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Protocols

A rapid immunochromatographic test strip for detecting rabies virus antibody

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A B S T R A C T

An immunochromatographic test strip (ICTS) for detecting antibodies to rabies virus was developed, using colloidal gold particles labeled with rabies virus glycoprotein as the tracer. The assay was evaluated using sera from dogs immunized with various commercial rabies vaccines, or from dogs in the clinics and sera from dogs immunized with vaccines against pathogens other than rabies virus, and negative sera from a wide variety of animal sources, including dogs, mice, and cats which had never been vaccinated. The ICTS was found to be highly specific for antibodies against rabies virus, with a detection limit of 0.5 IU/ml as measured by the fluorescent antibody virus neutralization (FAVN) test. Compared with the FAVN test, the specificity and sensitivity of ICTS were 98.2% and 90.4%, respectively. There was an excellent agreement between results obtained by the ICTS and FAVN tests (kappa = 0.888). Strips stored at 4 °C in a plastic bag with a desiccant retained their specificity and sensitivity for at least 15 months, and strips stored at ambient temperature remained stable for 12 months. The immunochromatographic test strip may therefore be useful for clinical laboratories lacking specialized equipment and for diagnosis in the field for rapid detection of rabies virus-specific antibodies.

1. Introduction

The measurement of seroneutralizing antibodies has been used commonly for many years to assess the level of immunity to rabies (Aubert, 1992; Cliquet et al., 1998) and a virus-neutralizing antibody titer of 0.5 IU/ml in sera is the threshold of seroconversion to rabies vaccination as recommended by the World Health Organization (WHO). Detection and quantitation of antibodies against rabies virus (RABV) are also of prime importance for assessing the efficacy of rabies vaccination campaigns. The rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973) and the fluorescent antibody virus neutralization (FAVN) test (Cliquet et al., 1998) are the only in vitro methods recommended by the World Organization for Animal Health (OIE) for measuring virus neutralizing antibodies. However, such assays require a considerable level of expertise and are generally undertaken only in reference laboratories with suitable biocontainment facilities. In addition, the procedures are time-consuming, and only a limited number of samples can be processed at one time. They are therefore inefficient and expensive for human and animal clinics in developing countries without trained personnel. The challenges in the 21st century for diagnostic test developers are two-fold: first, to achieve internationally accepted validation of a test; and second, to overcome financial and logistical barriers that prevent implementation of a test in the developing countries with the greatest need (Fooks et al., 2009). A more convenient and affordable diagnostic test for antibodies against rabies virus should be developed in countries where rabies is endemic and where resources are limited.

The immunochromatographic assay, also referred to as lateral flow device or simply a strip assay, is a technique in which a cellulose membrane is used as the carrier and a colloidal gold-labeled antigen or antibody is used as the tracer (Zhang et al., 2006, 2009a,b). Although this method has only been used for qualitative analysis so far, it provides rapid detection of antigens and antibodies, and thus possesses advantages over the conventional immunoassays, such as low cost, inexpensive equipment, simplicity of procedure, rapid operation, and long-term stability over a wide range of environmental conditions. These characteristics render a test ideally suited for on-site testing by untrained personnel (Peng et al., 2007). Immunogold labeling has been applied

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widely for diagnosis of infection and for antibody detection (Cui et al., 2008; Hedstrom et al., 1998; Mikawa et al., 2009; Oku et al., 2001; Smits et al., 2001; Yang et al., 2010). A rapid immunochromatographic test kit for rabies virus detection was developed and evaluated using clinical samples. The kit provides a simple and rapid method for detection of infection with rabies (Kang et al., 2007; Nishizono et al., 2008). In the present study, a rapid, one-step immunochromatographic test strip capable of detecting specifically rabies virus antibodies in serum was developed.

2. Materials and methods

2.1. Cells and virus

Mouse neuroblastoma (MNA) cells were maintained in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). The Challenge Virus Standard strain (CVS-11) of RABV was propagated in MNA cells.

2.2. Reagents

Chloroauric acid was purchased from Sigma Company (St. Louis, MO, USA). Nitrocellulose membranes, fiberglass, and filter paper were purchased from Millipore Corporation (Billerica, MA, USA).

