The fate of urinary tract virus BK human polyomavirus in source-separated urine

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Running Head: BK human polyomavirus in source-separated urine

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Abstract

Human polyomaviruses are emerging pathogens that infect a large percentage of the human population and are excreted in urine. Consequently, urine that is collected for fertilizer production often has high concentrations of polyomavirus genes. We studied the fate of infectious double-stranded DNA (dsDNA) BK human polyomavirus (BKPyV) in hydrolyzed source-separated urine with infectivity assays and qPCR. Although BKPyV genomes persisted in the hydrolyzed urine for long periods of time (T_{90} >3 weeks), the viruses were rapidly inactivated (T_{90} = 1.1-11 hours) in most of the tested urine samples. Interestingly, the infectivity of dsDNA bacteriophage surrogate T3 was much more persistent than BKPyV (T_{90} = 24-46 days), highlighting a major shortcoming of using bacteriophages as human virus surrogates. Pasteurization and filtration experiments suggest BKPyV virus inactivation was due to microorganism activity in the source-separated urine, and SDS-PAGE western blots showed that BKPyV protein capsid disassembly is concurrent with inactivation. Our results imply that stored urine may not pose a substantial risk of BKPyV transmission, that qPCR and infectivity of the dsDNA surrogate do not accurately depict BKPyV fate, and that microbial inactivation may be driven by structural elements of the BKPyV capsid.

Importance

We demonstrate that a common urinary tract virus has a high susceptibility to the conditions in hydrolyzed urine and consequently would not be a substantial exposure route to humans using urine-derived fertilizers. The results have significant implications for understanding virus fate. First, by demonstrating that the dsDNA (double-stranded DNA) genome of the polyomavirus lasts for weeks despite infectivity lasting for hours to days, our work highlights the shortcomings of using qPCR to estimate risks from unculturable viruses. Second, commonly used dsDNA surrogate viruses survived for weeks in the same conditions that BK polyomavirus survived for only hours, highlighting issues with using virus surrogates to predict how human viruses will behave in the environment. Finally, our mechanistic inactivation analysis provides strong evidence that microbial activity drives rapid virus inactivation, likely through capsid disassembly. Overall, our work underlines how subtle structural differences between viruses can greatly impact their environmental fate.

54 **Introduction**

55 Enteric viruses that arise from fecal contamination have long been known to be of
56 serious concern for public health. Viruses that infect the urinary tract of humans can also
57 be shed in high quantities yet their concentrations and fate in the environment are unclear.
58 Zika virus, for example, is excreted in urine (1) and can cause microcephaly in newborn
59 children of infected mothers (2). Similarly, cytomegalovirus (CMV) is shed in the urine
60 of infected individuals and is a risk to infants of infected mothers as the virus can cause
61 hearing and vision loss and other developmental disabilities (3).

62 Polyomaviruses are another class of emerging pathogens that commonly infect the
63 urinary tract of humans (4–6). These non-enveloped, dsDNA viruses readily infect a vast
64 majority of the public asymptomatically (7,8), but can also cause severe diseases in
65 immunocompromised individuals (9). Primary infection occurs in childhood, and the
66 viruses persist for the entire life of the individual, mainly in epithelial cells in the kidneys
67 and urinary tract and leukocytes in the blood (6,7,9–12). BK polyomavirus (BKPyV) and
68 JC polyomavirus (JCPyV) are most commonly found excreted in urine (13,14). The
69 excretion of BKPyV by healthy individuals is asymptomatic, but in transplant patients,
70 replication can cause severe disease (15–17). JCPyV and BKPyV concentrations have
71 been reported as high as 10^{10} gene copies/mL in the urine of sick individuals, with
72 healthy adults typically excreting lower concentrations (5,18).

73 Despite the potential for abundant polyomavirus gene copies in excreted urine, its
74 transmission pathways have not yet been fully determined. Respiratory and fecal-oral
75 routes of transmission have been proposed for BKPyV (19–22), and urine may play a role
76 (23). Ingestion of contaminated water and food has been implicated as an exposure route

(21), indicating the potential significance of polyomavirus transmission via the environment.

The need to better understand polyomavirus transmission by urine is underscored by the growing trend of diverting urine from the waste stream and capturing nutrients in urine-derived fertilizers. Urine diversion can provide several environmental benefits, including a sustainable source of phosphorus (24–26), reduction in costs and pollution associated with wastewater treatment (27,28), a potential reduction of water usage (29), and more efficient treatment of contaminants. Despite the benefits of diverting urine, biological contaminants need to be managed before urine can be reused. Biological contaminants in urine are mitigated with a number of treatment technologies, including long-term storage for several months, pasteurization, or by nutrient precipitation (*e.g.,* struvite) (30–32).

When urine is stored in sealed containers to inactivate biological contaminants, the urea in urine is hydrolyzed, resulting in high pH (~9) and an increase in aqueous ammonia concentrations (2000-8000 mg N/L) (33,34). This transition to hydrolyzed urine can occur within a few hours or days depending on urease enzyme activity in the urine. The high pH and high aqueous ammonia levels have a biocidal impact on indicator organisms (35–37).

