Emerging Problems in Infectious Diseases

Evaluation of a rapid immunochromatographic diagnostic test (RIDT) for diagnosis of rabies in samples from Argentina

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Abstract

Introduction: Rabies is a globally widespread zoonosis of viral origin that causes fatal encephalitis in humans and animals. In countries where rabies is endemic and there is a lack of well-equipped diagnostic laboratories, a rapid immunochromatographic diagnostic test (RIDT) for detection of rabies could be an indispensable tool. In this study we evaluated the limit of detection, as well as specificity and sensitivity of RIDT, compared to the standard fluorescent antibody test (FAT).

Methodology: A total of 174 samples were diagnosed by both RIDT and FAT. Fresh clinical samples, poorly conserved samples and brains in advanced state of decomposition generated under laboratory conditions were used to resemble field conditions. The sensitivity of RIDT was evaluated with CVS fixed strain of rabies virus (RABV), previously titrated in 21-day old albino mice and compared with the Reverse Transcription – Polimerase Chain Reaction (RT-PCR) technique in parallel. Additionally, the Mouse Inoculation Test (MIT) was used to perform the antigenic characterization of Rabies virus variants.

Results: The limit of detection of RIDT was 100 LD50 / 0.03 mL and its performance, as compared to that of FAT, showed a sensitivity of 97.96%, a specificity of 100% and a concordance by the Kappa test of 0.98 with 95% CI.

Conclusions: RIDT provides results comparable to those of FAT and this test can be considered as an appropriate method under the field conditions, even in samples that are not suitable for FAT due to their state of decomposition.

Key words: Immunochromatography; rapid test; rabies; samples.

Introduction

Rabies is a globally widespread zoonosis of viral origin that causes fatal encephalitis in humans and animals. This disease is caused by the rabies virus (RABV), which belongs to the genus *Lyssavirus*, family *Rahbdoviridae*.

In Africa and Asia canine rabies continues to be a serious problem, concentrating the highest global mortality rates, especially in children. Bite incidents range from 100 to 120 events per 100,000 people in both urban and rural areas [1-3]. In the Americas, the control and vaccination programs initiated by Pan American Health Organization (PHAO) in 1983 have been effective in reducing the impact of the disease [4]. However, each country in the region continues to report cases of rabies detected in many different wild reservoirs, especially in bats, where the highest number of cases and variety of variants have been identified so far [5]. In Argentina, RABV variants occur in fairly defined regions. In the northwest (NW), the street virus is important because of the transit of people and dogs that cross the border with Bolivia, being able to sporadically cause the disease in humans [6]. In addition, the paralytic rabies caused by subvariant 3[a], affects the cattle production in Salta province and north of Córdoba province [7]. In the northeast (NE), mainly in suburban localities of the provinces of Chaco, Formosa and Misiones near the border with Paraguay, circulates variant 2, which is associated with foxes, wild animals and dogs. As in the NW, there are also cases of rabies due to variant 3, which extend to the provinces of Corrientes and Santa Fe [7,8].

Since the 1990s, the increased occurrence of positive cases throughout the country is associated with the different species of insectivorous bats. [9]. In large urban centers such as the city of Buenos Aires, contacts of bats with pets and people and biting accidents occur daily. Bats represent approximately 60% of the samples that are received at the Instituto de Zoonosis Luis Pasteur, with a historical prevalence of 3%, being *Tadarida brasiliensis* the species that groups 90% of the positive bats [10].

The objective of this study was to evaluate the performance of a rapid immunochromatographic diagnostic test (RIDT) and compare it to the standard fluorescent antibody test (FAT), on fresh clinical specimens, as well as on samples decomposed under laboratory conditions and natural conditions.

Methodology

Samples

Fresh samples

In total, we analyzed 167 fresh samples. A group of 165 specimens of different species such bats, dogs, cats and bovine were taken at random during the period 2011-2015, and two RABV strains isolated from suckling mouse brain and antigenically characterized as V6 (*Lasiurus* spp) and V2 (*Cerdocyon thous*) were used to complete the evaluation of the RIDT kit on all the autochthonous variants of RABV.

Samples in advanced state of decomposition

For our study we used 7 samples in advanced state of decomposition. Among them, five original dog samples of RABV positive brains from an outbreak of urban rabies occurred between 2002 and 2008, which had been antigenically characterized as V1 and conserved at -70ºC, were used to simulate samples in an advanced state of decomposition. To this end, samples were subjected to decomposition in plastic collectors at

room temperature (20 ± 2 °C) and protected from light, for a period between 30 and 120 days.

