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## Field postmortem rapid immunochromatographic rabies diagnostic test for resource-limited settings with further molecular applications
### --Manuscript Draft--

1 **TITLE:**
2 Field Postmortem Rapid Immunochromatographic Rabies Diagnostic Test for Resource-Limited
3 Settings with Further Molecular Applications
4
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53     **KEYWORDS:**
54     Rabies, diagnosis, field, postmortem, brain RIDT, lateral flow device, low- and middle-income
55     countries, rapid, remote areas, RT-qPCR, genotyping

56

57     **SUMMARY:**
58     We present a complete protocol for postmortem diagnosis of animal rabies under field conditions
59     using a rapid immunochromatographic diagnostic test (RIDT), from brain biopsy sampling to final
60     interpretation. We also describe further applications using the device for molecular analysis and
61     viral genotyping.

62

63     **ABSTRACT:**
64     Functional rabies surveillance systems are crucial to provide reliable data and increase the
65     political commitment necessary for disease control. To date, animals suspected as rabies-positive
66     must be submitted to a postmortem confirmation using classical or molecular laboratory
67     methods. However, most endemic areas are in low- and middle-income countries where animal
68     rabies diagnosis is restricted to central veterinary laboratories. Poor availability of surveillance
69     infrastructure leads to serious disease underreporting from remote areas. Several diagnostic
70     protocols requiring low technical expertise have been recently developed, providing opportunity
71     to establish rabies diagnosis in decentralized laboratories. We present here a complete protocol
72     for field postmortem diagnosis of animal rabies using a rapid immunochromatographic diagnostic
73     test (RIDT), from brain biopsy sampling to the final interpretation. We complete the protocol by
74     describing a further use of the device for molecular analysis and viral genotyping. RIDT easily
75     detects rabies virus and other lyssaviruses in brain samples. The principle of such tests is simple:
76     brain material is applied on a test strip where gold conjugated antibodies bind specifically to
77     rabies antigens. The antigen-antibody complexes bind further to fixed antibodies on the test line,
78     resulting in a clearly visible purple line. The virus is inactivated in the test strip, but viral RNA can
79     be subsequently extracted. This allows the test strip, rather than the infectious brain sample, to
80     be safely and easily sent to an equipped laboratory for confirmation and molecular typing. Based
81     on a modification of the manufacturer's protocol, we found increased test sensitivity, reaching
82     98% compared to the gold standard reference method, the direct immunofluorescence antibody
83     test. The advantages of the test are numerous: rapid, easy-to-use, low cost and no requirement
84     for laboratory infrastructure, such as microscopy or cold-chain compliance. RIDTs represent a
85     useful alternative for areas where reference diagnostic methods are not available.

86
87     **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[4–6]. 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[10–13]. 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 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

132  interpretation compared to the gold standard tests DFAT and DRIT. This allows for on-site testing
133  by personnel with limited technical expertise.

134

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

140

141  RIDT techniques for detection of RABV and other lyssaviruses have been evaluated. One of the
142  first studies was conducted by Korean researchers in 2007[10]. Compared to the DFAT method, in
143  51 animal samples and 4 RABV isolates, the RIDT showed a sensitivity and specificity of 91.7%
144  and 100%, respectively. These results were later confirmed with 110 animal brain samples from
145  Korea, with sensitivity and specificity, compared to DFAT, of 95% and 98.9%, respectively[15]. More
146  recently, other studies assessed the performance of this RIDT using virus isolates and/or infected
147  brain samples from various animals with different geographical origins. A panel of 21 samples,
148  including African RABV and other African lyssaviruses (Duvenhage virus (DUVV), Lagos bat virus
149  (LBV) and Mokola virus (MOKV)), were successfully detected, with sensitivity of 100% compared
150  to the DFAT[16]. Similar high sensitivity (96.5%) and specificity (100%) values were obtained from
151  a panel of 115 brain samples from Ethiopia[17]. Another study evaluated European RABV isolates,
152  two other European lyssaviruses (European bat lyssavirus type 1 (EBLV-1) and type 2 (EBLV-2)),
153  and the Australian bat lyssavirus (ABLV)[18]. Based on analysis of 172 animal brain samples, the
154  RIDT kit had 88.3% sensitivity and 100% specificity compared to DFAT, and the three rabies-
155  related lyssaviruses were successfully detected. In this study, some of the false negative results
156  came from brain samples stored in glycerol buffer, suggesting that improper glycerol removal
157  influenced capillary flow or antibody binding. A recent analysis of 43 clinical samples from
158  Australian bats confirmed previous test results, with complete concordance to DFAT[19]. Two
159  studies were conducted in India using the RIDT on a limited number of clinical samples (11 and
160  34 samples). Compared to DFAT, sensitivity was between 85.7% and 91.7% and specificity was
161  100%[20,21]. Another evaluation of this kit using 80 animal brain samples from Africa, Europe and
162  the Middle East obtained complete concordance with DFAT for specificity (100%) but a higher
163  sensitivity (96.9%) compared to the previous studies[22]. In a recent inter-laboratory comparison
164  of this RIDT performed in 22 different laboratories using a panel of 10 samples, overall
165  concordance was 99.5%[23].

166

167  Only one recent multicentric study showed unsatisfactory overall RIDT performance[24]. Samples
168  from three different datasets were tested and provided variable sensitivity and specificity values
169  compared to DFAT. For example, sensitivity and specificity obtained with the first panel (n= 51)
170  and the second panel (n=31) of samples from experimental infected animals, all tested in
171  laboratory A, gave a sensitivity of 16% and 43%, respectively, whereas the specificity was 100%
172  for both. Conversely, the results of the third panel (n=30) of field clinical samples analyzed by
173  laboratory B provided a complete concordance with the results of DFAT, which was further nearly
174  completely confirmed by laboratory A (85% sensitivity and 100% specificity). Batch-to-batch

175     variation was suggested as a possible explanation for the fluctuating relatively low sensitivity with
176     RIDT[24].

177

178     At the same time, another study performed a similar validation process of the above described
179     RIDT, with a modification of the manufacturer recommended protocol[14]. The pre-dilution step
180     (1:10) in PBS was omitted during preparation of the brain material. Based on this simpler
181     modified protocol, the authors obtained sensitivity and specificity of 95.3% and 93.3%,
182     respectively, compared to DFAT by testing, under laboratory conditions, a dataset of 73 animal
183     brain samples, naturally or experimentally infected with various RABV strains. The study
184     presented the first evaluation of this RIDT in a field setting (Chad, Africa). In 48 clinical brain
185     samples, sensitivity and specificity were 94.4% and 100%, respectively. The discrepancies
186     between DFAT and RIDT were due to false positive results with DFAT, determined after
187     confirmation with RT-PCR. When these results were deleted, there was complete concordance,
188     and it demonstrated that the RIDT was more reliable that DFAT under these field conditions[14].
189     No batch-to-batch variation was observed using the modified protocol. When the modified
190     protocol was applied to a small number of the DFAT/ RIDT divergent samples (n=8) in the study
191     of Eggerbauer et al.[24], all were found concordant (100% sensitivity).

192

193     Another major advantage of the RIDT is secondary use for detecting viral RNA fixed on the strip
194     using molecular techniques (such as RT-PCR) and subsequent genotyping[14,24]. Following an
195     extraction step, Léchenne et al.[14] demonstrated viral RNA fixed on the Anigen device membrane
196     using RT-PCR with 86.3% sensitivity in a panel of 51 samples (including 18 samples tested and
197     shipped from Chad at ambient temperature). Subsequent genotyping was possible in 93% of the
198     14 samples tested. Sanger sequencing of PCR amplicons of at least 500 nucleotides in length were
199     used. In addition to RABV isolates, the test detected four other lyssavirus species, DUVV, EBLV-1,
200     EBLV-2 and Bokeloh bat lyssavirus (BBLV), during a fully concordant international inter laboratory
201     test[14]. The sensitivity of viral RNA detection was even higher (100%) in the study of Eggerbauer
202     et al., based on laboratory samples examination[24]. The latter study also demonstrated that the
203     buffer used in the RIDT kit inactivated virus. Thereby, the devices can be shipped easily, at
204     ambient temperature without specific biosafety precautions to reference laboratories, for
205     molecular confirmation and genotyping.

206

207     Based on the previous evaluations, RIDT tools offer numerous advantages for use in field settings,
208     especially when the reference diagnostic techniques are not available. However, this test also
209     has some limitations, in particular, low sensitivity of antigen detection[14,24]. The test is applicable
210     for samples containing high quantities of viral antigens, such as brain samples. However, it is not
211     appropriate for other samples such as saliva or other body fluids. Another drawback is cost of the
212     device (around 5-10 Euros in Europe), which is less expensive compared to the cost of performing
213     DFAT, RT-PCR or DRIT, but which still remains high for LMICs. However, future development and
214     validation of similar RIDTs from other companies could lead to a price decrease. One study
215     reported batch-to-batch variations. Although not reported by others, strict quality controls
216     should nevertheless be performed when testing a new batch, as for any reagent used in a quality
217     management environment. The use of the modified protocol was not altered when using
218     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 DFA 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.