Research on biological contaminants in source-separated urine has primarily focused on the presence and fate of enteric pathogens (35,38–43). Many enteric viruses are single-stranded RNA (ssRNA) viruses, so ssRNA viral surrogates are often used to predict enteric virus fate in urine. Inactivation of the ssRNA bacteriophage MS2, for example, correlated well with aqueous base (*e.g.* NH_3, OH^-) activity, suggesting that

100 inactivation is caused by transesterification of the ribose in RNA (44). Other ssRNA
101 viruses are susceptible to ammonia activity, whereas the single-stranded DNA (ssDNA)
102 bacteriophage ΦX174, the double-stranded RNA (dsRNA) reovirus, and the double-
103 stranded DNA (dsDNA) human adenovirus and bacteriophage T4 were not susceptible to
104 the same transesterification inactivation pathway (45). These results suggest that although
105 common enteric ssRNA viruses are susceptible to the conditions in hydrolyzed urine,
106 viruses commonly found in the urinary tract (polyomavirus, cytomegalovirus, etc.) may
107 be stable in the high aqueous ammonia concentrations found in hydrolyzed urine and
108 could therefore pose risks in urine-derived fertilizers.

109 To identify the potential transmission risks that polyomavirus may pose in source-
110 separated urine and urine-derived fertilizer production, we tracked the presence and fate
111 of human polyomavirus in fresh and hydrolyzed urine using molecular and culture-based
112 methods. We compared these results to the behavior of common bacteriophage surrogates
113 in an effort to better understand how well surrogate infectivity predicts environmental
114 virus fate and how capsid characteristics may influence inactivation in environmental
115 matrices.

116

117 **Materials and Methods**

118 *Urine collection and characterization*

119 Urine was collected from men and women at nine public events in Vermont,
120 Massachusetts, and Michigan, USA. After collection, urine was stored in sealed
121 containers at room temperature to minimize ammonia off-gassing. Specific urine samples
122 were also pasteurized by heating to 80° C for at least 3 minutes. Nutrient content (total

123 ammonia nitrogen, total phosphorus, total nitrogen) of hydrolyzed urine was evaluated
124 using Standard Methods (4500-NH_3 F; 4500-P J and 4500- P E; 4500-N C and 4500-NO_3^-
125 C) (46). Details on collection location, event type, number of donors, and characteristics
126 of the urines used in this study are provided in Table 1. Experiments with urines are
127 described by the collection event (A-I), followed by the treatment the urine has
128 undergone (fresh, hydrolyzed, pasteurized), and the length of time after that treatment.
129 For example, urine collected from a rural festival in Vermont (event A; Table 1) and used
130 for an experiment 10 months after it underwent hydrolysis was referred to as "A –
131 hydrolyzed 10 months."
132
133 *Bacteriophage and virus strains*
134 Bacteriophages used as surrogates for human viruses included *Escherichia coli*
135 non-enveloped bacteriophages T3, MS2, and Qβ. Bacteriophage T3 (38.2 kbp) is a
136 dsDNA virus that has dimensions of 50 × 20 nm in size and was used as a surrogate for
137 polyomaviruses, which are dsDNA viruses. Bacteriophages MS2 (3.6 kbp) and Qβ (4.2
138 kbp) are both ssRNA viruses and 25 nm in diameter, but Qβ has disulfide bonds in its
139 capsid, and MS2 does not. These two viruses were studied to help explain differences
140 observed in the inactivation kinetics of BKPyV and T3.
141 The BK polyomavirus Dunlop variant was used to study the fate of BKPyV. This
142 genetic variant was chosen since it can be propagated at a high titer (~10^8 IU (infectious
143 unit) mL^{-1}) to provide maximal experimental sensitivity and range. BKPyV (dsDNA) has
144 a 5.1 kbp genome and is approximately 45 nm in diameter (47).

145 Bacteriophages MS2 and T3 were propagated in their *E. coli* hosts (ATCC 15597
146 and 11303). The viruses were extracted from cellular material with a chloroform
147 extraction method (48) and purified with an Econo Fast Protein Liquid Chromatography
148 system (BioRad, USA) equipped with a HiPrep Sephacryl S-400 column (GE, USA).
149 The purified virus fraction was concentrated with 100 kDa Amicon ultracentrifugal filters
150 and filter-sterilized with 0.22 μm polyethersulfone (PES) membrane filters (Millipore,
151 USA). The final MS2 and T3 stocks (~10^{11} PFU mL^{-1}) were stored in phosphate buffer
152 (5 mM NaH_2PO_4 and 10 mM NaCl, pH 7.5) at 4 °C. Bacteriophage Qβ was propagated
153 in its *E. coli* host (ATCC 15597) and purified similar to MS2 and T3, except the protein
154 chromatography step was excluded. The Qβ stocks (~10^{11} PFU mL^{-1}) were stored in
155 phosphate buffer at 4 °C for immediate use in infectivity experiments. The
156 bacteriophages were enumerated by the double layer plaque assay (48). Briefly, aliquots
157 of each virus were serially diluted, and 100 μL of final serial dilutions were combined
158 with 100 μL of an overnight culture of their respective *E. coli* hosts and 5 mL of soft
159 agar. Plaques were enumerated after overnight incubation at 37 °C.
160 BKPyV was propagated in Vero and 293TT cells using previously published
161 methods (49,50). Briefly, Vero and 293TT cells were grown to 70% confluency, infected
162 with BKPyV crude lysate at MOI 0.1 IU $cell^{-1}$, and incubated at 37 °C for three weeks
163 (Vero cells) or 10 days (293TT cells). Virus lysates were purified over density CsCl
164 gradient centrifugation, and the collected virus fraction was dialyzed overnight in buffer
165 (10 mM HEPES, 1mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.9).
166 Infectious BKPyV was enumerated in renal proximal tubule epithelial (RPTE)
167 cells with immunofluorescence assays (IFAs) (49). When RPTE cells in the wells of 24

