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Fate of the Urinary Tract Virus BK Human Polyomavirus in Source-Separated Urine

Heather E. Goetsch,[a] Linbo Zhao,[b] Mariah Gnegy,[a]* Michael J. Imperiale,[b] Nancy G. Love,[a] Krista R. Wigginton[a]

[a]Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, Michigan, USA
[b]Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan, USA

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 quantitative PCR (qPCR). Although BKPyV genomes persisted in the hydrolyzed urine for long periods of time (T_{90} [time required for 90% reduction in infectivity or gene copies] of >3 weeks), the viruses were rapidly inactivated (T_{90} of 1.1 to 11 h) in most of the tested urine samples. Interestingly, the infectivity of dsDNA bacteriophage surrogate T3 (T_{90} of 24 to 46 days) was much more persistent than that of BKPyV, highlighting a major shortcoming of using bacteriophages as human virus surrogates. Pasteurization and filtration experiments suggest that 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 does 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 is 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 under 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.

KEYWORDS BK polyomavirus, nutrient recovery, source-separated urine

Enteric viruses that arise from fecal contamination have long been known to be of serious concern for public health. Viruses that infect the urinary tract of humans can also be shed in large quantities, yet their concentrations and fate in the environment are unclear. Zika virus, for example, is excreted in urine (1) and can cause microcephaly in newborn children of infected mothers (2). Similarly, cytomegalovirus (CMV) is shed in

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Address correspondence to Krista R. Wigginton, kwigg@umich.edu.

* Present address: Mariah Gnegy, Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

the urine of infected individuals and is a risk to infants of infected mothers, as the virus can cause hearing and vision loss and other developmental disabilities (3).

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

Despite the potential for abundant polyomavirus gene copies in excreted urine, its transmission pathways have not yet been fully determined. Respiratory and fecal-oral routes of transmission have been proposed for BKPyV (19–22), and urine may play a role (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 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 (2,000 to 8,000 mg N/liter) (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 and OH^-) activity, suggesting that inactivation is caused by transesterification of the ribose in RNA (44). Other ssRNA viruses are susceptible to ammonia activity, whereas the single-stranded DNA (ssDNA) bacteriophage ΦX174, the double-stranded RNA (dsRNA) reovirus, and the double-stranded DNA (dsDNA) human adenovirus and bacteriophage T4 were not susceptible to the same transesterification inactivation pathway (45). These results suggest that although common enteric ssRNA viruses are susceptible to the conditions in hydrolyzed urine, viruses commonly found in the urinary tract (polyomavirus, cytomegalovirus, etc.) are stable at the high aqueous ammonia concentrations found in hydrolyzed urine and therefore could pose risks in urine-derived fertilizers.

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

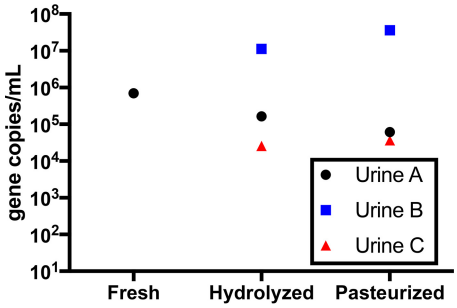

FIG 1 BKPyV gene copy (152 bp) concentrations detected by qPCR in urine A (fresh, hydrolyzed for 10 months, and pasteurized after being hydrolyzed for 10 months) and urine samples B and C (hydrolyzed for 1 month and 10 months, respectively, and pasteurized after the same hydrolysis times). Fresh urine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

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 concentrations in the collected urine before and after hydrolysis and pasteurization were enumerated by quantitative PCR (qPCR). The endogenous BKPyV DNA concentration in freshly collected source-separated urine sample A prior to hydrolysis was 7.0×10^5 gene copies ml^{-1} (Fig. 1), which is consistent with reported concentrations in urine of healthy individuals (5×10^0 to 1.24×10^8 gene copies ml^{-1}) (5). These data are based on one fresh urine sample, as it is difficult to collect large fresh urine samples since hydrolysis can happen quickly. The average BKPyV gene copy concentration in hydrolyzed urine samples A, B, and C was 3.8×10^6 gene copies ml^{-1} and 1.2×10^7 gene copies ml^{-1} in pasteurized urine samples A, B, and C (Fig. 1) These data suggest that the polyomaviruses, or at least the polyomavirus DNA, survive the harsh conditions of hydrolyzed urine storage and pasteurization.