1.1. 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.

1.2. 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.

263

264 1.3. Optionally and in addition of brain stem (medulla oblongata), collect other parts of the
265 brainstem or the brain (cerebellum, hippocampus, thalamus and cortex) by the same occipital
266 route by pushing and rotating the plastic pipette or straw towards the eye socket (**Figure 3**).
267

268 1.4. If using a straw or pipette, gently squeeze it to deposit the brain sample (0.5-2 g) in a tube
269 for subsequent analysis and/or biobanking.
270

271 NOTE: Sample storage in glycerol is not recommended, as it seems to affect capillary flow or the
272 antibody binding step of the RIDT[18].
273

274 **2. Execution of the modified RIDT protocol[14]**
275

276 NOTE: This modification omits a dilution step (1:10) into PBS, as specified in manufacturer
277 protocol (all versions), and can be implemented under laboratory or field settings.
278

279 2.1.1. Use the swab/dropper to collect the equivalent of half a peanut or pea (0.1-0.5 g) of brain
280 material and place it in the buffer sample tube.
281

282 NOTE: For the modified protocol, all reagents/consumables are included in the kit (no PBS or
283 additional tube is needed) (**Figure 4**). Document the batch number of the kit and check validity
284 of the expiration date.
285

286 2.2.1. Carefully crush the brain material directly in the tube with the swab for about 30 s until a
287 homogeneous suspension is obtained.
288

289 NOTE: The buffer reaction inactivates the infectivity of the virus in the conditions of the
290 manufacturer's protocol[24].
291

292 2.3.1. Using the dropper, deposit four drops (approximately 100 µL) of the suspension in the
293 sample inlet on the test device.
294

295 2.4.1. Wait for complete sample migration (1-5 min) before reading the test device. The
296 migration should start rapidly after deposit of the sample (1-5 min).
297

298 2.5.1. In case of delay (due to high viscosity suspension), gently scratch the bottom of the
299 deposit site of the device with the dropper (1-5 times) and add 1-2 more drops. Migration should
300 start immediately thereafter.
301

302 2.6.1. Read the test result in the detection window after 5-10 min, and no more than 20 min,
303 after the end of the migration.
304

305 2.7.1. Interpret the result based on presence or absence of the control line (C-line) and test line
306 (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%.

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

3.1. 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].

3.1.1. Carefully open the device and remove the filter paper.

3.1.2. 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.

3.1.3. Perform the extraction in accordance with manufacturer recommendations, as previously described[27]. At this step, the exogenous internal control can be added.

3.1.4. During the process, add 2 µL of glycogen for facilitating precipitation of RNA, according to the manufacturer recommendations.

3.1.5. 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].

3.2. 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 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].

3.2.1. 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 in duplicate.

3.2.2. 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.**

3.2.3. 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.

3.2.4. 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.

3.2.5. Analyze the results obtained with the three assays according to **Table 4**.

**4. Genotyping after RNA extraction from the RIDT device**

**4.1.** Reverse transcription RT[27,29]

4.1.1. 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.

4.1.2. Incubate at 65 °C for 10 min in a heat-block and then store on ice.

4.1.3. 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

395 μM) and complete with nuclease-free water to obtain a final volume of 20 μL for each sample.
396
397 4.1.4. Add the master mix (20 μL) to the sample (10 μL) (final volume of 30 μL) and incubate at 42
398 °C for 90 min in a heat-block.
399
400 4.1.5. Proceed to the next step with PCR amplification or store the cDNA at -20 °C.
401
402 4.2. Conventional PCR[27,29,31]
403
404 NOTE: Different techniques of conventional PCR are available for genotyping. Two are presented,
405 both hemi-nested PCR, targeting a part of the nucleoprotein or a part of the viral protein of the
406 lyssavirus. The protocol is the same for each of these assays, except for the primers and cycling
407 conditions. Positive (positive RNA) and negative (negative cDNA and/or nuclease-free water)
408 controls should be included in each series and each round of PCR.
409
410 4.2.1. Prepare for each sample in a 0.2 mL microtube a master mix reaction solution for the first
411 PCR step. This mix contains 5 μL of 10x NH4 Reaction Buffer, 2.5 μL of $MgCl_2$ solution (50 mM), 1
412 μL of dNTP Mix (10 μM), 1 μL of each primer (10 μM), 0.2 μL (1 U) of Biotaq DNA polymerase and
413 37.3 μL of nuclease-free water (final volume of 48 μL). The primers are indicated in **Table 5**.
414
415 4.2.2. Add 2 μL of cDNA in every tube and cycle on a separate conventional PCR thermal cycler
416 for each assay, according to **Table 6**.
417
418 4.2.3. Prepare a second master mix reaction solution identical to the previous one with using the
419 appropriate primers (**Table 5**) for the hemi-nested PCR reaction.
420
421 4.2.4. Add 2 μL of the first round PCR product and cycle on a conventional PCR thermal cycler
422 using the cycling parameters indicated in **Table 6**.
423
424 4.2.5. Visualize the different PCR products (first and second round PCR) after loading them on a
425 1% agarose gel (100 mL of Tris-acetate EDTA buffer 1x – TAE 1x) with ethidium bromide (final
426 concentration around 0.01%) and run the gel during 30 min at 120 V. A positive PCR result is
427 observed in the form of a bright band of the expected size (**Table 5**).
428
429 4.3. Sanger sequencing
430
431 4.3.1. Perform a Sanger sequencing of the amplicons obtained with the pan-lyssavirus hemi-
432 nested PCR and complete the genotyping analysis.
433
434 **REPRESENTATIVE RESULTS:**
435 As with any diagnostic method, sample collection is of paramount importance for reliability of
436 the results, especially when performed in field settings. The collection process needs to be as
437 simple as possible to guarantee collection of high-quality samples. The collection of a brain biopsy
438 (brainstem with medulla oblongata) via the foramen magnum route for postmortem diagnosis of

439     animal rabies fulfills this requirement, as indicated in **Figures 2A–D**[25].

440

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

445

446     This modified protocol was implemented and evaluated in five different laboratories, including
447     one WHO collaborative center on rabies (Lab 1, France), one FAO reference center for rabies (Lab
448     5, Italy) and three reference laboratories located in enzootic African countries, Chad (Lab 2), Ivory
449     Coast (Lab 3) and Mali (Lab 4). In Chad, an evaluation of the RIDT was done in both laboratory
450     and field settings.

451

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

461

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

465

466     **Table 9** (from Léchenne 2016[14]) shows an example of results obtained after RNA detection by the
467     dual combined pan-lyssavirus RT-qPCR targeting the viral polymerase of lyssavirus. A panel of 51
468     positive RIDT tests performed in laboratory conditions (Lab 1, n=32) or in Chad (Lab 2, n=19) and
469     then shipped at ambient temperature to Lab 1, was tested. Positive detection was obtained for
470     18 (94.7%), 26 (81.2%) and 44 (86.3%) samples from Lab 1, Lab 2 and the two combined,
471     respectively. In addition, genotyping was performed for 14 of these samples (10 from Lab 1 and
472     4 from Lab 2) using the hemi-nested PCR targeting the partial nucleoprotein gene and was
473     successful for 13 of them (93%) (from Léchenne et al. 2016[14]).

