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NO EXCESS RISK FOR COLORECTAL CANCER AMONG SUBJECTS SEROPOSITIVE FOR THE JC POLYOMAVIRUS

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ABSTRACT

The human polyomaviruses JC virus (JCV) and BK virus (BKV) are oncogenic in experimental systems and commonly infect humans. JCV DNA has been reported to be present in human colon mucosa and in colorectal cancers.

To investigate whether the risk for colorectal cancer is associated with JCV or BKV infection, we performed a case-control study nested in the Janus biobank, a cohort of 330,000 healthy Norwegian subjects. A 30 year prospective follow-up using registry linkages identified 386 men with colorectal cancer who had baseline serum samples taken >3 months before diagnosis. Control subjects were matched for sex, age and date of blood sampling and county of residence. Seropositivity for JCV or BKV had high (97-100%) sensitivity for detection of viral DNA-positive subjects and discriminated the different polyomaviruses. Seropositivity was mostly stable over time in serial samples.

The relative risk for colorectal cancer among JCV seropositive subjects was 0.9 (95% CI: 0.7 - 1.3) and the BKV-associated relative risk was 1.1 (95% CI: 0.8 - 1.5). Determining seropositivity using alternative cut-offs also found no evidence of excess risk.

In summary, this prospective study found no association between JCV or BKV infections and excess risk for colorectal cancer.

INTRODUCTION

The human polyomaviruses BK virus (BKV) and JC virus (JCV) commonly infect humans[1]. Initial infection rarely causes clinical disease, although respiratory symptoms or urinary tract disease is sometimes found in the case of BKV[2]. JCV and BKV can be detected in tonsillar tissue and suggesting that the respiratory tract is the primary site of infection[3]. JC viral particles are found in urban sewage, suggesting that virus-contaminated water and food may be a source of infection[4]. Following primary infection, both BKV and JCV persist as latent infections in kidney epithelial cells and B lymphocytes[1, 5]. Under conditions of severe immunosuppression such as leukaemia, acquired immunodeficiency syndrome (AIDS) and organ transplantation both viruses may be reactivated and cause disease. BKV reactivation is related to urinary tract diseases such as haemorrhagic cystitis and ureteric stenosis[6, 7], whereas JCV reactivation can induce progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system[8].

About 70–90% of healthy adults are seropositive for BKV and JCV. Seroconversion for BK infection occurs in early childhood and JC seroconversion occurs in late childhood[5].
BKV seropositivity increases rapidly with increasing age, reaching 98 % seroprevalence at 7–9 years of age, followed by a minor decrease. JCV seroprevalence increases more slowly with age, reaching 50 % positivity among children between 9-11 years[9].

The human polyomaviruses have in vitro transforming abilities, similar to the mouse polyomavirus and simian virus 40 (SV40)[6]. The virally encoded T antigens of both BKV and JCV are essential for transformation and bind to the p53 and pRb tumor suppressor proteins[10, 11]. Both BK and JC virus infections can induce chromosomal aberrations in human cells[12-14].

Possible associations of polyomaviruses with human cancer have been reported. JCV has been detected in certain brain tumours, in particular oligoastrocytoma[15, 16]. JCV DNA has been found in the upper and lower parts of the human gastrointestinal tract, particularly in the mucosa of the human colon and in colorectal cancers[17, 18] and expression of JCV proteins in colon cancer cells has been reported[19]. Molecular studies have shown presence of JCV in colon neoplasms, and that the virus can interact with cellular proteins to transform the cells[18, 19]. However, other studies have not confirmed presence of JCV in colon cancer[20].

The objective of this study was to investigate if there is an association between JCV infection and risk of colon cancer, using a method of JCV detection that is based on a different principle and can be efficiently used in epidemiologically strong study designs. Serology with virus like particles (VLP) containing the VP1 major capsid proteins of JC virus and BK virus for detection of specific IgG antibodies to JCV and BKV was validated and subsequently used to measure JCV-associated colorectal cancer risks in a prospective cohort study.

