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Use of a Direct, Rapid Immunohistochemical Test for Diagnosis of Rabies Virus in Bats

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Abstract: Rabies, a zoonotic encephalitis due to transmission of a lyssavirus, such as rabies virus (RABV), has the highest case fatality of any infectious disease. A global program for the elimination of human rabies caused by dogs is proposed for realization by 2030. Sensitive, specific, and inexpensive diagnostic tests are necessary for enhanced surveillance to detect infection, inform public health and veterinary professionals during risk assessments of exposure, and support overall programmatic goals. Multiple laboratory techniques are used to confirm a suspect case of rabies. One method for the detection of lyssavirus antigens within the brain is the direct rapid immunohistochemical test (dRIT), using light microscopy, and suitable for use under field conditions. Besides dogs, other major RABV reservoirs reside among mammalian mesocarnivores and bats. To date, use of the dRIT has been applied primarily for the diagnosis of RABV in suspect mesocarnivores. The purpose of this study was to assess the usefulness of the dRIT to the diagnosis of rabies in bats, compared to the gold-standard, the direct fluorescent antibody test (DFAT). Brains of 264 suspect bats, consisting of 21 species from Arizona and Texas, were used in the evaluation of the dRIT. The overall sensitivity of the dRIT was 100% (0.969–1.0, 95% CI) and the specificity was 94.6% (0.896–0.976, 95% CI), comparable to the DFAT. This preliminary study demonstrated the utility of the dRIT in the confirmation of RABV infection in bats. Future studies should include additional geographic, lyssavirus, and mammalian species representations for broader application during enhanced rabies surveillance, with incorporation of any potential adjustments to standard protocols, as needed.

Keywords: bat; diagnosis; direct rapid immunohistochemical test; encephalitis; lyssavirus; rabies; surveillance; wildlife; zoonosis

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1. Introduction

Rabies is an acute, progressive encephalitis caused by infection with highly neurotropic RNA viruses in the genus Lyssavirus, Family Rhabdoviridae [1]. This zoonosis is global in distribution and causes millions of exposures and tens of thousands of human fatalities annually [2]. More than 16 different lyssaviruses were described over the past 60 years, with several others under taxonomic consideration as additional species [3]. Globally, most deaths are due to bites from dogs infected with rabies virus (RABV). However, all mammals are believed susceptible to RABV infection [4]. Significant reservoirs of RABV include mesocarnivores, such as canines (e.g., *Canis* spp; *Lupulella* spp.), ferret-badgers (e.g., *Melogale* spp.), foxes (e.g., *Urocyon cinereoargenteus*; *Vulpes* spp.), mongooses (e.g., *Cynictis penicillate*; *Urva auropunctata*), procyonids (e.g., *Procyon lotor*), and skunks (e.g., *Mephitis mephitis*), as well as multiple bat spp. Within the Americas, RABV is the only lyssavirus characterized to date.

Typically, as quintessential neurotropic pathogens, a diagnosis of rabies in animals consists of the postmortem demonstration of specific viral antigens or nucleic acids within the central nervous system [5,6]. The direct fluorescent antibody test (DFAT) is a gold-standard method, used for the detection of lyssavirus antigens in the brains of suspect cases [5–7]. Besides the DFAT, the direct rapid immunohistochemistry test (dRIT) is also a recommended confirmatory method, using light microscopy and biotinylated antibodies for the observation of intra-neuronal inclusions within the central nervous system [5,6]. In recent decades, the dRIT has been applied to enhance rabies surveillance in Africa, Eurasia, and the Americas [8–18].

