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Field Postmortem Rabies Rapid Immunochromatographic Diagnostic Test for Resource-Limited Settings with Further Molecular Applications

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Abstract

Functional rabies surveillance systems are crucial to provide reliable data and increase the political commitment necessary for disease control. To date, animals suspected as rabies-positive must be submitted to a postmortem confirmation using classical or molecular laboratory methods. However, most endemic areas are in low- and middle-income countries where animal rabies diagnosis is restricted to central veterinary laboratories. Poor availability of surveillance infrastructure leads to serious disease underreporting from remote areas. Several diagnostic protocols requiring low technical expertise have been recently developed, providing opportunity to establish rabies diagnosis in decentralized laboratories. We present here a complete protocol for field postmortem diagnosis of animal rabies using a rapid immunochromatographic diagnostic test (RIDT), from brain biopsy sampling to the final interpretation. We complete the protocol by describing a further use of the device for molecular analysis and viral genotyping. RIDT easily detects rabies virus and other lyssaviruses in brain samples. The principle of such tests is simple: brain material is applied on a test strip where gold conjugated antibodies bind specifically to rabies antigens. The antigen-antibody complexes bind further to fixed antibodies on the test line, resulting in a clearly visible purple line. The virus is inactivated in the test strip, but viral RNA can be subsequently extracted. This allows the test strip, rather than the infectious brain sample, to be safely and easily sent to an equipped laboratory for confirmation and molecular typing. Based on a modification of the manufacturer's protocol, we found increased test sensitivity, reaching 98% compared to the gold standard

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reference method, the direct immunofluorescence antibody test. The advantages of the test are numerous: rapid, easy-to-use, low cost and no requirement for laboratory infrastructure, such as microscopy or cold-chain compliance. RIDTs represent a useful alternative for areas where reference diagnostic methods are not available.

Introduction

Canine rabies is the main cause of human rabies, globally responsible for approximately 59,000 human deaths per year, nearly all occurring in low- and middle-income countries (LMICs) in Asia and Africa[1]. The main etiological agent is a neurotropic canine-associated classical rabies virus (RABV, family *Rhabdoviridae*, genus *Lyssavirus*, species *Rabies lyssavirus*). However, other rabies-related lyssaviruses, mostly circulating in bat species, also cause disease[2,3]. In affected regions, disease surveillance and control are often hampered by low level political commitment likely due to lack of reliable data[4,5,6]. One reason for disease underreporting is the absence of laboratory diagnosis, due in part to limited access to equipped laboratories and trained staff as well as the difficulties of shipment of the specimens. Laboratory diagnosis is necessary to confirm rabies cases and additionally allows for genetic characterization of the involved strains, providing insight on virus transmission at the regional level[4,5,7].

The current gold standards for postmortem rabies diagnosis, approved by both the World Health Organization (WHO) and the World Organization for Animal Health (OIE), are the direct fluorescent antibody test (DFAT), the direct rapid immunohistochemistry test (DRIT) and molecular methods (e.g., reverse transcription polymerase chain reaction (RT-PCR))[4,8]. However, proper application in LMICs remains limited due to inadequate laboratory facilities with inconsistent power supply, uncooled sample transportation, and lack of a quality management system. Because animal rabies diagnosis is typically only conducted at central veterinary laboratories in LMICs, existing surveillance data mainly reflects the rabies situation in urban areas.

Recently developed low technology diagnostic alternatives offer opportunities to establish rabies diagnosis in remote areas and decentralized rabies laboratories[4,8,9]. The rapid immunochromatographic diagnostic test (RIDT) is a lateral flow test based on immunochromatography using gold conjugated detector antibodies and is a very promising rabies diagnostic tool[10,11,12,13]. The principle is simple: after dilution, brain material is mixed in the provided buffer, and a few drops are applied on the test strip where gold conjugated monoclonal antibodies bind specifically to rabies antigens, mainly the nucleoproteins (**Figure 1**). The antigen-antibody complexes then undergo lateral flow migration, binding at the test line (T-line) to fixed antibodies against rabies antigens, resulting in a clearly visible purple line. The remaining gold conjugated antibodies not bound to rabies antigens continue migrating and fix to the membrane through additional targeting antibodies, resulting in a clearly visible purple control line (C-line).

The one-step, low cost method is rapid, extremely easy and does not require expensive equipment or special storage conditions. With modification of the manufacturer protocol to eliminate the dilution step, nearly all equipment and reagents required to perform the test are included

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in the kit[14]. The result is read after 5-10 minutes without a microscope. This is a major advantage over the DFAT test, which requires a fluorescence microscope and immunofluorescence conjugate, along with refrigerated transportation and sample storage. Even the DRIT test, which can be performed using a light microscope, requires a continuous cold chain to store the anti-rabies antibodies, which are also not yet commercially available. In comparison to the DRIT, the RIDT requires no toxic chemicals, a particular advantage in countries where waste disposal is poorly regulated. The rapid test is less time-consuming with much easier interpretation compared to the gold standard tests DFAT and DRIT. This allows for on-site testing by personnel with limited technical expertise.

Based on these test properties, prompt diagnosis of suspected animals in remote areas becomes feasible, facilitating implementation of post exposure prophylaxis (PEP) for exposed people as soon as possible. In addition, distance transport of rabies samples is not necessary, resulting in better sample quality at the time of testing. However, the results obtained with the RIDT tests should currently be confirmed using a reference diagnostic test such as DFAT or DRIT.

RIDT techniques for detection of RABV and other lyssaviruses have been evaluated. One of the first studies was conducted by Korean researchers in 2007[10]. Compared to the DFAT method, in 51 animal samples and 4 RABV isolates, the RIDT showed a sensitivity and specificity of 91.7% and 100%, respectively. These results were later confirmed with 110 animal brain samples from Korea, with sensitivity and specificity, compared to DFAT, of 95% and 98.9%, respectively[15]. More recently, other studies assessed the performance of this RIDT using virus isolates and/or infected brain samples from various animals with different geographical origins. A panel of 21 samples, including African RABV and other African lyssaviruses (Duvenhage virus (DUVV), Lagos bat virus (LBV) and Mokola virus (MOKV)), were successfully detected, with sensitivity of 100% compared to the DFAT[16]. Similar high sensitivity (96.5%) and specificity (100%) values were obtained from a panel of 115 brain samples from Ethiopia[17]. Another study evaluated European RABV isolates, two other European lyssaviruses (European bat lyssavirus type 1 (EBLV-1) and type 2 (EBLV-2)), and the Australian bat lyssavirus (ABLV)[18]. Based on analysis of 172 animal brain samples, the RIDT kit had 88.3% sensitivity and 100% specificity compared to DFAT, and the three rabies-related lyssaviruses were successfully detected. In this study, some of the false negative results came from brain samples stored in glycerol buffer, suggesting that improper glycerol removal influenced capillary flow or antibody binding. A recent analysis of 43 clinical samples from Australian bats confirmed previous test results, with complete concordance to DFAT[19]. Two studies were conducted in India using the RIDT on a limited number of clinical samples (11 and 34 samples). Compared to DFAT, sensitivity was between 85.7% and 91.7% and specificity was 100%[20,21]. Another evaluation of this kit using 80 animal brain samples from Africa, Europe and the Middle East obtained complete concordance with DFAT for specificity (100%) but a higher sensitivity (96.9%) compared to the previous studies[22]. In a recent inter-laboratory comparison of this RIDT performed in 22 different laboratories using a panel of 10 samples, overall concordance was 99.5%[23].