168 well plates reached 70-80% confluency, serial dilutions of the BKPyV virus samples
169 were added to the cells. Following a one-hour infection at 4° C, the cells were further
170 incubated at 37° C for two days. Cells were then fixed with 4% paraformaldehyde for 20
171 minutes, washed three times with phosphate-buffered saline (154 mM NaCl, 5.6 mM
172 Na_2HPO_4, 1.06 mM KH_2PO_4, pH 7.4, PBS), washed with a 0.1% Triton detergent
173 solution, rinsed with PBS, and then washed with a 5% goat serum blocking solution. To
174 identify infected cells, the cells were treated first with a 1:200 dilution of antibody
175 pAB416 (51) in 5% goat serum, and then with a 1:200 dilution of polyclonal goat anti-
176 mouse IgG FITC antibody (Sigma) in 5% goat serum. The virus titer was determined by
177 counting the individual fluorescent cells. At least nine random fields of view with at least
178 five positive cells each were averaged to obtain the titer values (IU mL^{-1}) in each well.
179 Duplicate wells were prepared for each sample and their titer values were averaged (50).
180
181 *Virus inactivation in urine and buffer*
182 All inactivation experiments were conducted at room temperature in the dark. T3
183 bacteriophage was spiked into 10-50 mL of hydrolyzed urine (n=3) at concentrations of
184 $10^8 – 10^9$ PFU mL^{-1} to evaluate the inactivation rate of the human dsDNA virus surrogate.
185 Bacteriophages MS2 and Qβ were spiked into 10 mL of hydrolyzed urine (n=2) to
186 evaluate the inactivation rates of the bacteriophages with (MS2) and without (Qβ)
187 disulfide bridges in the protein capsid. Both surrogates were spiked into hydrolyzed urine
188 at an initial concentration of $10^8 – 10^9$ PFU mL^{-1}. Infectious particles were quantified
189 over time with plaque assays.

In the BKPyV inactivation experiments, 50 μL of stock BKPyV was spiked into 1 mL of urine or buffer at a concentration of 5×10^5- 1×10^6 IU mL^{-1}. At various time points, 50 μL aliquots were removed and spiked into 450 μL of tissue culture media (renal epithelial growth medium, REGM). The samples were then frozen at -80° C until enumerated with the tissue culture assays. Infectious BKPyV could be detected at concentrations as low as 3×10^2 IU mL^{-1}.

The BKPyV stock was spiked into buffers, hydrolyzed urine, pasteurized urine, and filtered urine to evaluate the role of solution characteristics on inactivation rates. Pasteurization consisted of heating urine to 80° C for at least 3 minutes. Urine was filtered through a 0.22 μm PES syringe filter. BKPyV inactivation was also measured in buffer solutions with various pH and ammonia concentrations (Table S2). These included phosphate carbonate buffers with and without ammonia (147 mM, consistent with levels found in hydrolyzed urine) and adjusted to pH 7 and 9, described as PC7, PC9, AmPC7, and AmPC9 (45).

Polyomavirus qPCR conditions

Endogenous BKPyV DNA concentrations in collected urine samples and Dunlop BKPyV DNA concentrations in spiked urine were evaluated using qPCR (conditions described in Table S1). DNA was extracted from all urine samples (100-300 μL) for qPCR analysis with Maxwell Total Viral Nucleic Acid Extraction kits (Promega) using the Maxwell 16 instrument (Promega). Primers (5' to 3') specific for endogenous BKPyV were selected to target the large T-antigen (152 bp; forward: AAGGAAAGGCTGGATTCTG; reverse: TGTGATTGGGATTCAGTG (52)) and

primers specific for the Dunlop BKPyV strain were designed to target the VP2 protein region of the Dunlop strain (900 bp; forward: ATTTCCAGGTTCATGGGTGCT; reverse: AGGCAACATCCATTGAGGAGC). The 10 μL reactions included 5 μL 2X Biotium Fast-Plus EvaGreen Master Mix, 0.5 μM primers, 0.625 mg/mL bovine serum albumin (BSA), and 1 μL of DNA template (0.5 to 5 ng). Standard curves were prepared between 10^1-10^8 gene copies mL^{-1}. All efficiencies were greater than 70%, and R^2 values were greater than 0.99.

Experiments to study polyomavirus sorption

Control experiments were conducted to determine if the measured decreases in BKPyV concentrations were due to the sorption of viruses to particles found in hydrolyzed urine. In these experiments, 50 μL of stock BKPyV was spiked into 1 mL of urine at a concentration of 5×10^5-1×10^6 IU mL^{-1} and briefly mixed. The solution was then incubated for various amounts of time to allow particles in the urine to settle. At predetermined times, two 50 μL aliquots were removed, including one from the top of the sample to avoid settled particles and one of the mixed sample. All aliquots were spiked into 450 μL of tissue culture media (REGM). Infectious BKPyV levels in the supernatant were compared to levels in the mixture.