BKPyV and bacteriophage T3 inactivation in hydrolyzed urine. The gene copy concentrations measured by qPCR do not necessarily correspond to the concentrations of infectious viruses. Although infectivity assays for the polyomaviruses found in urine are not possible at this time, certain polyomavirus genome variants, such as BKPyV Dunlop, can be enumerated *in vitro*. BKPyV Dunlop is a rearranged variant of the archetype that is present in humans. This variant differs in the noncoding control region of the genome and is easily grown in cell culture models (46). The variant viral particles are structurally identical to the viruses found in urine. BKPyV Dunlop was spiked into various hydrolyzed urine samples, and inactivation kinetics were then measured. Source-separated urine characteristics vary depending on the donors' age, nutrition, amount of physical exercise, etc. (47). We therefore utilized a number of source-separated urine samples 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} to 0.90 h^{-1}, corresponding to T_{90} (time required for 90% reduction in infectivity) values from 1.1 to 210 h (Fig. 2 and see Table 2). Most of the hydrolyzed urine samples exhibited rapid inactivation rates (E, F, H, and I), with T_{90} of $<$11 h, but BKPyV was much more stable in hydrolyzed urine sample G. Hydrolyzed urine sample G did not differ from the other urine samples in its ammonia concentration (Table 1) but was collected from fewer donors than urine samples E, F, H, and I.

In addition to different hydrolyzed urine samples exhibiting different inactivation rates, the same collected urine sample hydrolyzed for different amounts of time exhibited different BKPyV inactivation rates (Fig. 2B and Table 2). Our previous work demonstrated that the bacterial community changes as the urine is stored (48). Other work has shown that microbial activity can play a role in virus inactivation,

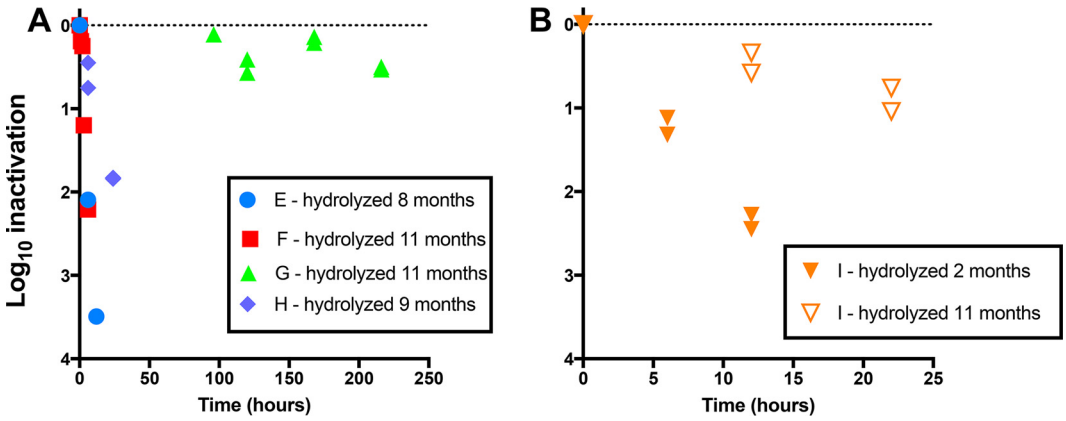

FIG 2 Infectivity of BK polyomavirus in hydrolyzed urine samples (E to I) measured over time. Initial infective virus concentrations in urine were 5×10^5 to 1×10^6 IU (infectious units) ml^{-1}. Experiments were conducted until the detection limit was reached (3×10^2 IU ml^{-1}). (A) Inactivation in different hydrolyzed urine samples. (B) Inactivation in the same urine sample that was hydrolyzed for different amounts of time.