Only one recent multicentric study showed unsatisfactory overall RIDT performance[24]. Samples from three different datasets were tested and provided variable sensitivity and specificity values compared to DFAT. For example, sensitivity

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and specificity obtained with the first panel (n=51) and the second panel (n=31) of samples from experimental infected animals, all tested in laboratory A, gave a sensitivity of 16% and 43%, respectively, whereas the specificity was 100% for both. Conversely, the results of the third panel (n=30) of field clinical samples analyzed by laboratory B provided a complete concordance with the results of DFAT, which was further nearly completely confirmed by laboratory A (85% sensitivity and 100% specificity). Batch-to-batch variation was suggested as a possible explanation for the fluctuating relatively low sensitivity with RIDT[24].

At the same time, another study performed a similar validation process of the above described RIDT, with a modification of the manufacturer recommended protocol[14]. The pre-dilution step (1:10) in PBS was omitted during preparation of the brain material. Based on this simpler modified protocol, the authors obtained sensitivity and specificity of 95.3% and 93.3%, respectively, compared to DFAT by testing, under laboratory conditions, a dataset of 73 animal brain samples, naturally or experimentally infected with various RABV strains. The study presented the first evaluation of this RIDT in a field setting (Chad, Africa). In 48 clinical brain samples, sensitivity and specificity were 94.4% and 100%, respectively. The discrepancies between DFAT and RIDT were due to false positive results with DFAT, determined after confirmation with RT-PCR. When these results were deleted, there was complete concordance, and it demonstrated that the RIDT was more reliable that DFAT under these field conditions[14]. No batch-to-batch variation was observed using the modified protocol. When the modified protocol was applied to a small number of the DFAT/ RIDT divergent samples (n=8) in the study of Eggerbauer et al.[24], all were found concordant (100% sensitivity).

Another major advantage of the RIDT is secondary use for detecting viral RNA fixed on the strip using molecular techniques (such as RT-PCR) and subsequent genotyping[14, 24]. Following an extraction step, Léchenne et al.[14] demonstrated viral RNA fixed on the Anigen device membrane using RT-PCR with 86.3% sensitivity in a panel of 51 samples (including 18 samples tested and shipped from Chad at ambient temperature). Subsequent genotyping was possible in 93% of the 14 samples tested. Sanger sequencing of PCR amplicons of at least 500 nucleotides in length were used. In addition to RABV isolates, the test detected four other lyssavirus species, DUVV, EBLV-1, EBLV-2 and Bokeloh bat lyssavirus (BBLV), during a fully concordant international inter laboratory test[14]. The sensitivity of viral RNA detection was even higher (100%) in the study of Eggerbauer et al., based on laboratory samples examination[24]. The latter study also demonstrated that the buffer used in the RIDT kit inactivated virus. Thereby, the devices can be shipped easily, at ambient temperature without specific biosafety precautions to reference laboratories, for molecular confirmation and genotyping.

Based on the previous evaluations, RIDT tools offer numerous advantages for use in field settings, especially when the reference diagnostic techniques are not available. However, this test also has some limitations, in particular, low sensitivity of antigen detection[14, 24]. The test is applicable for samples containing high quantities of viral antigens, such as brain samples. However, it is not appropriate for other samples such as saliva or other body fluids. Another drawback is cost of the device (around 5-10 Euros in Europe), which is less expensive compared to the cost of performing DFAT, RT-PCR or DRIT, but which still remains high for LMICs. However, future development and validation of similar RIDTs from other companies could lead to a price decrease.

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One study reported batch-to-batch variations. Although not reported by others, strict quality controls should nevertheless be performed when testing a new batch, as for any reagent used in a quality management environment. The use of the modified protocol was not altered when using different batches[14]. All except one study demonstrated that the sensitivity of RDIT was high compared to DFAT (around 90%-95%). Because rabies is always fatal, it is still strongly recommended to confirm any negative results with RDIT using a reference diagnostic test such as DFAT, DRIT or RT-PCR[14].

In this manuscript, we present a complete protocol for field postmortem diagnosis of animal rabies based on an example of a commercialized RIDT, from brain sample collection to application of a modified protocol compared to the manufacturer recommendations (which were previously validated[14]) and subsequent molecular analysis. This protocol was applied and validated many times under field conditions in West- and Central Africa, where the RIDT was used routinely for rabies diagnosis alongside the DFAT test. We additionally demonstrate a second application for the device, in laboratory settings, for extraction and detection using RT-PCR of viral RNA fixed on the device.

Protocol

1. Sample collection via the foramen magnum (occipital route)[25]

NOTE: This technique can be implemented under laboratory conditions or in field settings. Samples should be processed as soon as possible after death of the suspected animal or kept at cool temperature (refrigerated or frozen, if possible) to avoid decomposition which could affect the results. Similar to other reference techniques based on lyssavirus antigens detection such as DFAT and DRIT, decomposed samples should not be tested because it can affect the result (risk of false negative result).

CAUTION: All samples should be considered as potentially infectious. Safety regulations and procedures should be strictly followed, even in field settings[4]. In particular, wear appropriate personal protective equipment including mask, glasses, gloves and a lab coat. Use appropriate disinfectant for material and sample decontaminations (e.g., sodium hypochlorite with recommended manufacturer dilutions, 70% alcohol - ethanol or isopropanol, 1% soap solution). All personnel handling samples should be vaccinated against rabies.

1. Remove the animal head with a knife before the first cervical vertebra (atlas vertebra) to access the foramen magnum.
   NOTE: To minimize infective aerosol, avoid using a manual saw or similar tool.
2. Collect brainstem (medulla oblongata) sample using a disposable plastic pipette (**Figure 2A**), a drinking straw (**Figure 2B**), a clamp (**Figure 2C**) or a dropper (supplied with the RIDT) (**Figure 2D**).
   NOTE: Special attention must be paid when collecting the sample, because it is an utmost important step for the reliability of the results. In addition to the associated video which shows in a simple way how to collect the part of the brainstem of interest, a training step is highly recommended to make sure to collect the correct anatomical section.
3. Optionally and in addition of brain stem (medulla oblongata), collect other parts of the brainstem or the brain (cerebellum, hippocampus, thalamus and cortex)

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by the same occipital route by pushing and rotating the plastic pipette or straw towards the eye socket (**Figure 3**).

