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A simple and rapid immunochromatographic test kit for rabies diagnosis

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List of Abbreviations: BHK-21, Baby hamster kidney-21; CAV2, canine adenovirus type 2; CCV, canine coronavirus; cDNA, complementary deoxyribonucleic acid; CDV, canine distemper virus; CHV, canine hepatitis virus; CPV, canine parainfluenza virus; CPV, canine parvovirus; CVS, challenge virus standard; D-MEM, Dulbecco's modified Eagle's medium; dNTP, deoxyribonucleotide; ERA, Evelyn Rokitniko Abelson; FAT, fluorescent antibody test; FBS, fetal bovine serum; FFU, focus forming unit; G, glycoprotein; HEP-Flury strain, high egg passage-Flury strain; ICT, immunochromatographic test; LD50, 50% lethal dose; M, matrix; MAb, monoclonal antibody; MEM, minimum essential medium; MMLV, Moloney murine leukemia virus; N, nucleoprotein; NASBA, nucleic acid sequence based amplification; OIE, World Organization for animal health; P, nucleocapsid-associated phosphoprotein; PBS, phosphate buffer saline; PET, post exposure treatment; RABV, rabies virus; RNA, ribonucleic acid; RNP, ribonucleoprotein complex; RT-PCR, reverse transcription and polymerase chain reaction; TCID₅₀, 50% tissue infectious dose; VSV, vesicular stomatitis virus; WHO, World Health Organization.

Key words

immunochromatographic test, rabies, rapid diagnosis.

ABSTRACT

In rabies endemic countries, funds and infrastructure are often insufficient to employ the approved gold standard for the definitive diagnosis of rabies: the direct fluorescent test. In the present study, two types (type 1 and 2) of an ICT kit were evaluated for detection of rabies. These were developed using monoclonal antibodies which recognize epitope II and III of the nucleoprotein of rabies virus. Both kits specifically detected all rabies virus strains and there was no cross reactivity with *Lyssaviruses* (Lagos, Mokola and Duvenhage), *Rhabdovirus* (VSV and Oita 296/1972) and other common canine-pathogenic viruses. In type 1, a single type of monoclonal antibody was used. It was capable of detecting recombinant nucleoprotein and showed sensitivity of 95.5% (42/44) and specificity of 88.9% (32/36) using brain samples from rabid dogs. In contrast, type 2 which was made of two different monoclonal antibodies had a lower sensitivity of 93.2% (41/44) and higher specificity of 100% (36/36). These ICT kits provide a simple and rapid method for rabies detection. They need neither cold chain for transportation nor complicated training for personnel. This diagnostic test is suitable for rabies screening, particularly in areas with a high prevalence of rabies and where the fluorescent antibody test is not available.

Rabies is a fatal enzootic disease caused by the RABV, genus *Lyssavirus* of the *Rhabdoviridae* family. RABV has a single, negative-stranded RNA genome and consists of five structural proteins: a nucleoprotein (N protein), a nucleocapsid-associated phosphoprotein (P protein), a matrix protein (M protein), a glycoprotein (G protein) and a polymerase (L protein) (1). The N gene is highly conserved among *Lyssavirus* genotype 1 (RABV) and comparison of this gene has been employed in several epidemiological analyses. The envelope glycoprotein encoded by the G gene is responsible for receptor binding and viral invasion into host cells (2, 3). The glycoprotein is the main target for immune response and vaccine development (4–6).

RABV infects a wide range of mammalian species and causes fatal encephalitis. In Asia, the principal rabies reservoirs and/or transmitters are dogs, rather than wild animals such as foxes, bats, and raccoons. A recent estimate is that every year in Asia and Africa 55,000 people die of rabies (7). This is a modest estimate and the actual incidence of human rabies may be 100 times greater than officially recorded (7). The public health impact of rabies has been underestimated (8). Since rabies is fatal in all victims, diagnosis is often based on clinical manifestations only. However, definitive diagnosis of rabies can be obtained only by laboratory investigations, and can lead to the proper recommendation of PET using vaccine and globulin. Post-mortem diagnosis requires a FAT for detection of RABV in brain tissue. Definitive diagnosis of rabies infection using the “gold standard” of FAT as approved by both WHO and OIE is expensive and requires infrastructure and well-trained technicians (9, 10). Since many rabies endemic countries lack the above-mentioned requirements for the diagnosis of rabies, the exact number of rabies patients remains obscure. RT-PCR (11) and NASBA (12) methods have been successfully developed to detect rabies virus in tissue. However both of these techniques are out of reach in the areas of Asia and Africa where the virus is endemic.