Within the Americas, programs for the elimination of human rabies caused by dogs have resulted in significant regional progress [19]. Today, human and domestic animal cases in this region are caused primarily from RABV transmission via rabid wildlife, particularly from bats [20,21]. In North America, where canine RABV perpetuation has been eliminated, the majority of dRIT applications have been in support of enhanced rabies surveillance (ERS) activities focused upon the oral vaccination of free-ranging mesocarnivores, such as raccoons [22–24]. In contrast, most cases of bat rabies are detected by public health laboratories, after human exposure. Within the context of ERS and non-human exposures, the aim of this study was to evaluate the potential utility of the dRIT in the diagnosis of RABV in naturally infected rabid bats from the USA. Given progress to date during ERS for multiple species, we hypothesized that our findings would support the benefits of the dRIT as a useful diagnostic test as applied to other suspect wildlife, to confirm the presence of RABV in affected bat populations.

2. Materials and Methods

2.1. Samples

Brains originated from suspect bats that were collected as part of routine public health laboratory testing or ERS in Texas and Arizona, respectively, as described for the USA [24]. Brains were kept frozen (−20 to −80 °C) until analysis. The first preliminary evaluation was a retrospective examination of coded bat brain samples obtained from Texas, which had been evaluated via the DFAT by the Texas Department of Health during 2016. Findings by the dRIT were obtained and recorded, before unblinding of prior testing results.

The sample set from Arizona consisted of brains collected from bats as part of ERS, without a history of human or domestic animal exposure, during the period 2016–2020 and were tested prospectively by the dRIT. After evaluation, results were confirmed by the DFAT at the Wadsworth Center Rabies Laboratory, New York State Department of Health.

2.2. Protocol

The dRIT protocol was performed using the Wistar Institute purified and biotinylated anti-RABV ribonucleoprotein murine monoclonal antibodies (MAbs) 502 and 802, as described [25,26]. Briefly, touch impressions of bat brains were made on glass microscope slides. The slides were air-dried, fixed in 10% buffered formalin for 10 min, dip-rinsed in wash buffer of PBS with 1% Tween 80 (TPBS), immersed in 3% hydrogen peroxide for 10 min, and dip-rinsed in fresh TPBS. After dipping, excess buffer was shaken from the slides and blotted from the edges (repeated after each rinse). Slides were incubated in a humidity chamber (i.e., a plastic cover on a moistened paper towel) with the anti-RABV MAbs for 10 min, dip-rinsed in TPBS, incubated with a streptavidin-peroxidase complex for 10 min and dipped in TPBS. A 3-amino-9-ethylcarbazole (AEC) stock solution was prepared by dissolving one 20 mg AEC tablet in 4 mL of N, N-dimethylformamide. A working dilution was prepared by adding 1 mL of AEC stock solution to 14 mL of 0.1 mol/L acetate buffer and 0.15 mL 3% hydrogen peroxide. Slides were incubated with the AEC peroxidase substrate for 10 min, dip-rinsed in distilled water, and were counterstained with Gill's formulation #2 hematoxylin, diluted 1:2 with distilled water for 2 min and dip-rinsed in distilled water. Slides were mounted with a water-soluble mounting medium and examined by light microscopy (Leica Microsystems Inc., Buffalo Grove, IL, 60089, USA)

at magnifications of 200× to 400× for detection of typical RABV antigens (i.e., appearing as magenta-stained inclusions against a faint, bluish background, using the selected chromogen for this protocol), compared to positive and negative control brain samples. After generation of the dRIT data, the results of the DFAT upon bat brains were chosen as being informative as the gold standard, for definition of true positive, true negative, false positive, and false negative values.

2.3. Analysis

Based upon the comparison of the dRIT results with the findings from the DFAT, the diagnostic sensitivity (Se), specificity (Sp), positive predicted value (PPV), and negative predicted value (NPV) were calculated [27].

3. Results

Fifty brains from 9 species were received from Texas, consisting of 20 rabid and 30 non-rabid bats (Table 1). After unblinding of the line list, compared to the DFAT, the dRIT Se was 100% (0.832–1.0, 95% CI) and the Sp was 96.7% (0.828–0.992, 95% CI). A single false-positive result was obtained from an evening bat, *N. humeralis*. Upon re-testing of this sample, using a slightly thinner brain impression on the microscope slides, no RABV antigens were detected using the dRIT.