4. If using a straw or pipette, gently squeeze it to deposit the brain sample (0.5-2 g) in a tube for subsequent analysis and/or biobanking.

   NOTE: Sample storage in glycerol is not recommended, as it seems to affect capillary flow or the antibody binding step of the RIDT[18].

2. Execution of the modified RIDT protocol[14]

NOTE: This modification omits a dilution step (1:10) into PBS, as specified in manufacturer protocol (all versions), and can be implemented under laboratory or field settings.

1. Use the swab/dropper to collect the equivalent of half a peanut or pea (0.1-0.5 g) of brain material and place it in the buffer sample tube.

   NOTE: For the modified protocol, all reagents/ consumables are included in the kit (no PBS or additional tube is needed) (**Figure 4**). Document the batch number of the kit and check validity of the expiration date.

2. Carefully crush the brain material directly in the tube with the swab or the dropper for about 30 s until a homogeneous suspension is obtained.

   NOTE: The buffer reaction inactivates the infectivity of the virus in the conditions of the manufacturer's protocol[24].

3. Using the dropper, deposit four drops (approximately 100 µL) of the suspension in the sample inlet on the test device.

4. Wait for complete sample migration (1-5 min) before reading the test device. The migration should start rapidly after deposit of the sample (1-5 min).

5. In case of delay (due to high viscosity suspension) or to accelerate the start of the migration, gently scratch the bottom of the deposit site of the device with the dropper (1-5 times) and eventually add 1-2 more drops. Migration should start immediately thereafter.

6. Read the test result in the detection window after 5-10 min, and no more than 20 min, after the end of the migration.

7. Interpret the result based on presence or absence of the control line (C-line) and test line (T-line) (purple lines) in the detection window, according to **Figure 5**. Consider the sample positive when two lines are visible (**Figure 5A**), negative if only the C-line is present (**Figure 5B**) and invalid if only the T-line is present or if no lines are visible (**Figure 5C**).

   NOTE: Invalid results should be repeated at least once. Other techniques should be performed if results remain invalid. Negative results obtained with RIDT need to be subsequently confirmed using a gold standard reference method, like DFAT, DRIT and molecular methods (polymerase chain reaction or PCR). Even though the sensitivity of this test is high (see representative results), it is not 100%.

8. Store used devices at room temperature, or refrigerate/ freeze when possible, for subsequent molecular analysis (see section 4). Freeze the remaining sample suspension at -20 °C/-80 °C in the buffer tube to repeat the test if necessary or for subsequent molecular analysis.

3. RNA extraction and detection by RT-qPCR from the RIDT device

NOTE: This step can only be implemented under laboratory conditions with adapted environment and suitable equipment for molecular diagnosis. It can be done soon after the RIDT

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test or retrospectively on archived RIDT devices, stored at room temperature (15-30 °C), refrigerated or frozen.

1. RNA extraction

   NOTE: To monitor the extraction step, it is recommended to use an internal control that can be an endogenous mRNA (such as ß-actin) or an exogenous control (such as eGFP synthetic RNA) directly spiked into the sample during the first steps of the extraction[26, 27].

   1. Carefully open the device and remove the filter paper.
   2. Cut the deposit area of the sample and place it into a tube containing 1 mL of Tri-Reagent LS. Incubate at RT for 1 hour with gentle regular manual agitation.
   3. Perform the extraction in accordance with manufacturer recommendations, as previously described[27]. At this step, the exogenous internal control can be added.
   4. During the process, add 2 µL of glycogen for facilitating precipitation of RNA, according to the manufacturer recommendations.
   5. Adjust the final volume for RNA resuspension in nuclease-free water, with a volume of 50 µL generally used.

      NOTE: At the end of the centrifugation step for aqueous and organic phase separation (after addition of 200 µL of chloroform into the Tri-Reagent LS), the piece of membrane from the device will be at the bottom of the tube and not interfere with collection of the upper aqueous phase. Alternatively, other easy and rapid protocols can be used, for instance, using phenol-based reagents and silica membranes[28].

2. Detection by RT-qPCR[26]

   NOTE: Detection of potential viral RNA present in extracted samples can be done using different molecular techniques, such as reverse-transcription PCR, conventional (endpoint) or real time PCR (qPCR). Several methods are available, such as conventional RT-PCR[27, 29] or RT-qPCR[26, 30] targeting the viral nucleoprotein or polymerase gene. One example will be presented below based on a dual combined pan-lyssavirus RT-qPCR targeting a conserved region among the viral polymerase. This RT-qPCR technique associates two different RT-qPCR: one based on the TaqMan probe technology (pan-RABV RT-qPCR) and the other using the SyBR Green detection (pan-lyssa RT-qPCR). In addition, the detection of an exogenous internal control (eGFP RNA) directly spiked during the extraction process is done by a specific TaqMan probe-based RT-qPCR (eGFP RT-qPCR). Careful on-site validation of the molecular techniques selected for detection of viral RNA is important, in particular, to verify that primers, and probes for real-time RT-PCR, are adapted for detection of the strains circulating in the region of interest[4].

   1. Dilute RNA sample to 1:10 in nuclease free water. Test each RNA sample in duplicate, using a 96-well reaction plate or other formats. Use positive and negative controls for each assay and test at least in duplicate.
   2. Prepare the master mix reaction solution for the three different RT-qPCR assays according to **Table 1**, and with the primers/probes indicated in **Table 2**.
   3. Add 5 µL of diluted RNA samples and 15 µL of master mix to each of the three different assays. The pan-RABV RT-qPCR assay and the eGFP RT-qPCR assay can cycle in the same plate.

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4. Run the different assays following the thermal cycling conditions indicated in **Table 3**. If only one PCR thermal cycler is available, start with the pan-RABV RT-qPCR and keep the plate for the pan-lyssa RT-qPCR at 4 °C until the end of the pan-RABV RT-qPCR.
5. Analyze the results obtained with the three assays according to **Table 4**.

