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Evaluation of a rapid immunochromatographic assay for the diagnosis of rabies in regional laboratories of Costa Rica

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Abstract

Rabies is a viral, lethal, and zoonotic disease with worldwide distribution, primarily transmitted through bites from infected dogs and bats. The Biosafety Laboratory (LSE-LANASEVE) of the Animal Health Service of Costa Rica (SENASA) serves as the national reference laboratory for diagnosing rabies in humans and animals. Since regional laboratories lack the equipment for the direct fluorescent antibody test (FAT), we evaluated the rapid immunochromatographic diagnostic test (RIDT) from BioNote, employing FAT as a reference, to improve rabies diagnosis. We analyzed 193 brain tissue samples between 2014 and 2019. Out of these, 174 came from species that RIDT has been validated for: bovines (162), dogs (10), and raccoons (2). The rest were from unvalidated species, including horses (7), humans (1), and others. Among the 174 validated samples, 26 bovine samples were positive for both RIDT and FAT. Reviewing all 193 samples, 28 were positive and 165 negative using both methods. Two horse samples presented inconsistencies, being positive on FAT but negative on RIDT; these were subsequently verified as false negatives by RT-PCR. RIDT exhibited a sensitivity of 94 % (CI95, 83.9-102.3), specificity of 100 %, positive predictive value (PPV) of 100 %, and negative predictive value (NPV) of 99 % (CI95, 97.1-100.5). RIDT has demonstrated reliability in quickly diagnosing rabies for validated species. We advise its application in SENASA's regional laboratories for those particular species. If there's uncertainty, samples should be sent to LSE-LANASEVE for FAT or RT-PCR confirmation.

Keywords: Costa Rica; Rabies; Rapid Immunochromatographic Diagnostic Test (RIDT); Direct Fluorescent Antibody Test (FAT); Comparison

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Study contribution

This study is important for regional laboratories in Costa Rica, it will provide an instrument for rapid diagnosis. Reliable information will be available to support the use of the test and will increase the evidence on the efficacy of the test. This study can be replicated in other geographic areas with different populations.

Introduction

Rabies is a zoonotic, highly lethal viral disease with a worldwide distribution,[1] This virus is a member of the *Rhabdoviridae* family, *Lyssavirus* genus according to the International Committee on Taxonomy of Viruses,[2] it infects mammals and is mostly transmitted by a bite from an infected animal. Each year, approximately 59 000 deaths worldwide are caused by rabies,[3] with dogs being the main reservoirs of the disease.[4]

In Latin America and the Caribbean, cases of both canine (furious) and bat-transmitted (paralytic) rabies have been reported. Several control programs have been implemented throughout the region to reduce the number of rabies cases transmitted by dogs. These programs include euthanasia of infected dogs, management of canine population density, and massive vaccination of animals in areas with outbreaks.[5, 6] Despite these efforts, dog-transmitted rabies is still present in some countries, whereas bat-transmitted rabies has become more relevant.[7]

Canine rabies in Costa Rica has been eliminated since 1987.[8, 9] Over the last decade, there have been three cases of rabies infection in humans. The first case was in 2014 when a boy from Palmar Norte, Osa, was presumably bitten by a squirrel, however, sequencing of the nucleoprotein revealed that the strain was from *Desmodus rotundus*. The second case was a girl from Nicaragua who was bitten by an opossum, and the strain was from a canine lineage. The last case was from the region of Dota in 2018 when an adult male was bitten by a bat and the strain was shown to be from *Eptesicus fuscus*, an insectivorous bat.[9] Notably, back in 2001 another boy and his grandmother died from rabies only 20 kilometers away from where the boy from 2014 was infected.[5]

Currently, the Laboratory (LSE-LANASEVE) of the Animal Health Service (SENASA) is responsible for rabies diagnosis. Samples from all over the country are received and screened using the Direct Fluorescent Antibody Test (FAT), which is considered the gold standard test by the World Organization for Animal Health (OIE) and the World Health Organization.[1, 10] Since 2013, rabies results have been confirmed by RT-PCR, and in 2014, a rapid immunochromatographic diagnostic test (RIDT) was used to compare sensitivity and specificity with FAT. RIDT is a lateral flow test (LFT) based on immunochromatography using conjugated antibodies that allows for results in a matter of minutes, with a low cost, and does not require specialized equipment. With this test, regional laboratories without a fluorescence microscope could directly analyze the raw samples.

