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Exposure to raccoon polyomavirus (RacPyV) in free-ranging North American raccoons (*Procyon lotor*)

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Abstract

There is evidence that raccoon polyomavirus is causative for neuroglial brain tumors in the western United States. It is unknown if infection is limited to geographic locales where tumors have been reported or is widespread, like human polyomaviruses. We demonstrate raccoons in western, eastern and midwestern states have been exposed to RacPyV by detection of antibodies to capsid protein, VP1. While raccoons in eastern and midwestern states are seropositive, exposure is lower than in the western states. Additionally, across geographic areas seropositivity is higher in older compared to younger raccoons, similar to polyomavirus exposure in humans. Serum titers are significantly higher in raccoons with tumors compared to raccoons without. Unlike polyomavirus-associated diseases in humans, we did not detect significant sequence variation between tumor and non-tumor tissue in raccoons with tumors compared to those without tumors. This warrants further investigation into co-morbid diseases or genetic susceptibility studies of the host.

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Keywords

Antibodies; polyomavirus seroprevalence; raccoons; sequencing; RacPyV; neuroglial brain tumors

INTRODUCTION

Raccoon polyomavirus (RacPyV) was discovered in 2010 in a cluster of free-ranging raccoons with morphologically similar neuroglial tumors affecting their olfactory tract and frontal lobe[1]. Every tumor (n=20) diagnosed to date has high quantities of RacPyV genomic DNA present as detected by real-time quantitative polymerase chain reaction (qPCR) (M Church, unpublished data). Despite extensive surveillance of raccoons (given their role as vectors of rabies) and communications with pathologists and wildlife rehabilitators regarding our research, tumor cases have only been found in the western United States (California, Oregon, and Washington). A crucial step in establishing whether RacPyV plays a causal role in these tumors is to determine if exposure to RacPyV is limited to locations where tumors occur.

As measured by seroprevalence studies, exposure to human polyomaviruses (PyVs) is widespread and, in general, PyVs cause lifelong, clinically silent infections[2]. However, polyomaviral-associated disease does occur - mainly in severely immune suppressed people following long term, persistent infection[3]. The majority of adult humans have antibodies to all thirteen identified human polyomaviruses[2,4–6]. Very little has been published regarding seroprevalence of naturally occurring polyomaviruses in animals, though one long-term study demonstrated high seroprevalence of avian polyomavirus in psittacines[7]. We hypothesize that exposure to RacPyV mirrors that of human polyomavirus, with geographically widespread seroprevalence not restricted to areas where tumors have been identified. Moreover, we postulate that while exposure is widespread, associated disease is rare. To examine the geographic distribution of RacPyV seroprevalence and correlate it to disease status, we compared seropositivity in raccoons sampled from eleven states within the U.S. and three Canadian provinces. We evaluated seropositivity and serological titers with respect to geography, disease status, age, sex, and habitat.

Sequence variation between polyomavirus genotypes has been demonstrated to have effects on pathological outcomes[8–10]. For example, sequences in the noncoding region of the polyomavirus genome differ between tumor tissue and non-tumor tissues (sites of viral persistence) in mouse polyomavirus[11] and Merkel cell polyomavirus[12], PyVs which infect mice and humans, respectively. In addition, single nucleotide polymorphisms in the capsid protein gene VP1 resulting in single amino acid differences, have been associated with tumorigenic and nonpathogenic strains of mouse polyomavirus[10]. Comparing RacPyV isolates from different geographic locales, as well as isolates from tumor or non-tumor tissues could reveal specific sequence variations associated with tumor formation.

MATERIALS AND METHODS

Serum collection

Serum samples were collected from 499 raccoons from eleven United States (CA, FL, GA, IL, KY, MA, MO, NJ, NY, WA, WV) and three Canadian provinces (NB, ON, QU). Samples were collected for either routine disease surveillance or part of unrelated investigations. Some samples were aliquots from blood taken for clinical evaluation at rehabilitation centers (CA, IL, MA, WA), while others were collected as part of infectious disease surveillance projects (CA, FL, GA, KY, MO, NJ, NY, WV, BN, ON, QU) including those monitoring rabies, canine distemper virus, and *Leptospira* spp. All samples were collected in accordance with the specific animal care and control guidelines established at each institution. Serum samples were initially stored at collaborator institutions at 4° C (up to 3 months). Samples were either shipped directly within that time period, or stored at −20° or −80° C until shipment. Samples were shipped on dry ice, and stored at −80° C at UC Davis until time of analysis.