particularly for DNA viruses that experience slower abiotic inactivation or in matrices with higher microbial activity (49). 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 hydrolyzed urine samples that exhibited slow and fast virus inactivation rates (urine sample G was hydrolyzed for 11 months, and urine sample I was hydrolyzed for 2 months) with BacLight viability staining. The average hydrolyzed urine sample I ratio of live/dead bacteria (0.66) was higher than the average hydrolyzed urine sample G ratio (0.085) ($P = 0.0194$). Interestingly, the higher ratio of live/dead bacteria was measured in the sample that inactivated the virus at a significantly higher rate. This supported our hypothesis that microbial activity played a role in the different observed polyomavirus inactivation rates.

Other dsDNA viruses are stable in human excreta and animal manure with high ammonia and high pH (45). T4 (dsDNA), ΦX174 (ssDNA), and rhesus rotavirus (dsRNA) are stable in urine, with T_{90} values of 12.5 days, 7 days (49), and 35 days at 20°C (38), respectively, while human adenovirus (dsDNA) is less stable, with T_{90} values ranging from 2 to 24 h (49). We used another dsDNA virus, bacteriophage T3, to confirm that our observation was not unique to the urine samples tested. The dsDNA bacteriophage T3 was spiked into various urine samples, and the concentration of infectious T3 particles was measured over time. T3 was much more stable than BKPyV in hydrolyzed urine (Table 2), with an average first-order rate constant of 1.6×10^{-3} h^{-1} and an average T_{90} equal to 630 h ($n = 3$). T3 is also stable in other aquatic environments, remaining infectious in a wide pH range (5 to 9.2) and in wastewater matrices for at least 48 h (50, 51). T3 and BKPyV exhibited very different inactivation kinetics despite

TABLE 1 Characterization of urine samples used in experiments

Urine sample	Collection location	Event type/location	No. of donors	Total phosphorus (mg P/liter)[a]	Total nitrogen (mg N/liter)[a]	Total ammonia nitrogen (mg N/liter)[a]
A	Vermont	Rural festival	300	410 ± 1	4,700 ± 170	5,000 ± 260
B	Massachusetts	Male rest stop	>100	240 ± 24	4,400 ± 170	4,300 ± 210
C	Vermont	Combination of parade and festival	>300	400 ± 9	7,100 ± 430	5,700 ± 60
D	Michigan	University	200	850 ± 210	7,400 ± 270	5,600 ± 200
E	Michigan	Outdoor theater	80	490 ± 4	6,300 ± 230	5,600 ± 250
F	Michigan	Outdoor theater	60	320 ± 1	4,600 ± 230	4,800 ± 640
G	Michigan	University	10	700 ± 10	9,700 ± 1,000	6,300 ± 110
H	Vermont	Urine community collection	>100	450 ± 1	6,400 ± 400	5,800 ± 320
I	Vermont	Urine community collection	>100	460 ± 10	4,900 ± 600	6,300 ± 100

[a]Standard deviations are reported for >2 measurements.

TABLE 2 First-order rate constants and T_{90} values for BKPyV and bacteriophages T3, Qβ, and MS2 in hydrolyzed urine samples[b]