Recently, we have developed a novel diagnostic test for RABV using immunochromatographic techniques. Our system can achieve rapid and sensitive detection for RABV using MAb which recognize the N protein of RABV. In this study we have shown the uniqueness of our rapid diagnostic test kit and evaluated its efficacy by comparing it with standard diagnostic techniques. The rapid diagnosis test is simple, time- and cost-saving. It can be used anywhere in the world and needs no special reagents or equipment. We anticipate this kit will be widely used in rabies-endemic areas and countries.

MATERIALS AND METHODS

Study samples

The diagnostic laboratories of the Queen Saovabha Memorial Institute (Thai Red Cross Society), National Institute of Health, the National Institute of Animal Health Laboratory and Chulalongkorn University serve as the main facilities for examining suspected rabid animal specimens in the central region of Thailand. The origin and the clinical history of each animal were recorded. Diagnoses were done using FAT on impression smears from hippocampus and brain stem. FAT-positive samples were stored at -80°C for further study.

Viruses and cells culture

Neuroblastoma NA cells of A/J mouse origin were grown at 37°C in MEM supplemented with 10% heat-inactivated FBS. BHK-21 cells were grown at 37°C in D-MEM supplemented with 10% FBS.

For RABV we used CVS-11, ERA, HEP-Flury and Nishigahara strains as laboratory strains of RABV plus the 1088 strain of street RABV (13–15). *Lyssaviruses* were represented by Lagos B-19, Mokola, and Duvenhage viruses which had previously been passaged in suckling mouse brain and stored in our laboratory. Similarly, vesicular stomatitis virus (VSV) and Oita 296/1972 virus isolated from horseshoe bat in Oita, Japan (16) were used to represent *Rhabdovirus*. Other viruses used in this study were as follows; canine adenovirus type 2 (CAV2), infectious canine hepatitis virus (CHV), canine parainfluenza virus (CPiV), canine coronavirus (CCV), canine parvovirus (CPV) and canine distemper virus (CDV).

Viral RNA extraction and RT-PCR

Total RNA was extracted from 0.2–0.5 g of brain specimen homogenates or cell-culture suspension using the acid-guanidinium thiocyanate phenol chloroform method (TRIzol; Gibco BRL, Gaithersburg, MD, USA). The N gene was amplified using a sense primer, designated as NF2850 (nt. 28–50, 5'-ACAGACAGCGTCAATGGCAGAGC-3') and an anti-sense primer, designated as N660 (nt. 660–676, 5'-GTTTGGTATAGTACTCC-3') (17). Primer position is indicated according to the N gene of CVS strain with the GenBank accession number DQ286762. One microgram of total RNA was reverse transcribed for synthesis of first strand cDNA using primer NF2850 and MMLV reverse transcriptase (Gibco BRL) at 37°C for 2 hr. First PCR reaction was done by using the NF2850/N660 primer set under the following conditions: briefly, 50- μl reaction

mixtures containing 5 μ l of sample cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 20 μ M each of the two primers, 200 μ M dNTP, and 2 U of *Taq* polymerase (Promega Corp, Madison, WI, USA). The reaction mixture was subjected to 35 cycles of denaturation at 95°C for 0.5 min, annealing at 50°C for 0.5 min and extension at 72°C for 1.5 min. One tenth of the first PCR product was applied to the semi-nested PCR step. For this reaction, 10 g (nt. 66–82, 5'-CTACAATGGATGCCGAC-3') was used for sense primer. The second PCR reaction was performed by using the 10 g/N660 primer set under the same conditions as the first PCR reaction except for annealing at 56°C. The amplicon was subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide.

ICT kit

The test strip was constructed on the principles of immunochromatography using colloidal-gold-labeled MAb. We used two clones of MAb generated against the N protein of RABV. MAb10-41-F2 (18) and 87-3E2 recognize epitope III and II of the N protein respectively. Epitope mapping of these MAb was performed by competitive inhibition assay using MAb (kindly provided by Professor Minamoto, Gifu University) recognizing antigenic sites of N protein (19). One microgram/ml of anti-RABV N MAb 10-41-F2 was used for type 1 and MAb 87-3E2 for type 2. MAb was immobilized onto a nitrocellulose membrane for the test line zone and goat anti-mouse immunoglobulin antibody was immobilized for the control line zone to capture unbound MAb. A reagent pad containing colloidal gold-labeled MAb 10-41-F2 was located in front of the sample hole (Fig. 1a).

