

## Evaluation of a Monoclonal Antibody–Based Rapid Immunochromatographic Test for Direct Detection of Rabies Virus in the Brain of Humans and Animals

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**Abstract.** Rabies diagnosis uses a direct fluorescent antibody test (FAT) that is difficult, costly, and time-consuming, and requires trained personnel. We developed a rapid immunochromatographic test (RICT) for the diagnosis of rabies. The efficacy of the RICT was compared with that of the FAT. Brain samples were collected from humans, dogs, cats, and other animals in Sri Lanka (n = 248), Bhutan (n = 27), and Thailand (n = 228). The sensitivity (0.74–0.95), specificity (0.98–1.0), positive predictive value (0.98–1.0), negative predictive value (0.75–0.97), accuracy (0.91–0.98), and kappa measure of agreement (0.79–0.93) were all satisfactory for animal samples and samples preserved in 50% glycerol saline solution. Because the RICT showed high sensitivity but low specificity with human brain samples, it is unsuitable for confirming rabies in humans. No amino acid substitutions were found in the antibody attachment sites of the nucleoprotein gene with FAT-positive, RICT-negative samples. The RICT is reliable, user friendly, rapid, robust, and can be used in laboratories with a modest infrastructure.

### INTRODUCTION

Rabies virus infects a wide range of mammals and causes fatal encephalitis. More than three billion persons continue to be at risk of rabies virus infection in more than 100 countries.<sup>1</sup> Latest global estimates indicate that 55,000 persons die each year of rabies, which may be 100 times less than the actual figure.<sup>2</sup> Widespread underreporting is attributable to the lack of the necessary infrastructure required for rabies diagnosis in many rabies-endemic countries. The gold standard for routine rabies virus detection is a direct fluorescent antibody test (FAT) that uses postmortem brain tissue. Acquiring and maintaining a fluorescence microscope and the associated reagents is difficult in developing country settings. Preventing sample degradation during transport to central laboratories is another concern in these countries because a cold chain cannot be maintained in most cases. Therefore, sample decomposition hinders accurate diagnosis. The FAT does not work well with decomposed tissue, which means that reverse transcription polymerase chain reaction (RT-PCR) must be used.<sup>3</sup> Thus, the lack of a rapid test impedes surveillance, research, and management of rabies patients. Thus, a simple, rapid, reliable, and cost-effective test for rabies is urgently needed.

Several laboratories are trying to achieve this goal. The RT-PCR<sup>4</sup> and nucleic acid sequence–based amplification<sup>5</sup> methods have been successfully developed for reliable detection of rabies virus. However, both of these techniques are prohibitively expensive in many countries in Asia and Africa to which the virus is endemic. Their applicability is also debatable in the laboratories of most rabies-endemic countries. It would be more practical to transfer currently validated, robust techniques to these regions, where they should be monitored by quality control and regular interlaboratory evaluations.<sup>6</sup> Cost and simplicity must be considered when the adoption of new technologies is planned.<sup>7</sup>

To avoid the use of fluorescence microscopy, a direct rapid immunohistochemical test (dRIT) was developed, and the sensitivity and specificity of this test approached 100% when compared with that of the FAT.<sup>8,9</sup> However, dRIT requires a light microscope and refrigerated reagent storage. It is difficult to maintain cold storage in developing countries because of frequent interruptions to the power supply and these interruptions can compromise reagent quality. A new highly specific and sensitive rapid immunodiagnostic test (RIDT) has also been developed.<sup>10</sup> Immunochromatographic assays are cheap, reliable, rapid, and easy to perform; therefore, many diagnostic tests are based on this method. However, the RIDT has not been tested in the field, and requisite sample storage conditions are unknown.