474

475     **FIGURE AND TABLE LEGENDS**
476     **Figure 1**: **Schematic representation of the structure of an RIDT for rabies diagnosis.**

477

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

482

**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.**

**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.

**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).

**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.

**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).

**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).**

**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).

**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).**

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

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

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

**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.**

**Table 8: Limit of detection of the RIDT using 8 different titrated rabies virus suspensions (from**

527 **14).** [a]CVS: Challenge virus strain, SAD: Street Alabama Dufferin, PV: Pasteur virus. [b]Number of
528 fluorescent focus-forming units (FFU) per mL. [c]Number of fluorescent focus-forming units (FFU)
529 deposited on the strip.
530
531 **Table 9**: **Detection of viral RNA with RT-qPCR on Anigen test strip used in laboratory conditions**
532 **(Lab 1), in field conditions and shipped at ambient temperature (Lab 2) or combined (from**
533 **Léchenne et al. 2106[14]).**
534
535 **Supplementary Table 1: Description of the 162 samples tested with the RIDT test for**
536 **determination of its intrinsic parameters presented in Table 7.**
537
538 **DISCUSSION:**
539 The RIDT is a simple, rapid and low-cost method for postmortem rabies diagnosis and a promising
540 field alternative to laboratory testing. The application of such a test, especially for decentralized
541 areas of low- and middle-income countries, would improve understanding of rabies virus
542 prevalence and transmission on a local and potentially national scale. When combined with the
543 rapid brain sample collection method (without full necropsy), a great advantage is that the test
544 can be entirely performed in the field setting, away from laboratory facilities. Brain samples
545 collected via the foramen magnum can be used for testing, thus it is not required to completely
546 open the animal skull. The test is simple to perform and interpret and is particularly suitable for
547 field surveillance activities[14]. Other advantages of the RIDT over the DFA or DRIT are no need for
548 positive and negative controls and kit storage at room temperature. In addition, the modified
549 protocol, where the dilution step (1:10) into PBS is omitted, does not require extra reagents to
550 perform the test and further simplifies the procedure under field conditions.
551
552 A key point is the quality of the brain samples. Samples should be collected and tested as soon
553 as possible after death of the suspected animal, or kept at cool temperature before testing, to
554 avoid degradation. Decomposed samples should not be tested because it can affect the result
555 (risk of false negative result). Although no data are yet available regarding the loss of sensitivity
556 of RIDT over time for brain samples, we hypothesize that it is similar compared to the DFAT test[32].
557 However, time between the death of the animal and performing the test can be reduced, as the
558 test can be done quickly and directly in the field. Thus, there is in general a lower risk of
559 decomposed samples.
560
561 Another critical step within the protocol is the sample suspension migration. The migration
562 should start directly after deposit of the sample (1-5 min). High viscosity of the suspension could
563 therefore negatively influence the migration. Gently scratching the bottom of the device deposit
564 site with the dropper and adding 1-2 more drops often solves this problem, and the migration
565 begins immediately after.
566
567 Most of the RIDT tests performed in African laboratories (Chad, Ivory Coast and Mali) were
568 performed at ambient temperature which can exceed 30 °C, whereas the range of temperature
569 for storage and use recommended by the manufacturer is 15 °C – 30 °C. Although we did not
570 identify any impact of high temperature on RIDT test performance, it is necessary to evaluate it

571     more carefully. Similarly, the impact of high temperature during storage and transportation of
572     the device after use for viral RNA detection and genotyping needs additional evaluation. The
573     sensitivity of the viral RNA detection by RT-qPCR from the RIDT strip can be affected by the quality
574     of the brain sample initially used in the test, but also by the condition of storage of the RIDT tests
575     after use. For example, the sensitivity of the RNA detection was higher when used RIDT tests
576     were stored under controlled laboratory conditions (94.7%) compared to under field conditions
577     (e.g., Chad) (81.2%)[14]. These conditions might also affect the integrity (especially the length) of
578     RNA fixed on the strip, possibly explaining the moderate sensitivity for genotyping based on
579     longer PCR amplicons (e.g., >500 nucleotides)[14]. The sensitivity of RT-qPCR performed on the test
580     strip was lower than that obtained using FTA Whatman cards (80.6%)[14]. Similar to other
581     molecular techniques, the viral load can also impact the success of genotyping based on RDIT
582     strips, with potential negative results for samples with low viral load[14].
583
584     The test is not currently recommended by WHO and OIE for routine diagnosis and disease
585     surveillance, and a result cannot be used on its own to guide PEP decision making. Further test
586     validation is still needed. However, accurate quick rabies diagnosis is a crucial element of well-
587     functioning continuous rabies surveillance systems and is instrumental to increase political
588     commitment, which is eminently important for successful sustainable rabies control[33]. RIDT tests
589     offer new rabies diagnostic opportunities in this context and are a useful tool to expand animal
590     rabies surveillance in the field in low- or middle-income enzootic areas.
591

601

602     **DISCLOSURES:**
603     The authors have nothing to disclose.
604

605     **REFERENCES:**
606     1. Hampson, K. et al. Correction: Estimating the Global Burden of Endemic Canine Rabies. *PLoS*
607        *Neglected Tropical Diseases*. **9** (5), e0003786 (2015).
608     2. Rupprecht, C., Kuzmin, I., Meslin, F. Lyssaviruses and rabies: current conundrums, concerns,
609        contradictions and controversies. *F1000Research*. **6**, 184 (2017).
610     3. Walker, P.J. et al. ICTV Virus Taxonomy Profile: Rhabdoviridae. *The Journal of General Virology*.
611        **99** (4), 447–448 (2018).
612     4. *WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018*
613        *(WHO Technical Report Series, No. 1012). Licence: CC BY-NC-SA 3.0 IGO (*2018).
614     5. Dacheux, L. et al. More Accurate Insight into the Incidence of Human Rabies in Developing

615    Countries through Validated Laboratory Techniques. *PLoS Neglected Tropical Diseases*. **4** (11),
616    e765 (2010).

6.  Welburn, S.C., Beange, I., Ducrotoy, M.J., Okello, A.L. The Neglected Zoonoses - The Case for
    Integrated Control and Advocacy. *Clinical Microbiology and Infection: The Official Publication
    of the European Society of Clinical Microbiology and Infectious Diseases* (2015).

7.  Dacheux, L., Bourhy, H. Diagnostic tests for human rabies. *Revue Scientifique Et Technique
    (International Office of Epizootics)*. **37** (2), 581–593 (2018).

8.  OIE Terrestrial Manual - Rabies (Infection with rabies virus and other Lyssaviruses). *Manual of
    Diagnostic Tests and Vaccines for Terrestrial Animals 2018*. at
    <http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.17_RABIES.pdf>
    (2018).

9.  Kasempimolporn, S., Saengseesom, W., Lumlertdacha, B., Sitprija, V. Detection of rabies virus
    antigen in dog saliva using a latex agglutination test. *Journal of Clinical Microbiology*. **38** (8),
    3098–3099 (2000).

10. Kang, B. et al. Evaluation of a rapid immunodiagnostic test kit for rabies virus. *Journal of
    Virological Methods*. **145** (1), 30–36 (2007).

11. Nishizono, A. et al. A simple and rapid immunochromatographic test kit for rabies
    diagnosis. *Microbiology and Immunology*. **52** (4), 243–249 (2008).

12. Kasempimolporn, S., Saengseesom, W., Huadsakul, S., Boonchang, S., Sitprija, V.
    Evaluation of a rapid immunochromatographic test strip for detection of Rabies virus in dog
    saliva samples. *Journal of Veterinary Diagnostic Investigation: Official Publication of the
    American Association of Veterinary Laboratory Diagnosticians, Inc*. **23** (6), 1197–1201 (2011).

13. Ahmed, K. et al. Evaluation of a monoclonal antibody-based rapid
    immunochromatographic test for direct detection of rabies virus in the brain of humans and
    animals. *The American Journal of Tropical Medicine and Hygiene*. **86** (4), 736–740 (2012).

14. Léchenne, M. et al. Validation of a Rapid Rabies Diagnostic Tool for Field Surveillance in
    Developing Countries. *PLoS neglected tropical diseases*. **10** (10), e0005010 (2016).

15. Yang, D.-K. et al. Comparison of four diagnostic methods for detecting rabies viruses
    circulating in Korea. *Journal of Veterinary Science*. **13** (1), 43–48 (2012).

16. Markotter, W. et al. Evaluation of a rapid immunodiagnostic test kit for detection of
    African lyssaviruses from brain material. *The Onderstepoort Journal of Veterinary Research*. **76**
    (2), 257–262 (2009).

17. Reta, T. et al. Evaluation of Rapid Immunodiagnostic Test for Rabies Diagnosis Using
    Clinical Brain Samples in Ethiopia. *Journal of Veterinary Science & Medical Diagnosis*. **2** (3), 1–
    3 (2013).

18. Servat, A., Picard-Meyer, E., Robardet, E., Muzniece, Z., Must, K., Cliquet, F. Evaluation of
    a Rapid Immunochromatographic Diagnostic Test for the detection of rabies from brain
    material of European mammals. *Biologicals: Journal of the International Association of
    Biological Standardization*. **40** (1), 61–66 (2012).

19. Certoma, A. et al. Assessment of a Rabies Virus Rapid Diagnostic Test for the Detection of
    Australian Bat Lyssavirus. *Tropical Medicine and Infectious Disease*. **3** (4) (2018).

20. Ahmad, A., Singh, C.K. Comparison of rapid immunodiagnosis assay kit with molecular and
    immunopathological approaches for diagnosis of rabies in cattle. *Veterinary World*. **9** (1), 107–
    112 (2016).

659 21. Sharma, P., Singh, C.K., Narang, D. Comparison of immunochromatographic diagnostic
660  test with Hheminested Reverse transcriptase polymerase chain reaction for detection of rabies
661  virus from brain samples of various species. *Veterinary World*. **8** (2), 135–138 (2015).

