Laboratory Diagnosis of Rabies: The Role of Molecular Testing

Recommendations and Considerations for Expanding Testing Options for Postmortem Diagnosis of Rabies Virus in Animals

APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

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Introduction

The purpose of this report is to document the outcomes and recommendations on the role of molecular methods in the postmortem diagnosis of rabies virus in animals in the US based on a literature review and scientific consultation.

In this document we report on the six identified key components or topics to evaluate the role of molecular-based assays in the rabies diagnostic testing algorithm. As part of this review and consideration we kept in mind that any primary diagnostic method for rabies virus must be able to detect all currently known and circulating lyssavirus strains as well as newly emerging strains with a high sensitivity and specificity that is comparable to or better than the current diagnostic standard. Additional considerations included evaluating the ability to standardize practices, ensuring all aspects of testing including pre-analytical (sampling) and analytical workflows were considered and addressed as well as overall quality, including proficiency testing (PT) and safety. The **Process Summary (page 4)** includes details on the literature review, consultation and report. The **Overall Recommendations (page 5)** are provided next, followed by a **Summary of Gaps (page 6)**. Lastly, more detailed recommendations including background, limitations and additional needs are then listed by topic.

Background on Rabies Testing

The accurate and rapid laboratory diagnosis of rabies is a critical public health need. Rabies, caused by *Lyssavirus rabies* or rabies virus, is nearly 100% fatal in humans when post-exposure prophylaxis (PEP) is not initiated before clinical signs and symptoms develop. While PEP initiation usually happens immediately for high-risk exposures, it is often delayed or avoided if the animal is available for testing. The post-mortem testing of suspect animals by public health laboratories must be completed quickly with a high-quality, accurate method. A negative result would potentially lead to PEP not being initiated or discontinued. Therefore, a false negative result could be fatal. Ensuring a robust rabies virus testing algorithm is crucial.

In 1999, the National Workgroup on Rabies Prevention and Control published the *Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody (DFA) Testing*[1] (we will use "National Standard Protocol" throughout this document, though it is also commonly referred to as the "National Protocol" or the "National Minimum Standard Protocol") to address the need for standardization of a rapid, readily available and accurate method for the laboratory diagnosis of rabies. The DFA method was established as the gold standard diagnostic test for determining patient treatment following a potential exposure and has been shown to be highly sensitive (96%), specific (99%) and accurate.[1] Laboratories in the US use DFA, recommended by the US Centers for Disease Control and Prevention (CDC),[1] World Health Organization (WHO)[2] and the World Organization for Animal Health (WOAH)[3] as the gold standard primary diagnostic method for testing animals with suspected rabies virus infection. However, maintaining DFA as the primary diagnostic assay presents challenges, including loss of expertise in performing the method and reduced availability of DFA reagents.

While all diagnostic testing requires trained and competent staff, it is especially important in DFA testing to ensure slides are properly read and interpreted. Changes in the public health workforce have made it more difficult to maintain capacity for DFA. These changes include the diminishing national capability and capacity for fluorescent microscopy as molecular methods such as nucleic acid amplification test (NAAT) are increasingly utilized for diagnostic testing. Additionally, loss of trained staff due to budget reductions and retirements is compounded by the lack of training opportunities on florescent microscopy methods for incoming medical laboratory scientists as they continue to prioritize molecular methods in formal laboratory science programs.

DFA, like most laboratory methods, requires a steady supply of reagents from manufacturers. The National Standard Protocol requires slides to be stained with anti-rabies conjugates from two sources: either two different monoclonal antibody pools or one monoclonal antibody conjugate and one hyperimmune serum conjugate. This is problematic because there have been periodic shortages of these products for years and there is growing concern that a product may be discontinued as diagnostic manufacturers consider streamlining product lines.

In recent decades, NAAT-based diagnostic methods have become widely utilized for the detection of many viral and bacterial pathogens as either the primary diagnostic method or a supplemental diagnostic method to detect the presence of a pathogen. Currently the National Standard Protocol suggests the use of alternative tests to rule out or confirm rabies virus when the DFA is inconclusive or non-diagnostic. The suggested alternative testing includes virus isolation or PCR (the protocol specifically says PCR although several different NAAT methods could be used). Additionally, the WHO *Laboratory Techniques in Rabies, Fifth Edition* includes language about the use of molecular assays. In Volume 1--Chapter 5, published in 2018 and focused on surveillance for rabies virus, WHO recognizes DFA as well as reverse-transcription PCR (RT-PCR, a type of NAAT) as a primary diagnostic test when decisions on PEP may be affected, as a part of active surveillance and for detection of human rabies cases. Specifically, real-time RT-PCR (rtRT-PCR) is highly recommended as useful for primary or confirmatory testing, while a conventional RT-PCR should be accompanied by sequencing if used as a primary diagnostic test method (Table 4.2).[2] The second volume of the same manual published in 2019 focused on the techniques themselves; Chapter 27 includes more conservative language about the use of RT-PCR, including the following discussion point: "Despite the highest level of sensitivity and their ever increasing important role in many countries, the use of molecular assays, including RT-PCR for routine post-mortem diagnosis of lyssaviruses is currently not recommended if brain tissue is available, especially for animal rabies."[4] There is a clear role for molecular methods in rabies diagnosis and evaluating the literature and conducting a scientific consultation was critical to move the field forward.

Process Summary

The Association of Public Health Laboratories (APHL) convened a Rabies Virus Diagnostic Working Group in 2018 comprised of subject matter experts (SMEs) from state and local public health laboratories and university veterinary laboratories. The working group addressed issues associated with rabies diagnostic testing in the US. This group was charged with evaluating the role of molecular-based assays in the rabies diagnostic algorithm and providing recommendations. The group identified six topics which were divided into key questions and assigned two SMEs from the working group to each topic along with a CDC staff person. The search terms for the literature review were determined by the working group and the literature search was conducted at CDC with the assistance of CDC librarians. Each topics' key questions, assigned SMEs and search terms can be found in **Appendix A (page 20)**.

On July 12, 2021, APHL convened the working group along with CDC observers to review the evidence compiled from each literature review. The SMEs for each topic reviewed the output of the literature review and the group discussed their recommendations.

This document summarizes the findings by SMEs, combined with the input from the attendees at the meeting. In this report, we describe the background and limitations, followed by a summary and then the recommendations for each topic area. In cases where the topic had multiple key questions the information is all presented together. In certain circumstances additional research, data or diagnostic development needs are outlined which would be required to address the topic more fully or make further recommendations. The recommendations contained within this document are reflective of the Rabies Diagnostic Working Group.