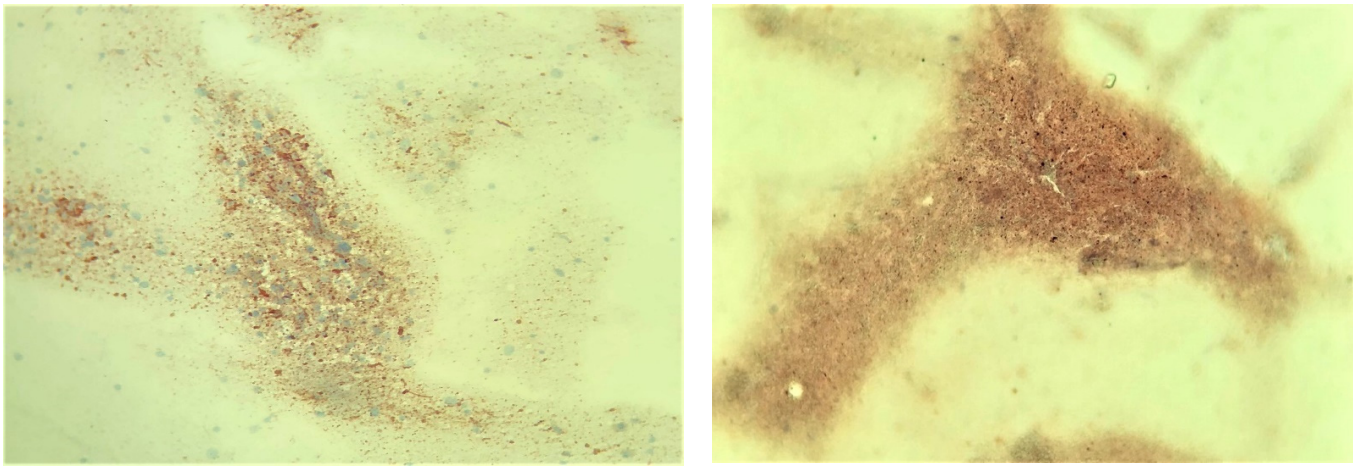
Table 1. Comparison of Results Obtained upon Suspect Rabid Bat Brain Samples from Texas by the Direct Fluorescent Antibody Test (DFAT) and the Direct Immunohistochemical Test (dRIT).

Bat	DFAT Positive	DFAT Negative	dRIT Positive	dRIT Negative
Mexican free-tailed bat, *Tadarida brasiliensis*	5	5	5	5
Evening bat, *Nycticeius humeralis*	5	5	6	4
Red bat, *Lasiurus borealis*	2	4	2	4
Northern yellow bat, *Lasiurus intermedius*	3	3	3	3
Tri-colored bat, *Perimyotis subflavus*	1	4	1	4
Hoary bat, *Lasiurus cinereus*	2	2	2	2
Cave bat, *Myotis velifer*	1	3	1	3
Seminole bat, *Lasiurus seminolus*	1	2	1	2
Southern yellow bat, *Lasiurus ega*	0	2	0	2
TOTALS	20	30	21	29

In Arizona, brains from 214 bats were tested by the dRIT, consisting of 15 species, plus five samples unidentified to species (Table 2). One brain sample from a Mexican free-tailed bat, *T. brasilensis*, was unsuitable for testing by both the dRIT and the DFAT, and was not included in these totals. Compared to the DFAT of 97 positive and 117 negative samples, the dRIT SE was 100% (0.963–1.0, 95% CI) and the Sp was 94% (0.881–0.976, 95% CI). There were 7 false-positive results: 2 Mexican free-tailed bats; 1 big brown bat; 1 canyon bat; 1 cave bat; and 1 lesser long-nosed bat (Figure 1). Negative, non-specific staining appeared as infrequent dull, pinkish, or reddish background staining of brain tissue, without distinctive magenta inclusions of various sizes and shapes, as detected in rabid animals by the protocol employed in this dRIT application (i.e., in contrast to the 'apple-green' inclusions observed by fluorescent microscopy in the DFAT) [5,6]. Combining the results of testing the 264 bat brain samples, representing 21 species from Arizona and Texas, provided a Se of 100% (0.969–1.0, 95% CI) and a Sp of 94.6% (0.896–0.976, 95% CI), with a PPV of 93.6% (0.878–0.972 95% CI) and a NPV of 100% (0.974–1.0, 95% CI).