4. Genotyping after RNA extraction from the RIDT device

1. Reverse transcription RT[27 , 29]
    1. Prepare a master mix with 6 µL of RNA, 2 µL of pd(N)6 random primers (200 µg/µL) and 2 µL of nuclease-free water for a final volume of 10 µL.
    2. Incubate at 65 °C for 10 min in a heat-block and then store on ice.
    3. Prepare a master mix with 6 µL of 5x First-Strand Buffer, 2 µL of 0.1 M dithiothreitol (DTT), 1 µL (200 U) of Superscript II reverse transcriptase, 2 µL (80 U) of RNasin, 2 µL of dNTP mix (10 µM) and complete with nuclease-free water to obtain a final volume of 20 µL for each sample.
    4. Add the master mix (20 µL) to the sample (10 µL) (final volume of 30 µL) and incubate at 42 °C for 90 min in a heat-block.
    5. Proceed to the next step with PCR amplification or store the cDNA at -20 °C.
2. Conventional PCR[27 , 29 , 31]

    NOTE: Different techniques of conventional PCR are available for genotyping. Two are presented, both hemi-nested PCR, targeting a part of the nucleoprotein or a part of the viral protein of the lyssavirus. The protocol is the same for each of these assays, except for the primers and cycling conditions. Positive (positive RNA) and negative (negative cDNA and/or nuclease-free water) controls should be included in each series and each round of PCR.

    1. Prepare for each sample in a 0.2 mL microtube a master mix reaction solution for the first PCR step. This mix contains 5 µL of 10x NH4 Reaction Buffer, 2.5 µL of $MgCl_2$ solution (50 mM), 1 µL of dNTP Mix (10 µM), 1 µL of each primer (10 µM), 0.2 µL (1 U) of Biotaq DNA polymerase and 37.3 µL of nuclease-free water (final volume of 48 µL). The primers are indicated in **Table 5**.
    2. Add 2 µL of cDNA in every tube and cycle on a separate conventional PCR thermal cycler for each assay, according to **Table 6**.
    3. Prepare a second master mix reaction solution identical to the previous one with using the appropriate primers (**Table 5**) for the hemi-nested PCR reaction.
    4. Add 2 µL of the first round PCR product and cycle on a conventional PCR thermal cycler using the cycling parameters indicated in **Table 6**.
    5. Visualize the different PCR products (first and second round PCR) after loading them on a 1% agarose gel (100 mL of Tris-acetate EDTA buffer 1x - TAE 1x) with ethidium bromide (final concentration around 0.01%) and run the gel during 30 min at 120 V. A positive PCR result is observed in the form of a bright band of the expected size (**Table 5**).
3. Sanger sequencing

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1. Perform a Sanger sequencing of the amplicons obtained with the pan-lyssavirus hemi-nested PCR and complete the genotyping analysis.

Representative Results

As with any diagnostic method, sample collection is of paramount importance for reliability of the results, especially when performed in field settings. The collection process needs to be as simple as possible to guarantee collection of high-quality samples. The collection of a brain biopsy (brainstem with medulla oblongata) via the foramen magnum route for postmortem diagnosis of animal rabies fulfills this requirement, as indicated in **Figure 2A-D**[25] .

After collection, the brain sample is submitted to the modified protocol of the RIDT, summarized in **Figure 6**. As indicated in the Protocol section, the major adaptation from the manufacturer provided protocol is omission of the dilution step in PBS, which simplifies the procedure and necessary consumables/reagents, thus all included in the kit (**Figure 4**).

This modified protocol was implemented and evaluated in five different laboratories, including one WHO collaborative center on rabies (Lab 1, France), one FAO reference center for rabies (Lab 5, Italy) and three reference laboratories located in enzootic African countries, Chad (Lab 2), Ivory Coast (Lab 3) and Mali (Lab 4). In Chad, an evaluation of the RIDT was done in both laboratory and field settings.

Compared to the reference technique DFAT, sensitivity and specificity of the RDIT were high for all laboratories, with 96% to 100% and 93.7% to 100%, respectively (**Table 7**). The lowest sensitivity and specificity of the RDIT was obtained for Lab 1 (France) during the laboratory validation step. Based on the cumulative number of tested samples (n=162) (**Supplementary Table 1**), the overall sensitivity and specificity compared to DFAT were 98.2% and 95.8%, respectively (**Table 7**). However, these preliminary but promising results were obtained on a limited sample dataset and need to be further confirmed on a large number of positive and negative samples, especially for those tested in enzootic areas, to avoid any potential underestimating or bias due to the current heterogenous datasets.

The RIDT test is suitable to detect lyssavirus in brain biopsies from infected animals, where the level of lyssavirus antigens is important. However, the test limit of detection remains high when testing titrated virus suspension (**Table 8**; **Figure 7**).

Table 9 (from Léchenne 2016[14]) shows an example of results obtained after RNA detection by the dual combined pan-lyssavirus RT-qPCR targeting the viral polymerase of lyssavirus. A panel of 51 positive RIDT tests performed in laboratory conditions (Lab 1, n=32) or in Chad (Lab 2, n=19) and then shipped at ambient temperature to Lab 1, was tested. Positive detection was obtained for 18 (94.7%), 26 (81.2%) and 44 (86.3%) samples from Lab 1, Lab 2 and the two combined, respectively. In addition, genotyping was performed for 14 of these samples (10 from Lab 1 and 4 from Lab 2) using the hemi-nested PCR targeting the partial nucleoprotein gene and was successful for 13 of them (93%) (from Léchenne et al. 2016[14]).

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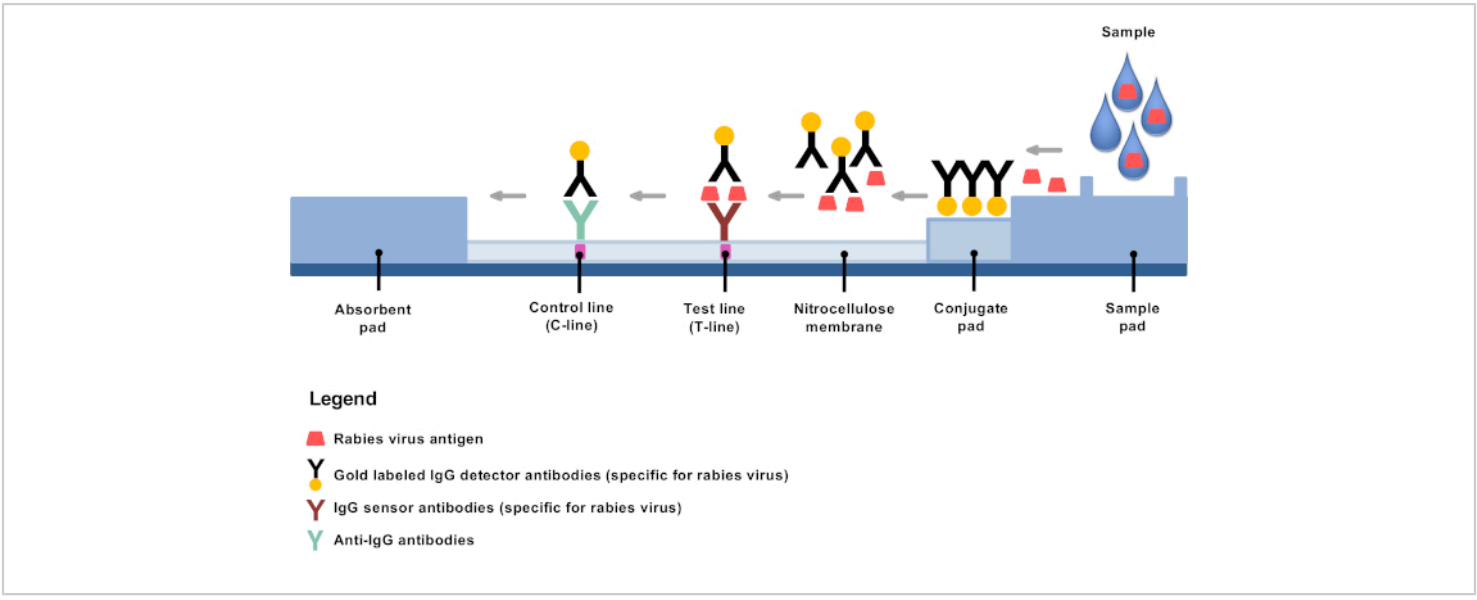