Overall Recommendations

For more complete details on the recommendations for each topic, please refer to each topic area later in the report. Additionally, there are a number of gaps (see ***Summary of Gaps, page 6****) in knowledge and necessary quality and safety standards that must be addressed prior to recommending NAAT as a primary diagnostic method for rabies virus.*

1. At this time, **DFA should remain as the primary diagnostic method for postmortem identification of rabies virus in animals** due to its extensively documented sensitivity and specificity, low cost of reagents and the well-established quality management systems associated with this method.

2. Laboratories with available capability and capacity are encouraged to **validate and implement a NAAT method**[a] **with high sensitivity, specificity, precision and accuracy**—such as the CDC LN34 or other NAAT method with equivalent performance—**in parallel with DFA** to be used for one or both scenarios below and depicted in the algorithms in Appendix C (page 22).

    a. **Scenario 1: Perform NAAT as an additional test method** when **DFA is unable to provide a conclusive, diagnostic result** or the **specimen conditions upon receipt are not satisfactory for DFA**.

    b. **Scenario 2:** Continue to **perform NAAT in parallel with DFA** to generate data for either a) all specimens received or b) for a pre-defined subset of specimens.

3. The following items should be established **prior to laboratories implementing NAAT as a primary diagnostic** for postmortem identification of rabies virus in animals:

    a. **A minimum standard protocol** for postmortem identification of rabies in animals by NAAT should be developed and published.

    b. **An external performance evaluation program** should be established.

a Note that there are significant biosafety concerns that should be addressed through a risk assessment during the validation process.

Summary of Gaps

Laboratories are strongly encouraged to examine and publish data to address these gaps, as applicable.

Gap/Limitation	Steps to Address Gap/Limitation	More Info
Insufficient/unclear data on the optimal range of storage conditions and acceptance criteria of specimens for NAAT.	Determine performance characteristics of NAAT methods on samples considered unsatisfactory for DFA (e.g., deteriorated, decomposed, desiccated).	Topic 1
Insufficient data on appropriate sampling for NAAT testing from a small vs. large animal to ensure consistent and robust test performance.	• Assess whether the sampling strategy for DFA is sufficient for NAAT including multiple dissections from a cross-section based on size. • Determine if there is a standard volume/mass or percentage of volume/mass (e.g., bat vs. horse) that should be utilized for animal testing. • Determine if multiple sections need to be tested. If so: ○ Are they extracted separately and treated as individual reactions? ○ Is there any pooling of extracted material? ○ How many reactions are needed to test a single animal?	Topic 1
Insufficient data on frequency of genetic mutations and extent of genetic changes for lyssaviruses and no systematic ongoing genomic surveillance plans.	• Examine the frequency and extent of genetic mutations through a comprehensive and systematic study of lyssavirus sequences from a diversity of specimens accounting for differences in species and geography. • Establish ongoing genomic surveillance for lyssaviruses in the US shared in publicly available databases.	Topic 2
Insufficient data to utilize NAAT as a primary diagnostic method.	Determine performance characteristics of NAAT as a primary diagnostic method by performing NAAT and DFA in parallel on all or a subset of submitted samples and publish in peer-reviewed journals.	Topic 2 Topic 4 Appendix C
Insufficient standardization of NAAT assay parameters.	Development and publication of a national minimum standard protocol for NAAT methods by an expert panel to mirror the National Standard Protocol for DFA.	Topic 2 Topic 4
Lack of PT program.	• Develop and establish a PT program that mirrors human clinical diagnostic testing requirements, including the frequency and complexity. • Mechanism to require participation in the PT program for all laboratories performing NAAT.	Topic 5
Biosafety concerns with handling of nucleic acid material/performing NAAT and whether that requires vaccination of staff.	• Demonstration of safe and efficient workflows for performing NAAT for rabies virus detection. • Depending on risk assessments and inactivation protocols, determination of appropriate vaccination status of staff handling nucleic acid extracts and performing NAAT methods.	Topic 6
Lack of published inactivation protocols and demonstration of complete inactivation of rabies virus in nucleic acid extracts.	• While each laboratory will likely need to perform their own verification of inactivation protocols, published literature demonstrating effective protocols are needed and would be a helpful tool. • Mechanisms for laboratories to confirm inactivation protocols when virus isolation is not available within their own laboratory.	Topic 6

Topic 1: Optimal Specimen Transport, Storage and Sampling Techniques

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

Proper specimen sampling, transport and storage are required to ensure the quality of all testing methods that influence clinical management of disease. Additionally, understanding the impact of suboptimal conditions on the different diagnostic methods is critical.

Peer-reviewed data directly comparing sampling approaches for DFA and, especially, NAAT were quite limited. The National Standard Protocol for DFA includes very detailed instructions for appropriate sampling for DFA that includes the dissected brain tissues that are required, where the cross-sections should be taken (including alternatives), how many different observations should be made and sufficient sampled brain to examine 40 separate views at 200X. However, there are no equivalent sampling recommendations for NAAT and the published studies on LN34 do not address this matter or provide sufficient details to direct laboratories how to appropriately sample for that method or NAAT more broadly. These details will be necessary to ensure high quality and reproducible testing results with NAAT. Specifically, data are needed to inform a robust sampling protocol for NAAT from a variety of animal sizes, similar to the National Standard Protocol, accounting for the likely small volume or mass that can be homogenized and extracted for NAAT.

There are few papers examining different specimen transport and storage conditions, and each had limitations, including limited sample numbers,[5] utilizing different diagnostic testing methods[5,6,7] and only analyzed either previously frozen canine brain tissue instead of fresh[5] or mouse infected brain tissue from laboratory experiments.[8] There was limited evidence that specimens can be preserved or stabilized by formalin fixation (DFA) or 50% glycerin/PBS (NAAT) if refrigeration is not available[8,9] and one paper described testing of specimens preserved in glycerin/PBS[8] but there was insufficient data to suggest fixed tissues are acceptable for testing. Therefore, no specific recommendation is included regarding fixed or preserved tissues. Additionally, the timeframe post-collection (or thawing) and the temperatures at which tissues were held varied considerably amongst the above-mentioned studies for DFA and NAAT (if included).

Summary

The ideal specimen sampling for DFA, per the current National Standard Protocol, should occur from fresh tissue absent decay or decomposition and include a full cross-section of brainstem and cerebellum (or hippocampus if the cerebellum is not available). If the specimen to be tested does not include a full cross-section of the brainstem and the cerebellum/hippocampus then negative results cannot be conclusively reported. In the absence of sampling recommendations for NAAT, laboratories should follow the sampling methods outlined in the National Standard Protocol, including using multiple dissections for larger cross-sections.