2.3. Sera and antisera

Fluorescein isothiocyanate (FITC)-conjugated antibody against the rabies viral N protein was purchased from Fujirebio (Melvin, PA). A mouse monoclonal antibody (MAbG63; IgG2a) recognizing epitopes at antigenic site II on the RABV G protein was selected by the Military Institute of Veterinary Science (Changchun). The neutralizing potency of the antibody was 18.6 IU/ml at 1 mg/ml for CVS-11 rabies virus detected by the FAVN test. Antisera for canine distemper virus (CDV), canine parvovirus (CPV), canine adenovirus (CAV-I), canine parainfluenza virus (CPIV), canine coronavirus (CCV) and rabies virus were prepared in the Military Institute of Veterinary Science (Changchun). Sera from dogs, cats, rabbits and mice that had never been vaccinated were used as negative controls. All animals were housed and handled following the ethical guidelines of the Regulations of Animal Ethics and Welfare of the Military Institute of Veterinary Science, Academy of Military Medical Sciences, China. A total of 756 clinic sera collected at random from 10 different provinces were evaluated. All serum samples were stored at –20 °C until used.

2.4. Isolation of rabies virus glycoprotein

The rabies virus glycoprotein (RABV G) was prepared by a modification of a technique which was described previously (Barton and Campbell, 1988; Cox et al., 1977; Smith et al., 2006). MNA cells were infected with CVS-11 at a multiplicity of infection (MOI) of 0.1, and incubated at 32 °C for 4 d. Culture fluid was collected, cells were removed by centrifugation (1500 × *g*, 20 min, 4 °C), and the virus was pelleted by ultracentrifugation at 45,000 × *g* for 2 h at 4 °C. Pellets were resuspended in PBS and left overnight at 4 °C. Beta-propiolactone (BPL, Sigma, Inc.) was added to the virus solution to a final BPL dilution of 1:3000. Incubation continued overnight at 4 °C, followed by 2 h at 37 °C to permit complete hydrolysis of the remaining BPL. Inactivated virus was then layered over a sucrose gradient and pelleted by ultracentrifugation (61,000 × *g*, 90 min, 4 °C). The pelleted virus was resuspended in Tris-buffered saline containing EDTA (0.13 M NaCl/0.05 M Tris, pH 7.2/0.001 M EDTA). The glycoprotein was dissociated from the virus by incubation (1 h, 24 °C) with 1% Triton X-100 in Tris-buffered saline. The soluble G protein fraction was separated from the residual suspension by sedimentation of the latter into a 25% sucrose cushion (55,000 × *g*, 160 min) and was collected by aspiration of the layer just above the cushion. After dialysis against a phosphate buffer (PB, 20 mM pH 7.2), Sephadex G-50 (GE Healthcare, UK) filtration was used to remove excess ampholine. Then the G protein was dialyzed extensively against phosphate buffer (PB, 20 mM pH 7.2) and stored in aliquots at −80 °C until used. Protein concentration was determined using a BCA kit (Pierce, Inc., Rockford, IL).

2.5. SDS-PAGE and Western blotting

The purified rabies virus glycoprotein was analyzed by immunoblot analysis. 20 μg of soluble protein was eletrophoresed on 12% denaturing gel and transferred to polyvinylidene difluoride membrane (Bio-rad, USA). The membrane was blocked for 2 h at room temperature in 5% non-fat dry milk (NFDM) in PBS (pH 7.4) followed by three washings with PBST (PBS containing 0.05% Tween-20) and incubated with anti-RABV G monoclonal antibodies (MAbG63) for 1 h at 37 °C. After three additional washes with PBST, the membrane was incubated with HRP-conjugated anti-mouse IgG (GE Healthcare, UK) at a 1:10,000 dilution in PBST for 1 h at room temperature. The membrane was washed three times with PBST and developed by HRP color development kit (Bio-rad, USA) as per the manufacturer's instructions.

2.6. Preparation of the conjugate pad

Colloidal gold particles with a mean diameter of 20 nm were prepared using a method described previously (Peng et al., 2007). Under constant stirring, 1.8 ml of 1% trisodium citrate (w/v) was added rapidly to 100 ml of 0.01% $HAuCl_4$ solution (w/v) at 100 °C and boiled for 5 min. As the resulting colloidal gold cooled gradually to ambient temperature with continuous stirring, the pH was maintained at 8.0 with 0.1 M potassium carbonate. Sodium azide was added to a final concentration of 0.01% (w/v) before storage at 4 °C in dark colored glass vessels.