Urine sample (hydrolysis time)	BKPyV		T3		Qβ		MS2	
	dsDNA, disulfide bonds		dsDNA		ssRNA, disulfide bonds		ssRNA	
	k (h^{-1})	T_{90} (h)	k (h^{-1})	T_{90} (h)	k (h^{-1})	T_{90} (h)	k (h^{-1})	T_{90} (h)
D (<1 wk)			$1.7 \times 10^{-3} \pm 3.1 \times 10^{-4}$	590				
E (4 mo)			$2.2 \times 10^{-3} \pm 9.5 \times 10^{-4}$	450				
E (8 mo)	0.67 ± 0.97[a]	1.5						
E (9 mo)					0.16 ± 0.33	6.3	0.11 ± 0.011	9.1
E (15 mo)	0.28 ± 0.51[a]	3.6						
F (4 mo)			$9.0 \times 10^{-4} \pm 5.0 \times 10^{-4}$	1,100				
F (8 mo)	0.40 ± 3.1[a]	2.5						
F (11 mo)	0.90 ± 0.41	1.1						
G (11 mo)	$4.7 \times 10^{-3} \pm 7.6 \times 10^{-3}$[a]	210						
H (3 mo)					0.13 ± 0.033	7.7	0.11 ± 0.019	9.1
H (9 mo)	0.17 ± 0.060	5.9						
I (2 mo)	0.45 ± 0.048	2.2						
I (2 mo), pasteurized	0.26 ± 0.045	3.8						
I (2 mo), filtered	0.31 ± 0.037	3.2						
I (10 mo)	0.16 ± 0.037	6.3						
I (10 mo), pasteurized	0.078 ± 0.16[a]	13						
I (10 mo), filtered	0.079 ± 0.21[a]	13						
I (11 mo)	0.094 ± 0.030	11						
I (11 mo), pasteurized	0.045 ± 0.027	22						
I (11 mo), filtered	0.036 ± 0.009	28						

[a]Linear regression not statistically different from zero.
[b]Ninety-five percent confidence intervals are reported for the linear regression analyses.

having the same genome type. This suggests that the inactivation mechanism for BKPyV is different from the inactivation mechanism for bacteriophage T3. We next sought to determine why BKPyV was susceptible to the conditions of hydrolyzed urine.

Attributes of polyomavirus leading to inactivation. Inactivation of nonenveloped viruses can be due to damage to the capsid structure or damage to the genome (52–55). To assess if inactivation in the urine was due to degradation of the polyomavirus genome, a 900-bp region of the BKPyV genome was monitored by qPCR as the virus was incubated in hydrolyzed urine sample I. The 900-bp amplicon covered ~20% of the BKPyV genome, and controls confirmed that unspiked urine did not contain the amplicon sequence. After 27 days we detected no significant decrease in gene copies based on both linear regressions of the entire data set and a Student t test of the gene copy concentrations at experiment times of 0 and 27 days. Our qPCR assay could effectively detect a 20% decrease in the initial gene copy concentration of BKPyV ($P = 0.0062$ by Student t test); this means that the reaction rate constant for the 900-bp amplicon in urine was <0.0083 day^{-1} ($T_{90} > 120$ days). Extrapolating this rate constant to the entire genome using equation 1 results in a genome rate constant, k, of <0.047 day^{-1} and a T_{90} value greater than 21 days. For comparison, the infectivity T_{90} for this same urine sample was 6.3 h (Table 2). These data verify that reactions in the dsDNA polyomavirus genomes are not responsible for virus inactivation in the hydrolyzed urine.

To investigate if capsid disassembly plays a role in polyomavirus inactivation, Western blots were employed to monitor the disulfide bonds that provide stability to the protein capsid structure. VP1 is the major structural protein in the polyomavirus capsid. The capsid is composed of 72 pentamers of this protein (56) connected with inter- and intrapentameric disulfide bridges (57). The presence of disulfide bridges sets polyomavirus apart from the other dsDNA viruses that have been tested in hydrolyzed urine and human excreta at combined high pH and ammonia levels. To investigate the stability of the capsid structure, SDS-PAGE Western blots were conducted on BKPyV proteins after the viruses were stored in urine sample I hydrolyzed for 10 months. If the disulfide bonds are intact, the virus is unable to enter the SDS-PAGE gel. Results confirmed protein structural changes following incubation in hydrolyzed urine (Fig. 3).