This study aimed to evaluate RIDT using FAT as a reference test to determine whether it can be used for the diagnosis of rabies in regional laboratories (where most of the cases are from) and to speed up the sample processing time. This would reduce the response time required to handle positive cases and the earlier detection of potential cases of canine rabies that may come from the borders of the country.

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Materials and methods

Ethical statement

This study did not require the approval of the Institutional Committee for the Care and Use of Experimental Animals. The practices performed in the present study are routine for the diagnosis of rabies according to the competencies of the institution according to the SENASA law 8495 and in compliance with the animal welfare law 7451 and its reforms in the law 9458 of the Costa Rican government.

Samples:

Between 2014 and 2019, 193 samples of brain tissue from different species received at LSE-LANASEVE were analyzed by both RIDT and FAT for rabies testing (Table 1).

- **Diagnostic tests:** For FAT, the protocol described by Kissling was followed., modified to maintain ratios of one in five normal brain-conjugate and positive brain-conjugate.[11] In the case of RIDT, the Anigen Rapid Rabies Ag Test Kit (BioNote Inc, Hwaseong-si, Korea) was used following the manufacturer's instructions with minor modifications.
- **Description - Direct fluorescent antibody test (FAT):** For this assay, an antibody from the Pasteur Institute, fluorescein isothiocyanate (FITC) conjugated monoclonal antibody was used.

  Touch impression smears on slides were prepared from brain tissue (cerebellum, caudate nucleus, pons, thalamus, and brain stem). Slides were fixed in acetone for 30 minutes, then added 10 µL of antibody conjugate, followed by a 30-minute incubation at 37 °C. After that, they were rinsed with 1X PBS and then distilled water. For each test, positive and negative controls were used. The touch imprint slides were observed under a fluorescence microscope. The presence or absence of typical granular intracytoplasmic apple-green fluorescence of aggregated nucleocapsids was used as a criterion for determining positive and negative samples, respectively.[11]
- **Description - Rapid Immunochromatographic Diagnostic Test (RIDT):** The Anigen Rapid Rabies Ag Test Kit was used according to the manufacturer's instructions with some minor modifications. The swab provided by the kit was introduced into at least four areas of the brain (thalamus, pons, brain stem, cerebellum, hippocampus, and caudate nucleus per sample) and then introduced into the kit buffer and mixed. Next, using a dropper pipette, four drops were deposited in the sample inlet of the test device. The test result was read in the detection window after 10 min.
- **RT-PCR:** Primers sense-504 and antisense-304 from Vieira et al.,[12] which generate a 248 bp amplicon targeting the nucleoprotein gene, were used. Modifications to the original protocol were as follows: RNA was extracted from 25 mg of brain tissue using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). RT-PCR was performed using a OneStep RT-PCR kit (Qiagen) with 0.6 µM of each primer and following the manufacturer's recommendations for the PCR reagent concentration. The PCR program was 56 °C for 30 min, 95 °C for 15 min, 40 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The products were run in a 2 % agarose gel with 1 TBE and dyed with GelRed (Biotium, Hayward, CA, USA) for 40 min at 125 V.

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Limit of detection (LOD):

LOD was established using the median lethal dose 50 (LD_{50}) as reference. A 1:10 dilution of brain tissue infected with the Challenge virus standard (CVS) strain was prepared in PBS and intracranially inoculated into mice. Serial 1:10 dilutions were made from 102 to 106, and 30 µL of each dilution was inoculated into six 21-day-old mice. The Reed and Muench method[13] was used to calculate LD_{50}. From each dilution, 20 µL were used for the RIDT test and 200 µL were extracted for RT-PCR.

Statistical analyses:

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were estimated for the Anigen Rapid Rabies Ag Test Kit (RIDT), using Direct Fluorescent Antibody Test (FAT) as a reference test with a two by two table on Microsoft® Excel 2019 (Microsoft Inc, Redmond, WA, USA). To determine the statistical differences and correlations between RIDT and FAT, the McNemar test coefficient was calculated using Win Episcope 2.0.[14]

Results

A comparison between RIDT and FAT by species is shown in Table 1. Most of the animals were bovine (84 %), with ages ranging between three months and thirteen years, but the majority were between 3 and 6 years old. Similarly, horse samples were obtained from animals 3 months to 15 years, for the rest of the animals, ages ranged from three months to nine years. The human sample was from an eleven-year-old.

The Brunca region had the most cases comprising 70 %, followed by the Central region with 10 %, and the Chorotega, Huetar Norte, and Pacific regions had 6.7 % each. Of all positive cases, 87 % were diagnosed in bovines, 7 % in horses, 3 % in water buffaloes, and 3 % in humans.