MATERIALS AND METHODS

Study population

The Janus Project in Norway was started in 1973 and contains blood samples from about 330,000 subjects. The samples have been collected from men who participated in county health examinations, mostly for cardiovascular diseases, and from blood donors. The participants in the health examinations were recruited from several counties in Norway. The blood donors were from the Red Cross Blood Donor Centre in Oslo. The blood collection took place during office hours, participants were not required to fast and fasting times were not recorded. Serum samples were stored at-25°C. Incident cases of colorectal cancer and deaths were identified through linkage with the Norwegian cancer and all-cause mortality registries. Because the present study was coordinated with a study evaluating whether serum leptin levels predict colorectal cancer risk, only male cancer cases were selected. Leptin levels have been reported to be a risk marker in men, but not in women[21]. Among 1,105 incident male cases of colorectal cancer, 400 men with no previous malignancy who were diagnosed with colorectal cancer more than 3 months after recruitment were randomly selected. Samples from 14 subjects could not be found, resulting in that 386 case subjects were included (age range: 34-85 years, mean age: 59 years, median age: 60 years). Time between blood sampling and diagnosis ranged between 4 months and 28 years (mean: 15 years, median: 16 years). The 386 controls were free of cancer at the time of diagnosis of the matched index case and matched on age (+/- 1 year), the date at blood sampling (+/- 2 month) and county.

To assess reproducibility, two serial samples from 80 subjects in the cohort (unrelated to the case-control sets) that had been collected about 1 year apart (mean, 11.3 months; range 3.5-12.9) were selected. The same samples had also been used in previous studies investigating serum concentrations of hormones and colorectal cancer[21].

The study was approved by the steering group of the Janus Biobank, Norway and by the Ethical committee at Lund University, Sweden (Decision nr 53/2005).

Polyomavirus virus-like particles

Polyomavirus virus-like particles (VLPs) from JC virus and from the SB strain of BK virus were produced in yeast cells from *Saccharomyces cerevisiae* as previously described[22,23]. The VLPs are empty capsids that consist of the major capsid protein, VP1. The *VP1* gene was inserted into the yeast expression vector, pFX7. The pFX7-derived expression plasmids carrying the *VP1* genes were transformed into the yeast *S. cerevisiae* for cultivation and vector replication. Expression of VP1 proteins results in spontaneous assembly into virus like particles retaining sialic acid-binding and antigenic properties of native virions[9].

Serological analysis

Specific IgG antibodies to BK and JC virus were detected as previously described[9]. Briefly, purified VLPs of BKV SB or JCV were added to half area Costar 3690 plates at a concentration of 6.25 ng/well and incubated overnight at 4°C. After washing, 10% horse serum in phosphate-buffered saline (HS-PBS) was added and incubated for 1 hr at room temperature. Serum samples were diluted 1/40 in HS-PBS, added to the wells and incubated for 2 hr in room temperature. Anti-human IgG Mouse monoclonals (Eurodiagnostica, Arnhem, The Netherlands), diluted 1/800 in HS-PBS, were added and incubated for 90 min. Goat anti-Mouse IgG peroxidase conjugate (Southern Biotechnology, Birmingham, AL) diluted 1/2,000 in HS-PBS was reacted at room temperature for 60 min. The peroxidase substrate ABTS was added and incubated for 30 min and the absorbances were measured at 415 nm. The analysing laboratory was blinded to all identity of the samples. A blinded testing

order ensuring that case-control sets were analysed together was used. During the entire study, only one cycle of freezing and thawing was performed.

The mouse monoclonal antibody NCL-JCBK, which reacts with both JC and BK polyomaviruses, was purchased from ImmunKemi (Järfälla, Sweden)[24]. The antibody was used as positive control in dilution 1/10,000. Human reference sera from 3 renal transplant recipients, who tested positive for BKV DNA in urine by Polymerase Chain reaction (PCR), were used as positive controls. The sera were used at a dilution 2-fold lower than the endpoint titer, 1/10,240, 1/640 and 1/40,960, respectively. The reference sera were kindly provided by the Swedish Institute for Infectious Disease Control. A pool of sera from healthy blood donors obtained from the Blood Donor Centre, Karolinska Hospital, Sweden, was also used as a positive control serum in dilution 1/40. A serum sample from a child aged 1 year and 9 months was used as negative control serum in all assays.

For reference and validation of the serologic assay we tested a subsample of 51 sera used in a previous study of 126 subjects from a cohort of homosexual men (median age 37 years) in Washington, D.C., and New York city recruited in 1982[25] and sampled between 1986 and 1996. Forty-nine subjects (39%) were HIV-positive. There was a wide spectrum of CD4 counts, consistent with varying degrees of immune suppression (CD4 count 0-249 cells/mm^3, n = 11; CD4 count 250-499 cells/mm^3, n = 18; CD4 count 500+ cells/mm^3, n = 20). Urine specimens from these subjects had been tested previously by polymerase chain reaction for presence of JCV and BKV DNA [26]. Of 51 serum samples tested in present study, 37 sera were from subjects with viruria for only JCV, 12 cases with only BKV viruria and 2 samples from subjects who tested positive for both JCV and BKV viruria [26]. In the present study, we measured the JCV and BKV antibody levels in the serum samples obtained concomitantly with these urine specimens.