To prepare colloidal gold-RABV G, 10 ml purified G protein (1.0 mg/ml) was added to 100 ml gold colloidal solution with constant stirring, and incubated for 30 min at room temperature. Bovine serum albumin (BSA) was then added to a final concentration of 1% (w/v) to stabilize the conjugate solution, which was then centrifuged at 10,000 × *g* for 30 min at 4 °C. After washing twice in 1% BSA, the colloidal gold-RABV G particulate was suspended in 10 ml 10 mM PBS (pH 8.0) containing 1% BSA, 2% sucrose and 0.01% (w/v) sodium azide, and dispensed onto the fiberglass strips (0.5 cm × 30 cm, Billerica, MA, USA) at a speed of 50 μL/cm using a XYZ 3000 Dispensing Platform (Bio-Dot, Irvine, CA, USA) to produce the conjugate pad. After drying under vacuum at 4 °C, the pads were then sealed with a desiccant in plastic bags and stored at 4 °C.

2.7. Membrane blotting

On the 2 cm nitrocellulose membrane (2.0 cm × 30 cm, Millipore), different working concentrations of staphylococcal protein A (SPA, Sigma–Aldrich, St. Louis, MO, USA) and affinity-purified anti-RABV G monoclonal antibodies were dispensed as test and control lines using the XYZ3000 Dispensing Platform at 1.25 μL/cm. After being dried at room temperature, they were stored at 4 °C as described above.

2.8. Assembly of the immunostrip

Sample fiberglass pads were treated with 20 mM phosphate buffer containing 1% PEG, 0.5% Tween-20 and 0.05% sodium azide

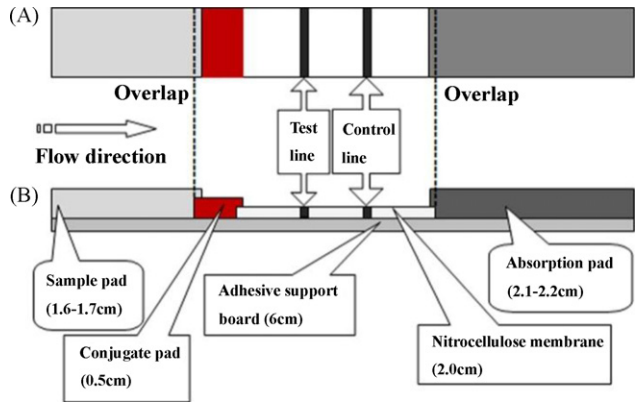

Fig. 1. Schematic diagram of the test strip showing the components, with their widths: (A) top view and (B) cross-section.

(pH 7.4), and dried at 37 °C. Fig. 1 provides a schematic diagram of the test strip. These were prepared on a 6 cm × 30 cm adhesive support board upon which were layered, in 1–2 mm overlapping positions, a fiberglass sample pad, fiberglass conjugate pad, nitrocellulose membrane, and absorption pad. The assembled boards were then cut into 4 mm wide pieces using a CM 4000 Cutter (Bio-Dot). The strip products were mounted in plastic cassettes with windows over the sample pad and the nitrocellulose membrane (Jincanhua Industry, Ltd., Shenzhen, China). The cassettes were then packaged in plastic bags with desiccant, and stored at 4 °C until used.

2.9. Principle of the test

A serum sample (50 μl) applied to the sample pad migrates into the conjugate pad, where any RABV-specific antibodies react with the colloidal gold-RABV G to form a complex which diffuses across the nitrocellulose membrane and reacts with SPA immobilized on the membrane forming a red-purple line (test line). Unreacted gold-RABV G complex migrates further and reacts with the anti-RABV G monoclonal antibodies, forming another red-purple line (control line). The test takes 10–15 min to complete, and a sample with a virus neutralizing antibody titer ≥ 0.5 IU/ml will produce two red-purple bands at both the test and control lines, whereas a sample with a titer < 0.5 IU/ml or negative for rabies virus will produce only one band at the control line (Fig. 2). A clearly visible red-purple band must appear at the control line for the test to be valid.