Brain tissue specimens were vigorously homogenized in mortar and 20% suspensions of the homogenate made in PBS and stored at -20°C until use. Before the test this homogenate was treated with an equal volume of B buffer containing 0.1% Triton-X, and 50 mM Tris-HCl, pH 7.0. Eighty μ l of the treated sample was added to the sample hole of the test strip. Purified recombinant N protein solution or culture supernatant of virus was applied directly without any treatment. The final results were available within 15 min as signified by the appearance of a red-colored band at the test zone as well as at the control zone. When the colloidal gold is prepared to a certain size, it is visible as a red color after formation of antigen-antibody complex on the test strip (Fig. 1b).

Evaluation of ICT and determination of specificity and sensitivity of the kit

For the evaluation of ICT, five strains of RABV, three strains of *Lyssavirus*, two strains of *Rhabdovirus*, and six

other viruses were examined. The sensitivity and specificity of ICT was determined by using 80 brain samples collected at Queen Saovabha Memorial Institute (Thai Red Cross Society) and comparing them with FAT as the reference method. Experimental infections or manipulation of RABV and *Lyssaviruses* were performed and stored in a Biosafety Level 3 facility in the Faculty of Medicine, Oita University, Japan.

RESULTS

Ability of ICT to detect RABV

Figure 1b shows a representative positive result of ICT after the appearance of a positive band in the test zone following the application of sample to the sample hole. First, we measured the detection limit of both kits using CVS-11 culture supernatant possessing an infectious titer of 4.0×10^7 FFU/ml ($=10^{4.28}$ TCID₅₀/50 μ l). Both kits detected approximately 10^{-3} fold diluted CVS-11 (4.0×10^3 FFU = $10^{1.28}$ TCID₅₀/50 μ l). For practical use, brain homogenate material will be used for test samples. Therefore, the utility of ICT was determined using rabid-brain samples. Ten percent brain homogenate obtained from suckling mouse brain infected with strain 1088 was used and the infectious titer of this material was 1.0×10^7 FFU/ml ($=10^{4.23}$ TCID₅₀/50 μ l). Type 1 and type 2 could detect up to maximal 3000 times dilution of the original material (approximately 3.3×10^2 FFU = $10^{0.75}$ TCID₅₀/50 μ l). Therefore, ICT showed a higher ability to detect RABV in brain homogenate than in culture supernatant. The detection limit of RT-PCR was $10^{-0.77}$ TCID₅₀/50 μ l which is approximately 30-fold more sensitive than the ICT kit.

Our preliminary study revealed that MAb used in the present kit recognizes an epitope on the N protein of RABV which is a well-conserved amino acid sequence among RABV. The intensity of the test line of the kit is reduced depending on the serial dilution of the supernatant containing the virus. ICT was capable of detecting not only N protein obtained from cell culture or brain sample but also recombinant protein purified by column from *Escherichia coli* DH-5 α transformed with pQE9-rNP harboring full length CVS N gene (20).

As shown in Table 1, we evaluated whether both kits could specifically detect laboratory strains and isolates of RABV (Fig. 1b), *Lyssavirus*, *Rhabdovirus*, and other viruses frequently infecting dogs. We found that both kits could specifically detect RABV and there was no cross reactivity with *Lyssaviruses* or *Rhabdoviruses*. Dogs suffering from