ELISA

RacPyV-specific immunoglobulin (IgG) was detected utilizing an indirect ELISA. 96-well Maxisorp plates (Fisher Scientific) were coated with recombinant RacPyV VP1 antigen, produced using a baculovirus expression system in insect cells[13]. Briefly, 10 ng purified recombinant RacPyV VP1 antigen per well was diluted in PBS and stored at 4°C overnight and/or for up to 4 weeks prior to use. Plates were washed in PBS with 0.1% Tween 20 (PBS-T) and blocked with 5% nonfat milk in PBS-T for two hours at room temperature. Serum samples were diluted 1:200 in 5% milk PBS-T and incubated for two hours at room temperature. Plates were washed in PBS-T and then incubated for one hour at room temperature with either mouse-anti-rabbit secondary antibodies (control; Bio-Rac, Hercules, CA, USA) or goat-anti-raccoon secondary antibodies conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA). ELISA plates were then washed in PBS-T and secondary IgG binding was visualized by addition of 100 μL of 2, 2′-Azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) HRP substrate solution while shaking (KPL Inc., Gaithersburg, MD, USA). Optical density (OD) was measured at 405 nm on a BioTek ELx808 absorbance microplate reader. Each plate contained a column with a positive control antibody (rabbit anti-VP1 polyclonal antibody) and a negative control antibody (pre-immune rabbit serum). For each sample, the corrected OD was calculated by subtracting the negative control antibody from the OD of the sample well for each serum sample. The cut-off value (CO) was calculated as follows: CO = A + 2SD, where A is the average OD of all dilutions of negative control antibody on the plate and SD is the standard deviation. Samples were considered positive when the corrected OD was above the cutoff, and titers were defined as the highest dilution past the cut-off.

DNA Extraction and nucleic acid amplification

Tumor tissue was collected from 16 raccoons in CA and 1 from WA for sequencing of the RacPyV genome. Persistent PyVs have been detected in many different tissues, including kidney, spleen, tonsil, oral cavity, skin, and gastrointestinal tract,[14–18] therefore a variety of non-tumor tissues were sampled for DNA extraction. Biological samples (oropharyngeal/

nasal swabs, tonsil, and spleen) from a subset of these tumor-bearing raccoons (3 from CA and 1 from WA) were also collected. Biological samples (urine, oropharyngeal/nasal swabs, feces, skin, olfactory bulb) from 6 raccoons in California that did not have tumors were collected as well. All samples were donated from wildlife rehabilitators in Washington (PAWS Wildlife Center in Snowach County) and from California including WildCare in Marin County, Lindsay Wildlife Museum in Contra Costa County, Sonoma County Wildlife Rescue, Grass Valley Wildlife Rehabilitation & Release and USDA APHIS Wildlife Services in Sacramento County. Spleen samples were collected from 32 raccoons in Georgia and kidney samples from 14 raccoons in Kentucky from the Southeastern Cooperative Wildlife Disease Study. Tissue samples were maintained at −80° C until DNA extraction. DNA from tumor tissue samples was extracted using Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions. Tissue samples from non-tumor samples were thawed in a chaotropic lysis buffer (DXB, Qiagen, Valencia, CA) and immediately homogenized in a GenoGrinder2000 (SpexCertiprep, Metuchen, NJ) for 2 min at 1000 strokes per minute. Homogenized tissue pieces were proteinase K digested at 56° C overnight. Total nucleic acids (including gDNA and RNA) were extracted from lysates with adapted standardized protocols as previously described[19]. Briefly, lysates were extracted on Whatman filters in a Corbett X-Tractor platform (Qiagen, Valencia, CA). Nucleic acids were eluted into 150 ul of PCR-grade nuclease-free water (Fisher) and 5 ul amplified in subsequent PCR reactions.

Extracted DNA from non-tumor samples were enriched for circular DNA using directed rolling circular amplification following a protocol outlined by Rockett et al., 2015 using Phi29 DNA polymerase. Circular template was initially primed with a primer set targeting the VP1 gene and modified with phosphorothioate on the two terminal 3′ bases to protect against phi29 single strand exonuclease activity. Primers (0.4μM) and 2 μL template were combined with 1× phi29 buffer (Thermo Scientific, Scoresby, Australia) and water up to 10 μL at 95 ° C for 3 min. The template was then amplified using 0.4 μM primer, 1× phi29 buffer, 2ng/μL bovine serum albumin, 15 mM dNTPs, 2U/μL phi29 DNA polymerase (Thermo Scientific, Scoresby, Australia), and water to a final reaction volume of 10 μL. This was combined with the pre-amplification mix and incubated at 30° C for 24 h, followed by phi29 inactivation at 65° C for 10 min and cooling to 4° C.