Specimens should be held at 4 °C prior to testing. However, if they cannot be kept at 4 °C, or if there will be a delay in delivery beyond 48 hours, the specimens can be frozen at -20 °C for transport. Specimens frozen for long-term storage should be held at -70 °C. Freeze-thaw cycles should be kept to a minimum. NAAT methods have been shown to be able to detect rabies virus in samples where cold chain has not been maintained or specimens have begun to decompose. Rabies virus RNA appears to be viable for at least 72 hours at 37 °C[10,11] and potentially for up to 15 days.[11] Viral antigen is typically not so robust. The addition of a NAAT method to the testing algorithm could enable a laboratory to accept specimens outside of the range required by DFA with a robust validation and stringent quality control.

Knowledge Gaps and Additional Considerations

Specimen Conditions

While molecular methods have been shown to detect virus for longer periods of time and in more degraded specimens than DFA, additional studies are needed to confirm these findings.[5,6,12,13] The additional studies should include:

- Demonstration of performance characteristics/limits of the NAAT method(s) by including:
  - Specimens collected and transported under "less than ideal" conditions, such as:
    - Overtly deteriorated/decomposed/desiccated specimens
    - Specimens not obviously in poor condition but received warm
    - Specimens stored unfrozen for extended periods of time
  - Specimens with diverse rabies virus variants and other *Lyssavirus* variants
  - Samples with low viral load and/or early infections (to the extent possible).
- Examination of the stability of viral RNA in decomposing specimens or those held at increased temperatures to demonstrate how long viral RNA (as compared to viral antigen detectable by DFA) is detectable in specimen types deemed unsuitable for testing by DFA.[9,13,14,15]

Sampling Approach

While the full cross-section of the brainstem and the cerebellum are required for testing, further studies are needed to determine the appropriate sampling method (e.g., the number of samples to test) for NAAT to ensure the assay performs consistently for all animals being tested, independent of their brain size. It is also important to know if there are limitations to the scalability if that is the case. Additional studies should examine:

- Are the recommendations for the National Standard Protocol regarding multiple dissections for larger cross-sections suitable and applicable for NAAT?
- Are there other/different parameters that should be used or calculated based on the size of the brain and what percentage (by mass or volume) should be sampled to ensure an adequate sample?
- Once extracted, would each tissue be handled as a separate amplification reaction and, therefore, how many reactions are needed per animal?

Recommendations for Optimal Specimen Transport, Storage and Sampling

1. Following the National Standard Protocol, freshly collected brain tissue—including a complete cross-section of the brainstem and cerebellum—should be used for all rabies diagnostic testing, including DFA and NAAT.

    a. The hippocampus can be used as an alternative tissue if the cerebellum is not available.[1]

    b. In the absence of any specific details (published studies or protocols) on sampling for NAAT, laboratories should follow the National Standard Protocol with regards to the sample types and inclusion of multiple dissections for larger cross-sections and subsequent extractions and amplification reactions.

    c. While best practice is to test the full cross-section of the brainstem and cerebellum, there may be times when that is not possible based on the sample received. If unable to test distinct brain sections, homogenization of the brain for NAAT might be an acceptable alternative to confirm rabies.[6] However, it cannot be used to rule out rabies.

2. Specimens, including whole brain or head, should be maintained at 4°C and tested within 48 hours for optimal detection of rabies virus for both DFA and NAAT.

    a. If the specimen(s) cannot be kept at 4°C or there will be a delay in delivery beyond 48 hours, the specimens can be frozen at -20°C for transport or -70°C for longer term storage.

    b. Freezing specimens may be appropriate in some situations to maintain sample integrity and reduce decomposition, but multiple freeze-thaw cycles should be avoided. Additionally, shipping frozen specimens could delay testing.[1]

    c. Proper packaging and shipping are essential to prevent contamination of the outside of the package as well as cross-contamination between specimens within the package.[1]

    d. Validation for a NAAT method could explore a wider acceptable temperature range for specimen transport and handling.

3. Laboratories should have a written policy on acceptance and rejection criteria for submitted specimens.

Topic 2: Minimum Standards for NAAT to be Used as a Primary Diagnostic Tool

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

As outlined in the introduction, there is precedent for the inclusion of NAAT methods in the diagnostic testing algorithm for rabies virus. It is included in the National Standard Protocol as one of two alternative tests (along with virus isolation) to verify results when DFA is unable to provide a conclusive, diagnostic result or in decomposing/non-intact samples.[1,6,14] RT-PCR (a specific NAAT approach) is recognized as primary and confirmatory diagnostic assay by international agencies.[2,4,15] While examining the role of NAAT as a primary diagnostic for rabies it is important to note that the need for proper specimen sampling and handling—including obtaining brain sections and necropsy (see **Topic 1, page 7**)—and having well-trained individuals would be equally important for both DFA and NAAT.

A primary diagnostic method for rabies virus must be able to detect all currently known and circulating lyssavirus strains as well as newly emerging strains and with a high sensitivity and specificity. This has been firmly established for DFA (96% and 99% respectively) along with an extensive history of performing at this level for a wide range of laboratories. There is also consensus that any method—but particularly one being used in a primary diagnostic role—would need to meet stringent quality standards, ideally through a minimum standard protocol and a robust PT program. Laboratories must have staff which are appropriately trained and have the necessary expertise to perform and report test results. Additionally, any method being used for primary diagnosis must be monitored to ensure that it continues to perform as expected and is able to detect known and emerging viruses.

NAAT-based diagnostic methods, including real-time PCR, are highly sensitive and specific with low limits of detection for rabies virus RNA.[10,11,16,17] Real-time PCR assays are consistently more sensitive than DFA for the use cases that have been described.[10,11,14,17,18,19] However, most studies compared NAAT in parallel with DFA or for samples not suitable for DFA, making a determination about its role as a primary diagnostic limited. Data presented in **Topic 1** also show that the specimen stability for RT-PCR is greater than for DFA, including detection between 3-15 days when held at 37 °C.[10,11] Additionally, while there is a role for highly sensitive rabies virus-specific assays which could be applied in an individual jurisdiction with sufficient resources and infrastructure for monitoring the assay, a broader pan-lyssavirus assay reduces risk of missed detections. Another important consideration for NAAT methods is the assay design to minimize the potential for false negatives. While there are different assay designs to mitigate this risk, the best practice in molecular assay design is to include multiple targets even if it doesn't increase the specificity and would only impact the sensitivity if there was a mutation. While most rabies virus NAAT methods are designed to the most conserved regions of the genome, it is still important to understand the frequency and extent of mutations which could be achieved through ongoing genomic surveillance. This is also critical for ongoing monitoring of the assay to ensure it will still detect known and potentially emerging strains.