Experiments to study polyomavirus genome degradation

To assess BKPyV genome stability, 50 μL of stock BKPyV was spiked into 1 mL of hydrolyzed urine at a concentration of 5×10^5-1×10^6 IU mL^{-1}. 50 μL aliquots were

235 removed from the mixture at different time points up to 27 days and added to 450 μL of
236 tissue culture media (REGM). The samples were stored at -80° C until viral DNA
237 extraction, and then qPCR was conducted on a 900 bp region of the genome (Table S1).
238 To estimate the reaction rate constants for the entire BKPyV genome, we first
239 extrapolated the concentrations measured for the 900 bp amplicon to the entire 5.1 kbp
240 genome with the following relationship (53):
241 $$log\frac{N}{N_0} = log\frac{n}{n_0} \times \frac{genome\ size}{amplicon\ size}$$ (Equation 1)
242 where $\frac{N}{N_0}$ is the extrapolated concentration of the entire genome and $\frac{n}{n_0}$ is the measured
243 concentration of the 900 bp amplicon. First order rate constants for the BKPyV genome
244 were estimated by conducting linear regressions of $\ln(N/N_0)$ versus time. This
245 extrapolation assumes that the reactions in the genome are uniformly distributed.
246 The ability of the BKPyV Dunlop strain qPCR method to detect small decreases
247 of the 900 bp gene copy concentrations in hydrolyzed urine was tested by diluting the
248 spiked urine solutions by 10% and 20% (i.e. 0.9× and 0.8×) with nuclease free water. The
249 undiluted, 0.9× and 0.8× samples were extracted five times each, and then the 900 bp
250 targets were quantified with qPCR. The measured gene copies in the 0.9× and 0.8×
251 samples were compared to the measured gene copies in the undiluted sample.
252
253 *Experiments to study polyomavirus capsid disassembly*
254 To evaluate protein capsid stability in hydrolyzed urine, BKPyV proteins were
255 separated with SDS-PAGE gels, and the VP1 capsid proteins were visualized by western
256 blotting (50). Specifically, 50 μL of stock BKPyV was spiked into 1 mL of hydrolyzed

urine at a concentration of 5×10^5-1×10^6 IU mL^{-1}. Immediately after adding the virus to the urine and then again after 24 hours, 80 μL aliquots were removed from the mixture. To preserve the disulfide bond configuration of BKPyV in the urine samples, 80 μL aliquots were combined with 1.6 μL of freshly prepared 1 M *N*-ethylmaleimide (Sigma), diluted in 100% ethanol. This mixture was incubated on ice for 45 minutes and stored at -80 °C until viral proteins from the BKPyV capsid were separated using 8% SDS-PAGE gels. In the SDS-PAGE analysis, 80 μL sample aliquots were added to 20 μL of reducing buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 250 mM dithiothreitol, 0.025% bromophenol blue) or 20 μL nonreducing buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.025% bromophenol blue). Samples in reduced conditions were diluted 1:20 to have similar signal strength as non-reduced samples for western blot visualization. After buffer addition, all samples were heated to 42° C for 5 minutes and resolved on an SDS-PAGE gel. Under non-reducing conditions, the capsid proteins will enter the gel only if the disulfide bridges were broken in the experiments. Control samples were prepared in reduced conditions to evaluate the total amount of VP1 proteins in the samples. After separation, VP1 proteins were wet-transferred to a nitrocellulose membrane in buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 60 V overnight. Membranes were blocked in 2% nonfat dry milk diluted in 0.1% Tween 20 prepared in phosphate buffer (PBS-T), stained with 1:2000 VP1 primary antibody diluted in 2% nonfat dry milk solution, stained with 1:2000 anti-mouse HRP secondary antibody (Amersham) diluted in 2% nonfat dry milk solution, and washed with PBS-T.

Viability of bacteria in urine

Bacteria with intact and damaged cell membranes in duplicate urine samples were quantified using BacLight "Live/Dead" stain (Molecular Probes) according to the manufacturer's protocol. Viable (fluorescent green) cells were counted and compared to cells with damaged membranes (fluorescent red) using fluorescence microscopy and averaging counts over 10 random fields of view.

Data Analysis

Virus inactivation was modelled with first order kinetics. Rate constants *k* were calculated with linear regressions of natural log concentration and time using the Data Analysis plugin for Microsoft Excel. The reported errors correspond to 95% confidence intervals. The T_{90} values, or time required for 90% reduction in infectivity or gene copies, were calculated as the reciprocal of the first order rate constants.

Student T-tests were used to compare bacteria live/dead ratios, changes in the 900 bp gene copy concentrations after storage in hydrolyzed urines, and to test the sensitivity of our DNA extraction and qPCR assays. ANOVA multiple linear regression analysis was used to compare the inactivation kinetics of two model viruses, MS2 and Qβ, and to assess the impact of urine pasteurization and filtration on virus inactivation kinetics. *p*-values are provided for all statistical analyses.

Results and Discussion

Polyomavirus concentrations in urine and urine-derived fertilizers

Infectious polyomaviruses present in urine cannot be enumerated due to a lack of a compatible tissue culture for studying urine isolates. Consequently, BKPyV DNA

303 concentrations in the collected urine before and after hydrolysis and pasteurization were
304 enumerated by qPCR. The endogenous BKPyV DNA concentration in freshly collected
305 source-separated urine A prior to hydrolysis was 7.0×10^5 gene copies mL^{-1} (Figure 1)
306 which is consistent with reported concentrations in urine of healthy individuals (5×10^0
307 – 1.24×10^8 gene copies mL^{-1}) (5). This data is based on one fresh urine sample, as it is
308 difficult to collect large fresh urine samples since hydrolysis can happen quickly. The
309 average BKPyV gene copy concentration in hydrolyzed urines A, B, C was 3.8×10^6
310 gene copies mL^{-1} and 1.2×10^7 gene copies mL^{-1} in pasteurized urines A, B, C (Figure 1)
311 These data suggest that the polyomaviruses, or at least the polyomavirus DNA, survive
312 the harsh conditions of hydrolyzed urine storage and pasteurization.
313
314 *BKPyV and bacteriophage T3 inactivation in hydrolyzed urine*
315 The gene copy concentrations measured by qPCR do not necessarily correspond
316 to the concentrations of infectious viruses. Although infectivity assays for the
317 polyomaviruses found in urine are not possible at this time, certain polyomavirus genome
318 variants, such as BKPyV Dunlop can be enumerated *in vitro*. BKPyV Dunlop is a
319 rearranged variant of the archetype that is present in humans. This variant differs in the
320 non-coding control region of the genome and is easily grown in cell culture models (54).
321 The variant viral particles are structurally identical to the viruses found in urine. BKPyV
322 Dunlop was spiked into various hydrolyzed urine samples, and inactivation kinetics were
323 then measured. Source-separated urine characteristics vary depending on the donors' age,
324 nutrition, amount of physical exercise, etc. (55). We therefore utilized a number of

source-separated urines collected from different regions of the United States and stored for different amounts of time to capture these variations.

