Lyssa excreta: Defining parameters for fecal samples as a rabies virus surveillance method

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ABSTRACT

It is not possible to systematically screen the environment for rabies virus (RABV) using current approaches. We sought to determine under what conditions RABV is detectable from feces and other accessible samples from infected wildlife to broaden the number of biological samples that could be used to test for RABV. We employed a recently-developed quantitative RT-PCR assay called the "LN34 panlyssavirus real-time RT-PCR assay", which is highly sensitive and specific for all variants of RABV. We harvested and tested brain tissue, fecal, and/or mouth swab samples from 25 confirmed RABV positive bats of six species. To determine if rabies RNA lasts in feces sufficiently long post-defecation to use it as a surveillance tool, we tested fecal samples from 10 bats at the time of sample collection and after 24 hours of exposure to ambient conditions, with an additional test on six bats out to 72 hours. To assess whether we could pool fecal pellets and still detect a positive, we generated dilutions of known positives at 1:1, 1:10, 1:50, and 1:200. For six individuals for which matched brain, mouth swab, and fecal samples were tested, results were positive for 100%, 67%, and 67%, respectively. For the first time test to 24 hours, 63% of feces that were positive at time 0 were still positive after 24 hours, and 50% of samples at

72 hours were positive across all three replicates. Pooling tests revealed that fecal positives were detected at 1:10 dilution, but not at 1:50 or 1:200. Our preliminary results suggest that fecal samples hold promise for a rapid and non-invasive environmental screening system.

Keywords: Lyssavirus, RABV, rabies, virus, Chiroptera, bat, non-invasive, disease surveillance, RT-PCR

INTRODUCTION

Rabies is a zoonotic disease of the central nervous system that invariably results in mortality [1]. It is caused by the RNA virus *Rabies lyssavirus* (RABV) and viruses from the *Lyssavirus* genus. RABV has the highest fatality rate of infectious diseases, with more than 59,000 human deaths globally each year [2]. Worldwide, dogs are the main RABV reservoir for human transmission, but in the Americas where vaccination of dogs is widespread, bats generate most of the human rabies cases [3, 4]. In Latin America, the primary species causing human infection is the common vampire bat (*Desmodus rotundus*) [5], while in the northwestern and southeastern U.S. tricolored (*Perimyotis subflavus*) and silver-haired (*Lasionycteris noctivagans*) bats have variants of rabies that are responsible for a higher proportion of human and terrestrial mammal deaths [6]. In Arizona, part of the American Southwest, suburban outbreaks of the disease occur regularly in wildlife populations, and interactions between wildlife and residents results in human exposures each year [7]. Arizona is one of the leading U.S. states for rabid wildlife, with bats, skunks, and gray fox the most common reservoir species [8]. Patyk et al. [9] found that among U.S. bat species, those in the Southwest were more likely to be rabid.

Despite its proximity and serious nature, it is not possible to systematically screen the environment for RABV. The gold standard for rabies diagnostics is the direct fluorescent antibody (DFA) test, which requires fresh brainstem tissues held at cold chain temperatures, requirements that prevent

47 surveillance using inexpensive, field-collected samples [10]. Additional testing by the US Department of
48 Agriculture, Animal and Plant Health Inspection Service, Wildlife Services is conducted through enhanced
49 rabies surveillance using the direct rapid immunohistochemical test (DRIT) [11, 12] or as part of the rabies
50 public health surveillance system. What is needed is a rabies detection approach based on readily
51 available, non-invasive samples that can be applied broadly.

52 A recently-developed quantitative RT-PCR assay called the "LN34 panlyssavirus real-time RT-PCR
53 assay" is highly sensitive and specific for all variants of rabies virus (RABV) [13, 14]. This assay was
54 developed by a Centers for Disease Control (CDC) research group [14] and was found to be as or more
55 sensitive than the DFA test [13]. It consists of a dual assay: the LN34 assay as well as a host species control
56 β-actin real-time RT-PCR assay that signals presence of RNA in a sample and indicates PCR inhibition,
57 extraction failure, or RNA degradation. Because the assay is highly sensitive, having succeeded with low
58 quality and formalin-fixed samples, there is promise for non-traditional sample types such as feces, which
59 contain intact and degraded nucleic acids [15]. Further, presence of RABV and other lyssaviruses in feces
60 and saliva [16, 17] presents a surveillance opportunity with the LN34 assay, which has been shown to
61 successfully detect RABV down to single digit copies of RNA [14].