The performance of a diagnostic test in one setting may also vary significantly from results reported elsewhere.<sup>11</sup> Two other tests, types I and II, were developed on the basis of the same immunochromatography principle.<sup>12</sup> Both tests use a monoclonal antibody against nucleoprotein (N), which is a highly conserved structural protein of rabies viruses and a reliable target for virus detection. The rabies virus has a single, negative-stranded RNA genome that consists of five structural proteins, i.e., N, matrix protein, glycoprotein, polymerase or large protein, and phosphoprotein.<sup>13</sup> Both types of test showed excellent sensitivity and specificity. However, type I showed a higher level of sensitivity and lower specificity compared with type II when tested using dog brain samples.<sup>12</sup> The current study improved type I kit to increase its specificity and we refer to this method as the rapid immunochromatographic test (RICT). The aim of this study was to determine the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa agreement of the RICT compared with that of the FAT when used for the diagnosis of rabies in different brain samples. The RICT is simple, and can be used anywhere in the world with no requirement for special reagents or equipment.

### MATERIALS AND METHODS

Standards for Reporting Diagnostic Accuracy guidelines were followed to ensure the accuracy and completeness of reporting.<sup>14</sup>

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**Ethical approval.** Ethical approval was obtained from the Ethical Committee of the Medical Research Institute, Colombo, Sri Lanka, for experiments on human brain samples. Consent was not obtained from the guardians of these deceased patients, but this procedure was approved by the Ethical Committee. In the present study, animal samples were not used for any purposes other than the RICT. Only a fraction of samples sent to the laboratory for routine diagnostic purposes, were used for the RICT, and local practices of handling animal samples were followed. The RICT results were not used for reporting the presence or absence of rabies. This test was not a commercial product and was not promoted for that purpose. Therefore, ethical approval was not required for animal samples according to local practices and it was not necessary to apply to the local Ethical Committee for approval.

**Collection of study samples and storage conditions.** Brain samples were collected in Sri Lanka, Bhutan, and Thailand. The method, period of collection, and storage conditions were as follows. After the death of a hospitalized rabies patient in Sri Lanka, the Judicial Medical Officer was asked to collect and send a sample. Animal samples were mainly sent by members of the public in Sri Lanka and Bhutan, and by veterinarians in some cases. Because autopsies of human rabies cases are not routinely conducted in Bhutan, human samples could not be collected in this country. Human samples could not be collected in Thailand because human rabies is rare in this country. It is also difficult to obtain permission to acquire samples from families of patients that died of rabies.

*Sri Lanka.* This study used animal and human samples submitted to the Rabies Laboratory, Medical Research Institute (Colombo, Sri Lanka). Animal brains were removed from skulls of suspected rabid animals by a trained person in the Rabies Laboratory. Human brain samples from suspected rabies patients were forwarded by the Judicial Medical Officers from different districts in Sri Lanka while maintaining a cold chain. Portions of all samples were stored at  $-70^{\circ}\text{C}$  until use. Human samples were collected during September 2008–November 2010, and animal samples were collected during October 2009–November 2010.

*Bhutan.* Animal brains were removed from skulls of suspected rabid animals at regional laboratories. Brains of suspected rabid dogs were removed by veterinarians at regional laboratories. Brains of suspected rabid cows were removed at site of death by a veterinarian. Brain samples were stored in 50% glycerol saline solution at  $4^{\circ}\text{C}$ . Samples were transported to the central laboratory at ambient temperature. Brain samples were stored at  $4^{\circ}\text{C}$  in the central laboratory. Samples were collected during January 2008–June 2010.

*Thailand.* Dog brain samples were acquired from the Queen Saovabha Memorial Institute (Thai Red Cross Society), the National Institute of Animal Health, the National Institutes of Health, and Chulalongkorn University. Brain samples of suspected rabid dogs were submitted to these facilities for the laboratory confirmation. All samples were stored at  $-70^{\circ}\text{C}$  until use. Samples were collected during 2003–2008.

**Reference test.** The FAT was used as the reference test in all cases to confirm the rabies diagnosis, and it was conducted immediately after receiving samples. In Sri Lanka, the FAT was performed after preparing slides using the smear method by crushing a small portion of the hippocampus and brain stem of animal and human brains, respectively. In this method,<sup>15</sup> a small section of tissue is placed at one end of the slide.