662 22. Voehl, K.M., Saturday, G.A. Evaluation of a rapid immunodiagnostic rabies field
663  surveillance test on samples collected from military operations in Africa, Europe, and the
664  Middle East. *U.S. Army Medical Department Journal*. 27–32 (2014).

665 23. Servat, A., Robardet, E., Cliquet, F. An inter-laboratory comparison to evaluate the
666  technical performance of rabies diagnosis lateral flow assays. *Journal of Virological Methods*.
667  **272**, 113702 (2019).

668 24. Eggerbauer, E. et al. Evaluation of Six Commercially Available Rapid
669  Immunochromatographic Tests for the Diagnosis of Rabies in Brain Material. *PLoS Neglected*
670  *Tropical Diseases*. **10** (6), e0004776 (2016).

671 25. Barrat, J. Simple technique for the collection and shipment of brain specimens for rabies
672  diagnosis. *Laboratory techniques in rabies eds F. X. Meslin, M.M. Kaplan, & H. Koprowski.*
673  *World Health Organization*. 425–432 (1996).

674 26. Dacheux, L. et al. Dual Combined Real-Time Reverse Transcription Polymerase Chain
675  Reaction Assay for the Diagnosis of Lyssavirus Infection. *PLoS neglected tropical diseases*. **10**
676  (7), e0004812 (2016).

677 27. Dacheux, L. et al. A reliable diagnosis of human rabies based on analysis of skin biopsy
678  specimens. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases*
679  *Society of America*. **47** (11), 1410–1417 (2008).

680 28. World Health Organization (WHO). Application of next generation sequencing to rabies
681  virus and other lyssaviruses. in *Laboratory techniques in rabies* Vol. 2 eds C. Rupprecht, A. R.
682  Fooks, & B. Abela-Ridder 49-61 (2019).

683 29. World Health Organization (WHO). Conventional pan-lyssavirus reverse transcriptase
684  polymerase chain reaction. in *Laboratory techniques in rabies* Vol. 2 eds C. Rupprecht, A. R.
685  Fooks, & B. Abela-Ridder 1-16 (2019).

686 30. World Health Organization (WHO). Rabies real-time reverse transcriptase polymerase
687  chain reaction. in *Laboratory techniques in rabies* Vol. 2 eds C. Rupprecht, A. R. Fooks, & B.
688  Abela-Ridder 17-34 (2019).

689 31. Talbi, C. et al. Evolutionary history and dynamics of dog rabies virus in western and central
690  Africa. *The Journal of General Virology*. **90** (Pt 4), 783–791 (2009).

691 32. McElhinney, L.M., Marston, D.A., Brookes, S.M., Fooks, A.R. Effects of carcase
692  decomposition on rabies virus infectivity and detection. *Journal of Virological Methods*. **207**,
693  110–113 (2014).

694 33. Vigilato, M.A.N. et al. Progress towards eliminating canine rabies: policies and
695  perspectives from Latin America and the Caribbean. *Philosophical Transactions of the Royal*
696  *Society of London. Series B, Biological Sciences*. **368** (1623), 20120143 (2013).
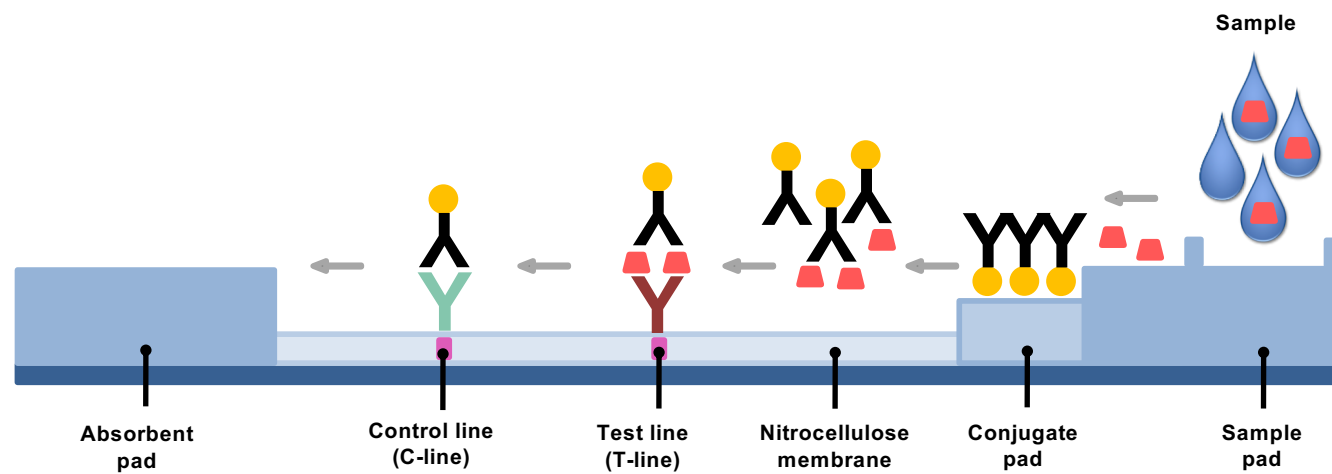
**Legend**

Rabies virus antigen

Gold labeled IgG detector antibodies (specific for rabies virus)

IgG sensor antibodies (specific for rabies virus)

Anti-IgG antibodies

Figure 3

Figure 4

Figure 5

Figure 6

**A**

Mix directly the brain sample into the assay diluent for 30 s with the swab

Take the homogenate with the disposable dropper

Add 4 drops vertically into the deposit area of the device

Interpret the results at 5-10 min, and not more than 20 min

**B**

Prepare 10% brain homogenate in PBS

Load the swab into the 10% brain homogenate in PBS

Mix the loaded swab into the assay diluent for 10 s

Take the supernatant with the disposable dropper

Add 4 drops vertically into the deposit area of the device

Interpret the results at 5-10 min, and not more than 20 min

Figure 7

Table 1

| 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** | |
| **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 2

| RT-qPCR assay | Name |
|---|---|
| pan-RABV RT-qPCR assay | Taq3long |
| | Taq17revlong |
| | RABV4 |
| | RABV5 |
| Pan-lyssa RT-qPCR assay | Taq5long |
| | Taq16revlong |
| eGFP RT-qPCR assay | EGFP1F |
| | EGFP2R |
| | EGFP |

| Type | Length |
| --- | --- |
| Primer | 22 |
| Primer | 25 |
| Probe (FAM/TAMRA) | 29 |
| Probe (FAM/TAMRA) | 32 |
| Primer | 23 |
| Primer | 25 |
| Primer | 20 |
| Primer | 19 |
| Probe (FAM/TAMRA) | 22 |

| Sequence (5'-3') |
| --- |
| ATG AGA AGT GGA AYA AYC ATC A |
| GAT CTG TCT GAA TAA TAG AYC CAR G |
| AAC ACY TGA TCB AGK ACA GAR AAY ACA TC |
| AGR GTG TTT TCY AGR ACW CAY GAG TTT TTY CA |
| TAT GAG AAA TGG AAC AAY CAY CA |
| GAT TTT TGA AAG AAC TCA TGK GTY C |
| GAC CAC TAC CAG CAG AAC AC |
| GAA CTC CAG CAG GAC CAT G |
| AGC ACC CAG TCC GCC CTG AGC A |

| Sense | Position |
| --- | --- |
| S | 7273-7294[a] |
| AS | 7390-7414[a] |
| AS | 7314-7342[a] |
| S | 7353-7384[a] |
| S | 7272-7294[a] |
| AS | 7366-7390[a] |
| S | 637-656[b] |
| AS | 768-750[b] |
| S | 703-724[b] |

Table 3

| Pan-RABV RT-qPCR and eGFP RT-qPCR assays | | | |
|---|---|---|---|
| **Step** | **Cycle** | **Temp** | **Time** |
| 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 |
| **Pan-lyssa RT-qPCR assay** | | | |
| **Step** | **Cycle** | **Temp** | **Time** |
| 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 |
| Dissociation curve | 1 | 95 °C | 15 s |
| | | 55 °C | 1 min |
| | | 95 °C | 15 s |
| | | 55 °C | 15 s |

| Data Collection |
|---|
| |
| |
| |
| End point |

| Data Collection |
|---|
| |
| |
| |
| End point |
| Increase 0.1 °C/s, 55–95 °C |

Table 4

| Assay | Analysis |
|---|---|
| eGFP RT-qPCR | Cq in the interval of acceptance |
| | Cq out of the interval of acceptance |
| pan-RABV RT-qPCR | Cq <38 |
| | Cq ≥38 |
| pan-lyssa RT-qPCR | Melting curve considered as positive |
| | Melting curve considered as negative |

| Results | Interpretation |
| --- | --- |
| Extraction validated | Analysis of other assays can be done |
| Extraction not validated | Retest the sample (repeat the run or/and the extraction), request another sample if necessary |
| Positive | Positive detection of viral RNA |
| Negative | Analysis the pan-lyssa RT-qPCR assay |
| Positive | Positive detection of viral RNA |
| Negative | Absence of detection of viral RNA |