Serum samples from a consecutive series of 1031 serum samples from children aged between 0 and 13 years submitted for clinical virological analyses to the Dept. of Clinical Virology, Karolinska Hospital, Sweden were stratified in age groups of 2 year intervals, whereafter a random subsample was selected from each age group. In the group of children 1.1-3.0 years old, 50 serum samples were selected and used as reference in a previous study [9]. For the present study, 44 of these samples were still available. The sera were tested in dilution 1/40 in HS-PBS.

Definition of cut-off values in our previous study was the mean value plus one standard deviation of the log absorbance values among the 1.1–3-year-old children [9]. Zero absorbance values were set to half of the lowest detectable absorbance before log transformation.

Statistical analyses

Odds ratios (OR) adjusted for age at serum sampling were estimated by conditional logistic regression with LogXact software version 6 (Cytel Software Corporation, Cambridge, MA). Reproducibility for the paired samples taken one year apart was evaluated using kappa statistics[26]. Odds ratios were estimated by conditional logistic regression[26]. Box plot diagrams of antibody levels were produced using Statistica software version 4,5 (StatSoft Inc, Tulsa, OK).

RESULTS

Assay validation

Sensitivity and specificity

For reference and validation of the serologic assay sera from viral shedders (individuals testing positive for JCV or/and BKV DNA in urine), non-shedders (testing negative for JCV and/or BKV in urine) and serum samples from children between 1-3 years of age (that should no longer have maternal antibodies present and presumably have had only limited exposure to the human polyomaviruses) were tested for JCV and BKV antibody levels.

The previously used cut-off value for determining seropositivity was based on distribution of seroreactivities in the children control group [9]. In the present study, we also calculated the cut-offs that gave maximum sensitivity for viruria and maximum specificity using either the children control group or the viruria-negative adult homosexual reference group that was positive for the other human polyomavirus as reference.

With the previously used cut-off, almost all positive control subjects with viral DNA in urine were seropositive (97% for JCV, 100% for BKV), with about 20% of children also testing positive (Table 1). Exploring different cut-offs found that it was possible to raise the cut-off somewhat without any loss of sensitivity, but with similar or identical specificity using the children reference group ("polyomavirus-specific cut-off"; Table 1). The adult homosexual group that was positive for one or the other human polyomavirus contained measurable antibody reactivity against both polyomaviruses (Table 1). However, the antibody levels for one of the polyomaviruses were considerably higher among the subjects who were shedding that virus at the time (Figure 1A and Figure 1B). Thus, the JCV antibody titres were higher among those testing positive for JCV DNA in urine, compared with the individuals testing BKV positive and JCV negative (Figure 1). The same pattern was seen for the BKV viruria

shedders who had higher BKV antibody titres compared to the subjects shedding JCV, but not BKV (Figure 1). It was possible to find a higher cut-off level that still had acceptable sensitivity for the corresponding polyomavirus (about 90%), while being reasonably specific (specificity 65-75%) using the adult homosexuals currently shedding only the other polyomavirus as reference group ("Virus-specific shedding cut-off").

The children group had much lower seroreactivity than the adult groups, but there was a minority of children that were strongly positive in particular for BKV (Figure 1). This is likely to reflect the established fact that JCV infections typically occur somewhat later in childhood than do BKV infections [5].

Stability over time

Eighty subjects who had two serum samples collected about 1 year apart were tested for polyomavirus antibodies to assess biological stability over time. Reproducibility assessed by Kappa statistics (k) between the two samples was high for JCV IgG positivity (k=0.83), but moderate for BKV (strain SB) IgG positivity (k=0.58). Four samples showed seroconversion for JCV antibodies and five samples for BKV antibodies. Only two samples demonstrated a seroreversion for JCV and 4 sera for BKV (Table 2). At the slightly higher "Polyomavirus specific cut off" the biological stability over time was about the same (JCV k=0.78; BKV k=0.57). At the highest "Virus specific shedding cut off" level JCV was less stable (k=0.68), but BKV perfectly stable (k=1.0).