In addition, one bovine and one feline brain samples from the NW of the country were received in a decomposed state under field conditions at an ambient temperature of 23-24 °C for that time of year.

FAT

Smears were made from aliquots obtained from the fresh brain samples and then fixed on slides in acetone at -20 ° C. Subsequently, the slides with samples and positive and negative controls were stained with an anti-nucleocapsid antibody (BIO-RAD, Marne-La-Coquette, France) according to the manufacturer's instructions.

RIDT

The RIDT kit (Rabies Ag Test Kit, Bionote Inc., Korea) was used for the detection of the viral nucleoprotein by binding to a conjugated antibody. Each strip of the kit contains a positive control line that is displayed when running the sample (Figure 1). The samples were prepared and run according to the manufacturer's instructions. Briefly, brain samples are homogenized with PBS to a 1:10 dilution. Subsequently, a swab is inserted into the tube containing the homogenate, and the swab is transferred to a tube containing a lysis buffer (diluent) provided by

Figure 1. RIDT for the detection of the RABV viral nucleoprotein.
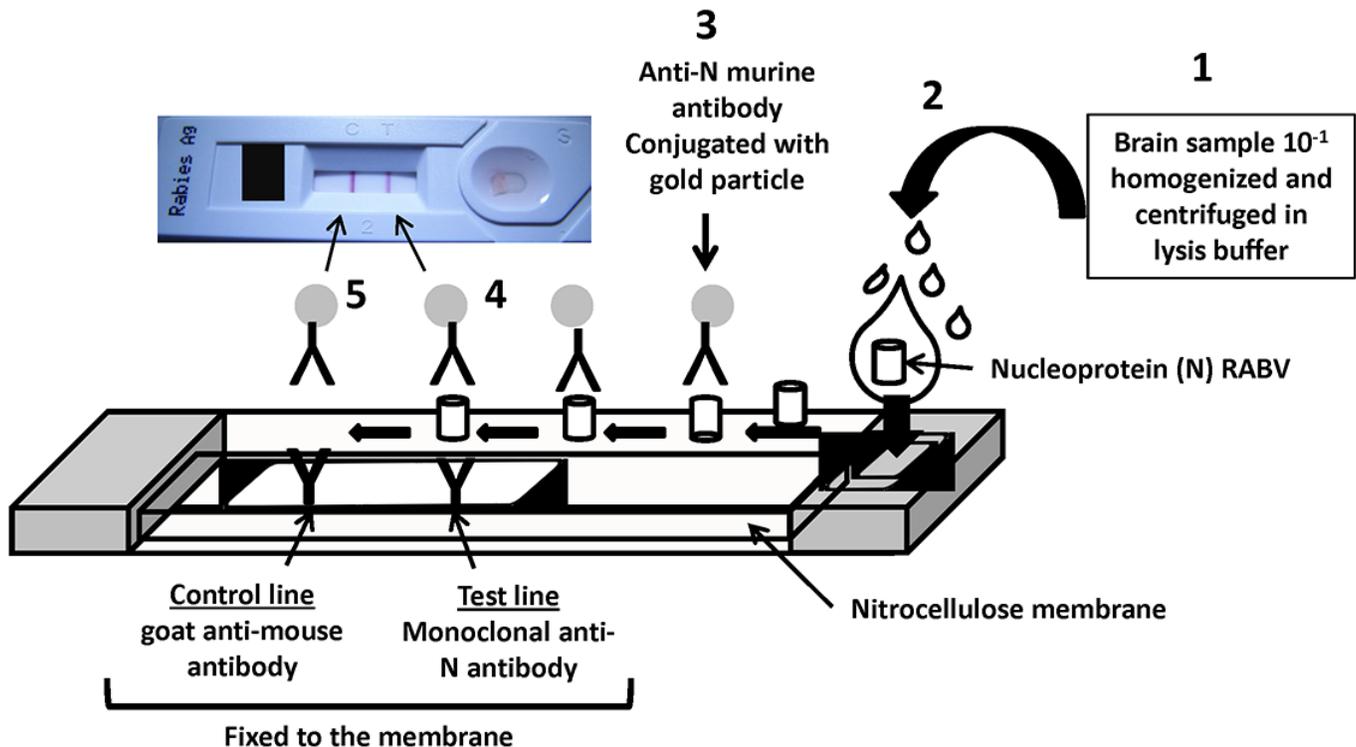
the manufacturer. Then, 4 drops of this tube are added to the sample hole of the test device. We introduced a modification, which consisted of homogenizing the brain sample directly with the lysis buffer provided by the kit. The sample was thoroughly vortexed and clarified by centrifugation. By using this modification, the difference was that, when clarifying the sample, the run was faster but the time for reading was similar.