Control experiments confirmed that the unspiked urine samples contained no culturable BKPyV. In the spiked hydrolyzed urine samples, BKPyV inactivation rates ranged from 4.7×10^{-3} – 0.90 $hour^{-1}$, corresponding to T_{90} values from 1.1 – 210 hours (Figure 2 and Table 2). Most of the urine samples exhibited rapid inactivation rates (E, F, H, and I), with T_{90} < 11 hours, but BKPyV was much more stable in urine G. Urine G did not differ from the other urine samples in its ammonia concentration (Table 1), but was collected from fewer donors than urines E, F, H, and I.

In addition to different urine samples exhibiting different inactivation rates, the same collected urine sample hydrolyzed for different amounts of time exhibited different BKPyV inactivation rates (Figure 2B and Table 2). Our previous work demonstrated that the bacterial community changes as the urine is stored (56). Other work has shown that microbial activity can play a role in virus inactivation, particularly for DNA viruses that experience slower abiotic inactivation or in matrices with higher microbial activity (57). We therefore hypothesized that the microbial activity was influencing inactivation, and that the variation of inactivation kinetics was due to different microbial activities in the urine samples.

Microbial activity was thus evaluated in urine samples that exhibited slow and fast virus inactivation rates (urines G – hydrolyzed 11 months and I – hydrolyzed 2 months) with BacLight viability staining. The average urine I ratio (0.66) was larger than the average urine G ratio (0.085, $p = 0.0194$). Interestingly, the higher live/dead bacteria ratio was measured in the sample that inactivated the virus at a significantly faster rate.

Applied and Environmental Microbiology

348 This supported our hypothesis that microbial activity played a role in the different
349 observed polyomavirus inactivation rates.

350 Other dsDNA viruses are stable in human excreta and animal manure with high
351 ammonia and high pH (45). T4 (dsDNA), ΦX174 (ssDNA), and rhesus rotavirus
352 (dsRNA) are stable in urine with T_{90} values of 12.5 days, 7 days (57), and 35 days at 20°
353 C (38), respectively, while human adenovirus (dsDNA) is less stable, with T_{90} values
354 ranging from 2-24 hours (57). We used another dsDNA virus bacteriophage T3 to
355 confirm that our observation was not unique to the urine samples tested. The dsDNA
356 bacteriophage T3 was spiked into various urine samples, and the concentration of
357 infectious T3 particles was measured over time. T3 was much more stable than BKPyV
358 in hydrolyzed urine (Table 2), with an average first order rate constant of 1.6×10^{-3} $hour^{-1}$
359 and an average T_{90} equal to 630 hours (n = 3). T3 is also stable in other aquatic
360 environments, remaining infectious in a wide pH range (5-9.2) and in wastewater
361 matrices for at least 48 hours (58,59). T3 and BKPyV exhibited very different
362 inactivation kinetics despite having the same genome type. This suggests that the
363 inactivation mechanism for BKPyV is different than the inactivation mechanism for
364 bacteriophage T3. We next sought to determine why BKPyV was susceptible to the
365 conditions of hydrolyzed urine.
366
367 *Attributes of polyomavirus leading to inactivation*
368 Inactivation of non-enveloped viruses can be due to damage to the capsid
369 structure or damage to the genome (60–63). To assess if inactivation in the urine was due
370 to degradation of the polyomavirus genome, a 900 bp region of the BKPyV genome was

371 monitored by qPCR as the virus was incubated in hydrolyzed urine I. The 900 bp
372 amplicon covered ~20% of the BKPyV genome, and controls confirmed that unspiked
373 urine did not contain the amplicon sequence. After 27 days we detected no significant
374 decrease in gene copies based on both linear regressions of the entire data set and a
375 student T-test of the gene copy concentrations at time = 0 and time = 27 days. Our qPCR
376 assay could effectively detect a 20% decrease in the initial gene copy concentration of
377 BKPyV (student T-test, p=0.0062); this means that the reaction rate constant for the 900
378 bp amplicon in urine was < 0.0083 day^{-1} (T_{90}>120 days). Extrapolating this rate constant
379 to the entire genome using equation (1) results in a genome rate constant $k < 0.047$ day^{-1}
380 and a T_{90} value greater than 21 days. For comparison, the infectivity T_{90} for this same
381 urine sample was 6.3 hours (Table 2). These data verify that reactions in the dsDNA
382 polyomavirus genomes are not responsible for virus inactivation in the hydrolyzed urine.
383 To investigate if capsid disassembly plays a role in polyomavirus inactivation,
384 western blots were employed to monitor the disulfide bonds that provide stability to the
385 protein capsid structure. VP1 is the major structural protein in the polyomavirus capsid.
386 The capsid is composed of 72 pentamers of this protein (64) connected with inter- and
387 intrapentameric disulfide bridges (65). The presence of disulfide bridges sets
388 polyomavirus apart from the other dsDNA viruses that have been tested in hydrolyzed
389 urine and human excreta at combined high pH and ammonia levels. To investigate the
390 stability of the capsid structure, SDS-PAGE western blots were conducted on BKPyV
391 proteins after the viruses were stored in urine. If the disulfide bonds are intact, the virus is
392 unable to enter the SDS-PAGE gel. Results confirmed protein structural changes
393 following incubation in hydrolyzed urine (Figure 3). Immediately after BKPyV was

