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). We sought to determine under what conditions RABV is detectable from wildlife feces 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 rabies virus (RABV). We harvested and tested brain tissue, fecal, and/or mouth swab samples from 25 confirmed RABV positive bats of six species. As a first assessment of whether rabies RNA lasts sufficiently long post-defecation to use it as a surveillance tool, we tested feces from 10 bats at the time of fecal harvesting and after 24 hours of exposure to ambient conditions. Due to the success of this test, we performed a second 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. For the second time test, all three

replicates were positive for 50% of samples at 72 hours. Pooling tests revealed that fecal positives were detected at 1:10, 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, 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 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, 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 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 the proximity and serious nature of this virus, it is not possible to systematically screen the environment for the disease. 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 obviate inexpensive, field-collected samples [10]. Additional testing by the US Department of Agriculture, Animal

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

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

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

70

71 **METHODS**

72 **Sample acquisition**

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

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

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

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

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

107 **RESULTS**

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

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

Bat species	Brainstem	Mouth swab	Feces
Western yellow	Positive (LN: 17.30, BA: 23.77)	Positive (LN: 25.15, BA: 31.58)	Positive (LN: 23.91, BA: 27.63)

Pocketed free-tailed	Positive (LN: 22.25, BA: 25.10)	Inconclusive (LN: *, BA: 32.33)	Positive (LN: 30.24, BA: 23.57)
Mexican free-tailed	Positive (LN: 21.31, BA: 21.96)	Positive (LN: 31.45, BA: 30.98)	Inconclusive (LN: 40.16, BA: 28.52)
Western pipistrelle	Positive LN: (22.35, BA: 25.08)	Inconclusive (LN: *, BA: 33.41)	Positive (LN: 27.06, BA: 24.03)
Southern yellow	Positive (LN: 26.27, BA: 31.76)	Positive (LN: 30.19, BA: 37.14)	Inconclusive (LN: 38.87, BA: 36.99)
Western pipistrelle	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 western pipistrelle 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 big brown bat (n = 1), Mexican free-tailed bat (n=3), pallid bat (n=1), and western yellow bat (n=1) bat.

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

124

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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	6		24	30.47		3	6
48	33.10	2.98	3	6		48	30.96	1.28	4	6
72	30.39		3	6		72	29.68		4	6

126

127 **Table 4**. Positivity and amplification success of two RABV fecal samples declined at dilutions exceeding
128 1:10. Dilutions positive for rabies are underlined, LN is mean Ct over three replicates for the LN34 assay,
129 the number of successful amplifications is in parentheses, and the positive control was a synthetic
130 sequence provided by the Center for Disease Control. RABV fecal 1 was from a Mexican free-tailed and
131 RABV fecal 2 was from a big brown bat.

	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)

132

133 **DISCUSSION**

134 Bat fecal samples hold promise as a surveillance method for rabies virus. Success with feces, for
135 which nucleic acids are naturally degraded and in an environment of inhibitors [15], is likely aided by a
136 short amplicon; the LN34 assay amplifies a 165 bp region. We found that fecal samples of postmortem

137 rabid bats yielded Ct values that were higher than that of brain tissue, but the majority remained
138 positive. Half of the fecal samples were positive to at least 72 hours at ambient temperatures, which
139 suggests that there is time post-defecation to collect feces and for rabies to still be detectable. Feces
140 could still be detected when pooled at a ratio of 1:10 (one fecal pellet with rabies virus collected
141 together with nine without rabies virus), which provides guidance for pooling of feces in the field.
142 Notably, we used the established thresholds for brain tissue, but it may be that new, higher thresholds
143 to assign positivity could be set for this sample type [13].

144 Fecal samples will allow determination of rabies presence at a site or region, and will do so at a
145 scale that is not currently possible with postmortem tissues. It will be possible, for example, to sample
146 bat roosts to determine the prevalence and seasonality of the disease. The assay is inexpensive, and
147 thus it is possible to sample broadly, and to do so in any country that has RT-PCR capability. Further, it is
148 likely that feces from mammals other than bats can be targeted. For instance, surveillance programs
149 using canine feces would benefit vaccinations campaigns and the effort to eliminate dog-mediated
150 human rabies deaths by 2030 [18]. Finally, it will be possible to explore pairing positive fecal samples
151 with sequencing methods to determine the geographical extent of circulation.

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