Table 5

| Hemi-nested conventional PCR assay | PCR round | Name | Length |
|---|---|---|---|
| Hemi-nested PCR targeting the polymerase gene | 1st round | PVO5m | 20 |
| | | PVO9 | 19 |
| | 2nd round | PVO5m | 20 |
| | | PVO8 | 22 |
| Hemi-nested PCR targeting the nucleoprotein gene | 1st round | N127 | 20 |
| | | N8m | 19 |
| | 2nd round | N127 | 20 |
| | | N829 | 19 |

| Sequence (5'-3') | Sense | Position[a] | Amplicon size (bp) |
|---|---|---|---|
| ATG ACA GAC AAY YTG AAC AA | S | 7170-7189 | 320 |
| TGA CCA TTC CAR CAR GTN G | AS | 7471-7489 | |
| ATGA CAG ACA AYY TGA ACA A | S | 7170-7189 | 250 |
| GGT CTG ATC TRT CWG ARY AAT A | AS | 7398-7419 | |
| ATG TAA CAC CTC TAC AAT GG | S | 55-74 | 1532 |
| CAG TCT CYT CNG CCA TCT C | AS | 1568-1586 | |
| ATG TAA CAC CTC TAC AAT GG | S | 55-74 | 845 |
| GCC CTG GTT CGA ACA TTC T | AS | 881-899 | |

Table 6

| 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 7

| Lab | Country | Period of evaluation | Nb of samples | DFAT | | RIDT | | 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 8

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

| Limit of detection (FFU/mL)[c] |
| --- |
| $10^6$ |
| $10^6$ |
| $>\ 8.1 \times 10^6$ |
| $10^7$ |
| $10^6$ |
| $> 2.4 \times 10^5$ |
| $10^5$ |
| $10^5$ |

Table 9

| | | RIDT performed in | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Lab 1 | | | Lab 2 | | | C |
| | | Positive | Negative | Total | Positive | Negative | Total | Positive |
| **Viral RNA detection** | Positive | 18 | 1 | 19 | 26 | 0 | 32 | 44 |
| | Negative | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| | Total | 18 | 1 | 19 | 26 | 0 | 35 | 44 |

**ombined**

| Negative | Total |
|----------|-------|
| 7        | 51    |
| 3        | 3     |
| 10       | 54    |

Table of Materials

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| Anti-Rabies Nucleocapsid Conjugate (lyophilizied, adsorbed) | Bio-Rad, France | 3572112 |
| Applied Biosystems 7500 Real-Time PCR System | Applied Biosystems, France | 4351104 |
| Disposable plastic pipette, drinking straw, clamp, dropper | - | - |
| Evans Blue Solution 1% | Bio-Rad, France | 3574911 |
| Primer eGFPF1 | Eurofins Genomics, Germany | - |
| Primer eGFPR2 | Eurofins Genomics, Germany | - |
| Primer Taq17 revlong | Eurofins Genomics, Germany | - |
| Primer Taq3 long | Eurofins Genomics, Germany | - |
| Probe eGFP FAM/TAMRA | Eurofins Genomics, Germany | - |
| Probe RABV4 FAM/TAMRA | Eurofins Genomics, Germany | - |
| Probe RABV5 FAM/TAMRA | Eurofins Genomics, Germany | - |
| Rapid Rabies Ag Test Kit | BioNote Inc., Republic of Korea | RG18-01DD |
| Recombinant RNasin Ribonuclease Inhibitor | Promega, USA | N2515 |
| SuperScript III Platinum One-Step qRT-PCR Kit | Invitrogen, France | 11732-020 |
| TRIzol Reagent | Invitrogen, France | 15596026 |

**Comments/Description**

Fluorescein-5-isothiocyanate (FITC) conjugated polyclonal antibody against the nucleocapsid of rabies virus. Use for the DFAT reference

Amplification of the viral RNA by RT-qPCR after extraction from the RIDT device.

Equipment used for the collection of the  brain stem (medulla oblongata) via the foramen magnus (occipital route).

Counter-coloration used for the DFAT to facilite the reading under UV microscope.
Forward primer for the amplification of the internal control eGFP by RT-qPCR after RNA extraction from the RIDT device, sequence: 5'-
Reverse primer for the amplification of the internal control eGFP by RT-qPCR after extraction from the RIDT device, sequence: 5'-GAAC
Reverse primer for the amplification of the viral RNA by RT-qPCR after RNA extraction from the RIDT device, sequence: 5'-ATGAGAAGT
Forward primer for the amplification of the viral RNA by RT-qPCR after RNA extraction from the RIDT device, sequence: 5'-GATCTGTCT
Forward primer for the amplification of the internal control by RT-qPCR after extraction from the RIDT device, sequence: 5'-AGCACCCA
Probe for the amplification of the viral RNA by RT-qPCR after RNA extraction from the RIDT device, sequence: 5'-AACACYTGATCBAGKA
Probe for the amplification of the viral RNA by RT-qPCR after RNA extraction from the RIDT device, sequence: 5'-AGRGTGTTTTCYAGRA

Rapid immunochromatographic diagnostic test (RIDT, also named lateral flow device or LFD) for the post-mortem diagnosis of rabies.

Enzyme used with the kit for the amplification of the viral RNA by RT-qPCR after extraction from the RIDT device.

Kit for the amplification of the viral RNA by RT-qPCR after extraction from the RIDT device.

Phenol/chloroforme based total RNA extraction using the cellulose membrane of the RIDT.

e tecnnique.

GACCACTACCAGCAGAACAC-3'.
CTCCAGCAGGACCATG-3'.
GGAAYAAYCATCA-3'.
GAATAATAGAYCCARG-3'.
GTCCGCCCTGAGCA-3'.
CAGARAAYACATC-3'.
CWCAYGAGTTTTTYCA-3'.

Ref: JoVE60008R2

Field postmortem rapid immunochromatographic rabies diagnostic test for resource-limited settings with further molecular applications

Rebuttal document

Dear Dr Steindel:

We were totally delighted to receive your comments in connection with the afore mentioned manuscript.

We thank again the editors and the external reviewers very much for reading the manuscript so carefully and for providing most useful comments and suggestions. We are grateful for the invitation to revise and re-submit our manuscript once again, and we apology for the delay of our reply.

We have carefully reworked our manuscript in light of the reports.

Below, please find our point-by-point response, clearly indicating how and where in the manuscript changes have been made. To further assist you in readily reviewing our changes made, we used track changes in the original manuscript. However, we attach in addition a cleaned version of the manuscript for easy readability and which underwent final formatting. The cleaned version can be used for further processing.

We look forward to your further disposition.

Yours sincerely,

Stephanie Mauti and Laurent Dacheux (on behalf of all the authors)

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**Point-by-point response:**

Comments from the editors and reviewers:

Editorial comments:

1. Please provide a key detailing exactly which steps/substeps in the protocol each video that you provided us corresponds to; e.g., it looks like P1060133 shows 1.1.

Details of the videos

- 293_0017_01.MOV: Interview about the advantages of the RIDT. Needs in addition subtitles. Would be nice if this part could be shown in the video in addition to the test protocol. Needs to be cut together appropriately.
- 293_0018_01.MOV: See comments of video 293_0017_01.MOV. Material which is needed for the conduction of the test (Protocol step 1., before step 1.1.).
- 293_0019_01.MOV: Step 1. Substep 1.1. Needs to be cut together appropriately.
- 293_0020_01.MOV: Step 1. and 2. Substeps. 1.2.-1.4. and 2.1.-2.3. Needs to be cut together appropriately.
- 293_0021_01.MOV: Step 2. Substeps 2.4.-2.7. Needs to be cut together appropriately.
- 293_0022_01.MOV: Step 2. Substeps 2.3.-2.8. Desinfection of the material and packing together. Needs to be cut together appropriately.

- P1060131.MP4: Step 1. Preparation of the field site. If used, this sequence needs to be cut together appropriately.
- P1060133.MP4: Step 1. Substep 1.1. Needs to be cut together appropriately.
- P1060134.MP4: Step 1. Substep 1.2. Needs to be cut together appropriately.
- P1060135.MP4: Step 1. Substeps 1.2.-1.4. Needs to be cut together appropriately.
- P1060136.MP4: Step 1. Substeps 1.2.-1.4. Needs to be cut together appropriately.
- P1060137.MP4: Step 1. Substeps 1.2.-1.4. Needs to be cut together appropriately.
- P1060138.MP4: Step 2. Test preparation. If used, this sequence needs to be cut together appropriately.
- P1060139.MP4: Step 2. Substeps 2.1.-2.3. Needs to be cut together appropriately.
- P1060140.MP4: Step 2. Substeps 2.1.-2.3. Needs to be cut together appropriately.
- P1060141.MP4: Step 2. Substeps 2.4.-2.6. Needs to be cut together appropriately.
- P1060142.MP4: Step 2. Substeps 2.6.-2.7. Needs to be cut together appropriately.
- P1060143.MP4: Step 2. Substeps 2.3.-2.7. Needs to be cut together appropriately.
- P1060144.MP4: Step 2. Substep 2.8. Desinfection of the material and packing together. Needs to be cut together appropriately.