Another slide is used to crush the section of tissue against the first slide and is then drawn along the length of the slide. In Bhutan, the FAT was performed after preparing slides using the smear method by crushing a small portion of dog hippocampus. Portions of the cerebellum were used with cattle, sheep, and goat brain samples. In Thailand, the FAT was performed on impression smears of the hippocampus.

**Index test.** In the current study, the type I kit<sup>12</sup> was further improved by replacing the absorbent paper and nitrocellulose membrane, increasing the width from 5 mm to 6 mm, and increasing the amount of antibody on the test line from  $0.5\ \mu\text{g}/\text{strip}$  to  $1\ \mu\text{g}/\text{strip}$ . The buffer used with the type I kit (0.1% Triton-X, 50 mM Tris-HCl, pH 7.0) was also replaced with a new buffer solution (20 mM Tris base, 1% Tween-20, and 0.1% sodium azide, pH 7.2) to enhance the specificity of the test.

A pea-sized brain sample was homogenized in 1 mL of buffer solution by using a mortar and pestle, and centrifuged in an Eppendorf (Hamburg, Germany) tube at a minimum of 3,000 rpm for 3 minutes at room temperature. A  $100\text{-}\mu\text{L}$  aliquot of the supernatant was applied to a sample hole in the test strip, and the result was viewed after 15 minutes. The presence of bands in the test and control areas of the strip confirmed a positive reaction and the integrity of the strip, respectively. No band in the test area but the presence of a band in the control area indicated a negative result. For any given test, different observers may have different thresholds for calling a result positive. Thus, blinding was conducted to evaluate the RICT results. Three persons, with no knowledge of the FAT results, read the RICT results. The result was considered positive or negative when three persons independently provided the same verdict. There were no disagreements in the reading of results in the present study.

**Cross-reactivity test.** To determine whether the RICT kit showed cross-reactivity with other viruses, culture supernatants (rabies virus, vesicular stomatitis virus, canine adenovirus type 2, infectious canine hepatitis virus, canine parainfluenza virus, canine coronavirus, canine parvovirus, canine distemper virus, Japanese encephalitis virus, chikungunya virus, West Nile virus, and dengue virus type 2) or the supernatants from suckling mouse brain homogenates (Lagos-B19, Mokola, Duvenhage, and Oita-296/1972) were applied directly to the test strip and results were read as described above. Suckling mouse brain inoculation was performed according to described methods.<sup>16</sup> The nonrabies viruses we tested may cause encephalitis and rabies-like illness.<sup>12</sup> *Lyssaviruses* were handled and stored in a Biosafety Level 3 facility at the Faculty of Medicine, Oita University.

**Robustness of the RICT kit.** Robustness was determined by confirming the capacity of RICT to correctly detect the presence or absence of rabies virus using individual test strips packed in an airtight packet that had been exposed to temperatures of 25, 37, and  $40^{\circ}\text{C}$  for 24, 48, and 72 hours by comparing their results using strips stored routinely at  $4^{\circ}\text{C}$ . We tested only dog brain samples that gave the same results with FAT and RICT strips that had been stored at  $4^{\circ}\text{C}$ . Five rabies-positive and three rabies-negative brain samples were used.

**Viral RNA extraction and RT-PCR.** Total RNA was extracted from 1 g of human brain specimen homogenates by using the acid-guanidinium thiocyanate-phenol-chloroform method (Trizol; Invitrogen, Carlsbad, CA). cDNA was synthesized by using random hexamer primers with a SuperScript III First-Strand Synthesis System (Invitrogen). The N gene was amplified

TABLE 1  
Results of cross-reactivity of rapid immunochromatographic test with different viruses\*