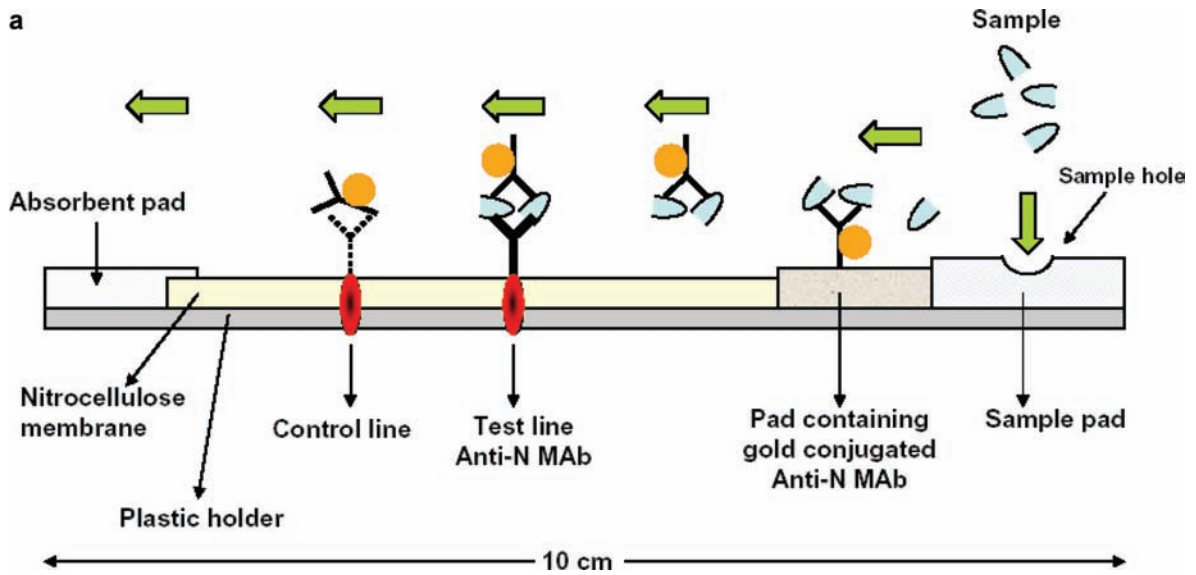


Fig. 1. (a) Diagram of rapid ICT strip for the detection of RABV. (b) ICT strip to detect RABV showing positive result. Positive bands were observed both in the test line and the control line. Lane 1: ERA, positive; lane 2: HEP-Flury, positive; lane 3: 1088, positive; lane 4: CVS-11, positive; lane 5: culture media, negative.

canine viral infections are often misdiagnosed as rabies. Our ICT showed negative reactions with CAV2, CHV, CPIV, CCV, CPV, and CDV.

During the preliminary analysis, both type 1 and type 2 showed false positive reactions with brain homogenate prepared only with PBS (data not shown). Sample treat-

ment with B buffer reduced false positive reactions significantly in type 1 and eliminated them totally in type 2. Therefore, rabid dog brain samples treated by B buffer were used to evaluate the efficacy of ICT. The positive band on the test strip did not fade at room temperature for over a month.

Table 1 Ability of ICT to detect RABV, *Lyssavirus*, *Rhabdovirus* and other canine viruses.

Virus	Origin	culture sup	Result	
			Type 1	Type 2
RABV	CVS-11	culture sup	+	+
	ERA	culture sup	+	+
	HEP-Flury	culture sup	+	+
	Nishigahara	culture sup	+	+
	1088	SMB	+	+
<i>Lyssavirus</i>	Lagos B-19	SMB	–	–
	Mokola	SMB	–	–
	Duvenhage	SMB	–	–
<i>Rhabdovirus</i>	VSV	culture sup	–	–
	Oita-296/1972	SMB	–	–
Other viruses	CAV2	culture sup	–	–
	CHV	culture sup	–	–
	CPIV	culture sup	–	–
	CCV	culture sup	–	–
	CPV	culture sup	–	–
	CDV	culture sup	–	–

SMB: 10% suckling mouse brain.

Efficacy of kits for clinical samples

To evaluate the efficacy of the kits for the diagnosis of RABV infections, eighty brain samples obtained from domestic dogs were tested. All tested samples were collected and stored for further analysis at the Queen Saovabha Memorial Institute (Thai Red Cross Society), National Institute of Health and the National Institute of Animal Health Laboratory of Thailand. Forty-four of the 80 samples were from rabid cases and the remaining 36 samples were from non-rabid cases as determined by FAT. Of the 44-rabies-positive samples, 42 cases tested positive and two negative using type 1. Of the 36 rabies-negative samples, four cases showed false positive results using type 1 (Table 2) in the form of very faint bands which could not easily be discriminated as negative results. Another two samples, which initially showed negative results using samples obtained from the hippocampus, showed positive results after repeated testing using samples from the brain stem. Furthermore, a few cases did not show positive results using 10% brain homogenate suspension but revealed positive results when 15–20% homogenate was used (data not shown). In terms of type 2, three cases of 44 rabies-positive samples tested negative but all rabies-negative samples showed negative results. Finally, with FAT as the reference method, results of tests on brain samples collected from the field indicated that the sensitivity and specificity of type 1 were 95.5% and 88.9% and that of type 2 were 93.2% and 100%, respectively.