While there are many published NAAT methods, there are a few that rise to the top. One method is the LN34 assay, a rtRT-PCR developed by CDC shown to detect all currently-known strains of lyssaviruses due to its targeting a highly conserved non-coding leader region and part of the nucleoprotein coding sequence of the *Lyssavirus* genome to maintain robustness.[14,20] In 2017, Wadhwa et al. published an evaluation of the LN34 assay, reporting a diagnostic sensitivity and specificity close to 100%.[14] Additionally, a large multi-center evaluation of the LN34 assay confirmed the low limit of detection (an estimated 8 RNA copies) and high specificity of 99.68% (95% CI: 99.29-99.88%) and sensitivity of 99.90% (95% CI: 99.47-100%) as compared to DFA.[20] This assay and the SYBR green assay based on Wakely et al. are included in the WHO document.[16] This assay has performance matching what would be ideal for a primary diagnostic method.

Summary

At the time of the consultation the only published data was the utilization of NAAT as an additional diagnostic method, not as the primary method. The LN34 assay performs well, but there is no minimum standard protocol, there is no PT program, there is not the decades long awareness of how the test performs and therefore also more limited expertise as compared to DFA. As described previously and given the high consequences of an inaccurate test result, additional real world performance data including potential limitations would need to be generated prior to utilizing this method as a primary diagnostic for rabies virus. We have described the role of NAAT in the diagnostic testing workflow in Topic 4 (page 14). There are also a number of quality and safety considerations when implementing a NAAT at any step in the workflow that are discussed in Topic 5 (page 16) and Topic 6 (page 17).

Knowledge Gaps and Additional Considerations

Standardization of Assay Parameters

In the absence of a standardized protocol, variation in procedures may result in decreased quality and overall assay performance. A national standard protocol for NAAT methods should be developed by an expert panel and should include:

- Minimum performance characteristics
- Standardized assay design features
- Other sections in the current protocol such as sample handling, sampling, test results and interpretation
- Expectations for participation in a robust performance evaluation program, as described in Topic 5, accessible to all facilities performing testing will be critical to assure quality and accurate testing results.

Determination of Genetic Changes

NAAT performance relies on the primers and probes binding to the nucleic acid target. While there may be minimal impact on DFA, a mutation in the primer binding site for the NAAT method limits the annealing of primers or probes in a NAAT method and decreases the sensitivity of the assay potentially resulting in false negatives, especially when the viral load is low. While risks associated with sporadic point mutations can never be fully mitigated, risks of more gradual changes associated with genetic drift could be partially alleviated by routine genomic surveillance. Potential approaches to address these gaps in knowledge include:

- Additional studies to examine the frequency and extent of genetic mutations through a comprehensive and systematic study of lyssavirus sequences from a diversity of specimens accounting for differences in species and geography.
- Ensure all known lyssavirus genome sequences are published in public and freely accessible databases.
- Establish ongoing genomic surveillance of circulating rabies viruses and ensure data is shared in public databases to perform in silico analysis of primer/probe designs against known sequences.

Recommendations for NAAT Minimum Standards

Currently there are significant gaps precluding the recommendation of NAAT as a primary diagnostic assay for the detection of rabies virus. Addressing the gaps in this document will be critical to re-evaluating the role of NAAT as a primary diagnostic. Review Topic 4 (page 14) for the role of NAAT in the rabies diagnostic testing algorithm.

Below are initial recommendations that should be addressed in a minimum standard for implementation of a NAAT as a primary diagnostic method.

1. The NAAT method should meet or exceed the performance characteristics of the DFA (> 96% specificity and > 99% sensitivity).[3]

2. The NAAT method should include a host target to serve as a specimen integrity and inhibition control, aiding in the interpretation of negative results (e.g., the LN34 assay uses beta-actin).

3. The NAAT method should be able to detect all currently-known and circulating lyssavirus strains (Appendix B, page 21) as well as newly emerging strains in the US, and there should be continuous monitoring of the assay design to ensure its ability to serve these two goals. One mechanism to accomplish this is through genomic surveillance (See Determination of Genetic Changes, page 11).

4. The NAAT method should be designed to minimize the likelihood of false negative results and best practice for molecular assays is to include at least two specific targets. As mentioned in Item 3 above, genomic surveillance is also important to monitor for genomic changes in the primer and probe binding regions.[21,22]

5. Prior to implementation, a robust method validation in alignment with robust quality management practices should be performed and demonstrate congruent or better results as compared to DFA prior to implementation. In most US laboratories this testing is not performed on human specimens but the test results may directly impact patient care, including treatment decisions for initiating PEP. Therefore, there must not be any doubt about the validity of the test results and the results must be timely.

6. Laboratories must retain well trained and competent personnel to perform NAAT testing and participate in a PT program (see Topic 5, page 16).

Topic 3: Define the Role of DFA in Laboratory Diagnosis of Rabies

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

While DFA has been the gold standard laboratory method for rabies diagnostic testing for many years, there are many challenges with maintaining DFA, as outlined in the introduction. When performed as written in the National Standard Protocol and tested from a properly collected specimen, users can expect a sensitivity and specificity of 96% and 99%, respectively.[3] However, a few important studies have demonstrated that a lack of standardization leading to variability in the protocol, equipment, biosafety standards or quality control will impact the performance of the assay.[23,24]

The reasons for lack of standardization or the inability to follow the National Standard Protocol exactly may be due to factors beyond the control of the laboratory. Examples include intermittent but ongoing challenges with access to commercially available monoclonal antibody conjugates necessary to perform testing, challenges with specimen collection, storage or transportation and lack of sufficient competent staffing as more experienced staff have left or retired from the field. **Topic 1 (page 7)** describes the ideal collection, storage and transport parameters. As the time between collection and testing increases, DFA sensitivity decreases which can increase the risk of false negative results. The need to optimize the conjugate for adequate coverage of variants in the geographic area and difficulty in maintaining the cold-chain storage of the samples present additional challenges to standardization of the assay.[25] Insufficient staff experience presents yet another challenge to the standardization of the DFA.[26] The performance of DFA is impacted by the experience of the microscopist and the number of different microscopists evaluating each slide.[26]

Summary

The DFA has been the gold standard and primary diagnostic test for rabies due to its well documented specificity and sensitivity on suitable specimens when tested with appropriately selected antibody conjugates covering all variants found in the US. There is a well-established National Standard Protocol, a well-established PT program[27] and standardized training provided by APHL and CDC.[1] However, maintaining DFA to this level of specificity and sensitivity also comes with significant requirements (which might also be limitations for some laboratories) including:

1. A properly maintained fluorescent microscope
2. Laboratory staff that are competent in testing and interpreting results
3. Steady access to monoclonal antibody conjugates.