Risk of future colorectal cancer in relation to baseline polyoma seropositivity

The JC virus IgG seroprevalence (using the previously used cut-off) was 72 % for subjects who later developed colon cancer compared with 74 % for matched control subjects who did not (OR = 0.91 ; 95% CI: 0.65 - 1.27). The BK virus IgG seroprevalence was 71% among cases and 69% among controls (OR = 1.09 ; 95% CI: 0.79 - 1.51) (Table 3).

Using the cut-off levels that gave maximum sensitivity and specificity ("polyomavirus-specific cut-offs"), JCV seropositive men had a significantly lower risk (OR = 0.69 ; 95% CI: 0.51 – 0.95) (Table 3). The confidence intervals have not been adjusted to reflect the fact that 2 different viruses were evaluated in 3 different cut-off levels, i.e. that 6 different testings were made.

Using the highest cut-off, that gave optimal virus specificity ("virus-specific shedding cut off"), the risks for colorectal cancer did not depart significantly from unity, neither among JCV nor among BKV seropositive subjects (Table 3).

DISCUSSION

We report a prospective population-based study that found no evidence for excess risk for colorectal cancer among men seropositive for JCV or BKV infection.

Several studies based on detection of viral DNA and/or studies on molecular transformation mechanisms have suggested an involvement of JCV in human cancers[18],[19]. However, one study found that among 233 colorectal cancer/normal tissue pairs none of the tumors and only one normal colon tissue specimen was JCV positive (<0.5%), while 70% of urine samples from healthy subjects were JCV positive by the same methods[20]. Also, while there are plausible mechanisms that could explain how JCV could have an effect on tumorigenesis, our finding that JCV-uninfected subjects have the same risk as JCV-exposed subjects suggests that JCV exposure is not a quantitatively important cause of these cancers,

Our observation that JCV seroprevalence was lower in cases than controls using one of the cut-off levels explored was unexpected. While random variability is one likely explanation, it is interesting to note that a lower JCV seroprevalence among cases has also been reported in a case-control study of non-Hodgkin´s lymphoma[25].

Polyomavirus serology is usually not used in diagnostics, but has been widely used in studies of the epidemiology of the infection[5]. Patients with the established JCV-associated disease PML are also known to have higher antibody levels than controls[27-29].

The serologic assay used in the present study was validated using an independent set of validation samples and found to have very high sensitivity for detecting subjects with polyomavirus shedding. The exact specificity of the assay can not be ascertained, as it is not possible to obtain samples established to be truly negative by independent methods (Only serology is able to detect latent infection with these polyomaviruses). However, the assays did have a substantial capacity to discriminate subjects with known infection from subjects with

no evidence of current shedding of virus or from subjects in age groups less likely to be infected (children below 3 years of age).

Epidemiological studies based on serology have several advantages. The sampling is readily standardised and measures exposure to the body as a whole, thus minimizing risks for differential detection in tissues taken from case and controls and/or non-representative samples. Also, this approach is essentially independent of the hypotheses on how viral carcinogenesis could occur. While a number of mechanisms whereby the polyomaviruses could increase the risk for cancer are possible, they all share the feature that the cancer risks should be lower among subjects who are not infected with these viruses.

The prospective biobank-based study design, when used in countries with complete nationwide case ascertainment, minimises most of the epidemiological sources of bias. For example, reverse causality biases are not likely to occur with the long follow-up times between blood draw and diagnosis of cancer, and selection biases due to incomplete attendance rate or inadequate study base definition are also unlikely[30].

Because tumour viruses are promising targets for cancer prevention, performing systematic seroepidemiological evaluation of as yet not established associations between tumour viruses and human cancer is important for future cancer research. Major associations between infections and cancer have been confirmed in similarly designed prospective biobank-based seroepidemiological studies, e g helicobacter pylori and stomach cancer[31], Epstein-Barr virus and Hodgkin´s lymphoma[32], non-Hodgkin´s lymphoma[33] and nasopharyngeal carcinoma[34] as well as papillomaviruses and cervical[35], anal[36], vulvar/vaginal[37] and oropharyngeal cancer[38]. Equally important, the biobank-based prospective seroepidemiological study design has also provided clear negative results for several claimed virus-tumour associations such as herpes

simplex and cervical cancer[39], human herpes virus 8 and myeloma[40] or BKV polyomavirus and neuroblastoma[41]. Our previous negative findings regarding BKV and neuroblastoma are of relevance to the present study, as the strength of evidence associating BKV with neuroblastoma was rather similar to the evidence associating JCV with colon cancer. BKV DNA was found in tumours by several methods, viral T antigen was found in the tumour cells and the BKV T antigen induced aberrant expression of p53 in these cells[42].