2.10. Fluorescent antibody virus neutralization (FAVN) test

Virus neutralizing antibody titers were measured by the FAVN test as described by Cliquet et al. (1998). Serial three-fold dilutions of the tested serum samples and controls were prepared in the microplate wells in 100 μl volumes. 100 $TCID_{50}$ of CVS-11 in 50 μl were added to each well and incubated at 37 °C for 60 min. MNA cells (0.2×10^5 cells in 50 μl) were added into each well, and the plates were incubated at 37 °C for 48 h. The wells were fixed in ice-cold 80% acetone and stained with FITC-conjugated anti-RABV N antibodies for 0.5 h. The plated cells were examined by fluorescent microscope (Olympus Corporation, Tokyo, Japan). Absence and presence of fluorescent foci in the cells was recorded. The 50% endpoint of the antibody was calculated according to the Spearman–Kärber formula (Kärber, 1931; Spearman, 1908). The values were compared with that of a reference serum (National Institute for Biological Standards and Control, Herts, UK) and normalized to international units (IU/ml).

3. Results

3.1. Purification of RABV G protein

It was found that rabies virus G protein could be isolated from Triton X-100-disrupted rabies virus, without losing its biological and serological properties (Atanasiu et al., 1974, 1976; Cox et al., 1977; Smith et al., 2006). In this study, the inactivated rabies virus was purified by sucrose gradient centrifugation, then soluble glycoprotein was dissociated from the virus by incubation with 1% Triton X-100 and purified by sucrose centrifugation and Sephadex G-50 filtration. About 12% of total protein was recovered after purification. To characterize the purified protein, purity and biological activity of the rabies virus G proteins were analyzed by SDS-PAGE (Fig. 3A) and Western blotting (Fig. 3B). As shown in Fig. 3A, the purified proteins displayed a dominant protein band that represented approximately 95% of the purified protein. Western blotting using anti-RABV G monoclonal antibody identified the purified rabies virus G proteins. The data showed that the rabies virus G proteins were purified highly and not degraded during the purification procedure.

3.2. Determination of working concentrations of captured reagents for immobilization

Staphylococcal protein A (SPA) and affinity-purified anti-RABV G monoclonal antibodies were diluted to 1.0, 1.5, 2.0, and 2.5 mg/ml with 20 mM PBS (pH 7.4). The various working concentrations were

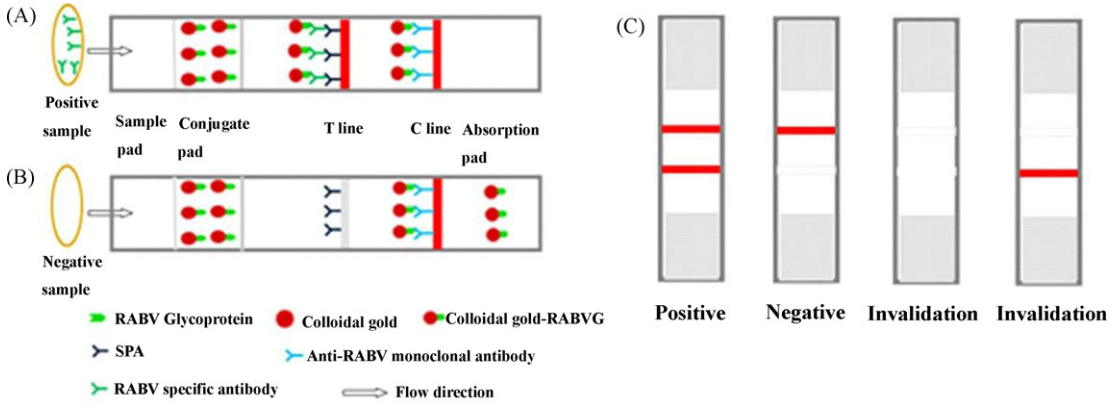

Fig. 2. The principle of the ICTS for detection of rabies virus antibody. (A) Serum sample with virus neutralizing antibody (VNA) titer ≥ 0.5 IU/ml. (B) Negative sample or serum sample with VNA titer < 0.5 IU/ml. (C) Read out.