Virus	Strain	Titer	Origin	Result	Cell line used
Rabies virus	CVS-N2	$2.0 \times 10^7$ FFU/mL	Culture sup	Pos	NA (C1300)
	1088-N1	$2.0 \times 10^7$ FFU/mL	Culture sup	Pos	NA (C1300)
	Nishigahara	$1.0 \times 10^7$ FFU/mL	Culture sup	Pos	BHK
	ERA-C1300	$3.9 \times 10^7$ FFU/mL	Culture sup	Pos	NA (C1300)
	HEP-Flury	$3.8 \times 10^7$ FFU/mL	Culture sup	Pos	BHK
Lyssavirus	Lagos-B19	ND	SMB	Neg	
	Mokola	ND	SMB	Neg	
	Duvenhage	ND	SMB	Neg	
Vesicular stomatitis virus	Indiana	$5.0 \times 10^7$ PFU/mL	Culture sup	Neg	BHK
		ND	SMB	Neg	
Oita-296/1972					
Canine adenovirus type 2		$4.0 \times 10^5$ TCID <sub>50</sub> /mL	Culture sup	Neg	MDCK
Infectious canine hepatitis		$4.0 \times 10^5$ TCID <sub>50</sub> /mL	Culture sup	Neg	MDCK
Canine parainfluenza virus		$4.0 \times 10^5$ TCID <sub>50</sub> /mL	Culture sup	Neg	MDCK
Canine coronavirus		$4.0 \times 10^5$ TCID <sub>50</sub> /mL	Culture sup	Neg	CRFK
Canine parvovirus		$4.0 \times 10^6$ TCID <sub>50</sub> /mL	Culture sup	Neg	CRFK
Canine distemper virus		$4.0 \times 10^{5.75}$ TCID <sub>50</sub> /mL	Culture sup	Neg	Vero
Japanese encephalitis virus	Peking	$2.0 \times 10^8$ PFU/mL	Culture sup	Neg	C6/36
Chikungunya virus	BaH306	$1.5 \times 10^8$ PFU/mL	Culture sup	Neg	C6/36
West Nile virus	NY99-6922	$1.55 \times 10^5$ PFU/mL	Culture sup	Neg	C6/36
Dengue virus type 2	ThNH 7/93	$2.1 \times 10^7$ PFU/mL	Culture sup	Neg	C6/36

\*FFU = focus-forming units; Culture sup = culture supernatant; Pos = positive; NA = neuroblastoma; BHK = baby hamster kidney; ND = not determined; SMB = 10% suckling mouse brain; Neg = negative; PFU = plaque-forming units; TCID<sub>50</sub> = 50% tissue culture infectious dose; MDCK = Madin-Darby canine kidney; CRFK = Crandell Rees feline kidney.

and its nucleotide sequence was determined by using described methods.<sup>17</sup>

**Data collection and analysis.** Sensitivity, specificity, PPVs, NPVs, and the kappa measure of agreement were determined by using GraphPad InStat 3 (GraphPad Software Inc., La Jolla, CA). Kappa is a measure of agreement that shows whether a test correctly predicts an outcome. The kappa value of agreement levels was interpreted as follows: poor agreement = < 0.20, fair agreement = 0.20–0.40, moderate agreement = 0.40–0.60, good agreement = 0.60–0.80, and very good agreement = > 0.80.<sup>18</sup>

Sequences were manually edited and compiled. The deduced amino acid sequences of N genes were compared with those of rabies viruses that produced the same or different results with the FAT and RICT. Multiple sequence alignment was performed by using ClustalW version 2 (www.clustal.org).

## RESULTS

Results of the cross-reactivity tests with other viruses are shown in Table 1. The test only produced positive results with

the rabies virus (CVS-N2, 1088-N1, Nishigahara, ERA-C1300, and HEP-Flury). Negative results were produced with other lyssaviruses (Lagos-B19, Mokola, and Duvenhage), vesicular stomatitis virus, Oita-296/1972,<sup>19</sup> canine adenovirus type 2, infectious canine hepatitis, canine parainfluenza virus, canine coronavirus, canine parvovirus, canine distemper virus, Japanese encephalitis virus, chikungunya virus, West Nile virus, and dengue virus type-2.

A total of 248 samples were collected in Sri Lanka, including 115 brain samples from cats, 86 from humans, and 47 from other animals (goat, wild cat, mongoose, grey mongoose, ruddy mongoose, squirrel, rock squirrel, civet cat, rabbit, cow, buffalo, pig, goat, loris, rat, and monkey). In Bhutan, 27 brain samples were collected from cows and dogs. In Thailand, 228 brain samples were collected from dogs.