Test comparison

Of the 193 samples, 174 were from species currently validated for the RIDT test. Positive and negative results matched in both tests for all samples, except two horses. In these two samples, the results for FAT were positive and negative for RIDT. Both samples were analyzed using RT-PCR and were confirmed positive; therefore, RIDT results were deemed as false negatives.

RIDT had a sensitivity of 94 % (CI95, 83.9–102.3) and a specificity of 100 %. The positive predictive value (PPV) was 100 % and the negative predictive value (NPV) was 99 % (CI95, 97.1–100.5). The median lethal dose 50 (LD_{50}) was 3.7×10^7 UI/mL, and the LOD for RIDT was 3.7×10^4 UI/mL, for RT-PCR, LOD was 37 UI/mL (Figure 1).

Countries with endemic rabies may have insufficient funds and infrastructure to perform FAT or RT-PCR, such as Costa Rica, where bat-transmitted rabies is endemic, not every animal health laboratory is equipped for such tests. RIDT provides a simple and quick method for rabies detection without the need to preserve cold

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Table 1. Samples by species and comparison of the results of FAT and RDIT

	N	FAT+/RIDT+	FAT-/ RIDT -	FAt+/ RIDT -
Bovine	162	26	136	0
Water buffalo	2	1	1	0
Equine	7	0	5	2
Canine	10	0	10	0
Feline	2	0	2	0
Raccoon	2	0	2	0
Coati	1	0	1	0
Squirrel	3	0	3	0
Sloth	2	0	2	0
Human	1	1	0	0
Ovine	1	0	1	0
Total	193	28	163	2

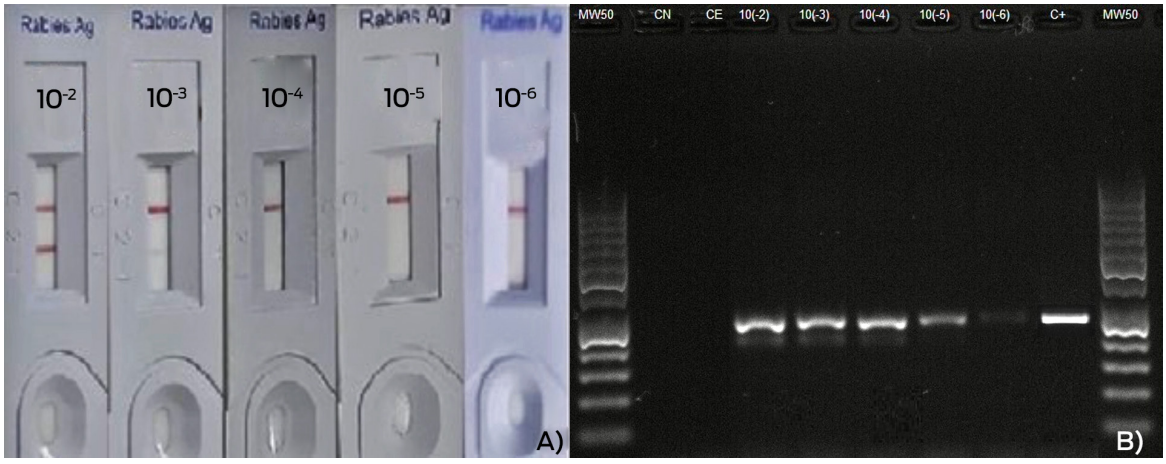

Figure 1. Limit of detection for RIDT and RT-PCR. A) Five RIDT from 10^{-2} to 10^{-6}, the limit of detection 10^{-3} equivalent to 3.7×10^{4} UI/mL where a faint band is observed, 10^{-2} two strong lines are observed. B) Electrophoresis agarose gel, 10^{-2} to 10^{-6}, the limit of detection 10^{-6} equivalent to 37 UI/mL band still observed.

chain during transportation, sophisticated equipment requiring regular and costly maintenance, or well-trained personnel. Several studies have compared RIDT with FAT to validate and improve the immunochromatographic test, for example, using two monoclonal antibodies instead of one[15] or testing its performance in *ante-mortem*[16] and *postmortem*[17] samples.