Figure 1: Schematic representation of the structure of an RIDT for rabies diagnosis. Please click here to view a larger version of this figure.

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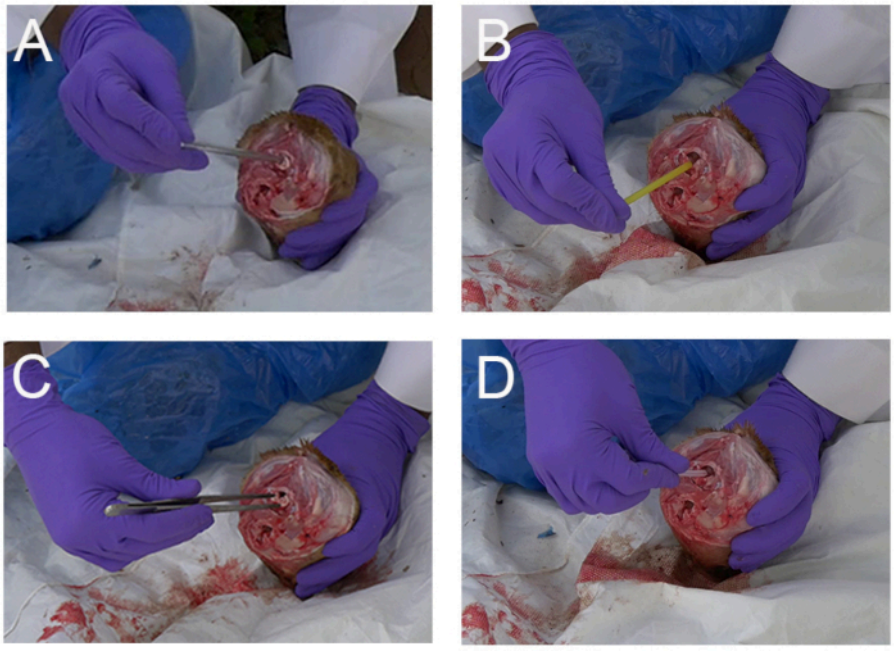

Figure 2: Examples of rapid simple techniques for collection of brain samples (brainstem with medulla oblongata) in animals (dog shown here) via the occipital foramen in field settings (Mali). (**A**) Collection with a disposable plastic pipette (**B**) Collection with a plastic drinking straw (**C**) Collection with a clamp (**D**) Collection with the disposal dropper provided in the RIDT kit. Please click here to view a larger version of this figure.

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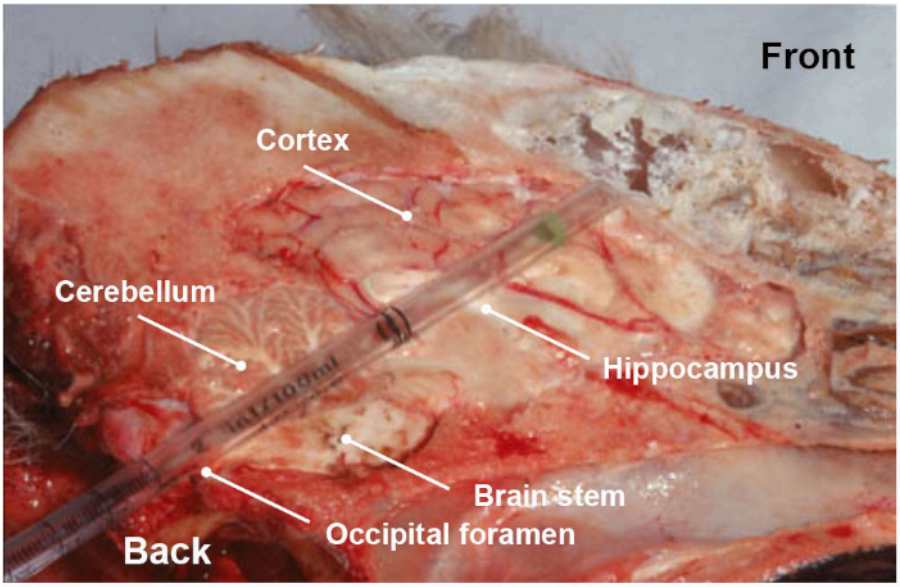

Figure 3: Longitudinal anatomical section of dog head, showing the different parts of the brain (brainstem, cerebellum, hippocampus, thalamus and cortex) collected when pushing, in a rotational movement, a disposable plastic pipette through the occipital foramen route. Please click here to view a larger version of this figure.

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Figure 4: Description of the contents of RIDT kit, including the device, a disposable plastic dropper, a disposable swab, and the assay diluent. The tube where the sample will be collected and stored is not provided. Please click here to view a larger version of this figure.