62 Our overarching goal was to define and illustrate an effective and inexpensive surveillance system
63 for rabies detection that can form the foundation of future statewide efforts to better understand public
64 health risks. To address the goal, an environmental screening system for detection of RABV from feces
65 first required evaluation of the strengths and limitations of feces as a potential sample type. We tested 1)
66 RABV quantity in brain stem, mouth swab, and fecal material from infected individual bats; 2) evaluated
67 RABV positivity of feces at ambient temperatures over 72 hours to better understand how long RABV may
68 be detectable post defecation; and, 3) calculated RABV positivity of pooled fecal samples to determine
69 how many fecal samples can be collected together to still return a positive result. We hypothesized that
70 bat fecal samples could be reliably employed to detect RABV using the LN34 assay.

71

72 **METHODS**

73 **Sample acquisition**

74 This study was approved by the Institutional Animal Care and Use Committee (IACUC) at
75 Northern Arizona University (Protocol 18-012). Carcasses and brain stems from bats found to be RABV
76 positive via DRIT were provided by USDA Wildlife Services. Arizona bats evaluated included *Lasiurus*
77 *xanthinus* (western yellow bat), *Eptesicus fuscus* (big brown bat), *Nyctinomops femorosaccus* (pocketed
78 free-tailed bat), *Tadarida brasiliensis* (Mexican free-tailed bat), *Parastrellus hesperus* (western
79 pipistrelle), *Lasiurus ega* (southern yellow bat), and *Antrozous pallidus* (pallid bat). Necropsies were
80 performed in a BSL3 facility by staff with pre-exposure rabies vaccinations. We harvested feces from the
81 intestines of bats using sterile scalpels and medical scissors, and used sterile cotton-tipped swabs to
82 collect saliva. Samples were deposited into DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and frozen
83 at -80°C until RNA extraction.

84 **LN34 panlyssavirus real-time RT-PCR assay**

85 We extracted RNA using the Zymo Direct-Zol RNA Miniprep Kit protocol. We performed the
86 LN34 and β-actin RT-qPCRs on a QuantStudio 7 Flex (ThermoFisher Scientific, Waltham, MA, USA) as
87 described previously [13, 14]. To summarize, for each sample, the LN34 assay targets the lyssavirus RNA
88 genome and the β-actin assay targets host β-actin mRNA. Each 10 µL reaction contained Luna Probe
89 One-Step RT-qPCR 4X Mix (New England Biolabs, Ipswich, MA, USA), primers (10 µM), probe (5 µM), and
90 2 µL RNA template. Samples were run as three replicates, and each assay run contained synthetic
91 positive control RNA provided by the Center for Disease Control (Atlanta, GA, USA) and no template
92 control reactions in triplicate. We used the LN34 assay diagnostic algorithm for post-mortem brainstem
93 samples to determine the positive/inconclusive/negative thresholds [13].

94 **Tissue types, fecal time tests, and pooling**

95 For individuals of five bat species (*Lasiurus xanthinus*, n = 1; *Lasiurus ega*, n = 1; *Nyctinomops*
96 *femorosaccus*, n = 1, *Tadarida brasiliensis*, n = 1; *Parastrellus hesperus*, n = 2), we tested three tissue
97 types (brainstem, saliva/mouth cells via mouth swab, and guano) with the LN34 assay. We also
98 performed two time tests to determine how long feces remained positive at ambient conditions. We
99 used feces of 1) ten RABV positive *Parastrellus hesperus* to 24 hours (0 and 24 hours), and, 2) six RABV
100 positive bats (*Eptesicus fuscus*, n = 1; *Antrozous pallidus*, n = 1; *Lasiurus xanthinus*, n = 1;, *Tadarida*
101 *brasiliensis*, n = 3) to 72 hours (0, 24, 48, and 72 hours). Each fecal sample was divided into two (for the
102 24 hour test) or four (for the 72 hour test) portions. When each time point was reached, we added 1 mL
103 of DNA/RNA Shield to the fecal matter and stored the samples at -80°C until RNA extraction. To
104 determine the extent to which fecal samples could be pooled in a field scenario (assuming the most
105 dilute case of only one fecal sample from a RABV positive bat), we tested two known positive fecal
106 samples (*Eptesicus fuscus* and *Tadarida brasiliensis*). We used 10 µL from 20 known RABV negative bat
107 fecal extractions to make a negative pool. This was used to dilute the positive samples to 1:1, 1:10, 1:50,
108 and 1:200.