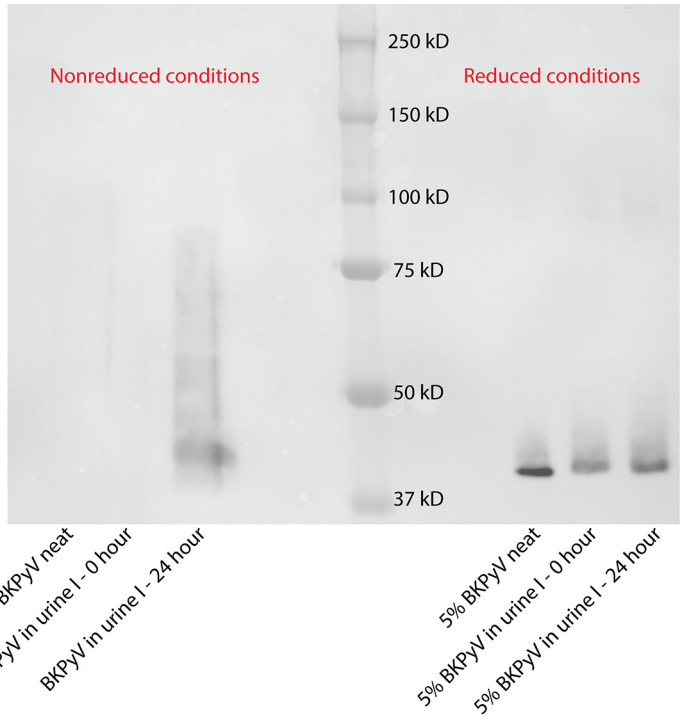

FIG 3 Western blot of BKPyV proteins separated under nonreducing (left) and reducing (right) conditions. BKPyV proteins were analyzed after addition to buffer (neat), immediately after addition to urine sample I hydrolyzed for 10 months (0 h), and after incubating for 1 day in urine sample I hydrolyzed for 10 months (24 h). The reduced samples were diluted to 5% of the experimental concentration to allow visualization on the same gel as that of the nonreduced samples. The BKPyV VP1 monomer size is 42 kDa.

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 nonreducing SDS-PAGE gel. After BKPyV was incubated in hydrolyzed urine for 24 h and inactivated, VP1 monomers were detected; therefore, the BKPyV capsid was disassembling. Control samples at a time of 0 and after 24 h, in which the VP1 protein disulfide bonds were reduced experimentally prior to electrophoresis, confirmed that the increase in signal observed in Fig. 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 higher rate in hydrolyzed urine than MS2 ($P = 0.0011$) (Table 2; see also Fig. S1 in the supplemental material). The RNA genome of Qβ is longer than that of MS2 (4.2 kbp versus 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$\times$ larger than the $k_{\{NH3\}}$ for MS2. Our inactivation rate constant for Qβ was 1.32$\times$ larger than that of MS2. Consequently, if mechanisms beyond RNA transesterification inactivate Qβ, such as disruption of the capsid structure, these mechanisms are minor. Additional viruses that contain disulfide bonds will need to be studied in hydrolyzed urine to better define the role these bonds play in virus inactivation.

Hydrolyzed urine characteristics that influence BKPyV inactivation rates. To understand why different hydrolyzed urine samples exhibited different rates of BKPyV

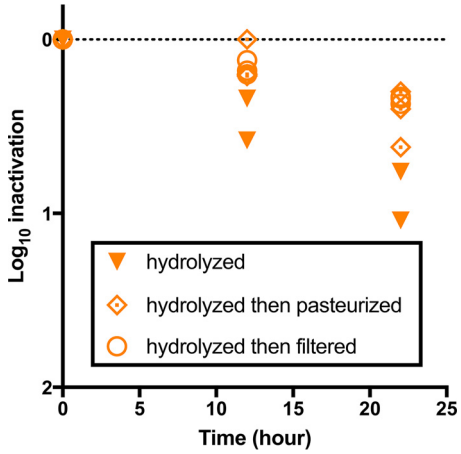

FIG 4 Infectivity over time of polyomavirus BKPyV spiked into urine sample I hydrolyzed for 11 months and then pasteurized or filtered. Initial BKPyV concentrations were 5×10^5 to 1×10^6 IU ml^{-1}.