Sequencing

Standard PCR with Accuprime DNA Taq polymerase (Life Technologies, Thermo Fisher Scientific) was then performed to amplify the RacPyV genome from the product of the dRCA reactions for sequencing. Samples were sequenced by at the College of Biological Sciences ^{UC}DNA Sequencing Facility Core Facility using Sanger sequencing protocols (BigDye® Terminator v. 3.1 Cycle Sequencing Kit). Sequence reads were assembled using MacVector (Macvector, Inc) and VectorNTI (Life Technologies) software. A consensus sequence for RacPyV sequences from tumor samples was obtained using the following criteria: if all or all but one sample agree, the consensus was defined by the sequence contribution and any unconfirmed position will be designated N. This tumor consensus sequence was then compared to the segments of RacPyV sequenced from non-tumor tissues

from raccoons in California and Washington with and without tumors, and from the spleens of raccoons from Georgia.

Statistical analysis

R statistical software was used to perform statistical analyses (significance level set at 5%) and to create maps and graphs. Comparisons between seroprevalence rates between different groups of raccoons (sex, age, geography, ecosystem type) were made using Fisher's exact test. In order to compare antibody titers between groups, a one-way ANOVA was applied using GraphPad Prism software. P values less than 0.05 were considered significant.

Statistical analysis to determine the significance of the nucleotide differences between RacPyV sequences was performed using an exact test of goodness of fit (using number of single nucleotide polymorphisms) and a Fisher's exact test (using percentage of nucleotide differences from the tumor consensus sequence). Sequence variation was compared between the following cohorts: 1) tumor tissue 2) non-tumor tissue from tumor-bearing raccoons 3) non-tumor tissue from non-tumor bearing raccoons in California, and 4) non-tumor tissue from non-tumor bearing raccoons in Georgia.

RESULTS

Seroprevalence of RacPyV

A total of 182 serum samples from 11 American states and 3 Canadian provinces were positive for antibodies to RacPyV VP1 when analyzed by ELISA (from a total of 499 samples analyzed). Numbers of available samples per state ranged from 3 (WA) to 75 (GA), and antibodies to RacPyV were detected in serum samples from all states and provinces examined (TABLE 1). Results of the seroprevalence survey demonstrate that raccoons from 11 states across the U.S. have antibodies to the VP1 capsid protein of RacPyV (FIGURE 1). The seropositivity in areas where tumors have been diagnosed (CA and WA) was greater than 60%, and ranged in other states from 5% in WV to 57% in NY. Percentage of positive samples from Canada ranged from 43 to 95%. The seroprevalence of raccoons in CA was compared to that of raccoons in other states (FIGURE 2). The proportion of seropositive raccoons in CA was significantly higher than the proportion in a majority of the states examined (FL, GA, IL, KY, NJ, WV; $P<0.05$, Fisher's exact test). The most striking difference in seropositivity was between raccoons with and without tumors (FIGURE 3). In addition, a significant difference between the seroprevalence of adults and juveniles was determined ($P<0.05$, Fisher's exact test). Juvenile raccoons had a seropositivity of 16.8%, while 45.7% of samples adult raccoons were seropositive. No significant difference was detected between proportions of seropositive samples from females compared to males nor of raccoons from rural habitats compared to those from suburban or urban habitats.

RacPyV antibody titers

RacPyV antibody titer values ranged from 200 to 102400, and varied by state (FIGURE 4 and TABLE 1). Highest titer values 1:102400 were detected in samples from CA, GA, and MO. The mean titer values varied significantly between CA and the following states: FL, NY, KY and the province of NB ($P < 0.0001$, Kruskall-Wallis). In addition, the mean titer

values of serum samples from raccoons with tumors was significantly higher than from without tumors (P = 0.0065, Mann-Whitney) (TABLE 2).

RacPyV Consensus Sequence

The consensus RacPyV sequence derived from the alignment of 17 tumor-derived sequences with whole viral genome coverage (Rac 2, 3, 4, 5, 8–20) is given in supplemental FASTA file. The sequences obtained from tumor tissue from the fourth (Rac 4) and seventeenth (Rac 17) tumor-bearing raccoons matched the consensus sequence identically. The RacPyV sequences derived from the remaining California tumor samples exhibit a range of 1 to 11 single nucleotide polymorphisms (SNPs) that varied from the consensus sequence (average = 5.1, standard deviation = 4.1, see TABLE 3 and Supplemental Table 1). In addition, seven sequences (Rac 5, 9, 10, 12, 14, 19 20) had deletions of 1 (Rac 10, 14) to 349 (Rac 9) nucleotides. Sequence identities ranged from 93.04% (Rac 9) to 99.98% (Rac 2, 3, 11, 14, 18).

The sequence from the tumor identified in Washington contained 24 SNPs, two of which (base pair (bp) 1503 and 2172) were unique to that sample and not shared with any other sequence from any other tumor sequences. Two SNPs in the T antigen (TAg) coding region at (bp 18 and 2145), and four in the VP1 coding region (bp 2866, 3223, and 3328) were present in sequences from seven tumors (Rac 5, 8, 12, 13, 15, 16, and 19). Sequence identity to the consensus sequence was 99.52%.