added to urine, minimal VP1 pentamers, dimers, or monomers were visible; therefore, most of the BKPyV particles were intact and too large to enter the non-reducing SDS-PAGE gel. After BKPyV was incubated in hydrolyzed urine for 24 hours and inactivated, VP1 monomers were detected; therefore, the BKPyV capsid was disassembling. Control samples at time = 0 and after 24 hours, in which the VP1 protein disulfide bonds were reduced experimentally prior to electrophoresis, confirmed that the increase in signal observed in Figure 3 was due to disassembly of BKPyV in urine and not due to different amounts of added virus capsids. These results, coupled with the relatively slow T3 inactivation kinetics and the relatively slow genome reaction kinetics, suggest that something about the capsid structure of BKPyV renders it susceptible to disassembly and rapid inactivation in hydrolyzed urine.

To explore the role of disulfide bonds when viruses are inactivated in hydrolyzed urine, two related model viruses (MS2 and Qβ) were added to hydrolyzed urine, and infectivity was measured over time. These two ssRNA viruses have similar RNA sequences (up to 80% similarity in the replicase subunit) and capsid size (25 nm), but differ in that the capsid proteins of Qβ are connected with disulfide bridges. Bacteriophage Qβ inactivated at a significantly faster rate in hydrolyzed urine than MS2 (p = 0.00105) (Table 2 and Figure S1). The RNA genome of Qβ is longer than MS2 (4.2 kbp vs. 3.6 kbp), and based on a model developed by Decrey et al., we compared the expected ssRNA transesterification rates in Qβ and MS2 based on their genome sizes (44). The $k_{\{NH3\}}$ for Qβ was predicted to be 1.17× larger than the $k_{\{NH3\}}$ for MS2. Our inactivation rate constant for Qβ was 1.32× larger than that of MS2. Consequently, if mechanisms beyond RNA transesterification inactivate Qβ, such as disruption of the

417 capsid structure, these mechanisms are minor. Additional viruses that contain disulfide
418 bonds will need to be studied in hydrolyzed urine to better define the role these bonds
419 play in virus inactivation.
420
421 *Hydrolyzed urine characteristics that influence BKPyV inactivation rates*
422 To understand why different hydrolyzed urine samples exhibited different rates of
423 BKPyV inactivation, we explored which characteristics of the hydrolyzed urine
424 contributed to BKPyV inactivation. We first hypothesized that the BKPyV particles
425 adsorbed to large particulates in the urine and then settled out rapidly before aliquots
426 were collected for culturing. Control experiments were therefore conducted in which
427 particulates in the urine were allowed to settle and inactivation of BKPyV was measured
428 in the supernatant. The rate constant with particulates (0.40 ± 0.12 hour^{-1}) was not
429 statistically different from the rate constant without particulates (0.39 ± 0.12 hour^{-1}, p =
430 0.90). Sorption to particulates and settling was therefore ruled out as a significant
431 contributing factor in the observed inactivation rates.
432 We next tested the impact of the high pH and ammonia levels in the hydrolyzed
433 urine samples, as these conditions are biocidal to many organisms, including RNA
434 viruses (44). Linear regressions conducted on BKPyV concentrations over time in buffers
435 with pH and ammonia levels similar to hydrolyzed urine were not significantly different
436 from zero (n=3). This demonstrated that the BKPyV was not losing infectivity due to the
437 elevated pH and high ammonia concentrations in hydrolyzed urine.
438 Finally, we tested the role of microbial activity. Microorganisms can contribute to
439 virus inactivation in some environments (57,66,67). To evaluate if the microorganisms

present in hydrolyzed urine impact the infectivity of BK polyomavirus, BKPyV was added to hydrolyzed urine, hydrolyzed urine that was recently pasteurized, and hydrolyzed urine that was recently filtered through filters with 0.22 μm pores. ANOVA multiple linear regression analyses suggested that BKPyV was inactivated at slower rates when urine I (hydrolyzed 11 months) was either pasteurized or filtered (Table 2, Figure 4, $p = 0.0014$ for pasteurized urine, $p = 9.7 \times 10^{-5}$ for filtered urine). This experiment was repeated in urine I (hydrolyzed 2 months) and urine I (hydrolyzed 10 months) with similar results (Table 2). Inactivation was not completely prevented after filtration and pasteurization; therefore, additional unknown factors contributed to BKPyV inactivation in the hydrolyzed urine.

This work demonstrates that the urinary tract virus BKPyV is rapidly inactivated in most hydrolyzed urine samples. It is therefore likely that short-term storage of urine (< 1 month) is adequate for mitigating risks associated with polyomavirus. We ruled out the role of elevated ammonia and pH levels in BKPyV inactivation and provided evidence for the role of microbial activity. The specific inactivation mechanism most likely involves capsid damage, since the DNA was not degraded over long periods of time and the capsid proteins disassembled. We hypothesize that the disulfide bonds in BKPyV make it more susceptible to inactivation in the hydrolyzed urine because the dsDNA bacteriophage T3 was very stable. Inactivation experiments with MS2 and Qβ were not able to support this hypothesis due to the rapid RNA transesterification reactions that inactivated both viruses.