109 **RESULTS**

110 The six individuals for which we tested matched brain, mouth swab, and fecal samples, results
111 were RABV positive for 100%, 67%, and 67% (Table 1), respectively. For the 24 hour time test, 63% of
112 feces that were positive at time 0 were still positive after 24 hours (Table 2). For the 72 hour time test,
113 all three replicates were positive for 50% of samples at 72 hours (Table 3). Pooling tests revealed that
114 fecal RABV positives were detected at 1:10, but not at 1:50 or 1:200 (Table 4).

115 **Table 1.** Using the LN34 assay, rabies virus was detected in the brainstem, mouth swab, and feces of
116 rabid bat carcasses. Values are Ct means of three replicates. LN=LN34, BA=β-actin.

Bat species	Brainstem	Mouth swab	Feces
Lasiurus xanthinus	Positive (LN: 17.30, BA: 23.77)	Positive (LN: 25.15, BA: 31.58)	Positive (LN: 23.91, BA: 27.63)
Nyctinomops femorosaccus	Positive (LN: 22.25, BA: 25.10)	Inconclusive (LN: *, BA: 32.33)	Positive (LN: 30.24, BA: 23.57)
Tadarida brasiliensis	Positive (LN: 21.31, BA: 21.96)	Positive (LN: 31.45, BA: 30.98)	Inconclusive (LN: 40.16, BA: 28.52)
Parastrellus hesperus	Positive LN: (22.35, BA: 25.08)	Inconclusive (LN: *, BA: 33.41)	Positive (LN: 27.06, BA: 24.03)
Lasiurus ega	Positive (LN: 26.27, BA: 31.76)	Positive (LN: 30.19, BA: 37.14)	Inconclusive (LN: 38.87, BA: 36.99)
Parastrellus hesperus	Positive (LN: 19.32 BA: 27.04)	Positive (LN: 33.71, BA: 31.84)	Positive (LN: 31.09, BA: 31.50)

*Undetermined

Table 2. Positivity of fecal samples harvested from rabid *Parastrellus hesperus* bat carcasses and tested at time 0 and after 24 hours at ambient conditions, with mean Ct in parentheses.

	Time 0	24 hours
1	Positive (LN: 24.51, BA: 25.25)	Positive (LN: 27.43, BA: 26.24)
2	Positive (LN: 31.98, BA: 30.51)	Positive (LN: 31.98, BA: 30.10)
3	Positive (LN: 32.00, BA: 30.10)	Positive (LN: 33.15, BA: 37.50)
4	Positive (LN: 30.61, BA: 25.15)	Positive (LN: 31.81, BA: 35.23)
5	Positive (LN: 24.89, BA: 28.80)	Positive (LN: 33.74, BA: 38.36)
6	Positive (LN: 33.61, BA: 28.12)	Inconclusive (LN: 35.54, BA: 35.40)
7	Inconclusive (LN: 37.18, BA: 29.55)	Inconclusive (LN: 37.56, BA: 29.22)
8	Inconclusive (LN: 37.01, BA 28.86)	Positive (LN: 32.51, BA: 34.46)
9	Positive (LN: 33.46, BA: 33.03)	Inconclusive (LN: 44.04, BA: 39.14)
10	Positive (LN: 32.46, BA: 31.36)	Inconclusive (LN: 36.82, BA: Undetermined)