In four tumor-bearing raccoons where viral sequences could be derived from non-tumor tissue, all raccoons exhibited identical RacPyV sequences between tumor and non-tumor tissue (California Rac 14, 16, and 17 and Washington Rac 13). The RacPyV sequence obtained from tonsil tissue from one California raccoon (Rac 16) did not match the sequence from the tumor tissue from that animal. The sequence from the tonsil tissue from this raccoon matched that of the RacPyV consensus sequence.

RacPyV segments sequenced from non-tumor tissue from three of the six raccoons in California without tumors shared SNPs with sequences from tumor tissue. In the other three cases, there were unique SNPs identified in each sample (1 unique SNP in 2012F20Rac1Mar, 1 unique SNP in 2013D29Rac1CC, and 2 unique SNPs in 2012E29Rac1Pla), however, none of the SNPs did not result in an amino acid difference from the consensus.

Across all 23 raccoons examined, the VP1 coding region contained the most SNPs detected (67 SNPs) compared to other coding regions (TAg: 46 SNPs, VP2: 12 SNPs, NCRR: 2 SNPs). The numbers of SNPs in distinct areas of the RacPyV genome were not statistically significant as determined by Chi-square analysis.

Amino acid variation between RacPyV sequences

Tumor sequences—Within viral genes, eight (Rac 3, 5, 8, 12, 13, 15, 16, 19) of the sequences from tumors had SNPs in the TAg region, ten (Rac 2, 5, 8, 10, 11, 12, 13, 15, 16, 19) had SNPs in the VP1 region, and three (Rac 13 and 20) had SNPs in VP2. Of the SNPs found in the TAg region two were classified as point mutations resulting in amino acid

changes in the predicted large T protein (Rac 3 and 13) and one resulted in amino acid changes in the predicted small T protein (Rac 13). All ten SNPs in the VP1 region were synonymous, while both of the SNPs in the VP2 region were nonsynonymous point mutations that resulted in changes in amino acid sequences from the consensus.

In addition to SNPs, sequences from seven tumors (Rac 5, 9, 10, 12, 14, 19, 20) had deletions of one to 25 nucleotides. Two of the sequences resulted in a 4 amino acid truncation of the LT protein (Rac 9) and in an in-frame 4 amino acid deletion (Rac 19) 21 amino acids from the C terminus of the LT protein. Deletions in the T antigen region resulted in amino acid changes in predicted small T protein amino acid sequences in four tumor sequences. Nucleotide deletions in Rac 10 and 20 resulted in single amino acid changes in the sT protein. The sT protein of Rac 12 was truncated by 7 amino acids at the C terminus, and the sT protein of Rac 5 had an 8 amino acid deletion mid-protein. Sequences from two tumors (Rac 9, 12) had deletions in the VP1 gene resulting in a 107 amino acid truncation of the predicted protein (Rac 9) and in in-frame mid-protein deletion of 16 amino acids (Rac 12). A single nucleotide deletion in Rac 14 resulted in an amino acid change in VP2 protein.

Non-tumor sequences—Nucleotide sequences obtained from non-tumor tissue in four raccoons with tumors did not result in any amino acid sequence variation from the consensus sequence derived from tumor sequences.

Sequence variation from only one of the six nucleotide sequences obtained from non-tumor tissues from raccoons were synonymous. Feces from raccoon 2013D29Rac1CC, had a sequence that shared many SNPs in the sequence from tumor tissue from Rac 13, which resulted in two amino acid changes detected in Rac 13 as well: one amino acid change in the sT protein and one amino acid change in the VP2 protein.

CONCLUSIONS

Exposure to RacPyV is widespread and not limited to tumor endemic areas

To assess the geographical seroprevalence of RacPyV, we developed an indirect ELISA utilizing a recombinant capsid protein, RacPyV VP1, and analyzed sera from 499 raccoons across North America. We found that although RacPyV-associated neuroglial tumors have only been detected in raccoons in California, Oregon and Washington, raccoons across North America are exposed to RacPyV. This is similar to widespread exposure patterns reported for human polyomaviruses[2,20,21]. Seropositivity percentages in raccoons from Canadian provinces were just as high or higher compared to California, and these data warrant further investigations into tumor incidence in this region. The seropositivity percentage was significantly higher in American western states (where tumors have been diagnosed) compared to the eastern states (where no tumors have been diagnosed). Several scenarios could explain this discrepancy. First, this could represent lower exposure to RacPyV in eastern states, coinciding with a lower/undetected incidence of associated brain tumors in the east. Second, it is possible there is a yet-undiscovered polyomavirus endemic in raccoons in eastern states with a VP1 sequence that varies from the RacPyV isolates sequenced in the western US. This could result in a different amino acid sequence, allowing

for cross-reactivity by antibodies and account for the lower seropositivity and the lower median titer values in the eastern U.S. compared to samples from the western U.S. We obtained spleen and kidney samples from raccoons in GA and KY, respectively, in an attempt to evaluate RacPyV sequences from east coast raccoons. We were unsuccessful in obtaining interpretable sequence information, which may be due to several reasons: 1) none of the samples contained RacPyV, 2) RacPyV was present in too small a quantity to amplify by directed rolling circle amplification, or 3) RacPyV sequence in these tissues varied too much from the consensus sequence to be detected by directed rolling circle amplification. Future studies to examine RacPyV in these tissues or additional prospectively collected samples might be informative utilizing more sensitive techniques like deep sequencing.