Our results are a reminder that positive qPCR measurements do not indicate the presence of infective viruses. BKPyV DNA genes persisted for months, but infective

463 viruses persisted for only hours to days. Employing bacteriophage surrogates to predict
464 pathogen behavior is ideal for viruses that are not culturable; however, our results
465 underscore the fact that bacteriophages are often inappropriate surrogates for human
466 viruses even when they contain the same genome type. Using the inactivation behavior of
467 bacteriophage T3 to predict polyomaviruses would greatly overestimate the BKPyV risk
468 posed by urine-derived fertilizer.

469 It is, of course, impractical to test the survivability of every human virus in every
470 environmental condition with culture assays, especially when a number of human viruses
471 do not have readily available culture systems (e.g., human norovirus). Instead, the
472 environmental virology community should aim to understand how the chemical,
473 structural, and biological characteristics of viruses impact their environmental fate. This
474 requires studying the mechanistic fate of a broad range of viruses in various
475 environmental conditions. Our results suggest that the capsid of BKPyV is disassembled
476 in hydrolyzed urine samples despite the common assumption that disulfide bridges have a
477 stabilizing effect on protein structures. It remains to be seen whether this effect influences
478 the stability of other important human viruses with disulfide bonds in capsid proteins
479 including HIV, Hepatitis B, and Hepatitis C (68–70). Once we identify the protein
480 characteristics that drive inactivation in hydrolyzed urine and other environmental
481 matrices, we will be able to select more appropriate process surrogates for specific human
482 viruses.

483 Finally, microbial activity appears to contribute to the BKPyV inactivation,
484 although the specific mechanism by which microorganisms inactivate the virus remains
485 unclear. Viral inactivation in environmental matrices often depends on a variety of

factors, and the components leading to inactivation can vary from sample to sample. The varied inactivation rates observed amongst the urine samples may be due to the prokaryotic and eukaryotic levels and communities in the urine samples. Our earlier work revealed that bacterial communities in several collected urine samples converge to have similar structures at the 16S level when stored (56). Identifying the specific prokaryotic and eukaryotic organisms responsible for the inactivation of viruses in urine samples and other environmental matrices will be necessary to more systematically evaluate the risks that they pose.

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735 **Table 1. Characterization of urines used in experiments. Standard deviations are reported for >2 measurements.**

Urine Label	Collection Location	Event Type	No. of Donors	Total Phosphorus (mg P/L)	Total Nitrogen (mg N/L)	Total Ammonia Nitrogen (mg N/L)
A	Vermont	Rural Festival	300	410±1	4700±170	5000±260
B	Massachusetts	Male rest stop	>100	240±24	4400±170	4300±210
C	Vermont	Combination of parade and festival	>300	400±9	7100±430	5700±60
D	Michigan	University	200	850±210	7400±270	5600±200
E	Michigan	Outdoor Theater	80	490±4	6300±230	5600±250
F	Michigan	Outdoor Theater	60	320 ±1	4600±230	4800±640
G	Michigan	University	10	700±10	9700±1000	6300±110
H	Vermont	Urine Community Collection	>100	450±1	6400±400	5800±320
I	Vermont	Urine Community Collection	>100	460±10	4900±600	6300±100

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738 **Table 2. First order rate constants and T_{90} values for BKPyV and bacteriophages T3, Qβ, and MS2 in hydrolyzed urines. 95% confidence intervals are reported for the**
739 **linear regression analyses.**

	BKPyV		T3			Qβ		MS2	
	dsDNA – disulfide bonds		dsDNA			ssRNA – disulfide bonds		ssRNA	
Urine (hydrolysis time)	k ($hour^{-1}$)	T_{90} (hours)		k ($hour^{-1}$)	T_{90} (hours)	k ($hour^{-1}$)	T_{90} (hours)	k ($hour^{-1}$)	T_{90} (hours)
D (< 1 week)			1.7	10^{-3} ±3.1	10^{-4} 590				
E (4 months)			2.2	10^{-3} ±9.5	10^{-4} 450				
E (8 months)	0.67±0.97*	1.5							
E (9 months)						0.16±0.33	6.3	0.11±0.011	9.1
E (15 months)	0.28 ±0.51*	3.6							
F (4 months)			9.0	10^{-4} ±5.0	10^{-4} 1100				
F (8 months)	0.40 ±3.1*	2.5							
F (11 months)	0.90±0.41	1.1							

G (11 months)	4.7 ±7.6	10^{-3} 10^{-3}*	210				
H (3 months)				0.13±0.033	7.7	0.11±0.019	9.1
H (9 months)	0.17±0.060		5.9				
I (2 months)	0.45±0.048,		2.2				
I (2 months), pasteurized	0.26±0.045		3.8				
I (2 months), filtered	0.31±0.037		3.2				
I (10 months)	0.16 ±0.037		6.3				
I (10 months), pasteurized	0.078±0.16*		13				
I (10 months), filtered	0.079±0.21*		13				
I (11 months)	0.094±0.030		11				
I (11 months), pasteurized	0.045±0.027		22				
I (11 months), filtered	0.036±0.009		28				

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742 *Linear regression was not statistically different from zero.