Recommendations for DFA's Diagnostic Role

1. Laboratories should maintain DFA as the primary diagnostic method, performing it as written in the National Standard Protocol. This includes ensuring laboratory staff are engaged in standardized training and are assessed for competency in the method.
   a. Two independent readers are necessary, especially if the readers are inexperienced or the specimen is weakly positive or displays an increase in nonspecific staining.[1,26]
   b. Laboratory must participate in a PT program.
2. Laboratories must ensure access to properly manufactured, quality checked and titrated antibody conjugates for testing.
3. Laboratories must ensure that the microscope is properly maintained.

Topic 4: Algorithm for Confirmation of Rabies Infection

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

Laboratory detection of rabies virus must be rapid because the results are frequently used to determine the need for life-saving PEP. A clinician may wait, if possible, until the test result is available because a negative rabies virus test result would avoid unnecessary administration of PEP. If DFA results are non-diagnostic or inconclusive, or in cases where the sample lacks distinct brain sections and is not acceptable for DFA, a NAAT can confirm a rabies infection if the test is positive. Historically, results were confirmed by virus isolation in cell culture or bioassay in animals. However, in the past two decades NAAT methods have become more commonly used for rabies testing—they are highly sensitive and specific, reproducible and have a much shorter turnaround time.[10,11,12,13,14,16,17,20,28,29,30] Published studies demonstrate the utility of NAAT for rabies virus diagnosis, particularly in decomposed specimens.[10,11,16,18,23,28,31] And there is robust support for NAAT as a complement to the current diagnostic approach (i.e., DFA).[2,4,14,30,31] See Appendix C (page 22) for a proposed diagnostic workflow.

Lastly, the use of a NAAT in the diagnostic algorithm lends itself well to the addition of downstream applications such as next generation sequencing for strain typing, ongoing monitoring of mutations in primer/probe sites and even testing for other pathogens.

As with any new assay or algorithm, laboratories should establish a baseline for expected turnaround time from specimen receipt to results, including the time needed for confirmatory testing as well as assessing any potential changes in the cost of testing for the algorithm as compared to the previous assay and/or algorithm.

Summary

Laboratories should continue to utilize the DFA as the gold-standard rabies diagnostic method and validate and implement a NAAT method to be used when DFA cannot be performed or is unable to provide a conclusive, diagnostic result. It is important to demonstrate that the NAAT method has congruent results with DFA, including on a full panel of rabies viruses identified through routine laboratory testing or that could be potentially detected, prior to utilization as a confirmatory test. Many public health laboratories are well-equipped to perform NAAT as it has been used as part of a testing algorithm for other infectious diseases and pathogens for many years. Most importantly for rabies virus diagnosis, the addition of a high-quality sensitive and specific NAAT method can improve diagnostic outcomes and decrease indeterminate or inconclusive results. Proposed interim algorithms have been included in this document (Appendix C) to address adding NAAT as an additional method as well as to collect performance data to reconsider its role as a primary diagnostic (Topic 2, page 10).

Recommendations for Confirmation Algorithms

1. Laboratories should continue to follow the National Standard Protocol, as written.

2. As with DFA, when rabies is unable to be ruled out with the available sample, test results should be considered in conjunction with clinical and epidemiological evidence.

3. Laboratories with available capability and capacity should validate and implement a NAAT method,[a] such as the LN34 or other NAAT method with equivalent performance to be used for one or both scenarios below (**Appendix C**).[14,20,32]

   a. **Scenario 1:** Perform NAAT as an additional test method when:
      i. DFA is unable to provide a conclusive, diagnostic result (e.g., non-specific staining or unsatisfactory result) or
      ii. Specimen conditions upon receipt are not satisfactory for DFA (e.g., incomplete cross-section of brainstem and cerebellum, liquified, desiccated or the gross anatomy is unrecognizable).

   > **Reporting/Interpretation:** *Positive NAAT results can be reported as positive, but any other NAAT result should be reported as unsatisfactory and/or with a statement that rabies infection cannot be ruled out. Due to the absence of a minimum standard protocol for NAAT and lack of a PT program, these should not be reported as negative for rabies.*

   b. **Scenario 2:** Continue to perform NAAT in parallel with DFA to collect additional performance data for either:
      i. All specimens received or
      ii. A pre-defined subset of specimens.

a There are significant biosafety concerns with implementation of a NAAT method that are addressed in Topic 6 (page 17)

Topic 5: Optimal Proficiency Testing

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

Publications describing PT specific for rabies molecular testing were rare. Two studies involving interlaboratory comparisons were published but neither represented a true PT.[24,26] Federal regulations for PT programs for testing human clinical specimens in the US require that proficiency panels should consist of five specimens per testing event and that there should be at least two events per year at regular intervals.[33] Most rabies diagnostic testing in public health laboratories is performed on animal specimens rather than human specimens and therefore is not subject to human testing regulations. However, the clinical relevance of this testing and the results are of such significant consequence that following best practices for testing and PT are imperative. While a formal PT program exists for rabies DFA, there are currently no PT programs available for rabies NAAT.

Summary

Laboratories performing rabies NAAT as part of the diagnostic algorithm should participate in a PT program that mirrors clinical diagnostic testing requirements such as two testing events at approximately equal intervals per year. Ideally, a single panel should be used to test laboratory proficiency for both DFA and NAAT and include representative rabies variants seen across the US (**Appendix B, page 21**) and include both weak and strong positive specimens and negative specimens. A PT administrator for rabies NAAT will need to be identified and metrics will need to be established ahead of time to determine an acceptable passing score.

Recommendations for Optimal PT

1. Laboratories performing NAAT as part of a rabies diagnostic algorithm should participate in a PT program that mirrors clinical diagnostic testing requirements.

2. Until the time that this can be established, public health laboratories implementing a NAAT method should follow best practices for internal blinded PT or intra-laboratory comparison.