Interestingly, prevalence of seropositive raccoons was highest in samples from New Brunswick, which is geographically disparate from where RacPyV associated tumors have been diagnosed. This discrepancy could be due to a number of reasons. First, there might be tumors that have previously gone undiagnosed in New Brunswick. Additionally, signalment was not provided in the data gathered from this location, meaning it is possible that adult animals were overrepresented compared to California or other states. Finally, New Brunswick was also the only state or province with samples collected from 2008. Therefore this could represent a year of viral introduction or rapid spread through raccoon populations, however we would need samples from other areas in that year to confirm this.

Seropositivity is higher in older raccoons compared to young raccoons

Seropositivity is greater in adult raccoons than in young raccoons, which is a trend that is also seen in seroprevalence studies of human polyomaviruses[22–25]. Polyomavirus infection in humans occurs in childhood, though the exact mode of transmission unknown, and for BK virus, has been suggested to occur vertically as well as horizontally[26,27]. The mode of transmission of RacPyV is still unknown, though our finding that older raccoons are exposed to RacPyV at a greater percentage than young raccoons suggests it could be similar to other polyomaviruses. No significant differences in exposure were detected between sexes or between raccoons in different habitats (suburban vs. rural), which is similar to what has been shown in human polyomaviruses[2].

Elevated titers in tumor-affected raccoons

Antibody titers are higher in raccoons with tumors and in raccoons in tumor endemic areas. Elevated antibody titers associated with polyomavirus induced disease have been reported in studies of progressive multifocal leukoencephalopathy caused by JC virus[28,29] and Merkel cell carcinoma caused by Merkel cell polyomavirus[30]. This increased humoral response in tumor-affected raccoons suggests a prolonged exposure or an increase in viral replication during development of disease, followed by a humoral response and further supports previous work demonstrating the role of RacPyV in causation of these tumors[31].

RacPyV sequence variation is minor between groups of raccoons

Geographically discrete disease outbreaks, similar to the localized occurrence of these RacPyV-associated tumors are attributed to a number of causes, including: a change in the genetic makeup of the causative pathogen, a unique genomic susceptibility marker of the

host, or a unique co-morbidity[2]. Here, we examined RacPyV sequences from tissues of raccoons with and without tumors and have demonstrated that there is no compelling genetic variation between RacPyV isolates from these raccoons (e.g. in tumor versus non-tumor tissue). In addition, full necropsies were performed on all raccoons with tumors and no concurrent infections were diagnosed at the time of death by gross or histological examination. Thus, while previous infection or unidentified co-morbid conditions might have predisposed raccoons to tumor formation, no such conditions were identified at the time of necropsy in this investigation, although we cannot rule out the immune status of the affected animals at the time that they were infected with RacPyV. Another important possibility is that there is something inherent to the raccoon genome within the western United States (i.e. MHC haplotype) that contributes to tumor formation following RacPyV infection. Further investigation into the genetic identity of raccoons from disparate geographic locations might elucidate a genetic predisposition to viral associated transformation. This work suggests that RacPyV-associated disease is more complex than the variation in viral sequence (either by integration or mutation) seen with other polyomaviruses (i.e. JC virus[32,33] and mouse polyomavirus[34]) and may involve a complex interplay between environment and co-pathogens. RacPyV might represent a newly emergent infection in raccoons in the western US, and by continuing to study the genomic drift, we will be able to identify specific changes that are unique to, or necessary for disease causation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- Established seroprevalence for raccoon polyomavirus (RacPyV) in the United States and Canada
- Demonstrated that infection with RacPyV is not limited to raccoons affected by neuroglial tumors
- Identified and annotated a consensus sequence for RacPyV

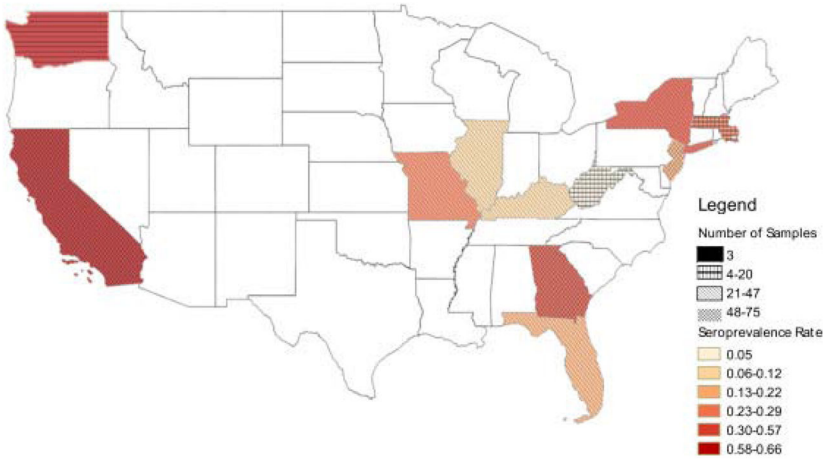

Fig. 1.