Table 3. Positivity of fecal samples harvested from rabid bat carcasses and tested at time 0 and after 24, 48, and 72 hours at ambient conditions. At 72 hours, at least one replicate was positive for 4 of 6 samples (a) and all three replicates were positive for 3 of 6 samples (b). Bat species tested included *Eptesicus fuscus* (n = 1), *Tadarida brasiliensis* (n=3), *Antrozous pallidus* (n=1), and *Lasiurus xanthinus* (n=1).

a)	**At least one replicate was positive**

LN34					β-actin				
Time	Mean Ct	Ct Range	# Samples Positive	Total # Samples	Time	Mean Ct	Ct Range	# Samples Positive	Total # Samples
24	35.45	1.03	5	6	24	29.25	3.51	4	6
48	34.61		5	6	48	32.77		5	6
72	34.42		4	6	72	29.68		4	6

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b) All three replicates were positive									
LN34					β-actin				
Time	Mean Ct	Ct Range	# Samples Positive	Total # Samples	Time	Mean Ct	Ct Range	# Samples Positive	Total # Samples
24	33.37	2.98	2	6	24	30.47	1.28	3	6
48	33.10		3	6	48	30.96		4	6
72	30.39		3	6	72	29.68		4	6

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130 **Table 4**. Positivity and amplification success of two RABV fecal samples declined at dilutions exceeding
131 1:10. Dilutions positive for rabies are underlined, LN is mean Ct over three replicates for the LN34 assay,
132 the number of successful amplifications is in parentheses, and the positive control was a synthetic
133 sequence provided by the Center for Disease Control. RABV fecal 1 was from a *Tadarida brasiliensis* and
134 RABV fecal 2 was from an *Eptesicus fuscus*.

	1:1	1:10	1:50	1:200
RABV fecal 1	LN: 31.25, BA: 26.52 (3)	LN: 34.12, BA: 29.73 (3)	LN: 36.56, BA: 30.56 (1)	Undetermined (0)
RABV fecal 2	LN: 32.13, BA: 35.71 (3)	LN: 34.82, BA: 31.93 (3)	LN: 35.56, BA: 32.17 (1)	Undetermined (0)
Positive control	LN: 27.65, BA: undetermined (3)	LN: 30.46, BA: 31.8 (3)	LN: 32.32, BA: 31.81 (3)	LN: 34.14, BA: 31.84 (3)

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136 **DISCUSSION**

137 Bat fecal samples hold promise as a surveillance method for rabies virus. The successful testing
138 of feces, for which nucleic acids are naturally degraded and in a matrix containing multiple inhibitors
139 [15], was likely aided by a short amplicon; the LN34 assay amplifies a 165 bp region. We found that fecal
140 samples of postmortem rabid bats yielded PCR Ct values that were higher than that of brain tissue (*i.e.*,
141 lower quantity), but the majority remained positive. Half of the fecal samples were positive to at least
142 72 hours at ambient temperatures, which suggests that there is time post-defecation to collect feces
143 and for rabies to still be detectable. Feces could still be detected when pooled at a ratio of 1:10 (one
144 fecal pellet with rabies virus collected together with nine without rabies virus), which provides guidance
145 for pooling of feces in the field. Notably, we used the established Ct thresholds for brain tissue, but it
146 may be that new, higher thresholds to assign positivity could be set for this sample type [13].

147 Fecal samples will allow determination of rabies presence at a site or region, and will do so at a
148 scale that is not currently possible with postmortem tissues. It will be possible, for example, to non-
149 destructively sample bat roosts to determine the enzootic prevalence and seasonality of RABV. The
150 assay is inexpensive, and thus it is possible to sample broadly, and to do so in any locale that has RT-PCR
151 capability. Further, it is likely that feces from mammals other than bats can be targeted. For instance,
152 surveillance programs using canine feces would benefit vaccinations campaigns and the effort to
153 eliminate dog-mediated human rabies deaths by 2030 [18]. Finally, it will be possible to explore pairing
154 positive fecal samples with sequencing methods to determine the phylogeographic dynamics of species
155 specific variants and better understand the evolving risk of zoonotic expansion.

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