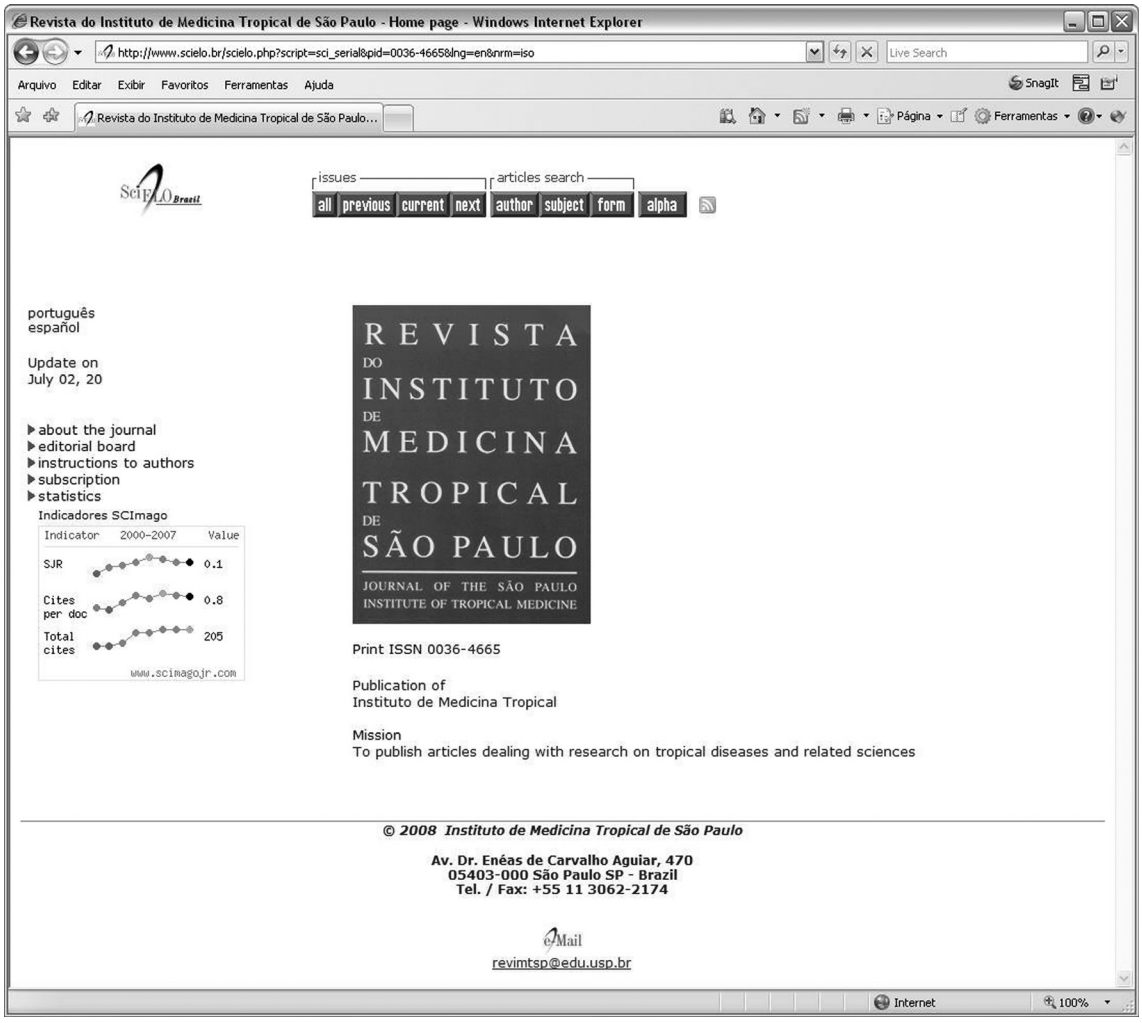

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