inactivation, we explored which characteristics of the hydrolyzed urine contributed to BKPyV inactivation. We first hypothesized that the BKPyV particles adsorbed to large particulates in the urine and then settled out rapidly before aliquots were collected for culturing. Control experiments were therefore conducted in which particulates in the hydrolyzed urine were allowed to settle and inactivation of BKPyV was measured in the supernatant. The rate constant with particulates (0.40 ± 0.12 h^{-1}) was not statistically different from the rate constant without particulates (0.39 ± 0.12 h^{-1}) ($P = 0.90$). Sorption to particulates and settling was therefore ruled out as a significant contributing factor in the observed inactivation rates.

We next tested the impact of the high pH and ammonia levels in the hydrolyzed urine samples, as these conditions are biocidal to many organisms, including RNA viruses (44). Linear regressions conducted on BKPyV concentrations over time in buffers with pH and ammonia levels similar to those of hydrolyzed urine were not significantly different from zero ($n = 3$). This demonstrated that the BKPyV was not losing infectivity due to the elevated pH and high ammonia concentrations in hydrolyzed urine.

Finally, we tested the role of microbial activity. Microorganisms can contribute to virus inactivation in some environments (49, 58, 59). 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. Analysis of variance (ANOVA) multiple linear regression analyses suggested that BKPyV was inactivated at lower rates when urine sample I hydrolyzed for 11 months was either pasteurized or filtered (Table 2 and Fig. 4) (P value of 0.0014 for pasteurized urine, P value of 9.7×10^{-5} for filtered urine). This experiment was repeated with urine sample I hydrolyzed for 2 months and with urine sample I hydrolyzed for 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 infectious viruses. BKPyV DNA genes persisted for months, but infectious viruses persisted for only hours to days. Employing bacteriophage surrogates to predict pathogen behavior is ideal for viruses that are not culturable; however, our results underscore the fact that bacteriophages are often inappropriate surrogates for human viruses even when they contain the same genome type. Using the inactivation behavior of bacteriophage T3 to predict polyomaviruses would greatly overestimate the BKPyV risk posed by urine-derived fertilizer.

It is, of course, impractical to test the survivability of every human virus under every environmental condition with culture assays, especially when a number of human viruses do not have readily available culture systems (e.g., human norovirus). Instead, the environmental virology community should aim to understand how the chemical, structural, and biological characteristics of viruses impact their environmental fate. This requires studying the mechanistic fate of a broad range of viruses under various environmental conditions. Our results suggest that the capsid of BKPyV is disassembled in hydrolyzed urine samples despite the common assumption that disulfide bridges have a stabilizing effect on protein structures. It remains to be seen whether this effect influences the stability of other important human viruses with disulfide bonds in capsid proteins, including HIV, hepatitis B, and hepatitis C (60–62). Once we identify the protein characteristics that drive inactivation in hydrolyzed urine and other environmental matrices, we will be able to select more appropriate process surrogates for specific human viruses.

Finally, microbial activity appears to contribute to BKPyV inactivation, although the specific mechanism by which microorganisms inactivate the virus remain 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 among 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 (48). 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.

MATERIALS AND METHODS

Urine collection and characterization. Urine was collected from men and women at nine public events in Vermont, Massachusetts, and Michigan. After collection, urine was stored in sealed containers at room temperature to minimize ammonia off-gassing. Specific urine samples were also pasteurized by heating to 80°C for at least 3 min. Nutrient content (total ammonia nitrogen, total phosphorus, and total nitrogen) of hydrolyzed urine was evaluated using standard methods (63). Details on collection location, event type, number of donors, and characteristics of the urine samples used in this study are provided in Table 1. Experiments with urine samples are described by the collection event (samples A to I), followed by the treatment the urine has undergone (fresh, hydrolyzed, and/or pasteurized) and the length of time after that treatment. For example, urine collected from a rural festival in Vermont (Table 1, event A) and used for an experiment 10 months after it underwent hydrolysis was referred to as "urine sample A hydrolyzed for 10 months."