In summary, JCV or BKV seropositive men are not at increased risk for colorectal cancer arguing against a role of these infections in the etiology of this tumor.

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Table 1A. JCV seropositivity rates at different cut off levels for seropositivity among men with JCV DNA detected in urine, men without JCV DNA in urine and among healthy children 1-3 years of age.

Cut-off, OD-values for JCV	**JC DNA positive men (n=39) No. seropositive (%)**	**JC DNA negative men (n=12) No. seropositive (%)**	**Children (n=44) No. seropositive (%)**
Previously used cut off: OD =0.371	38 (97.4%)	10 (83.3%)	8 (18.2%)
Polyomavirus specific cut off: OD =0.550	38 (97.4%)	9 (75.0%)	7 (15.9%)
Virus specific shedding cut off: OD =0.850	35 (89.7%)	3 (25.0%)	5 (11.4%)

Table 1B. BKV seropositivity rates at different cut off levels for seropositivity among men with BKV DNA detected in urine, men without BKV DNA in urine and among healthy children 1-3 years of age.

Cut-off, OD-values for BKV	**BK DNA positive men(n=14) No. seropositive (%)**	**BK DNA negative men (n=37) No. seropositive (%)**	**Children (n=44) No. seropositive (%)**
Previously used cut off: OD = 0.450	14 (100%)	27 (73.0%)	9 (20.5%)
Polyomavirus specific cut off: OD = 0.500	14 (100%)	23 (62.2%)	9 (20.5%)
Virus specific shedding cut off: OD =0.700	13 (92.9%)	3 (35.1%)	6 (13.6%)

Table 2. Biological stability over time of JCV and BKV antibodes in serial samples taken 1 year apart from 80 healthy adult members of the biobank cohort

	Pos sample I Pos Sample II (+,+)	**Neg sample I Pos sample II (-,+)**	**Pos sample I Neg sample II (+,-)**	**Neg sample I Neg sample II (-,-)**
JCV	54 67.5 %	4 5%	2 2.5%	20 25%
BKV SB	63 78.8%	5 6.2%	4 5%	8 10%

Table 3. Relative risk for development of colorectal cancer during follow-up among healthy subjects participating in a population-based biobanking project.

Antibody status	Cut off	Index cases (n=386)	Matched controls (n=386)	OR (95% CI)	P-value
JCV IgG neg	Previously used[a]	109 (28.2%)	102 (26.4%)	1[r]	
JCV IgG pos	Previously used[a]	277 (71.8%)	284 (73.6%)	0.91 (0.65-1.27)	0.57
BKV IgG neg	Previously used[a]	113 (29.3%)	120 (31.1%)	1[r]	
BKV IgG pos	Previously used[a]	273 (70.7%)	266 (68.9%)	1.09 (0.79-1.51)	0.58
JCV IgG neg	Polyomavirus specific[b]	161 (41.7%)	131 (33.9%)	1[r]	
JCV IgG pos	Polyomavirus specific[b]	225 (58.3%)	255 (66.1%)	0.69 (0.51-0.95)	0.02
BKV IgG neg	Polyomavirus specific[b]	128 (33.2%)	146 (37.8%)	1[r]	
BKV IgG pos	Polyomavirus specific[b]	258 (66.8%)	240 (62.2%)	1.27 (0.92-1.75)	0.14
JCV IgG neg	Virus specific shedding[c]	234 (60.6%)	218 (56.5%)	1[r]	
JCV IgG pos	Virus specific shedding[c]	152 (39.4%)	168 (43.5%)	0.82 (0.61-1.12)	0.21
BKV IgG neg	Virus specific shedding[c]	231 (59.8%)	228 (59.1%)	1[r]	
BKV IgG pos	Virus specific shedding[c]	155 (40.2 %)	158 (40.9%)	0.96 (0.70-1.32)	0.81

[r] Reference category

a) Preassigned cut-off level for assigning seropositivity

b) Cut-off level for assigning seropositivity set to maximize sensitivity and specificity for JCV or BKV DNA shedding, using the healthy children as control group.

c) Cut-off level for assigning seropositivity set to maximize sensitivity and specificity for JCV or BKV DNA shedding, using subjects form the same cohort shedding the other polyomavirus as control group.