The sensitivity, specificity, PPV, NPV, accuracy, and kappa measure of agreement for the RICT when compared with the FAT are shown in Table 2.

The optimal temperature for the storage of the RICT was 4°C. Five FAT-positive and three FAT-negative dog brain samples produced the same results when the RICT was stored

TABLE 2

Sensitivity, specificity, positive predictive value, negative predictive value, accuracy, and kappa measure of agreement of rapid immunochromatography test compared with fluorescent antibody test for detection of rabies virus\*

Characteristic	Country				
	Thailand	Bhutan	Sri Lanka	Sri Lanka	Sri Lanka
Species	Dog	Cow, dog	Cat	Other animals	Human
No. samples	228	27	115	47	86
Sensitivity	0.95 (0.89–0.98)	0.90 (0.70–0.99)	0.74 (0.57–0.87)	0.89 (0.52–1.0)	0.99 (0.93–1.0)
Specificity	0.98 (0.93–1.0)	1.0 (0.54–1.0)	1.0 (0.95–1.0)	1.0 (0.91–1.0)	0.08 (0.0–0.38)
PPV	0.98 (0.94–1.0)	1.0 (0.82–1.0)	1.0 (0.88–1.0)	1.0 (0.63–1.0)	0.87 (0.78–0.93)
NPV	0.94 (0.88–1.0)	0.75 (0.35–0.97)	0.88 (0.80–0.94)	0.97 (0.86–1.0)	0.50 (0.01–0.99)
Accuracy	0.96 (0.93–0.98)	0.93 (0.74–0.99)	0.91 (0.82–0.96)	0.98 (0.83–1.0)	0.86 (0.74–0.93)
KMA	0.93 (0.88–0.98), Very good	0.81 (0.55–1.06), Very good	0.79 (0.66–0.91), Good	0.93 (0.79–1.07), Very good	0.17 (–0.36 to 0.58), Poor

\*Values in parentheses are 95% confidence intervals. PPV = positive predictive value; NPV = negative predictive value; KMA = kappa measure of agreement.

at 25°C and 37°C for 48 hours. The RICT strips stored at 37°C for 72 hours produced a positive reaction with one rabies-negative sample, i.e., 33.3% of the FAT-negative samples were positive when using the RICT in these conditions. The RICT strips stored at 40°C for 24, 48, and 72 hours produced positive reactions with two rabies-negative samples, i.e., 66.7% of the negative samples were positive when using the RICT at these conditions. All other rabies-positive samples produced a positive reaction in strips stored at different temperatures for different periods. Therefore, we do not recommend storing RICT strips at 37°C for more than 24 hours, or at 25°C for more than 72 hours.

The complete N gene was determined for five strains that produced positive results by using the FAT and RICT (strain numbers H-08-1320, H-557-10, H-951-09, H-219-08, H-1282-09). Three strains produced negative results using the FAT but positive results using the RICT (strain numbers H-457-09, H-1123-08, H-1125-08), whereas one strain (strain number H-74-10) produced a positive result with the FAT but a negative result with the RICT.

Alignment of the deduced amino acid sequence of the N genes indicated substitutions in two strains that produced positive results using the FAT and RICT. In one strain, Lys was substituted with Glu at residue 5, and in the other strain, Ala was substituted with Gly at residue 371. There were no substitutions in other three strains.

## DISCUSSION

Rabies is still neglected after 125 years of vaccine development, and it is considered one of the most neglected diseases in the developing countries, which experience the greatest burden in poor rural communities.<sup>20</sup> Surveillance is a basic but important process when trying to understand the magnitude of rabies frequency in different countries and within individual countries. However, surveillance is hampered by a lack of equipment, reagents, and experienced personnel in countries where rabies is widely prevalent.