Seroprevalence of antibodies to RacPyV VP1 protein in serum samples from raccoons in the United States. Number of samples in states ranged from 3 (in WA) to 75 (in GA). Seroprevalence in states with tumors (CA and WA) are greater than 0.6, with other states ranging from 0.05 (in WV) to 0.57 (in NY).

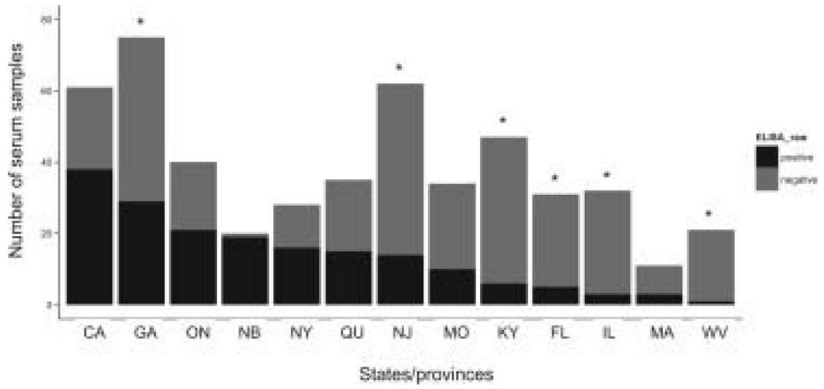

Fig. 2.

Number of positive and negative serum samples from raccoons in ten states and three provinces. States with significantly lower seroprevalence percentages than CA are indicated by an asterisk ($p<0.05$).

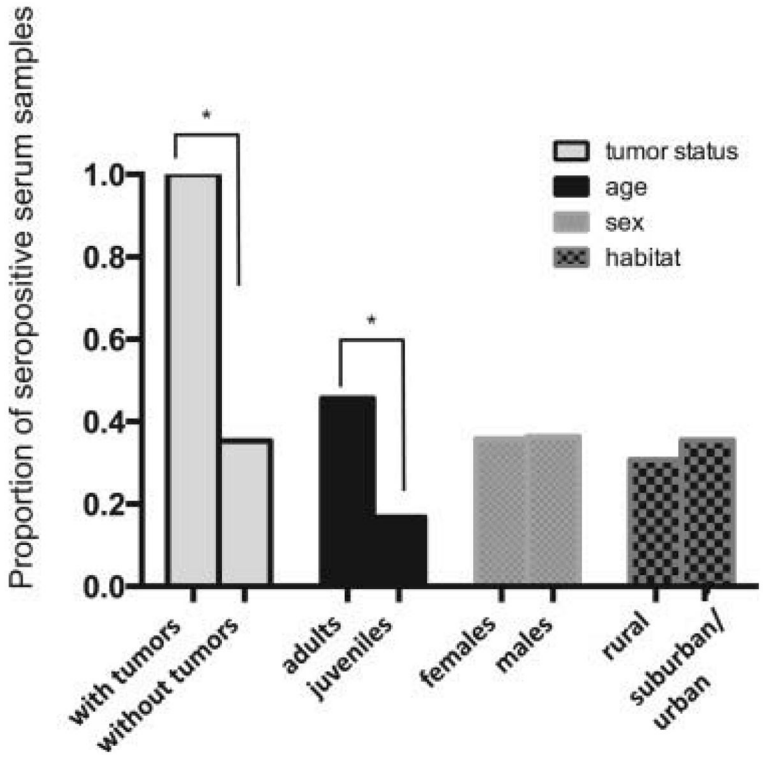

Fig. 3.

Proportions of seropositive serum samples from 1) raccoons with and without tumors, 2) adult and juvenile raccoons, 3) female and male raccoons, and 4) raccoons from rural and suburban or urban environments. Statistically significant differences of seropositive proportions between groups are indicated by asterisks ($p<0.05$).