Table 2 Concordance of ICT kit and FAT

	Type 1		Type 2	
	+	–	+	–
FAT +	42/44	2/44	41/44	3/44
FAT –	4*/36	32/36	0/36	36/36

*Very faint band, clearly identified as a negative.

DISCUSSION

In the present study, we describe a simple and rapid diagnostic test for rabies infection based on the principle of immunochromatography on filter paper. The assay system was initially developed as a lateral-flow immunoassay for the detection of *Rotavirus* in fecal samples (21). Thereafter, this method has been widely accepted in various fields of laboratory diagnosis, including infectious diseases (22, 23).

Definitive *intra vitam* or postmortem diagnosis of rabies infection requires FAT detection of RABV in brain autopsy, skin biopsy, and corneal impression samples. FAT is approved by both WHO and OIE as the “gold standard” and available in several developing countries. FAT is a simple and reliable technique. Nevertheless, it requires expensive infrastructure and well-trained technicians, especially for postmortem diagnosis of rabid animals in endemic countries. More advanced techniques such as RT-PCR and NASBA have been successfully developed to detect RABV and approved for higher sensitivity. These are an alternative rapid way to confirm the diagnosis of rabies; however both methods need further skills and incur additional costs. More recently, a latex agglutination test (24) and direct rapid immunohistochemical test (25) have also been developed and evaluated using rabid samples. However these techniques are not yet widely accepted as an on-site diagnostic method in the areas of Asia and Africa where the virus is endemic. Since alternative detection systems for RABV such as RT-PCR and NASBA are based on the principal of gene amplification, they require several hours to obtain final results. Moreover, specific reagents and instruments are needed. PCR techniques are globally available for the diagnosis of various diseases; however socio-economical reasons limit the availability of these laboratory diagnostic methods in most rabies-endemic countries.

Surveillance data is the most important information for rabies evaluation, successful animal control and rabies elimination from endemic areas. The cost and need for well trained personnel are obstacles to the availability of FAT laboratories in such areas. Therefore a rapid, accurate and low-cost means of laboratory diagnosis is

both necessary and desirable in order to make an on-site diagnosis of suspected cases, especially for *intra vitam* diagnosis of rabid animals. The rabies infection status of suspected animals is crucial for a rapid decision on PET, which should be started as soon as possible in any rabies exposed cases, but preferably only on the basis of a definite diagnosis, as the supply of rabies tissue culture vaccine is limited.

The ICT kit can detect RABV by recognizing the N protein, which is the most abundant in the virion or in RABV-infected tissue. Interestingly, the test was capable of detecting N protein without any treatments, although N protein is an internal protein of the virion. Why is the internally located N protein detected by the test? One possibility is that the MAb used in the ICT might recognize N protein which has leaked from the destroyed virion or infected tissue. Electron microscopic observation revealed that RABV particles contain several damaged virions and/or virions with protruding N protein (data not shown). Leakage of N protein from damaged RABV-infected brain tissue might be detected by the ICT. Therefore, detergent in B buffer possibly exposes N protein in the infected tissue in addition to reducing the incidence of non-specific reactions.

Regarding the diagnosis of rabies for PET, false negatives have more serious consequences than false positives, because misdiagnosis might have fatal consequences in rabies-exposed individuals. False negative results could occur due to the sequence of viral infection and distribution in the early stages of infection. For detection of rabies, selection of the brain region from which to obtain samples is important. The brain stem and hippocampus provide best results. Similar to FAT, any doubtful or negative results need to be confirmed by animal history or other laboratory techniques, such as RT-PCR, NASBA, cell culture or mouse inoculation tests depending on the facilities available in the local laboratory.

Evaluation of the ICT kit for the diagnosis of rabies using saliva or cerebrospinal fluid from living animals requires further study. The possibility of using this kit as an *intra vitam* diagnostic test might help to avoid the unnecessary killing of dogs in endemic countries.

In conclusion, our diagnostic test can provide results within 15 min, and the interpretation of the results does not require expertise and expensive equipment. This test does not need cold chain for transportation. Thus the kit is suitable for screening and surveillance of a large number of rabies-suspected animals in laboratories with proper facilities for biohazard in endemic areas because of its simple, rapid, reliable, and cost-saving properties. It will provide helpful information for decisions on the need for PET.

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