Table 2. Comparison of Results Obtained upon Suspect Rabid Bat Brain Samples from Arizona by the Direct Fluorescent Antibody Test (DFAT) and the Direct Rapid Immunohistochemical Test (dRIT).

Bat	DFAT Positive	DFAT Negative	dRIT Positive	dRIT Negative
Mexican free-tailed bat, *Tadarida brasiliensis*	26	21	28	19
Big brown bat, *Eptesicus fuscus*	20	18	21	17
Canyon bat, *Parastrellus hesperus*	33	18	34	17
California myotis, *Myotis californicus*	2	3	2	3
Cave bat, *Myotis velifer*	4	20	5	19
Pallid bat, *Antrozous pallidus*	6	14	6	14
Lesser long-nosed bat, *Leptonycteris yerbabuena*	0	7	1	6
Western yellow bat, *Lasiurus xanthinus*	2	4	2	4
Yuma myotis, *Myotis yumanensis*	1	2	2	1
Hoary bat, *Lasiurus cinereus*	2	0	2	0
Western red bat, *Lasiurus blossevillii*	0	2	0	2
Arizona myotis, *Myotis occultus*	0	1	0	1
Western small-footed myotis, *Myotis ciliolabrum*	0	1	0	1
Big free-tailed bat, *Nyctinomops macrotis*	1	0	1	0
Townsend's big-eared bat, *Corynorhinus townsendii*	0	1	0	1
Not identified	0	5	0	5
TOTAL	97	117	104	110



(**a**) (**b**)

Figure 1. Representative microscopic observations of rabies virus-suspect bat brains by the direct rapid immunohistochemical test (dRIT). (**a**) Example of a positive test sample, with specific staining (i.e., detection of frequent magenta inclusions) in the brain of a rabid California myotis (*Myotis californicus*) by the dRIT (400×), as confirmed by the gold-standard direct fluorescent antibody test (DFAT). (**b**) Example of a negative test sample, with infrequent non-specific staining in the brain of a non-rabid lesser long-nosed bat (*Leptonycteris yerbabuena*) by the dRIT (400×), in agreement with the DFAT.

4. Discussion

Prior research, focused primarily upon mesocarnivores, outlined the core benefits of the dRIT during large-scale ERS in support of national programs for the oral vaccination of wildlife [22,23]. The individual and combined Se/Sp of the dRIT in this study, obtained upon a sample of naturally infected rabid bats, were comparable to prior findings and the expectations obtained with the gold-standard comparator, the DFAT. For example, in a recent summary of general DFAT findings, among 70 participants during routine laboratory proficiency testing, the Se was 97.9% (ranging from 66.7% to 100%), while the Sp was 96.1% (ranging from 33.3% to 100%) [28].