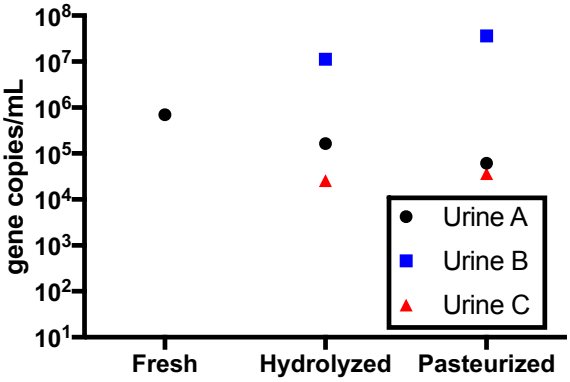

Figure 1. BKPyV gene copy (152 bp) concentrations detected by qPCR in urine A (fresh, hydrolyzed for 10 months, and pasteurized after hydrolyzed for 10 months) and urines B and C (hydrolyzed 1 month and after 10 months, respectively, and pasteurized after the same amount of hydrolysis time). Fresh urine was only available for urine A due to the rapid hydrolysis in urines B and C.

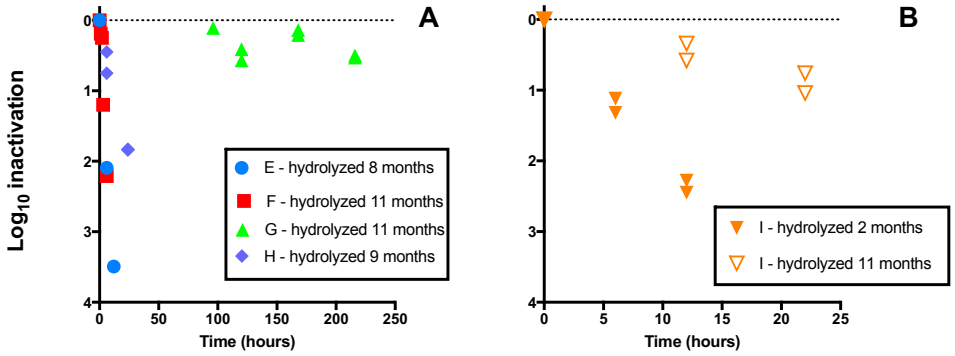

Figure 2. Infectivity of BK polyomavirus in hydrolyzed urine samples (E - I) measured over time. Initial infective virus concentrations in urine were 5 10^5-1 10^6 IU (infectious units) mL^{-1}. Experiments were conducted until the detection limit was reached (3×10^2 IU mL^{-1}). Left panel A shows variability in kinetics across different urine aged between 8-11 months. Right panel B shows kinetic changes with hydrolysis time.

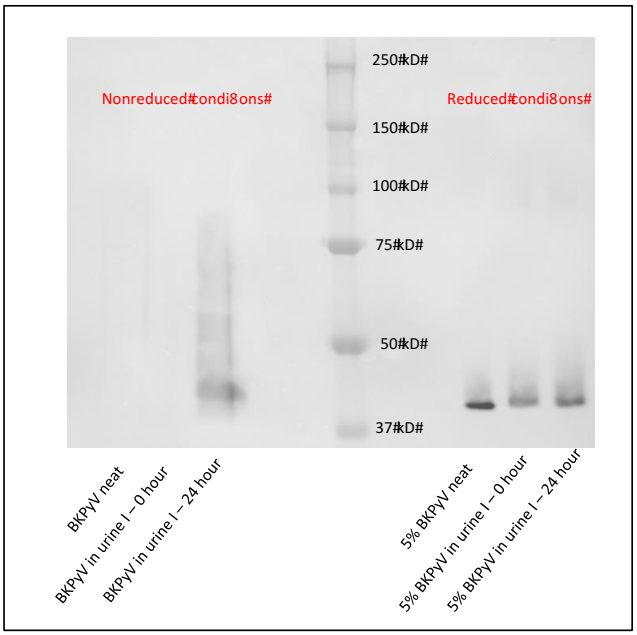

Figure 3. Western blot of BKPyV proteins separated under non-reducing (left) and reducing (right) conditions. BKPyV proteins were analyzed after addition to buffer (neat), immediately after addition to hydrolyzed urine (0 hour) and after incubating for one day in hydrolyzed urine (24 hour). The reduced samples were diluted to 5% of the experimental concentration to allow visualization on the same gel as the non-reduced samples. The BKPyV VP1 monomer size is 42 kD.

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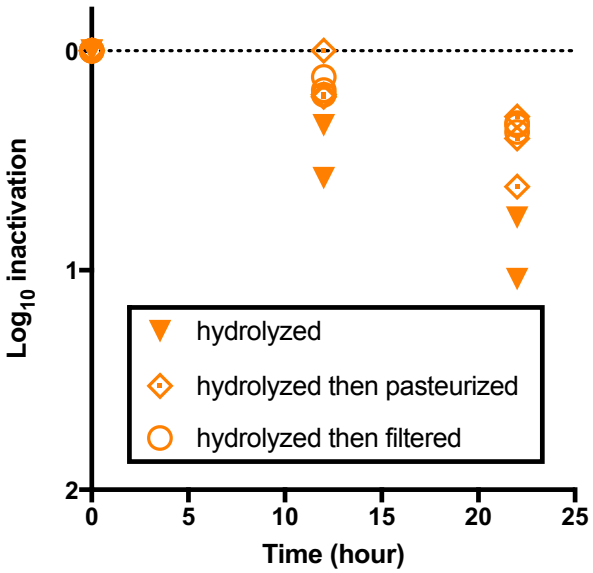

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765 **Figure 4. Infectivity over time of polyomavirus BKPyV spiked into urine I samples that had been hydrolyzed for**

766 **11 months, pasteurized, and filtered. Initial BKPyV concentrations were 5×10^5-1×10^6 IU mL^{-1}. Experiments**

767 **were conducted until the detection limit was reached (3×10^2 IU mL^{-1}).**

768