Antigenic characterization

The samples were antigenically characterized by using a panel of 19 murine monoclonal antibodies produced and assigned by Atlanta's CDC (USA). To this end, suckling mice were intracerebrally inoculated with viral strains isolated from original brain samples positive by FAT as described previously [11]. Subsequently, smears of these isolates were fixed on slides in acetone at -20 ° C and the indirect fluorescent antibody test (IFAT) was performed using the panel of monoclonal antibodies. The monoclonal antibodies were used at a working dilution of 10^{-3} and a confirmatory dilution of 10^{-2} in Eagle's essential medium (GIBCO-Thermofisher, Waltham, USA) using strains of the 2-4-6-ERA and DR19 variants as controls at each typing. Smears of RABV negative brains, which were then stained with fluorescein isothiocyanate-conjugated anti-mouse antigammaglobulin (BIO-RAD, Hercules, USA) and observed under a 40X blue light epifluorescence microscope (Olympus CX31, Tokyo, Japan), were used as negative controls.

In this way, the strains belonging to genotype 1 circulating in Argentina were typified [12,13] according to their antigenic patterns as: 1, 2, 3, 4, 6, *Eptesicus*, *Myotis* and *Histiotus* and their respective subvariants. These corresponded to:

- variant 1: dog and mongoose
- variant 2: dog, fox and wild animals
- variant 3a: vampire (*Desmodus rotundus*)
- variant 4: insectivorous bat (*Tadarida brasiliensis*)
- variant 6: insectivorous bat (*Lasiurus* spp)
- variant insectivorous bat (*Eptesicus* spp)
- variant insectivorous bat (*Myotis* spp)
- variant insectivorous bat (*Histiotus* spp).

Virus control

A stock of the Challenge Virus Standard (CVS) strain of RABV was titrated using tenfold dilutions in 21-day-old albino mice brains. The resulting titer was $10^{6.0}$ LD50 / 0.03 mL. Aliquots of each dilution were also used to perform RT-PCR to determine and compare the limit of detection of both RT-PCR and

RIDT. To determine the RIDT kit limit of detection, the stock was subjected to serial tenfold dilutions starting from: A) all dilutions were carried out using the lysis buffer provided with the kit; B) a first dilution in PBS (which was not run in the kit) and the following dilutions made in the lysis buffer provided with the kit. As a result, the starting dilution run by the kit was 10^{-1} in treatment A, and 10^{-2} in treatment B (Figure 2).

RT-PCR

RNA extraction was performed from a 30 µL homogenate of each dilution used to obtain the viral titer of the CVS RABV control strain, using the column method (Zymmo Research Corp, Irvine, USA), in a final elution volume of 20 µL. For samples which could not be diagnosed by FAT (ND), extraction was performed from 200 µL of 10% homogenate, using the same column method.

The RT-PCR reaction was performed using a One-Step RT-PCR Kit (QIAGEN, Hilden, Germany), where 5 µL of RNA was added to a mixture containing: 3 µL of 5X buffer, 0.5 µL of dNTPs at a concentration of 10 mM, 3 µL Q- 5X, 0.3 µL of Enzyme Mix, 0.15 µL of each primer 504 (Forward: 5' TATACTCGAATCATGATGAATGGAGGTCGACT 3') and 304 (Reverse: 5' TTGACGAAGATCTTGCTCAT 3') [14] at a concentration of 50 pMoles and 4.9 µL of DEPC water for a final reaction volume of 15 µL. Amplification was

Figure 2. Limit of detection of RIDT with the CVS strain.

Column (A): serial dilutions in base 10 in the lysis buffer of the kit. Column (B): base 10 serial dilutions, in which the first dilution in PBS was performed. The band of the first dilution is most reinforced in the test line of column A. The arrows indicate the cutting line of both columns.