Regardless of the suggested additional supportive application for the critical utility of the dRIT in ERS of affected wildlife, this preliminary study had several limitations. More than 40 different bat species occur throughout the USA, but our catchment area only included two states in the southwestern portion of the country, restricted to a small fraction of submitted samples, and reflective of less than 50% of the continental species diversity. Considering state-wide totals, during 2019 alone, Arizona and Texas reported 138 and 565 rabid animals, including 55 and 289 rabid bats, respectively [24]. Moreover, samples were not collected randomly. In Texas, specimens consisted of bats submitted to the state health laboratory, primarily due to human or domestic animal exposures during 2016. These included convenience samples of frozen brains that had been subject previously to the DFAT, which were adequate in both relative quality and amount, for re-testing by the dRIT. In Arizona, in contrast to retrospective testing of the Texas samples with a prior known outcome in the DFAT, this activity involved prospective testing of bats without known human or domestic animal exposure (e.g., animals found dead; bats provided after pest control removal). In addition, most of the Arizona samples originated in the southeastern portion of the state, primarily from a single county (i.e., Pima), and sample sizes were constrained based upon dependence of those ERS specimens from a limited variety of sources. Not unexpectedly, repercussions from the COVID-19 pandemic limited availability of bat samples from cooperators during the period 2019–2020. Considering that each year in the USA, tens of thousands of bats are submitted for laboratory testing, this study contained only a very small cumulative fraction of this annual collection. Moreover, for both state samples, in contrast to the multiple dRIT publications of alternative methodologies used to date, a single harmonized protocol was performed in this investigation for RABV detection and alternate outcomes might be expected, based upon the use of other MAb or polyclonal conjugates, chromogens, equipment, technical expertise, species compositions, and lyssavirus representations.

One other obvious drawback for general disease surveillance in bats is their relatively small body size compared to mesocarnivores. Whereas recommendations for collection of suspect mesocarnivores are explicit to avoid damaging the head, widespread guidelines for humane killing of bats are often lacking, resulting in crushing and other severely traumatic injuries, when submitted by the public. Consequently, tissue specimen quantity and quality will vary greatly, due to predation, putrefaction, mummification, or other environmental and ecological factors, such that identification of specific anatomical areas of the central nervous system critical for definitive rabies diagnosis (i.e., the brainstem and cerebellum) may be missing (Figure 2).

Despite these epidemiological biases, testing issues, and logistical concerns as encountered under many ERS conditions, technically the basic Se, Sp, PPV and NPV outcomes obtained using the dRIT applied to this set of samples from bats were favorable. No false negatives were recorded, and false positive results were limited to ~3% of total samples, a finding which declined with the experience gained over time with routine dRIT use. Negative, non-specific staining was uncommon, and may be related in part to tissue quality, impression thickness, bacterial contamination, or other variables.

Figure 2. Example of a submitted fringed myotis bat (*Myotis thysanodes*) sample, with no adequate brain tissue availability for testing by the dRIT.

Although molecular testing by real-time PCR and other modern methods is encouraged by central laboratories, the use of rapid RABV antigen detection assays by local point-of-care facilities is a key advantage of the dRIT, usually occurring in less than 1 h. Just as the DFAT served as a confirmatory test to the dRIT during this study, the reverse could also be envisioned, whenever equivocal results are obtained in routine diagnostic testing for rabies. Additionally, the dRIT is economical to perform compared to the DFAT or molecular tests, which require more expensive equipment and maintenance [5,16,26]. Given the historical role of wildlife as lyssavirus reservoirs throughout Africa, Australia, Eurasia and the Americas, incorporation of sensitive, specific, economical, practical, and decentralized diagnostic methods for rabies surveillance are needed for broader application to a diverse array of mammalian species, especially under field conditions.

5. Conclusions

In addition to the use of the dRIT for ERS of rabies in mesocarnivore reservoirs, the test is also suitable for RABV detection in bats. Inclusion of the dRIT for bats broadens pathogen detection beyond traditional, centralized public health testing alone, improves the opportunity for identification of cross species transmission events and the emergence of novel RABV variants, with consequent host shifts and potential long-term perpetuations [24,29,30]. To this effect, continued application in areas such as Arizona is warranted, where realization of the extension of bat RABV to meso-carnivores prompted timely management actions towards active disease prevention and control, including the use of oral and parenteral wildlife vaccination [29]. Considering its perpetuation among diverse wildlife such as bats, rabies is not a candidate for eradication. However, routine extension of the dRIT and ERS to other diverse geographic regions, mammalian species and lyssaviruses is encouraged,

especially in tandem to the current focus upon the global elimination of human rabies caused via dogs by 2030 [3,13–18].

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