Bacteriophage and virus strains. Bacteriophages used as surrogates for human viruses included *Escherichia coli* nonenveloped bacteriophages T3, MS2, and Qβ. Bacteriophage T3 (38.2 kbp) is a dsDNA virus that has dimensions of 50 by 20 nm in size and was used as a surrogate for polyomaviruses, which are dsDNA viruses. Bacteriophages MS2 (3.6 kbp) and Qβ (4.2 kbp) are both ssRNA viruses and 25 nm in diameter, but Qβ has disulfide bonds in its capsid and MS2 does not. These two viruses were studied to help explain differences observed in the inactivation kinetics of BKPyV and T3.

The BK polyomavirus Dunlop variant was used to study the fate of BKPyV. This genetic variant was chosen since it can be propagated at a high titer ($\sim 10^8$ IU [infectious units] ml^{-1}) to provide maximal experimental sensitivity and range. BKPyV (dsDNA) has a 5.1-kbp genome and is approximately 45 nm in diameter (64).

Bacteriophages MS2 and T3 were propagated in their *E. coli* hosts (ATCC 15597 and 11303). The viruses were extracted from cellular material with a chloroform extraction method (65) and purified with an Econo fast protein liquid chromatography system (Bio-Rad, USA) equipped with a HiPrep Sephacryl

S-400 column (GE, USA). The purified virus fraction was concentrated with 100-kDa Amicon ultracentrifugal filters and filter sterilized with 0.22-μm polyethersulfone (PES) membrane filters (Millipore, USA). The final MS2 and T3 stocks ($\sim 10^{11}$ PFU ml^{-1}) were stored in phosphate buffer (5 mM NaH_2PO_4 and 10 mM NaCl, pH 7.5) at 4°C. Bacteriophage Qβ was propagated in its *E. coli* host (ATCC 15597) and purified similarly to MS2 and T3, except the protein chromatography step was excluded. The Qβ stocks ($\sim 10^{11}$ PFU ml^{-1}) were stored in phosphate buffer at 4°C for immediate use in infectivity experiments. The bacteriophages were enumerated by the double-layer plaque assay (65). Briefly, aliquots of each virus were serially diluted, and 100 μl of final serial dilutions were combined with 100 μl of an overnight culture of their respective *E. coli* hosts and 5 ml of soft agar. Plaques were enumerated after overnight incubation at 37°C.

BKPyV was propagated in Vero and 293TT cells using previously published methods (66, 67). Briefly, Vero and 293TT cells were grown to 70% confluence, infected with BKPyV crude lysate at a multiplicity of infection (MOI) of 0.1 IU cell^{-1}, and incubated at 37°C for 3 weeks (Vero cells) or 10 days (293TT cells). Virus lysates were purified over density CsCl gradient centrifugation, and the collected virus fraction was dialyzed overnight in buffer (10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.9).

Infectious BKPyV was enumerated in renal proximal tubule epithelial (RPTE) cells with immunofluorescence assays (IFAs) (66). When RPTE cells in the wells of 24-well plates reached 70 to 80% confluence, serial dilutions of the BKPyV virus samples were added to the cells. Following a 1-h infection at 4°C, the cells were further incubated at 37°C for 2 days. Cells were then fixed with 4% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline (PBS; 154 mM NaCl, 5.6 mM Na_2HPO_4, 1.06 mM KH_2PO_4, pH 7.4), washed with a 0.1% Triton detergent solution, rinsed with PBS, and then washed with a 5% goat serum blocking solution. To identify infected cells, the cells were treated first with a 1:200 dilution of antibody pAB416 (68) in 5% goat serum and then with a 1:200 dilution of polyclonal goat anti-mouse IgG fluorescein isothiocyanate antibody (Sigma) in 5% goat serum. The virus titer was determined by counting the individual fluorescent cells. At least nine random fields of view with at least five positive cells each were averaged to obtain the titer values (infectious units per milliliter) in each well. Duplicate wells were prepared for each sample and their titer values were averaged (67).