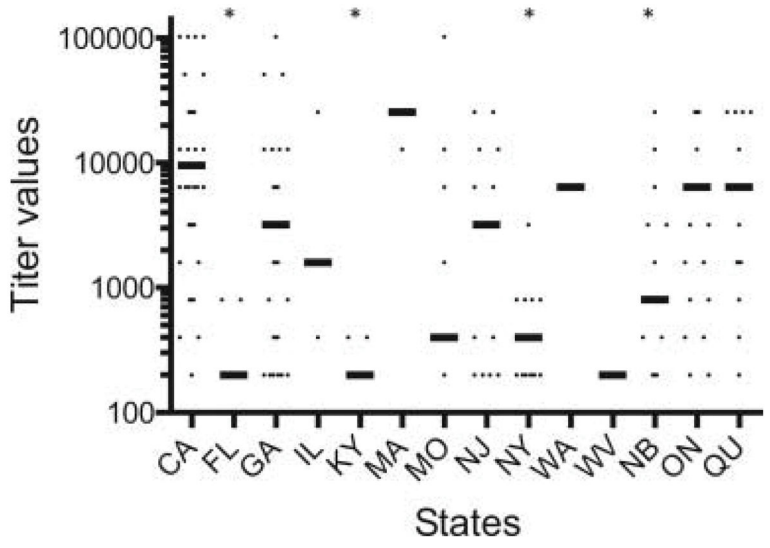

Fig. 4.

Titer values for serum samples from raccoons in the United States and Canada analyzed by indirect ELISA. Highest titer values (1:102,400) were detected in samples from CA, GA, and MO. The mean titer values varied significantly between CA and FL, KY, NY, and NB ($p<0.0001$). Median titers are represented by the black horizontal bar in each column.

Table 1

Serum samples collected from free ranging raccoons in North America and analyzed for seroreactivity to RacPyV VP1 protein by indirect ELISA.

State or Province	Number of samples	% Positive samples	Median titer value	Range of titer values
CA	61	0.62	9600	200–102400
FL	31	0.16	200	200 – 800
GA	75	0.39	3200	200 – 102400
IL	32	0.09	1600	400 – 25600
KY	47	0.13	200	200 – 400
MA	11	0.27	25600	12800 – 25600
MO	34	0.29	400	200 – 102400
NJ	62	0.23	3200	200 – 25600
NY	28	0.57	400	200 – 3200
WA	3	0.67	6400	6400 – 6400
WV	20	0.05	200	200 – 200
NB	20	0.95	800	200 – 25600
ON	40	0.525	6400	200 – 25600
QU	35	0.43	6400	200 – 25600
total	499	0.36	1600	200 – 102400

Table 2

Mean titer values of serum samples from raccoons with tumors were significantly higher (P = 0.0065, Mann Whitney) compared to samples from raccoons with tumors.

Titer values	Tumor	Non-Tumor
Number of values	7	176
Minimum	3200	200
25% Percentile	6400	400
Median	12800	1600
75% Percentile	25600	12800
Maximum	102400	102400
Mean	26971	9930
Std. Deviation	34359	19218
Std. Error of Mean	12986	1449

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Table 3

Summary of sequencing data from 1) tumor samples, 2) non-tumor tissue from raccoons with tumors, and 3) non-tumor tissue from raccoons without tumors. No significant differences were detected between sequences from any of the groups.

Raccoon	Tumor	State	Tissue sequenced	regions amplified	size of amplicon	No. of SNPs	SNP position(s)	Deletion positions	% sequence identity to consensus
2010D29Rac1Mar (Rac 4)	yes	CA	tumor	entire RacPyV genome	5016		**consensus**		100.00%
2014D09Rac1Mar (Rac 17)	yes	CA	tumor	entire RacPyV genome	5016		**consensus**		100.00%
2011F09Rac1Mar (Rac 2)	yes	CA	tumor	entire RacPyV genome	5016	1	3295 (VP1)		99.98%
2011H23Rac1Mar (Rac 3)	yes	CA	tumor	entire RacPyV genome	5016	1	2696 (TAg)		99.98%
2012F19Rac1Mar (Rac 11)	yes	CA	tumor	entire RacPyV genome	5016	1	3358 (VP1)		99.98%
2014G29Rac1Mar (Rac 18)	yes	CA	tumor	entire RacPyV genome	5016	1	4279 (VP2)		99.98%
2013F12Rac1Mar (Rac 14)	yes	CA	tumor	entire RacPyV genome	5015	single nucleotide deletion		4564 (VP2)	99.98%
2015C27Rac1Mar (Rac 20)	yes	CA	tumor	entire RacPyV genome	4991	1 (1 deletion)	4252 (VP2)	290–314 (TAg)	99.48%
2012D18Rac1Son (Rac 9)	yes	CA	tumor	entire RacPyV genome	4718	2 deletions		2692 – 2742 (TAg-VP1), 2744 – 3041 (VP1)	93.04%
2012E04Rac1Mar (Rac 10)	yes	CA	tumor	entire RacPyV genome	5015	1 (1 deletion)	3160 (VP1), 4843 (NCRR)	385 (TAg)	99.94%
2012K15Rac1Yolo (Rac 12)	yes	CA	tumor	entire RacPyV genome	4945	7 (2 deletions)	18 (TAg), 2145 (TAg), 2776 (VP1), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3328 (VP1)	523 – 545 (TAg), 3640 – 3687 (VP1)	98.44%
2012C07Rac1CC (Rac 8)	yes	CA	tumor	entire RacPyV genome	5016	8	18 (TAg), 2145 (TAg), 2776 (VP1), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3328 (VP1), 3640 (VP1)		99.84%
2013G02Rac1Mar (Rac 15)	yes	CA	tumor	entire RacPyV genome	5016	9	18 (TAg), 1401 (TAg), 1665 (TAg), 2145 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3346		99.82%