Table 1. FAT and RIDT diagnostics results from different sources employed in this study.

Species	Result	FAT	RIDT
Bats (n = 94)	Positive	32	31
	Negative/ND* (n = 1)	62	63
Dogs (n = 46)	Positive	9	9
	Negative/ND* (n = 2)	37	37
Cats (n = 26)	Positive	0	0
	Negative/ND* (n = 2)	26	26
Cattle (n = 6)	Positive	6	6
	Negative	0	0
Isolates (n = 2)	Positive	2	2
	Negative	0	0
TOTAL		**174**	**174**

* Five ND (Non-diagnosable samples by FAT and negative by RT-PCR).

performed using the following conditions: reverse transcription at 50 °C for 30 minutes, followed by denaturation at 95 °C for 15 minutes, 40 cycles of denaturation at 94 °C for 1 minute, annealing at 50 °C for 1 minute, elongation at 72 °C for 1 minute and final extension at 72 °C for 7 minutes. Electrophoresis was performed on a 2% agarose gel with ethidium bromide, using 1X TAE buffer for 25 minutes at 100 V.

RIDT detection limit

Five drops of each dilution made in A and B treatments (see virus control section) were placed in the well of the immunochromatographic plate of the kit, visualizing until the highest dilution at which the virus was detected by the appearance of a positive band and comparing with the RT-PCR results.

Sensitivity and specificity of RIDT

The results obtained by RIDT and FAT were compared using the Kappa test (statistical software program Epidat 3.1, available at https://www.sergas.es/Saude-publica/Epidat-3-1-descargar-Epidat-3-1-(espanol)). To ensure comparable results, a piece of brain from the same anatomical region that was used to prepare the homogenate subjected to RIDT, was collected to perform the FAT.

Results

Table 1 depicts the results obtained by FAT and RIDT methodologies, summarizing positive and negative samples. Decomposed samples that could not be diagnosable by FAT, were subjected to RT-PCR, all of which resulted negative (Table 2).

FAT
Fresh samples
43/167 (25.74%) of the fresh samples were positive.

Samples in advanced state of decomposition
6/7 (86%) of the samples in advanced state of decomposition were positive (Table 2) of which 5 samples corresponded to dogs and one to cattle. A cat sample resulted non-diagnosable for which it was subjected to RT-PCR for diagnosis and was confirmed negative.

Antigenic characterization

Positive samples and isolates were characterized antigenically, showing the following patterns: *Histiotus macrotus* (n = 1), *Myotis levis* (n = 1), *Eptesicus furinalis* (n = 1) and *Tadarida brasiliensis* (n = 29); bovine (*Desmodus rotundus*-V3[a] n = 5 and V1 n = 1); dogs (V1 n = 5 and *Eptesicus furinalis* n = 1); isolates

Table 2. FAT and RIDT results (+: Positive, -: Negative, ND: non-diagnosable) corresponding to samples in advanced state of decomposition. The cat sample was confirmed negative by RT-PCR.

Sample	Species	Procedence	FAT	RIDT
537-04	Dog	Jujuy	+	+
588-04	Dog	SS. Jujuy	+	+
673-04	Cattle	SS. Jujuy	+	+
909-04	Dog	SS. Jujuy	+	+
695-08	Dog	SS. Jujuy	+	+
1057-14	Cat	SS. Jujuy	ND	-
850-14	Dog	S.Mazza-Salta	+	+

Table 3. RIDT vs FAT results.

		IFD		Total
		Positive	Negative	
RIDT	Positive	48	0	48
	Negative	1	125	126
	Total	**49**	**125**	**174**

(*Lasiurus cinereus* n = 1 and *Cerdocyon thous* V2 n = 1).

RIDT

The results obtained by RIDT in fresh samples and in samples in an advanced state of decomposition were compared to those obtained with FAT. A total of 42 out of 43 fresh samples resulted positive by RIDT in relation to FAT. The 5 decomposed samples that could be diagnosed as positive by FAT were confirmed by RIDT (Table 2). In turn, the cat sample, negative by RT-PCR was confirmed negative by RIDT. The RIDT and FAT tests were also compared regarding the total samples (n = 174), obtaining a kappa concordance of 0.9857 IC (95%), a high sensitivity of 97.96% and a high specificity of 100% (Table 3). One false negative was recorded for a bat *Tadarida brasiliensis*, which resulted negative by RIDT while positive by FAT (Table 3).