FAT is an ideal methodology for the surveillance, control, and eradication of canine rabies, considering that there are reports of seemingly healthy dogs with detectable amounts of virus in saliva[18] and that in experimentally challenged dogs, the virus can be detected up to 13 days before nervous signs become clear.[19] However, because of the intermittent transmission of the virus, rabies diagnosis from saliva is not recommended.[4] RIDT, on the other hand, has several advantages for samples that require further testing, such as PCR, because the buffer used in Bionote kits inactivates the virus and preserves the viral RNA, allowing for further

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characterization. Using RT-PCR, rabies RNA was detected even after six weeks in samples stored at room temperature in RIDT buffer.[20] Other studies have compared rabies in-house[15, 21, 22] and commercial RIDT tests against RT-PCR, FAT, or both;[17, 20, 21, 23–26] therefore, we will only compare our results with those reports using the same kit (BioNote's Anigen Rapid Rabies Ag Test Kit).

According to our results, the samples from species validated for the RIDT test had a 100 % correlation with FAT (174 samples). However, when analyzing all 193 samples, two-horse samples had a mismatching result for an overall RIDT test sensitivity of 94 % (CI95, 83.9–102.3), specificity of 100 %, PPV of 100 %, and NPV of 99 % (CI95, 97.1–100.5). A similar study from India using the cerebellum and hippocampus from 34 samples of different species, including dogs, buffaloes, cows, and others, estimated a sensitivity of 91.7 % and a specificity of 100 %, where 22 of the 24 positive animals were detected by their RIDT test.[26] Another study from Bhutan, which analyzed 179 samples from dogs, bovines, cats, and other species, had a RIDT test sensitivity of 92 % and specificity of 100 %, after 10 false-positive samples (8 dogs and 2 bovines) compared to FAT.[24] An important difference between this study and ours is the brain regions that were sampled. We used the thalamus, pons, brain stem, cerebellum, hippocampus, and caudate nucleus per sample when available, whereas the study from Bhutan used the brain stem, hippocampus, and cerebellum.

Stein et al.[27] determined that the best region to sample for immunohistochemistry (IHC) in cats and dogs was the hippocampus, followed by the bovine brain stem, cervical spinal cord, and adjacent brainstem. Bassuino et al.[28] reached a similar conclusion for horses, where they found that IHC spinal cord sections have 3.5X higher odds of detecting rabies lesions than brain tissue alone. In two of our horse samples, weak positives for FAT were negative for RIDT, so this and the fact that brain tissue is not ideal could explain the two false-negative results in our study. In raccoons and skunks, Stein et al.[27] reported positive results throughout different brain regions, suggesting that sample selection may be less important for these wildlife reservoir species.

A negative result from an RIDT test should be interpreted with caution and always be weighed in with relevant information from the case, such as bites and scratches, symptoms, FAT, and/or RT-PCR results, especially in species that are not validated for RIDT. Eggerbauer et al.[20] compared six different RIDT kits and obtained poor results with many false positives following the manufacturer's instructions. Even Bionote's kit had a low sensitivity (41.4 %), contradicting their results from 2008 and previous studies, which estimated sensitivities above 88 %.[29–31] They attributed this difference partially to changes in the leaflet instructions and kits' batch-to-batch variation. They later modified the protocols to skip the first dilution step and improved the sensitivity of some samples with a previous negative result becoming positive and matching the FAT result. Note that the sensitivity of an assay is also influenced by the sample type and degree of autolysis.[10]

Based on our results, Bionote's rabies RIDT test has high sensitivity and specificity compared with FAT when analyzing postmortem brain tissue. Considering that this test is not intended for rabies diagnosis in horses, we can conclude that our results are satisfactory. When owners avoid euthanizing the animals during the early stages of the disease, the viral load in the brain can build up to detectable levels by

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FAT or RIDT, which are less sensitive than RT-PCR. PCR should be used to confirm negative results for animals with neurological symptoms that have died.

We estimated a McNemar p-value of 0.4795; therefore, there was no significant statistical difference between the RIDT and FAT results in our samples. This means that RIDT is reliable, shows consistent results, and can indeed be used for quick rabies diagnosis.

Conclusions

Based on this, we recommend that this test be used in the regional laboratories of SENASA, but only for species validated for the kit. If there are further doubts, a sample can be sent to LANASEVE for confirmation using FAT or RT-PCR. However, it is recommended to continue applying both tests to increase the database because the statistical analysis carried out in this study is not sufficient to validate it as the only diagnostic method.

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Data availability

The data can be found in SENASA's epidemiological surveillance system software (SIVE).

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All the resources used were provided by SENASA, as the data were extracted from the routine work of this institution.

Conflicts of interest

The authors declare no conflict of interest.

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