The FAT is the gold standard for routine rabies diagnosis, but it is cumbersome and requires expensive equipment, reagents, and well-trained personnel for the interpretation of the test. Therefore, the search for an easy and rapid diagnostic test for rabies has continued. An enzyme-linked immunosorbent assay (ELISA) known as rapid rabies enzyme immunoassay was developed, but it is no longer available commercially.<sup>21</sup> A new ELISA known as WELYSSA was recently developed and provides high specificity (99.9%) and sensitivity (97.0%).<sup>21</sup> The ELISA may be easier to perform than the FAT, but requires equipment for reading the test results, and the reagents should be stored at cold temperatures. A latex agglutination test was developed to diagnose rabies by using dog saliva, and the sensitivity, specificity, PPV, and NPV were 97.6%, 97.4%, 95.2%, and 98.7%, respectively.<sup>22</sup> This test appears to be promising for use in field laboratories in developing countries. However, further studies are required to confirm whether this test can be used for brain samples because the presence of rabies virus in saliva is not a reliable<sup>7</sup> indicator because of the intermittent shedding of rabies virus.

The recently developed dRIT has high sensitivity and specificity, but it is not clear whether this test can be applied on samples preserved in 50% glycerol for more than 15 months,

and it has also not been tested on human samples.<sup>8</sup> The new RIDT has only been tested on animal samples,<sup>10</sup> and its performance with human samples remains unknown. A variety of animal samples has not been tested in countries to which the disease is endemic, and the robustness of the kit is also unknown. In the current study, the RICT did not provide the same specificity as the dRIT, but its sensitivity was high. High sensitivity is important for a disease screening test where a missed diagnosis has serious consequences.<sup>23</sup> The dRIT requires a microscope and a refrigerator for storing reagents, but the turnaround time of this test is only just over one hour, which is faster than the FAT, where results are available within three hours if a fresh brain sample is used in the analysis.<sup>24</sup> The RICT identifies rabies within 20 minutes, which is a practical and realistic delivery time for results. The high level of sensitivity and specificity of the RICT when using samples preserved in glycerol indicates that this method can be used with confidence in the peripheral laboratories of developing countries where facilities are relatively modest.

Overall, the RICT yielded a high sensitivity and specificity when testing for animal rabies. We achieved one of our objectives by significantly improving the sensitivity of our kit from 88.9%<sup>12</sup> and maintaining a similar sensitivity to the previous version. Only cat brains produced a lower sensitivity. However, the kappa measure of agreement was good for cat samples, which indicated that results were satisfactory. With human brain samples, the kit showed low specificity but high sensitivity, which indicated that it should not be used as a confirmatory test for human brain samples. Only human samples produced a poor agreement when our kit was used.

We detected no substitutions in the antibody-binding sites of N genes. Therefore, the low specificity was not attributable to mutations that affected the antigenic site binding capacity of the antibody. This discrepancy may be caused by the low nonrabies human sample size. Because there was some cross-reactivity, the RICT showed less specificity with human brain samples. The exact reason for this cross-reactivity is unknown but it may be caused by a host-related factor. Our observations of cerebrospinal fluid from patients with encephalitis showed that a few samples had a positive reaction with the RICT. However, these patients were negative for rabies when tested by PCR, but positive for dengue virus IgM, and they recovered from their illness.

Some disparity prompted questions about the limitations of the FAT because some positive RICT results were associated with negative FAT but RT-PCR positive results. Because a study of a large number of samples confirmed there were no false-positive results with the FAT for nondecomposed samples,<sup>24</sup> our samples might not have been sufficiently fresh to show FAT-positive results. In addition, the origin of the specimen might affect the sensitivity of the RICT. Further analyses are needed to examine the factors associated with human brain samples that contribute to low specificity with the RICT. This analyses will facilitate improvement of assays that can be used for the surveillance of human brain samples.

In conclusion, the RICT has the advantages<sup>25</sup> that it is user-friendly, rapid, robust, easily deliverable, and requires no specialist equipment. Given its greater sensitivity and that it can be easily performed anywhere, the RICT will be a valuable adjunct to the FAT if used appropriately. Molecular tests and the FAT can be used as periodic confirmatory tests to ensure good laboratory practice.

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