Topic 6: Recommended Biosafety Standards and Practices for DFA and NAAT Rabies Testing

*See **Appendix A (page 20)** for more information on the key questions and search terms used to investigate this topic.*

Background and Limitations

Biosafety in the microbiology laboratory is critically important. Ensuring staff safety when working with rabies virus (or extracted nucleic acid) is even more critical, since rabies virus is 100% fatal in humans who do not receive proper PEP. While there are only two documented cases of laboratory-acquired infection from rabies virus (both the result of a presumed exposure to high concentrations of infectious aerosols[34,35]), all activities associated with rabies testing including known or potentially infectious materials or animals are considered high risk and should be performed using Biosafety Level 2 (BSL-2) practices, containment equipment and facilities.[35]

One study reported complete inactivation using 3% H_2O_2 within two hours.[36] A later publication reported inadequate inactivation by acetone fixation.[37] Despite these few studies there is still a significant gap in documented procedures for and data from inactivation studies of rabies virus. Additional precautions, such as BSL-2 with BSL-3 precautions, should be considered when working with lyssaviruses other than rabies virus.[35]

Laboratories performing rabies testing should be familiar with and refer to the following documents that also include relevant information:

- National Standard Protocol (necropsy and DFA-specific items)
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition, pages 299-301 (rabies virus and related lyssaviruses)
- Use of a Modified Preexposure Prophylaxis Vaccination Schedule to Prevent Human Rabies: Recommendations of the Advisory Committee on Immunization Practices — United States, 2022 (pre-exposure vaccination and monitoring)

Summary

All individuals performing rabies testing are considered a Tier 1 Risk Category which means that they have an elevated risk of unrecognized and recognized exposures.[38] These individuals should receive pre-exposure vaccination following Advisory Committee on Immunization Practices (ACIP) recommendations prior to working with rabies virus or any potentially infected animals. In addition, the presence of antibodies, including a titer check, should be determined prior to allowing staff to enter the rabies laboratory and perform any rabies testing and every six months thereafter.[38] Furthermore, each laboratory should perform a thorough biosafety risk assessment prior to beginning any work and with any modification of protocols, workflows or as needed. In this case the implementation of a NAAT method as part of the rabies diagnostic workflow would require a new risk assessment be performed and should include an evaluation of staff. Laboratories considering implementing NAAT procedures where removal of either specimens or nucleic acid extracts from the laboratory dedicated to rabies testing to shared spaces and/or handled by unvaccinated laboratory staff should complete a risk assessment and virus inactivation study prior to implementation.

Knowledge Gaps and Additional Considerations

Virus Inactivation

A common consideration with implementing a NAAT method for rabies is to utilize shared equipment and cross-trained molecular staffing. However, to consider this approach, it is essential to confirm viral inactivation following nucleic acid extraction. Some of the challenges with performing viral inactivation studies are:

- Every laboratory that wants to remove extracted nucleic acid from suspect rabies specimens will likely have to conduct inactivation studies.
- The gold standard method is to inoculate viral cultures with the extracted nucleic acid and demonstrate no viral growth. However, very few laboratories still maintain viral culture capability.
- Published inactivation studies are helpful and needed for reference, however a mechanism still likely needs to be created to enable laboratories without viral culture capability to demonstrate virus inactivation.
- Supplemental guidance on this critical step is necessary. This could include existing methods for related or similar viruses or potentially using a surrogate virus with markers to assess different protocols.

Recommendations for Biosafety Standards

1. Laboratories should follow the BMBL-6, which states that rabies testing should occur in a BSL-2 laboratory.
    a. Specifically:
        i. Protective gear, protection from bone dust and proper ventilation systems are needed to protect against aerosol particles reaching the respiratory tract.
        ii. Engineering controls should be considered to further reduce risk during rabies testing, such as a necropsy table that is designed to hold larger animal heads in place.
    b. Necropsy for specimens suspected to be infected with rabies should occur in a biosafety cabinet within the BSL-2 laboratory. Heavy and/or slice resistant gloves should be worn.
    c. If unable to be performed in a biosafety cabinet, PPE for necropsy should include dedicated laboratory clothing, heavy gloves and an N95 mask with a face shield or a powered air purifying respirator (PAPR).
2. Laboratories should follow current Rabies ACIP Vaccine Recommendations including pre-exposure vaccination, minimum acceptable rabies antibody titers and frequency of titer checks for all individuals working in the laboratory and involved in specimen preparation and testing.[38]
3. A risk assessment that includes biosafety considerations should be conducted by each facility prior to redesigning their workflow to accommodate NAAT methods. To determine appropriate handling of specimens and vaccination of staff this should also include the area(s) and equipment where the NAAT method would be performed and whether an inactivation study would be needed.

Additional Review

The working group recognizes that additional publications have become available since the group performed their literature review and wanted to note the relevant findings, though they have not been included in the report nor impacted the overall recommendations.

- In 2022, Dettinger et al. published a study in which the LN34 assay was used to identify early rabies virus infections and concluded that if LN34 were to be performed on all samples along with DFA, sensitivity may be improved, increasing the ability of all laboratories to detect rabies cases with low numbers or atypical antigen.[39]
- In 2022, Minozzo et al.evaluated a modified LN34 (a duplex LN34 and Beta-actin RT-qPCR) against the DFA and mouse inoculation test (MIT).[40] The novel duplex RT-qPCR protocol demonstrated sufficient diagnostic performance such that researchers concluded it could replace the MIT.
- In 2023, WOAH updated their recommendations to include language supporting the use of NAAT (specifically RT-PCR) as a primary diagnostic assay.[15] They include the caveat that RT-PCR assays should be performed on a composite sample of brain tissue which includes the brain stem and cerebellum. It adds that RT-PCR assays with reduced sensitivity and specificity or are unable to detect all lyssaviruses should be considered as a confirmatory assay following a primary diagnostic assay.
- In 2023, CSTE updated their case definition to include rabies cases detected by positive pan-lyssavirus probe-based rtRT-PCR; or detection of lyssavirus nucleic acid by genomic sequencing.[41] However, case definitions are not testing recommendations but rather a means to capture positive cases by all possible testing schema.
- In a 2024 study, Chierato et al. found the LN34 assay to demonstrate 100% sensitivity and 98% specificity compared to DFA.[42] They additionally found the LN34 to detect positive samples which were missed by DFA. Specificity was confirmed by Sanger sequencing. The researchers ultimately concluded the LN34 assay is useful as a confirmatory assay.
- In 2025, Gigante et al. published an update to the 2018 multi-site evaluation of the LN34 pan-lyssavirus assay.[32] An updated LN34 assay was evaluated in two US laboratories. This evaluation yielded a sensitivity of 99.72-100% and a specificity of 99.99-100% when compared to the gold standard DFA test. Additionally, greater than 90% of DFA indeterminate results were negative by LN34 testing. The LN34 optimization included increased primer concentration and updated primer formulation which contributed to the increased sensitivity. In addition, the assay combined the LN34 and internal control targets into a single well (LN34M). The LN34M assay showed comparable results to the singleplex assay.