2. Please provide only one file per table (e.g, Table 1a and 1b should be in one table, or be split up into Table 1 and Table 2). Please also use a degree symbol ('°') instead of a superscript 'o'.

Done. Table 1a and 1b are split up in Table 1 and 2. We also replace the superscript 'o' with the degree symbol ('°') in the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The revised manuscript by Mauti and colleagues is significantly improved in clarity and precision of claims. It describes a lateral flow assay for detection of rabies and select other lyssaviruses that does not require cold chain or extensive laboratory skills. It now includes methods for qRT-PCR that in some sense cloud the original purpose of developing a low technology field method for rabies diagnosis. The addition of qRT-PCR does allow for central laboratory confirmation of the original method and skirts the problem that both DFAT (their gold standard) and RIDT show limited sensitivity relative to RT-PCR methods.

We thank reviewer 1 for his comments. We added the qRT-PCR as an added value of the test, demonstrating also a complementary approach between peripheral diagnosis and central laboratory confirmation and/or molecular analysis. However, the focus still remains on the usability in rural areas of low- and middle-income countries. The first two steps of the protocol were filmed in Mali and it is planned that the final steps will be filmed at Institut Pasteur Paris, which underlines the usefulness of the test in low resource settings.

Major Concerns:

My major concern is that the field testing appears to look under the lamppost. There is considerable ascertainment bias that favors the RIDT assay. There are very few negatives provided from African test sites, so that the medically and epidemiologically important parameter of test specificity cannot be confidently determined. Test sensitivity is probably overestimated.

Specifically, during test development in Lab 1 (France), there are 58 DFA-positive samples. (As far as I can tell, there are no DFA-indeterminate and DFA-negative samples, yet these occur 20% of the time.) There are 32 true negative samples.

In contrast, in field work in Lab 2 (Chad) and other sites, there are 56 DFA-positive samples but only 16 negative samples. Chi-square shows trend between France and other sites (p=0.065) that becomes significant if you remove ONE negative from the other sites, OR remove the 4 lyssas, OR remove 9 mouse brains that are hardly field isolates (p<0.46). This is heterogeneous, therefore not an ideal dataset. There needs to be some acknowledgement that test performance is likely over-estimated.

We agree with Reviewer 1 and we acknowledge that the sample datasets, especially the ones from the field work, are not ideal. Unfortunately, for the latter, they represent the only datasets we were able to obtain from the field during the implementation step of the RIDT in the three African countries, where the animal rabies surveillance was low. We are confident that the use of this implemented RIDT will help to reinforce the surveillance and increase the number of animals tested in the future. However, the data we present here reflect also the difficulty of obtaining such samples in enzootic areas.

However, we considered the remark of Reviewer 1 and precise in the text of the manuscript that these results are preliminary and need to be further confirmed on a larger dataset on samples, especially to avoid any underestimation due to the samples limitation and heterogeneity:

Lines 473-476: "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."

Minor Concerns:
addressed

Reviewer #2:
The authors have put a lot of effort into editing the manuscript and addressing the concerns of the editor and reviewers. But I still feel more editing needs to be done prior to publication.
- The edits made to the title, and within the text to incorporate RT-qPCR and genotyping are appreciated to clarify why those methods were incorporated into a paper about low-and middle-income countries.

See first comment to reviewer 1. In addition, we have incorporated RT-qPCR based on your last comment that 'running the lateral flow test is not complicated', so we thought adding further aspects of the RIDT will nicely complements the test procedure itself.

- Thank you for clarifying in the manuscript that this method may prompt the implementation of PEP since it certainly shouldn't be used to make the decision to not treat. However, the authors response to Reviewer 1 regarding line 99 concerns me and the authors true thoughts "For PEP decision of a fatal disease, animal rabies diagnosis is of utmost importance and luckily there are several tests, which can be used for animal diagnosis in low-and-middle income countries (DFAT, DRIT, RIDT, PCR)."

We agree with reviewer 2 that PEP decision should never be based only on the RIDT test result.

- Line 111: "DRIT" needs to be changed to "rapid immunochromatographic diagnostic test (RIDT)"

Done

- Both Reviewer 1 and I commented about sample condition, decomposition and/or inappropriate areas of brain. I am still not satisfied with the changes to the manuscript or the authors responses to

that topic. If there is no brain stem in the test sample and it is not positive it must be called unsatisfactory. Even with follow up with more sensitive PCR tests of the cassette, incorrect tissue tested cannot give a negative result. The authors state in the protocol 1.2 that medulla oblongata is to be collected, but then in 1.3 say that if medulla oblongata isn't available other parts of the brain can be collected. Step 1.3 and the authors response to Reviewer 1's minor concern line 233 is misleading implying that any of those tissues could be substitutions. They alone cannot substitute for brain stem.

We agree with Reviewer 2 and we modified the step 1.3 as following: "Optionally and in addition of brain stem (medulla oblongata), other parts of the brainstem or the brain (cerebellum, hippocampus, thalamus and cortex) can be collected by the same occipital route by pushing and rotating the plastic pipette or straw towards the eye socket (Figure 3)."

- Protocol Step 1.3 was used as their answer to many of my concerns. I do not feel it was an adequate answer as it does not address the importance of testing the correct tissue. I assume that killing stray aggressive/rabid dogs in a low-income community is done in a method (trauma) that may cause skull and brain tissue damage. This can result in unidentifiable and/or insufficient brain tissue.

We totally agree with Reviewer 2 as this problem is unfortunately affecting all types of techniques and not only RIDT (such as the reference techniques DFAT and DRIT). As stated above, we modified step 1.3.

In addition I am concerned with the line "Samples should be processed as soon as possible after death" as it does not address the issue of decomposition sufficiently. How quickly is the specimen being brought to a testing center? Even with the RIDT more available in remote areas, and the authors feeling that decomposition won't affect the test, it can make tissue identification difficult.

Unfortunately, it will be difficult and probably irrelevant to give a precise duration between the death and the processing of the test. Indeed, the environments are too different and the duration of sample decomposition in Ivory Coast is not the same than in France.
However, we modified the text lines 230-231 and lines 573-574.

- Both Reviewer 1 and I commented about further explaining what part of the "core sample" should be tested and were answered that Step 1.2 covers that. I disagree. When someone is using this video to train how to do the procedure, one small mention of the tissue to test could be easily forgotten. The implication in 1.3 that any of the tissue is fine to test decreases the importance of 1.2. I worry someone starting to push the sample out of the straw, will only use the first "pea sized" section that comes out (cerebrum) for the test and put the rest of the sample into the storage tube. Although in the results section it states that the brain biopsy method collects mostly brainstem and medulla oblongata, the introduction states personnel with limited technical experience could perform the test. Would these personnel be able to identify the medulla oblongata from a biopsy without being specifically shown that in the video?

As indicated previously, we modified the step 1.3. In addition, the video should clarify the concerns of Reviewer 2. Lastly, we added the specific Note for the step 1.2: " 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."

- Reviewer 1 mentions invalid tests/retests. Authors state repeated at least once. That would be fine if it was invalid due to a clogged test or a test that the control did not work. But I feel there should be more explanation. If the test is invalid on a normal-looking tissue then yes, repeating and accepting the result would be acceptable. However, for severely decomposed tissue I agree that a test should be tried, but I don't know if I personally would feel comfortable with the result if the first test came back invalid and the second test negative.

We indicated lines 231-233 in the Note of the Step 1 that "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)."
In addition, we already mentioned in the Note of the Step 2.7 that "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 section), it is not 100%."

- I agree with Reviewer 1 that Figure 7 adds little, I had intended to say that on my initial review as well.
We removed Figure 7.

I'm including several references regarding virus dissemination in brain and tissue requirements for testing for the editor's convenience.