Raccoon	Tumor	State	Tissue sequenced	regions amplified	size of amplicon	No. of SNPs	SNP position(s)	Deletion positions	% sequence identity to consensus
							(VP1), 3640 (VP1) (VP1), 3640 (VP1)		
2013I26Rac1Mar (Rac 16)	yes	CA	tumor	entire RacPyV genome	5016	9	18 (TAg), 1401 (TAg), 1665 (TAg), 2145 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3346 (VP1), 3640 (VP1)		99.82%
2011L28Rac1SF (Rac 5)	yes	CA	tumor	entire RacPyV genome	4978	9 (2 deletions)	18 (TAg), 1401 (TAg), 1665 (TAg), 2145 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3346 (VP1), 3640 (VP1)	250 – 275 (TAg), 4781 – 4792 (NCRR)	99.06%
2014H22Rac1Pla (Rac 19)	yes	CA	tumor	entire RacPyV genome	5004	11 (1 deletion)	18 (TAg), 1401 (TAg), 1665 (TAg), 2145 (TAg), 2328 (TAg), 2696 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3640 (VP1), 4886 (NCRR)	2668 – 2679 (TAg)	99.54%
2013A08Rac1SnoWA (Rac 13)	yes	WA	tumor	entire RacPyV genome	5016	24	18 (TAg), 51 (TAg), 306 (TAg), 382 (TAg), 1503 (TAg), 2145 (TAg), 2160 (TAg), 2172 (TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3172 (VP1), 3208 (VP1), 3505 (VP1), 3535 (VP1), 3568 (VP1), 3592		99.52%

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Raccoon	Tumor	State	Tissue sequenced	regions amplified	size of amplicon	No. of SNPs	SNP position(s)	Deletion positions	% sequence identity to consensus
							(VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI (VP1), 3730 (VP1), 4009 (VP2), 4019 (VP2), 4283 (VP2), 4828 (VI		
2013A08Rac1WA (Rac 13)	yes	WA	OPN swab	entire RacPyV genome	5016	no variation from sequence determined from tumor from same raccoon			99.52%
2013F12Rac1Mar (Rac 14)	yes	CA	tonsil	TAg: bp 1002–1676 VP: bp 2962–4209	1923	no variation from sequence determined from tumor from same raccoon			ND
2013I26Rac1Mar (Rac 16)	yes	CA	tonsil	TAg: bp 1002–1674 VP: bp 2509–4209	2374	1	1665 (TAg)		ND
2014D09Rac1Mar (Rac 17)	yes	CA	spleen	TAg: bp 1002–1675 VP: bp 2509–4209	2375	no variation from sequence determined from tumor from same raccoon			ND
2013B06Rac2Son	no	CA	feces	bp 1522–2974; 3031–4116	2538	1	2866 (VP1)		ND
2012E29Rac1Pla	no	CA	urine	entire RacPyV genome	5016	2	3166 (VP1), 3977 (VP1)		99.96%
2012F20Rac1Mar	no	CA	OPN swab	entire RacPyV genome	5016	2	3538 (VP1), 3703 (VP1)		99.96%
2013F10Rac1Sac	no	CA	olfactory bulb	bp 2037– 2974; 3038–3712	1611	6	2145 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3346 (VP1), 3640 (VP1)		ND
2013C30Rac1CC	no	CA	skin	entire RacPyV genome	5016	8	18 (TAg), 2145 (TAg), 2776 (TAg), 2866 (VP1), 3058 (VP1), 3223 (VP1), 3328 (VP1), 3640 (VP1)		99.84%
2013D29Rac1CC	no	CA	feces	bp 1–1149; bp 1503–5016	4662	22	18 (TAg), 51 (TAg), 382 (TAg), 2145 (TAg), 2160		ND

Raccoon	Tumor	State	Tissue sequenced	regions amplified	size of amplicon	No. of SNPs	SNP position(s)	Deletion positions	% sequence identity to consensus
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		
							(TAg), 2214 (TAg), 2586 (TAg), 2974 (VP1), 3058 (VP1), 3074 (V		