Limit of detection between RIDT and RT-PCR

The limit of detection was evaluated with the CVS control strain with a titer of $10^{6.0}$ LD50 in 0.03 mL (see virus control and RIDT detection limit). The detection limit of the RIDT was $10^{4.0}$ LD50 in 0.03 mL (Figure 2), whereas that of the RT-PCR technique was $10^{0.5}$ LD50 in 0.03 mL.

Discussion

The diagnosis of rabies by FAT and MIT constitutes the "Gold standard" [15] in fresh samples, with RT-PCR being the most suitable tool for deteriorated samples [16-18] and for the molecular characterization targeting the virus nucleoprotein.

Here, we analyzed a pool of fresh post-mortem samples from bats circulating in Argentina and from some domestic animals, as well as a group of canine samples in advanced decomposition stage under laboratory conditions and two samples (cattle and cat) degraded under natural conditions. According to our observations, the RIDT kit detected the nucleoprotein in all RABV variants circulating in Argentina, demonstrating that the variant should not be considered a factor that could affect the sensitivity or specificity of the kit, as suggested by other authors [19]. To facilitate the use of the kit, the first dilution was performed directly with the kit buffer, allowing the sample band to be displayed in a more reinforced form, improving its reading [20].

In poorly preserved samples, the sensitivity of FAT can be lower [21], and it is necessary to submit the material to another laboratory of greater complexity, extending the time of diagnosis. According to our results in post-mortem samples, the sensitivity of the RIDT was close to that of the FAT (97.9%). Similar to that, observed by other authors [22], the RIDT allows obtaining a first comparable result that must be confirmed by biological assays and RT-PCR, a fact especially useful in samples that have lost their anatomical limits by degradation. According to our results the RIDT kit is suitable for the use in decomposed samples, since it detected positive samples corroborated by FAT and by RT-PCR when the former was not applicable (Table 2). However, it is important to remark that the RIDT detection limit is considerably lower compared to the RT-PCR technique, and hence it could lead to false negative results when the sample has low viral load.

Table 2. FAT and RIDT results (+: Positive, -: Negative, ND: non-diagnosable) corresponding to samples in advanced state of decomposition. The cat sample was confirmed negative by RT-PCR.

Sample	Species	Procedence	FAT	RIDT
537-04	Dog	Jujuy	+	+
588-04	Dog	SS. Jujuy	+	+
673-04	Cattle	SS. Jujuy	+	+
909-04	Dog	SS. Jujuy	+	+
695-08	Dog	SS. Jujuy	+	+
1057-14	Cat	SS. Jujuy	ND	-
850-14	Dog	S.Mazza-Salta	+	+

In our study, the detection limit of the RIDT was much lower ($10^{4.0}$ LD50 / 0.03 mL) than that of the RT-PCR ($10^{0.5}$ LD50 / 0.03 mL). False negatives may be observed in samples with low viral load less than 100-125 LD$_{50}$ / 0.03 mL) [23]. In the present study, only one false negative was recorded for a bat *Tadarida brasiliensis* by the RIDT kit (Table 3), and it was not clear whether this was due to a fault in the kit or to a low viral load, and its titer could not be determined due to the low encephalic material. No false positive samples were obtained by RIDT compared to FAT (Table 3), which coincides with that described in a review of five pioneering studies carried out in different laboratories and with different strains of both RABV and *Lyssavirus* associated with rabies [19,22-24]. In those studies, the Kappa concordance was between 0.82 and 0.96 CI: 95%, whereas in our study it was 0.98 CI: 95%, as shown in the RIDT vs. FAT tables due to false negatives.

Conclusion

In countries where rabies is endemic and there are not enough well-equipped diagnostic laboratories, RIDT can be considered a useful tool when there is no reliable anti-rabies conjugate, high cold storage equipment for control strains, mice for the production of slides and very old microscopes without maintenance service. Having a rapid and easy-to-use diagnosis can be valuable in formulating a prevention strategy with limited resources through vaccination campaigns. In the field, particularly in areas with presence of vampires, RIDT must be used by personnel trained and qualified by the corresponding sanitary authority to take the appropriate measures in each case. As for its use in public health, it should be complemented with the reference techniques and follow the corresponding prophylaxis guidelines.

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