Centers for Disease Control and Prevention. Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing: a minimum standard for rabies diagnosis in the United States. https://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf. Published 2002

Bingham, J, van der Merwe, M. Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test. J Virol Methods. 2002;101(1-2):85-94

Appler, K, Brunt, S, Jarvis, J, Davis, A. Clarifying Indeterminate Results on the Direct Fluorescent Antibody Test Using Real-Time Reverse Transciptase Polymerase Chain Reaction. Public Health Reports, Volume 134, Issue 1, January/February 2019, Pages 57-62
https://doi.org/10.1177/0033354918810776

Hamir, AN, Moser,G. Morphologic and immunoperoxidase study of neurologic lesions in naturally acquired rabies of raccoons. Journal of Veterinary Diagnostic Investigation. Volume 4, Issue 4, October 1992, Pages 369-373

Beck, S, et.al. Pathobiological investigation of naturally infected canine rabies cases from Sri Lanka. BMC Veterinary Research. Volume 13, Article number 99 (2017)

Supplementary Table 1

| Lab | Country | Year (test) | Identification | Host |
|---|---|---|---|---|
| Lab 1 | France | 2015 | 150007 | Cat |
| Lab 1 | France | 2015 | 150036 | Dog |
| Lab 1 | France | 2015 | 150038 | Cat |
| Lab 1 | France | 2015 | 150041 | Cat |
| Lab 1 | France | 2015 | 150042 | Ferret |
| Lab 1 | France | 2015 | 150043 | Dog |
| Lab 1 | France | 2015 | 150044 | Dog |
| Lab 1 | France | 2015 | 150049 | Dog |
| Lab 1 | France | 2015 | 150050 | Dog |
| Lab 1 | France | 2015 | 150051 | Dog |
| Lab 1 | France | 2015 | 150052 | Cat |
| Lab 1 | France | 2015 | 150053 | Dog |
| Lab 1 | France | 2015 | 150054 | Cat |
| Lab 1 | France | 2015 | 150055 | Red fox |
| Lab 1 | France | 2015 | 150056 | Cat |
| Lab 1 | France | 2015 | 150057 | Dog |
| Lab 1 | France | 2015 | 150058 | Dog |
| Lab 1 | France | 2015 | 150059 | Dog |
| Lab 1 | France | 2015 | 150060 | Cat |
| Lab 1 | France | 2015 | 150061 | Cat |
| Lab 1 | France | 2015 | 150062 | Cat |
| Lab 1 | France | 2015 | 150119 | Ferret |
| Lab 1 | France | 2015 | 150125 | Cat |
| Lab 1 | France | 2015 | 150127 | Cat |
| Lab 1 | France | 2015 | 150129 | Cat |
| Lab 1 | France | 2015 | 150132 | Dog |
| Lab 1 | France | 2015 | 150133 | Dog |
| Lab 1 | France | 2015 | 150134 | Red fox |
| Lab 1 | France | 2015 | 150148 | Horse |
| Lab 1 | France | 2015 | 150230 | Red fox |
| Lab 1 | Nigeria | 2015 | 8670NIG | Human |
| Lab 1 | Greenland | 2015 | 8683GRO | Fox |
| Lab 1 | Greenland | 2015 | 8684GRO | Fox |
| Lab 1 | Egypt | 2015 | 8692EGY | Human |
| Lab 1 | Benin | 2015 | 8697BEN | Cat |
| Lab 1 | Saudi Arabia | 2015 | 8706ARS | Fox |
| Lab 1 | Cameroon | 2015 | 8801CAM | Dog |
| Lab 1 | Ethiopia | 2015 | 8807ETH | Hyena |
| Lab 1 | Ethiopia | 2015 | 8808ETH | Dog |
| Lab 1 | Ivory Coast | 2015 | 9003CI | Dog |
| Lab 1 | Niger | 2015 | 9010NIG | Dog |
| Lab 1 | Chad | 2015 | 9021TCH | Dog |

| Lab | Country | Year | ID | Host |
|---|---|---|---|---|
| Lab 1 | Guinea | 2015 | 9024GUI | Dog |
| Lab 1 | USA | 2015 | 9104USA | Skunk |
| Lab 1 | Mexico | 2015 | 9115MEX | Dog |
| Lab 1 | Mauritania | 2015 | 9136MAU | Goat |
| Lab 1 | Russia | 2015 | 9141RUS | Polar fox |
| Lab 1 | Germany | 2015 | 9217ALL | Red fox |
| Lab 1 | Chad | 2015 | 9218TCH | Dog |
| Lab 1 | Central African Republic | 2015 | 9228CAR | Dog |
| Lab 1 | Namibia | 2015 | 9231NAM | Jackal |
| Lab 1 | Somalia | 2015 | 9302SOM | Dog |
| Lab 1 | SeNegativeal | 2015 | 9305SEN | Dog |
| Lab 1 | Mauritania | 2015 | 9312MAU | Dog |
| Lab 1 | Iran | 2015 | 9319IRA | Jackal |
| Lab 1 | Hungary | 2015 | 9391HON | Fox |
| Lab 1 | Turkey | 2015 | 93101TUR | Fox |
| Lab 1 | Estonia | 2015 | 93105EST | Fox |
| Lab 1 | Zimbabwe | 2015 | 93119ZIM | Dog |
| Lab 1 | Rwanda | 2015 | 94289RWA | Dog |
| Lab 1 | Brazil | 2015 | 9522BRE | Dog |
| Lab 1 | Burkina Faso | 2015 | 9547HAV | Dog |
| Lab 1 | Chad | 2015 | 9609TCH | Dog |
| Lab 1 | Tanzania | 2015 | 9613TAN | Dog |
| Lab 1 | Poland | 2015 | 96178POL | Fox |
| Lab 1 | India | 2015 | 9702IND | Human |
| Lab 1 | French Guiana | 2015 | 9705FRA | Bovine |
| Lab 1 | Myanmar | 2015 | 9915BIR | Dog |
| Lab 1 | Cambodia | 2015 | 9916CAM | Dog |
| Lab 1 | China | 2015 | 02041CHI | Dog |
| Lab 1 | Afghanistan | 2015 | 02052AFG | Dog |
| Lab 1 | France | 2015 | 04031FRA | Dog |
| Lab 1 | Madagascar | 2015 | 04033MAD | Human |
| Lab 1 | Lab strain | 2015 | CVS 27 14-10 | - |
| Lab 1 | France | 2015 | GS7 | Red fox |
| Lab 1 | Greece | 2015 | DR627 | Red fox |
| Lab 1 | France | 2015 | 127900 | Bat (Myotis nattereri) |
| Lab 1 | South Africa | 2015 | DUVV 05-11 | Human |
| Lab 1 | France | 2015 | 122938 | Bat (Eptesicus serotinus) |
| Lab 1 | United Kingdom | 2015 | EBL2 RV1787 | Bat (Myotis daubentonii) |
| Lab 1 | France | 2015 | Negative 17-13 | Red fox |
| Lab 1 | France | 2015 | Negative 17-13 | Red fox |
| Lab 2 | Chad | 2012 | 342 | Dog |
| Lab 2 | Chad | 2012 | 344 | Dog |
| Lab 2 | Chad | 2012 | 345 | Dog |

| | | | | |
|-------|-------------|------|----------|-------|
| Lab 2 | Chad | 2012 | 346 | Dog |
| Lab 2 | Chad | 2012 | 347 | Dog |
| Lab 2 | Chad | 2012 | 348 | Dog |
| Lab 2 | Chad | 2012 | 349 | Dog |
| Lab 2 | Chad | 2012 | 350 | Dog |
| Lab 2 | Chad | 2012 | 354 | Dog |
| Lab 2 | Chad | 2012 | 355 | Dog |
| Lab 2 | Chad | 2012 | 356 | Dog |
| Lab 2 | Chad | 2012 | 357 | Dog |
| Lab 2 | Chad | 2012 | 358 | Dog |
| Lab 2 | Chad | 2012 | 359 | Dog |
| Lab 2 | Chad | 2012 | 361 | Dog |
| Lab 2 | Chad | 2012 | 363 | Dog |
| Lab 2 | Chad | 2012 | 364 | Dog |
| Lab 2 | Chad | 2012 | 365 | Dog |
| Lab 2 | Chad | 2012 | 366 | Dog |
| Lab 2 | Chad | 2013 | 367 | Dog |
| Lab 2 | Chad | 2013 | 368 | Dog |
| Lab 2 | Chad | 2013 | 369 | Dog |
| Lab 2 | Chad | 2013 | 371 | Dog |
| Lab 2 | Chad | 2013 | 372 | Dog |
| Lab 2 | Chad | 2013 | 373 | Dog |
| Lab 2 | Chad | 2013 | 379 | Dog |
| Lab 2 | Chad | 2013 | 380 | Dog |
| Lab 2 | Chad | 2014 | 381 | Dog |
| Lab 2 | Chad | 2014 | 383 | Dog |
| Lab 2 | Chad | 2014 | 384 | Dog |
| Lab 2 | Chad | 2014 | 386 | Dog |
| Lab 2 | Chad | 2014 | 387 | Ovine |
| Lab 2 | Chad | 2014 | 390 | Dog |
| Lab 2 | Chad | 2014 | 392 | Dog |
| Lab 2 | Chad | 2014 | 393 | Dog |
| Lab 2 | Chad | 2014 | 394 | Dog |
| Lab 2 | Chad | 2014 | 395 | Dog |
| Lab 2 | Chad | 2014 | 401 | Dog |
| Lab 2 | Chad | 2014 | 402 | Singe |
| Lab 2 | Chad | 2014 | 403 | Dog |
| Lab 2 | Chad | 2015 | 405 | Dog |
| Lab 2 | Chad | 2015 | 406 | Dog |
| Lab 2 | Chad | 2015 | 407 | Dog |
| Lab 2 | Chad | 2015 | 408 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 012 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 013 | Cat |