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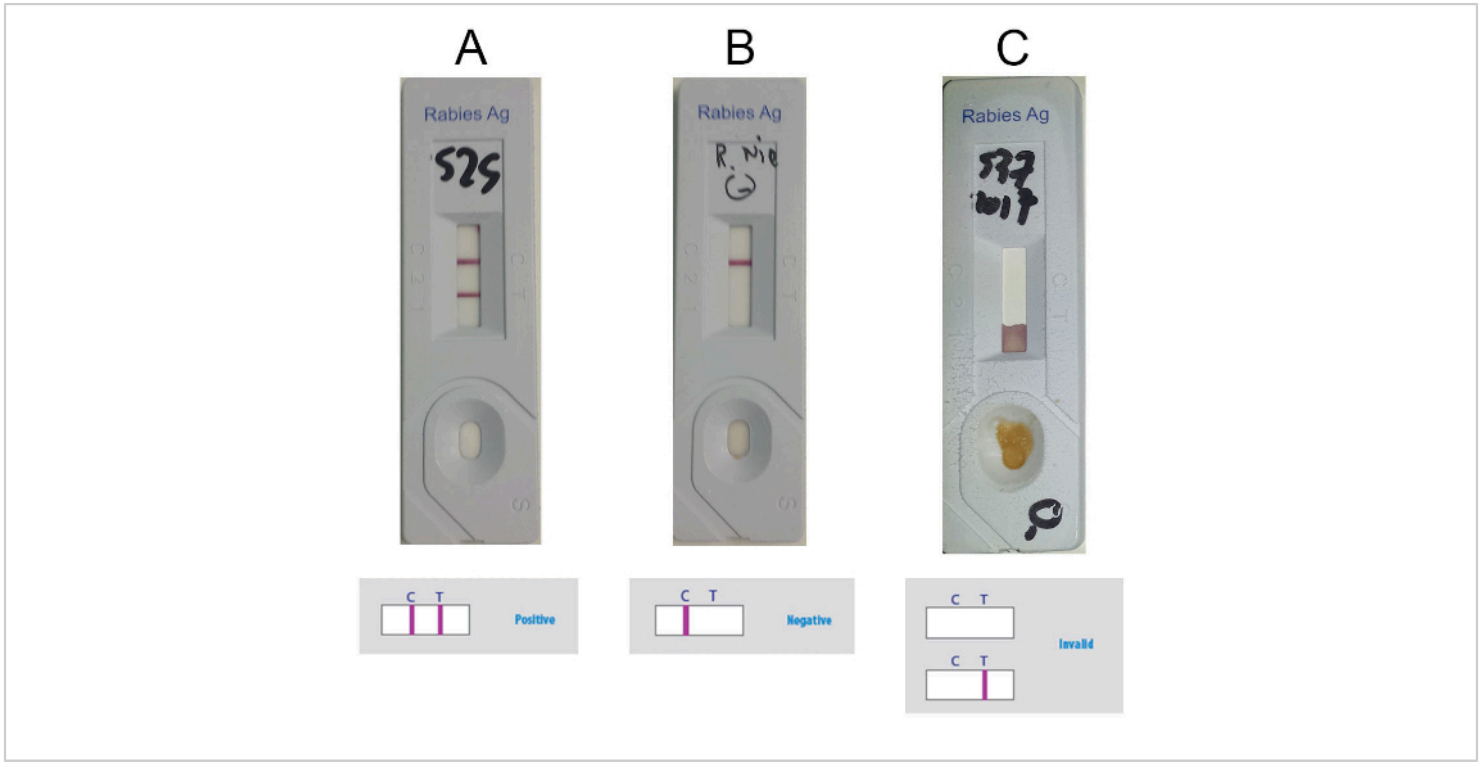

Figure 5: Representative results for interpretation of the Anigen RIDT. (**A**) Positive results (visible presence of two lines, C-line and T-line) (**B**) Negative results (visible presence of C-line only) (**C**) Invalid results (absence of visible C-line). Please click here to view a larger version of this figure.

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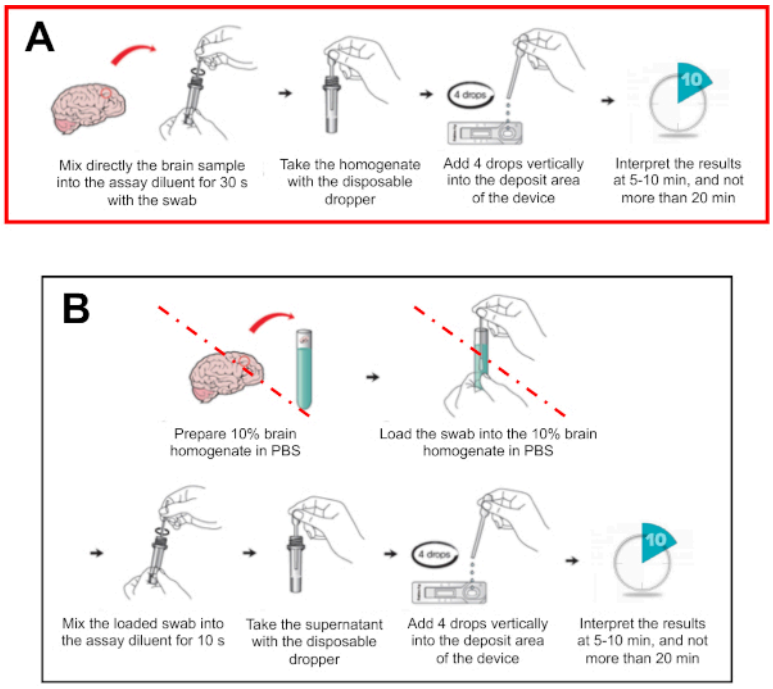

Figure 6: Schematic representation of RIDT protocol, adapted from manufacturer instructions. (**A**) Modified version of the protocol, with deletion of the dilution step recommended by the manufacturer (**B**) Initial protocol recommended by manufacturer, with a pre 1:10 dilution step in PBS of the brain samples. The steps deleted in the modified version of the protocol (presented in **Figure 6A**) are indicated with a red line. Please click here to view a larger version of this figure.

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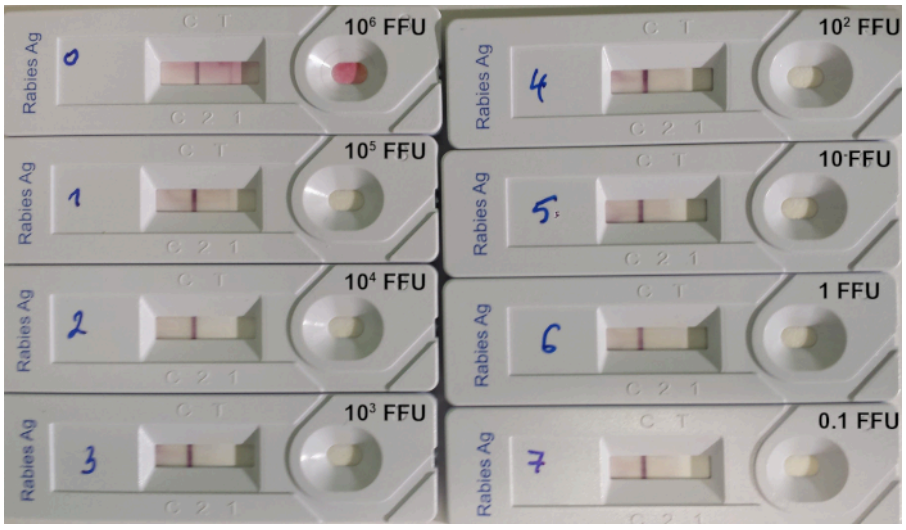

Figure 7: Example of determination of the limit of detection of RIDT[14]. A serial 10:1 dilution of a titrated rabies virus of the strain 9704ARG was used. The quantity of virus deposited on each device is indicated in FFU (fluorescent focus-forming units). Please click here to view a larger version of this figure.