Virus inactivation in urine and buffer. All inactivation experiments were conducted at room temperature in the dark. T3 bacteriophage was spiked into 10 to 50 ml of hydrolyzed urine ($n = 3$) at concentrations of 10^8 to 10^9 PFU ml^{-1} to evaluate the inactivation rate of the human dsDNA virus surrogate.

Bacteriophages MS2 and Qβ were spiked into 10 ml of hydrolyzed urine ($n = 2$) to evaluate the inactivation rates of the bacteriophages with (MS2) and without (Qβ) disulfide bridges in the protein capsid. Both surrogates were spiked into hydrolyzed urine at an initial concentration of 10^8 to 10^9 PFU ml^{-1}. Infectious particles were quantified 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 to 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 min. Urine was filtered through a 0.22-μm PES syringe filter. BKPyV inactivation was also measured in buffer solutions with various pHs and ammonia concentrations (see Table S2 in the supplemental material). 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 are described in Table S1). DNA was extracted from all urine samples (100 to 300 μl) for qPCR analysis with Maxwell total viral nucleic acid extraction kits (Promega) using a 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, TGTGATTGGGATTCAGTGCT [69]), 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 reaction mixtures included 5 μl 2× 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 and 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 to 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 medium (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 to 1×10^6 IU ml^{-1}. Fifty-microliter aliquots were removed from the mixture at different time points up to 27 days and

added to 450 μl of REGM. The samples were stored at −80°C until viral DNA extraction, and then qPCR was conducted on a 900-bp region of the genome (Table S1).

To estimate the reaction rate constants for the entire BKPyV genome, we first extrapolated the concentrations measured for the 900-bp amplicon to the entire 5.1-kbp genome with the following relationship (70):

$$\log\frac{N}{N_0} = \log\frac{n}{n_0} \times \frac{\text{genome size}}{\text{amplicon size}} \quad (1)$$

where N/N_0 is the extrapolated concentration of the entire genome and n/n_0 is the measured concentration of the 900-bp amplicon. First-order rate constants for the BKPyV genome were estimated by conducting linear regressions of $\ln(N/N_0)$ versus time. This extrapolation assumes that the reactions in the genome are uniformly distributed.

The ability of the BKPyV Dunlop strain qPCR method to detect small decreases of the 900-bp gene copy concentrations in hydrolyzed urine was tested by diluting the spiked urine solutions by 10% and 20% (i.e., 0.9× and 0.8×) with nuclease-free water. The undiluted, 0.9×, and 0.8× samples were extracted five times each, and then the 900-bp targets were quantified with qPCR. The measured gene copies in the 0.9× and 0.8× samples were compared to the measured gene copies in the undiluted sample.

Experiments to study polyomavirus capsid disassembly. To evaluate protein capsid stability in hydrolyzed urine, BKPyV proteins were separated with SDS-PAGE gels, and the VP1 capsid proteins were visualized by Western blotting (67). Specifically, 50 μl of stock BKPyV was spiked into 1 ml of hydrolyzed urine at a concentration of 5×10^5 to 1×10^6 IU ml^{-1}. Immediately after adding the virus to the urine and then again after 24 h, 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 min 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 under reduced conditions were diluted 1:20 to have signal strength similar to that of nonreduced samples for Western blot visualization. After buffer addition, all samples were heated to 42°C for 5 min and resolved on an SDS-PAGE gel. Under nonreducing conditions, the capsid proteins will enter the gel only if the disulfide bridges were broken in the experiments. Control samples were prepared under 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 1:2,000 VP1 primary antibody diluted in 2% nonfat dry milk solution, stained with 1:2,000 anti-mouse horseradish peroxidase 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 modeled 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 bacterial live/dead ratios and changes in the 900-bp gene copy concentrations after storage in hydrolyzed urine samples 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.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02374-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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