| Lab 3 | Ivory Coast | 2017 | Viro 014 | Dog |
|-------|-------------|------|----------|-----|
| Lab 3 | Ivory Coast | 2017 | Viro 68 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 71 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 89 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 97 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 006 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 020 | Dog |
| Lab 3 | Ivory Coast | 2017 | Viro 035 | Cat |
| Lab 4 | Mali | 2017 | 042/01/17 | Dog |
| Lab 4 | Mali | 2017 | 106/01/17 | Dog |
| Lab 4 | Mali | 2017 | 143/02/17 | Dog |
| Lab 4 | Mali | 2017 | 174/02/17 | Dog |
| Lab 4 | Mali | 2017 | 216/03/17 | Dog |
| Lab 4 | Mali | 2017 | 285/03/17 | Dog |
| Lab 4 | Mali | 2017 | 389/05/17 | Dog |
| Lab 4 | Mali | 2017 | 410/05/17 | Dog |
| Lab 4 | Mali | 2017 | 530/08/17 | Dog |
| Lab 4 | Mali | 2017 | 537/08/17 | Dog |
| Lab 4 | Mali | 2017 | 541/08/17 | Jackal |
| Lab 4 | Mali | 2017 | 581/08/17 | Dog |
| Lab 4 | Mali | 2017 | 610/09/17 | Dog |
| Lab 4 | Mali | 2017 | 640/09/17 | Dog |
| Lab 4 | Mali | 2017 | 646/09/17 | Dog |
| Lab 4 | Mali | 2017 | 663/10/17 | Dog |
| Lab 4 | Mali | 2017 | 682/10/17 | Dog |
| Lab 4 | Mali | 2017 | 692/11/17 | Dog |
| Lab 5 | Italy (ex-Nepal) | 2016 | 117/1996 | Human (ex-dog) |
| Lab 5 | Italy (ex-India) | 2016 | 3570/2011 | Italy (ex-India) |
| Lab 5 | Botswana | 2016 | 2871/2009 | Bovine |
| Lab 5 | Botswana | 2016 | 6665/2009 | Honey badger |
| Lab 5 | Brazil | 2016 | 351/2010 | Bovine |
| Lab 5 | Brazil | 2016 | 5B1/2011 | Kinkajou (Potos flavus) |
| Lab 5 | Brazil | 2016 | 343/2011 | Equine |
| Lab 5 | Italy | 2016 | 6944/2009 | Red fox |

| Lyssavirus species | Year (collection) | Type of sample | Origin of sample | DFAT result |
|---|---|---|---|---|
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| Negative | 2015 | Brain | Primary | Negative |
| RABV | NA | Brain | Experimental (mouse) | Positive |
| RABV | 1980 | Brain | Primary | Positive |
| RABV | 1981 | Brain | Primary | Positive |
| RABV | 1979 | Brain | Experimental (mouse) | Positive |
| RABV | 1986 | Brain | Primary | Positive |
| RABV | 1987 | Brain | Primary | Positive |
| RABV | 1987 | Brain | Primary | Positive |
| RABV | 1988 | Brain | Primary | Positive |
| RABV | 1987 | Brain | Primary | Positive |
| RABV | 1989 | Brain | Primary | Positive |
| RABV | 1990 | Brain | Primary | Positive |
| RABV | 1990 | Brain | Primary | Positive |

| | | | | |
|---|---|---|---|---|
| RABV | 1990 | Brain | Primary | Positive |
| RABV | 1991 | Brain | Primary | Positive |
| RABV | 1991 | Brain | Primary | Positive |
| RABV | 1991 | Brain | Primary | Positive |
| RABV | 1988-90 | Brain | NA | Positive |
| RABV | 1991 | Brain | Primary | Positive |
| RABV | 1992 | Brain | Primary | Positive |
| RABV | 1992 | Brain | Primary | Positive |
| RABV | 1992 | Brain | Primary | Positive |
| RABV | 1993 | Brain | Primary | Positive |
| RABV | 1992 | Brain | Primary | Positive |
| RABV | 1993 | Brain | Primary | Positive |
| RABV | NA | Brain | Primary | Positive |
| RABV | 1993 | Brain | Primary | Positive |
| RABV | 1993 | Brain | Primary | Positive |
| RABV | 1993 | Brain | Primary | Positive |
| RABV | NA | Brain | Primary | Positive |
| RABV | 1994 | Brain | Primary | Positive |
| RABV | 1995 | Brain | Primary | Positive |
| RABV | 1995 | Brain | Primary | Positive |
| RABV | 1996 | Brain | Primary | Positive |
| RABV | 1996 | Brain | Primary | Positive |
| RABV | 1994 | Brain | Primary | Positive |
| RABV | 1997 | Brain | Primary | Positive |
| RABV | 1997 | Brain | Primary | Positive |
| RABV | 1999 | Brain | Primary | Positive |
| RABV | 1999 | Brain | Primary | Positive |
| RABV | 1987 | Brain | Primary | Positive |
| RABV | 2002 | Brain | Primary | Positive |
| RABV | 2004 | Brain | Primary | Positive |
| RABV | 2004 | Brain | NA | Positive |
| RABV | - | Brain | Experimental (mouse) | Positive |
| RABV | 1986 | Brain | Experimental (mouse) | Positive |
| RABV | 2012 | Brain | Experimental (mouse) | Positive |
| BBLV | 2012 | Brain | Experimental (mouse) | Positive |
| DUVV | 1971 | Brain | Experimental (mouse) | Positive |
| EBLV-1 | 2002 | Brain | Experimental (mouse) | Positive |
| EBLV-2 | 2004 | Brain | Experimental (mouse) | Positive |
| Negative | 2012 | Brain | Primary | Negative |
| Negative | 2012 | Brain | Primary | Negative |
| RABV | 2012 | Brain | Primary | Negative |
| RABV | 2012 | Brain | Primary | Positive |
| RABV | 2012 | Brain | Primary | Positive |

| RABV | 2012 | Brain | Primary | Positive |
|------|------|-------|---------|----------|
| RABV | 2012 | Brain | Primary | Positive |
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| RABV | 2012 | Brain | Primary | Positive |
| RABV | 2012 | Brain | Primary | Positive |
| RABV | 2012 | Brain | Primary | Positive |
| RABV | 2012 | Brain | Primary | Negative |
| RABV | 2012 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Positive |
| RABV | 2013 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Positive |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Positive |
| RABV | 2014 | Brain | Primary | Positive |
| RABV | 2014 | Brain | Primary | Positive |
| RABV | 2014 | Brain | Primary | Negative |
| RABV | 2014 | Brain | Primary | Positive |
| RABV | 2015 | Brain | Primary | Positive |
| RABV | 2015 | Brain | Primary | Positive |
| RABV | 2015 | Brain | Primary | Positive |
| RABV | 2015 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |

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|------|------|-------|---------|----------|
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Negative |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Negative |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Negative |
| RABV | 2017 | Brain | Primary | Negative |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Negative |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | 2017 | Brain | Primary | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |
| RABV | -    | Brain | -       | Positive |

| Anigen RIDT result | Batch |
|---|---|
| Negative | 1801111 |
| Negative | 1801111 |
| Negative | 1801111 |
| Negative | 1801111 |
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| Positive | 1801111 |
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| Negative | 1801111 |
| Negative | 1801111 |
| Negative | 1801076 |
| Positive | 1801076 |
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| Positive | 1801076 |
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| Positive | NA |
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| Negative | NA |
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| Positive | NA |
| Negative | NA |
| Positive | NA |
| Positive | NA |
| Positive | NA |
| Positive | NA |
| Positive | NA |
| Positive | NA |
| Positive | 1801077/1801081 |
| Positive | 1801077/1801081 |
| Positive | 1801077/1801081 |
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| Positive | 1801077/1801081 |

**Comments**

Results from Léchenne et al., 2016
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Results from Léchenne et al., 2016, lyssavirus species not confirmed by molecular genotyping
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**Author(s):** Stephanie Mauti et al.

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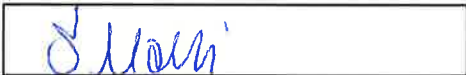
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