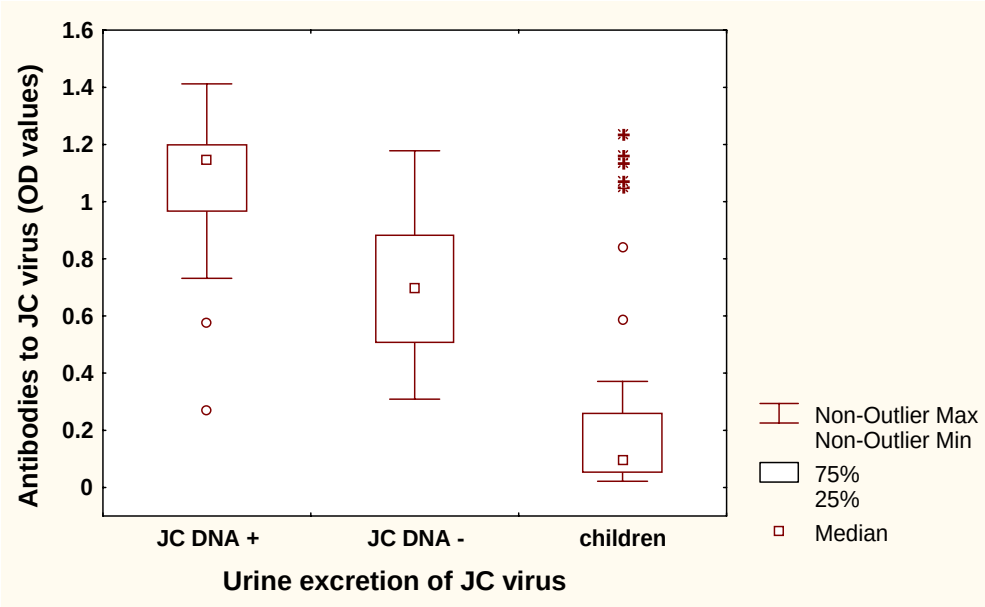

Figure 1A. JCV antibody levels among a group of men with JCV DNA detected in urine, men from the same cohort without JCV DNA in urine and among healthy children 1-3 years of age.

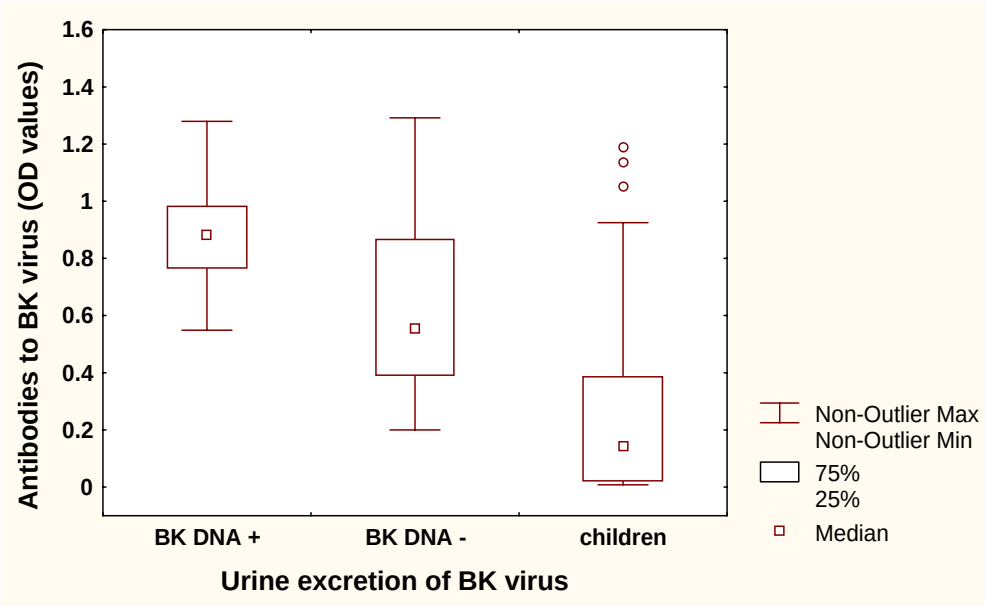

Figure 1B. BKV antibody levels among a group of men with BKV DNA detected in urine, men from the same cohort without BKV DNA in urine and among healthy children 1-3 years of age.