Conclusions

NAAT is a powerful and promising diagnostic tool in a rabies diagnostic testing algorithm. Laboratories that are currently utilizing NAAT as part of their rabies testing algorithm should make every effort to share their results in peer-reviewed publications as the role for NAAT in the diagnostic algorithm continues to be evaluated.

Laboratories that have not yet implemented a NAAT method are encouraged to do so as an additional test per the overall recommendations. All laboratories are encouraged to review the Summary of Gaps (page 6) and publish any completed studies that would address those gaps, including any demonstrating the performance of NAAT methods in a primary role in a testing algorithm.

Finally, some of the identified gaps *must* be addressed by the community prior to full implementation of rabies NAAT as the primary diagnostic assay:

- A national minimum standard protocol for rabies NAAT should be established by a panel of experts.
- A national plan for genomic surveillance to monitor genetic variability in rabies virus should be established.
- A robust performance evaluation plan should be established to monitor NAAT proficiency in laboratories who perform testing.

Appendices

Appendix A: Topics and Search Terms

Topic 1: Develop standards for quality specimen handling, storage and sampling to assure accurate diagnostic test results

Key Questions:

- What are the optimal specimen transport conditions to assure quality rabies testing results?
- What is the optimal specimen sampling technique to assure the most sensitive rabies test results?

Search Terms: This key component was added after the completion of the search. The results from the literature search for the remaining five key components were used to answer these questions.

Reviewed By: Lisa Dettinger and Anna Strain

Topic 2: Develop Minimum Standards for Reliable Real-time RT-PCR Diagnostic Assays to be Used as Primary Diagnostic Tools

Key Questions:

- What are the performance characteristics of published real-time RT-PCR diagnostic assay protocols for the detection of lyssavirus?
- What is the stability of rabies viral nucleic acid ex vivo?
- What are the minimum criteria for detection of lyssavirus subtypes in a rRT-PCR?
- What is the frequency of genetic drift/ shift of lyssavirus in the PCR target region?
- Would a two-target lyssavirus assay improve sensitivity or specificity?

Search Terms: Rabies, Lyssa, Lyssavirus, Pan-lyssavirus, Primary Diagnosis, Diagnostic Techniques and Procedures, Molecular Diagnostics, Pathology, molecular, Polymerase Chain Reaction, PCR, Virus Mutations, Genetic shift, Genetic drift, Sequencing, Next generation sequencing

Reviewed By: April Davis and Glen Gallagher

Topic 3: Define the Role of DFA in Laboratory Diagnosis of Rabies

Key Questions:

- What is the role of DFA in laboratory diagnosis of rabies?
- What are the performance characteristics of rabies DFA per the published National Standard Protocol?

Search Terms: Rabies (Rabies, Lyssa, Lyssavirus, Pan-lyssavirus), Diagnosis (Primary Diagnosis, Diagnostic Techniques and Procedures), Fluorescent antibody technique, Direct fluorescent antibody technique

Reviewed By: Susan Moore and Jerry Saliki

Topic 4: Develop Algorithm for Confirmation of Rabies Infection

Key Question: What combination of diagnostic assays maximizes correct detection of lyssavirus?

Search Terms: Rabies (Rabies, Lyssa, Lyssavirus), Infection, Algorithm, Confirmation

Reviewed By: Richard Greisser and Sharon Messenger

Topic 5: Develop Proficiency Testing Recommendations

Key Questions:

- How often should PT be conducted in laboratories conducting rabies testing?
- What should a sufficient PT panel for rabies be composed of?

Search Terms: Proficiency Testing, Quality Assurance, Health Care, Laboratory Proficiency Testing, Interlaboratory vs. intralaboratory, Molecular proficiency testing, Laboratory performance measures, Proficiency testing evaluation

Reviewed By: Michael Pentella and Jafar Razeq

Topic 6: Develop Recommendations on Relevant Biosafety Standards and Practices for Conducting DFA and Molecular Testing for Rabies

Key Questions:

- What are the minimum requirements for personal protective equipment (PPE) requirements when performing necropsy? When performing rabies testing?
- What procedures should be followed to ensure laboratory safety when removing specimens from the rabies laboratory for molecular testing or storage?
- What are the minimum equipment and facilities requirements to ensure laboratory safety when performing necropsy? When performing rabies testing?

Relevant Terms: Rabies (Rabies, Lyssa, Lyssavirus), Laboratory (Inactivation), Biosafety (Containment of Biohazards, Personal protective equipment, Biosafety cabinet, Containment, Biological)

Reviewed By: Michael Pentella and Jafar Razeq

Appendix B: Currently Known Circulating Rabies Virus Variants in the United States

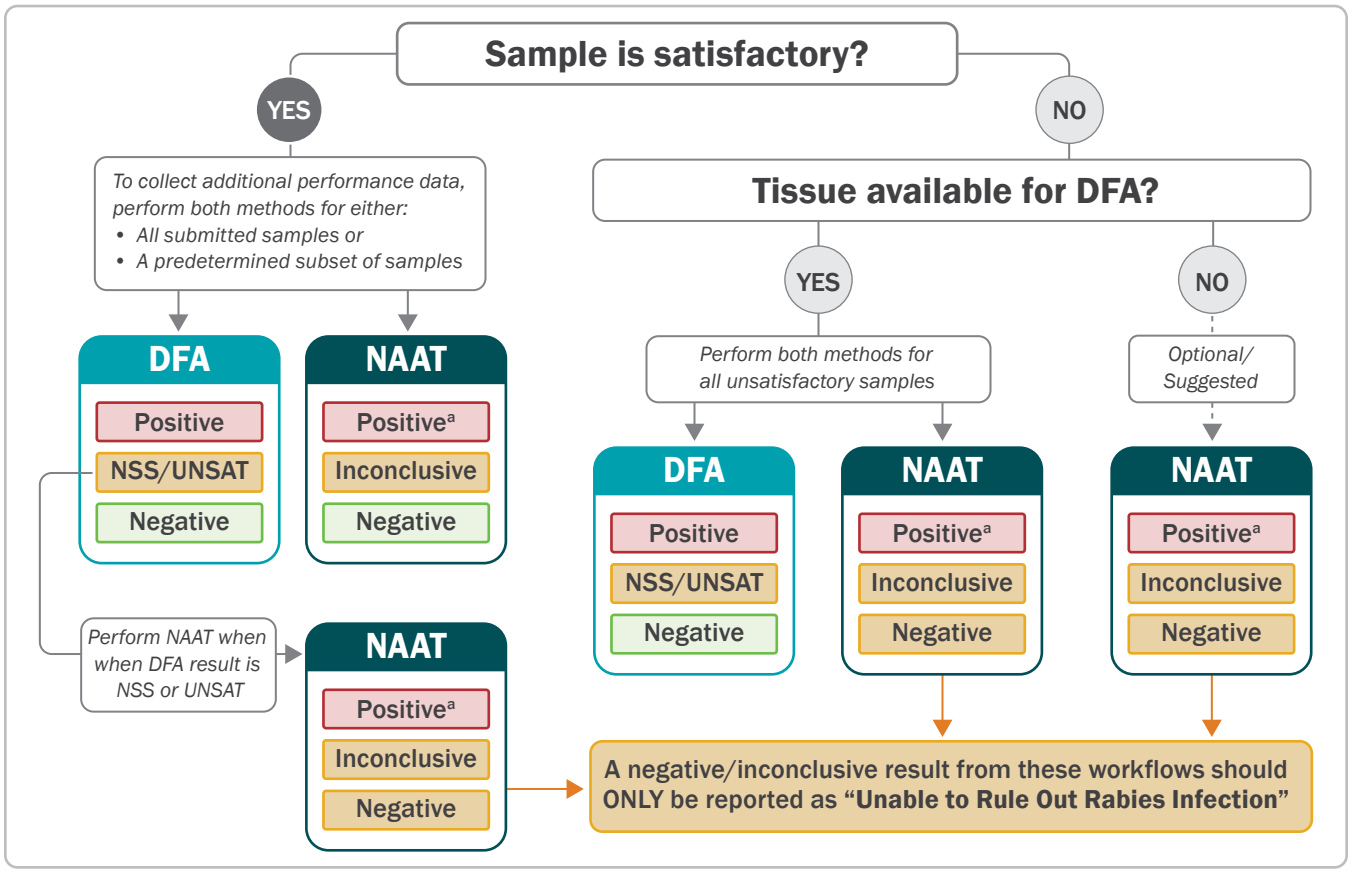
This list, based off a 2018 study,[43] is not necessarily all-inclusive and does not account for the possibility of newly-emergent strains that might arise within the US in the future or rabies virus variants that might be imported into the US from other countries or territories.