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Pan-RABV RT-qPCR assay	
Reagent	**µL/Reaction**
2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 6 mM MgSO4)	10
Nuclease free water	1.5
Taq3long (Forward) [10 µM]	1
Taq17revlong (Reverse) [10 µM]	1
RABV4 [10 µM]	0.3
RABV5 [10 µM]	0.3
$MgSO_4$ [50-mM] (provided in the kit)	0.25
ROX Reference Dye (25 µM) (provided in the kit)	0.05
RNasin (40U/µL) (Promega)	0.2
SuperScript III RT/Platinum Taq Mix	0.4
Total per reaction	15
eGFP RT-qPCR assay	
Reagent	**µL/Reaction**
2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 6 mM MgSO4)	10
Nuclease free water	2.8
EGFP1F (Forward) [10 µM]	0.5
EGFP2R (Reverse) [10 µM]	0.5
eGFP probe [10 µM]	0.3
$MgSO_4$ [50-mM] (provided in the kit)	0.25
ROX Reference Dye (25 µM) (provided in the kit)	0.05
RNasin (40U/µL) (Promega)	0.2
SuperScript III RT/Platinum Taq Mix	0.4
Total per reaction	15
Pan-lyssa RT-qPCR assay	

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Reagent	µL/Reaction
2x SYBR Green Reaction Mix	10
Nuclease free water	2.1
Taq5long (Forward) [10 µM]	1
Taq16revlong (Reverse) [10 µM]	1
$MgSO_4$ [50-mM] (provided in the kit)	0.25
ROX Reference Dye (25 µM)	0.05
RNasin (40U/µL) (Promega)	0.2
SuperScript III RT/Platinum Taq Mix	0.4
Total per reaction	15

Table 1: Description of the master mix reaction solution for the three different RT-qPCR assays (pan-RABV RT-qPCR, pan-lyssa RT-qPCR and eGFP RT-qPCR).

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RT-qPCR assay	Name	Type	Length	Sequence (5'-3')	Sense	Position
pan-RABV RT-qPCR assay	Taq3long	Primer	22	ATG AGA AGT GGA AYA AYC ATC A	S	7273-7294[a]
	Taq17revlong	Primer	25	GAT CTG TCT GAA TAA TAG AYC CAR G	AS	7390-7414[a]
	RABV4	Probe (FAM/ TAMRA)	29	AAC ACY TGA TCB AGK ACA GAR AAY ACA TC	AS	7314-7342[a]
	RABV5	Probe (FAM/ TAMRA)	32	AGR GTG TTT TCY AGR ACW CAY GAG TTT TTY CA	S	7353-7384[a]
Pan-lyssa RT-qPCR assay	Taq5long	Primer	23	TAT GAG AAA TGG AAC AAY CAY CA	S	7272-7294[a]
	Taq16revlong	Primer	25	GAT TTT TGA AAG AAC TCA TGK GTY C	AS	7366-7390[a]
eGFP RT-qPCR assay	EGFP1F	Primer	20	GAC CAC TAC CAG CAG AAC AC	S	637-656[b]
	EGFP2R	Primer	19	GAA CTC CAG CAG GAC CAT G	AS	768-750[b]

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	EGFP	Probe (FAM/ TAMRA)	22	AGC ACC CAG TCC GCC CTG AGC A	S	703-724[b]

Table 2: Description of the primers/probes for the three different RT-qPCR assays (pan-RABV RT-qPCR, pan-lyssa RT-qPCR and eGFP RT-qPCR). [a] According to the Pasteur virus (PV) RABV genome sequence (GenBank accession number M13215). [b] According to the cloning vector pEGFP-1 sequence (GenBank accession number U55761).

Pan-RABV RT-qPCR and eGFP RT-qPCR assays				
Step	**Cycle**	**Temp**	**Time**	**Data Collection**
Reverse Transcription	1	45 °C	15 min	
RT inactivation/ initial denaturation	1	95 °C	3 min	
Amplification	40	95 °C	15 s	
		61 °C	1 min	End point
Pan-lyssa RT-qPCR assay				
Step	**Cycle**	**Temp**	**Time**	**Data Collection**
Reverse Transcription	1	45 °C	15 min	
RT inactivation/ initial denaturation	1	95 °C	3 min	
Amplification	40	95 °C	15 s	
		55 °C	1 min	End point
Dissociation curve	1	95 °C	15 s	Increase 0.1 °C/s, 55–95 °C
		55 °C	1 min	
		95 °C	15 s	
		55 °C	15 s	

Table 3: Description of the thermal cycling conditions for the three different RT-qPCR assays (pan-RABV RT-qPCR, pan-lyssa RT-qPCR and eGFP RT-qPCR).

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Assay	Analysis	Results	Interpretation
eGFP RT-qPCR	Cq in the interval of acceptance	Extraction validated	Analysis of other assays can be done
	Cq out of the interval of acceptance	Extraction not validated	Retest the sample (repeat the run or/and the extraction), request another sample if necessary
pan-RABV RT-qPCR	Cq <38	Positive	Positive detection of viral RNA
	Cq ≥38	Negative	Analysis the pan-lyssa RT-qPCR assay
pan-lyssa RT-qPCR	Melting curve considered as positive	Positive	Positive detection of viral RNA
	Melting curve considered as negative	Negative	Absence of detection of viral RNA

Table 4: Overall interpretation of the dual combined pan-lyssavirus RT-qPCR assay.

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Hemi-nested conventional PCR assay	PCR round	Name	Length	Sequence (5'-3')	Sense	Position[a]	Amplicon size (bp)
Hemi-nested PCR targeting the polymerase gene	1st round	PVO5m	20	ATG ACA GAC AAY YTG AAC AA	S	7170-7189	320
		PVO9	19	TGA CCA TTC CAR CAR GTN G	AS	7471-7489	
	2nd round	PVO5m	20	ATGA CAG ACA AYY TGA ACA A	S	7170-7189	250
		PVO8	22	GGT CTG ATC TRT CWG ARY AAT A	AS	7398-7419	
Hemi-nested PCR targeting the nucleoprotein gene	1st round	N127	20	ATG TAA CAC CTC TAC AAT GG	S	55-74	1532
		N8m	19	CAG TCT CYT CNG CCA TCT C	AS	1568-1586	
	2nd round	N127	20	ATG TAA CAC CTC TAC AAT GG	S	55-74	845
		N829	19	GCC CTG GTT CGA ACA TTC T	AS	881-899	

Table 5: Description of the primers used for the conventional hemi-nested PCR.