- California Skunk
- Eastern Raccoon
- North Central Skunk
- South Central Skunk
- Arizona Fox
- Arctic Fox
- Mongoose
- Bat[a]

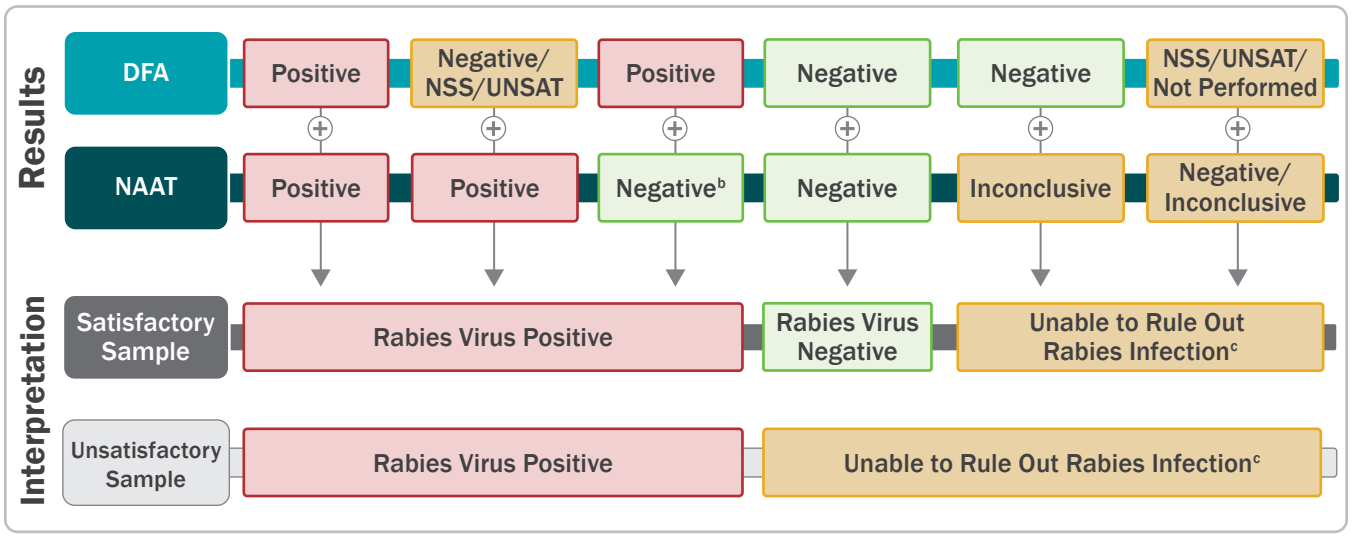
a Rabies virus variants in bats account for the leading cause of human rabies deaths in the US. At least 20 bat species serve as reservoirs for rabies virus variants in the US, and for some species of bat (e.g., *Eptesicus fuscus*), more than one genetically-distinct rabies virus variant has been detected.

Appendix C: Proposed Interim Rabies Diagnostic Workflow

Workflow Part A. Diagnostic Process Flow



Workflow Part B. Results and Interpretations



a Depending on the NAAT, a diagnostic cutoff value may need to be determined.

b If sample is satisfactory, additional followup testing should be performed (e.g., repeat NAAT, attempt strain typing and sequencing).

c Recommend consultation with infectious diseases and/or public health epidemiology with expertise in rabies.

Abbreviations: **NSS:** Nonspecific Staining **UNSAT:** Unsatisfactory

Appendix D: Glossary of Abbreviations

APHL..... Association of Public Health Laboratories

ACIP..... Advisory Committee on Immunization Practices

BSL..... Biosafety level

BMBL..... Biosafety in Microbiological and Biomedical Laboratories

CDC..... US Centers for Disease Control and Prevention

CSTE..... Council for State and Territorial Epidemiologists

DFA..... Direct Fluorescent Antibody, also referred to as DFAT or FAT

MIT..... Mouse inoculation test

NAAT..... Nucleic acid amplification testing

PAPR..... Powered Air Purifying Respirator

PBS..... Phosphate buffered saline

PCR..... Polymerase chain reaction

PEP..... Post-exposure prophylaxis

PHL..... Public health laboratory

PPE..... Personal protective equipment

PT..... Proficiency testing

RFIT..... Rapid Fluorescent Focus Inhibition Test

RNA..... Ribonucleic acid

RT-PCR..... Reverse transcription polymerase chain reaction

rtRT-PCR... Real-time reverse transcription polymerase chain reaction

SME..... Subject matter expert

US..... United States of America

WHO..... World Health Organization

WOAH..... World Organization for Animal Health

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