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	Hemi-nested PCR targeting the polymerase gene			
	Step	**Cycle**	**Temperature**	**Time**
First and second rounds	Initial denaturation	1	94 °C	3 min
	Denaturation	35	94 °C	30 s
	Hybridation		56 °C	45 s
	Elongation		72 °C	40 s
	Final elongation	1	72 °C	3 min
	Hemi-nested PCR targeting the nucleoprotein gene			
	Step	**Cycle**	**Temperature**	**Time**
First round	Initial denaturation	1	94 °C	3 min
	Denaturation	35	94 °C	30 s
	Hybridation		56 °C	30 s
	Elongation		72 °C	45 s
	Final elongation	1	72 °C	3 min
Second round	Initial denaturation	1	94 °C	3 min
	Denaturation	35	94 °C	30 s
	Hybridation		58 °C	30 s
	Elongation		72 °C	30 s
	Final elongation	1	72 °C	3 min

Table 6: Description of the thermal cycling conditions for the conventional hemi-nested PCR.

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Lab	Country	Period of evaluation	Nb of samples	DFAT results		RIDT results		Sensitivity	Specificity
				Pos	Neg	Pos	Neg		
Lab 1	**France**	2015	82	50	32	50	32	96%	93.7%
Lab 2	**Chad**	2012-2015	44	33	11	33	11	100%	100%
Lab 3	**Ivory Coast**	2017	10	8	2	8	2	100%	100%
Lab 4	**Mali**	2017	18	15	3	15	3	100%	100%
Lab 6	**Italy**	2016	8	8	0	8	0	100%	-
All		**2015-2017**	**162**	**114**	**48**	**114**	**48**	**98.2%**	**95.8%**

Table 7: Determination of the intrinsic parameters (sensitivity, specificity) of the RIDT test compared to the reference DFAT method, based on the analysis of a total of 162 samples and with the participation of 5 different laboratories.

Virus strain[a]	Original host	Location	Initial concentration (FFU/mL)[b]	Limit of detection (FFU/mL)[c]
9147FRA	Red fox	France	3.1×10^7	10^6
CVS	Lab isolate	-	1.6×10^7	10^6
8743THA	Human	Thailand	8.1×10^7	$> 8.1 \times 10^6$
9508CZK (SAD)	Lab isolate	-	5.4×10^8	10^7
PV	Lab isolate	-	4.3×10^7	10^6
9001FRA	Dog	French Guiana	2.4×10^6	$> 2.4 \times 10^5$
9704ARG	Bat	Argentina	9.5×10^7	10^5
04030PHI	Human	Philippines	2.5×10^7	10^5

Table 8: Limit of detection of the RIDT using 8 different titrated rabies virus suspensions (from Léchenne et al. 2016[14]). [a] CVS: Challenge virus strain, SAD: Street Alabama Dufferin, PV: Pasteur virus. [b] Number of fluorescent focus-forming units (FFU) per mL. [c] Number of fluorescent focus-forming units (FFU) deposited on the strip.

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		RIDT performed in								
		Lab 1			Lab 2			Combined		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Viral RNA detection	Positive	18	1	19	26	0	32	44	7	51
	Negative	0	0	0	0	0	3	0	3	3
	Total	18	1	19	26	0	35	44	10	54

Table 9: Detection of viral RNA with RT-qPCR on Anigen test strip used in laboratory conditions (Lab 1), in field conditions and shipped at ambient temperature (Lab 2) or combined (from Léchenne et al. 2016[14]).

Supplementary Table 1: Description of the 162 samples tested with the RIDT test for determination of its intrinsic parameters presented in Table 7. Please click here to view this table (Right click to download).

Discussion

The RIDT is a simple, rapid and low-cost method for postmortem rabies diagnosis and a promising field alternative to laboratory testing. The application of such a test, especially for decentralized areas of low- and middle-income countries, would improve understanding of rabies virus prevalence and transmission on a local and potentially national scale. When combined with the rapid brain sample collection method (without full necropsy), a great advantage is that the test can be entirely performed in the field setting, away from laboratory facilities. Brain samples collected via the foramen magnum can be used for testing, thus it is not required to completely open the animal skull. The test is simple to perform and interpret and is particularly suitable for field surveillance activities[14]. Other advantages of the RIDT over the DFAT or DRIT are no need for positive and negative controls and kit storage at room temperature. In addition, the modified protocol, where the dilution step (1:10) into PBS is omitted, does not require extra reagents to perform the test and further simplifies the procedure under field conditions.

A key point is the quality of the brain samples. Samples should be collected and tested as soon as possible after death of the suspected animal, or kept at cool temperature before testing, to avoid degradation. Decomposed samples should not be tested because it can affect the result (risk of false negative result). Although no data are yet available regarding the loss of sensitivity of RIDT over time for brain samples, we hypothesize that it is similar compared to the DFAT test[32]. However, time between the death of the animal and performing the test can be reduced, as the test can be done quickly and directly in the field. Thus, there is in general a lower risk of decomposed samples.

Another critical step within the protocol is the sample suspension migration. The migration should start directly after deposit of the sample (1-5 min). High viscosity of the suspension could therefore negatively influence the migration. Gently scratching the bottom of the device deposit site with the dropper and adding 1-2 more drops often solves this problem, and the migration begins immediately after.

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Most of the RIDT tests performed in African laboratories (Chad, Ivory Coast and Mali) were performed at ambient temperature which can exceed 30 °C, whereas the range of temperature for storage and use recommended by the manufacturer is 15 °C - 30 °C. Although we did not identify any impact of high temperature on RIDT test performance, it is necessary to evaluate it more carefully. Similarly, the impact of high temperature during storage and transportation of the device after use for viral RNA detection and genotyping needs additional evaluation. The sensitivity of the viral RNA detection by RT-qPCR from the RIDT strip can be affected by the quality of the brain sample initially used in the test, but also by the condition of storage of the RIDT tests after use. For example, the sensitivity of the RNA detection was higher when used RIDT tests were stored under controlled laboratory conditions (94.7%) compared to under field conditions (e.g., Chad) (81.2%)[14]. These conditions might also affect the integrity (especially the length) of RNA fixed on the strip, possibly explaining the moderate sensitivity for genotyping based on longer PCR amplicons (e.g., >500 nucleotides)[14]. The sensitivity of RT-qPCR performed on the test strip was lower than that obtained using FTA Whatman cards (80.6%)[14]. Similar to other molecular techniques, the viral load can also impact the success of genotyping based on RDIT strips, with potential negative results for samples with low viral load[14].

The test is not currently recommended by WHO and OIE for routine diagnosis and disease surveillance, and a result cannot be used on its own to guide PEP decision making. Further test validation is still needed. However, accurate quick rabies diagnosis is a crucial element of well-functioning continuous rabies surveillance systems and is instrumental to increase political commitment, which is eminently important for successful sustainable rabies control[33]. RIDT tests offer new rabies diagnostic opportunities in this context and are a useful tool to expand animal rabies surveillance in the field in low- or middle-income enzootic areas.

Disclosures

The authors have nothing to disclose.

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