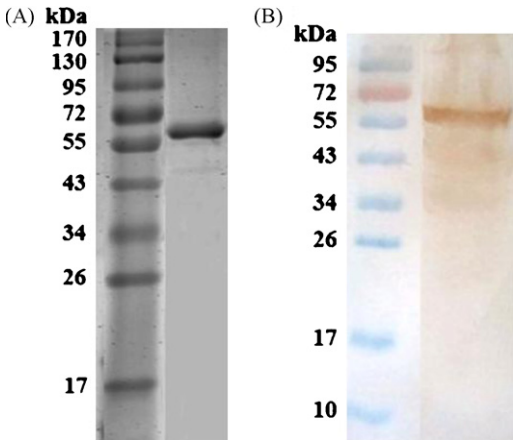

Fig. 3. SDS-PAGE and western blotting analysis of purified RABV G. (A) 20 μg of purified RABV G protein was seperated by SDS-PAGE and (B) analyzed by western blotting.

then dispensed onto nitrocellulose membrane for assembly of ICTS. The optimal concentration of SPA was achieved by testing the ICTS with different concentrations against 0.5 IU/ml WHO standard serum, and selecting the minimum concentration that produced a detectable line. The result showed that the optimal concentration of SPA that produced a detectable line with 0.5 IU/ml WHO standard serum but not reacted with sera of virus neutralizing antibody titers < 0.5 IU/ml, or negative sera, was 1.5 mg/ml. The optimal concentration of anti-RABV G monoclonal antibody was determined based on the red color of the control line both for positive and negative samples. Finally, the optimal concentrations of SPA and affinity-purified anti-RABV G monoclonal antibody for immobilization were 1.5 and 1.0 mg/ml, respectively (data not shown).

3.3. Specificity of the ICTS

The specificity of the ICTS was demonstrated by testing 185 sera from dogs, cats, rabbits and mice that had never been vaccinated, 115 different serum samples derived from dogs vaccinated with non-RABV pathogens (CDV, CPV, CAV, CCV, CPIV), different commercial rabies vaccines or virus strains maintained in the laboratory. The results were compared with the same samples analyzed by FAVN tests. All serum samples derived from non-immunized dogs, cats, rabbits and mice, and sera from dogs immunized with pathogens other than rabies virus were found to be negative for anti-RABV antibodies by both ICTS and FAVN tests. Visually, the tested samples appeared identical to the blank control, with only one red-purple band at the control line. All serum samples from dogs with virus neutralizing antibody titers ≥ 0.5 IU/ml determined by FAVN tests produced two bands by ICTS (Fig. 4). The ICTS was specific for sera positive for rabies virus and did not cross-react with other naïve animal sera or sera from animals immunized with other pathogens.

Table 1
The specificity and sensitivity of ICTS in assaying sera from dogs immunized with different types of vaccines.

Vaccine	FAVN test		ICTS	
	≥0.5 IU/ml	<0.5 IU/ml	Positive	Negative
Inactivated Evelyn-Rokitnicki-Abelseth (ERA)	20	13	20	13
Inactivated Pasteur virus (PV)	12	8	12	8
Flury-LEP	15	11	15	11
Commercial (Intervet nobivac)	8	7	8	7

Table 2
Sensitivity of the ICTS for sera with a relative low virus neutralizing antibody (VNA) titer.

FAVN test		ICTS		Positive %
VNA titer	Number	Positive	Negative	
0.20–0.49	44	0	44	0
0.50–0.69	86	78	8	90.7
0.70–0.79	22	22	0	100

3.4. Sensitivity of the ICTS

Sera positive for rabies virus from dogs immunized with various rabies vaccines were tested by ICTS with 3 different operators. When the virus neutralizing antibody titer of the serum samples was ≥0.5 IU/ml as determined by FAVN tests, the results by ICTS were all positive. The results were negative by ICTS when the serum samples were negative or with a titer < 0.5 IU/ml (Table 1). The sensitivity of the ICTS was also demonstrated by testing 152 selected serum samples with titers ranging from 0.2 IU/ml to 0.8 IU/ml as determined by FAVN tests (Table 2). Results showed that of the 108 sera with titers ≥ 0.5 IU/ml, 100 (92.6%) produced a distinct band on the test line, and 8 (7.4%) gave a negative response. Of the 8 sera giving a negative result, all of them had VNA titer ranging from 0.5 IU/ml to 0.7 IU/ml.

3.5. Correlation between ICTS and FAVN in clinical samples

A total of 756 clinical serum samples selected at random from 10 different provinces were tested by ICTS, and the results were

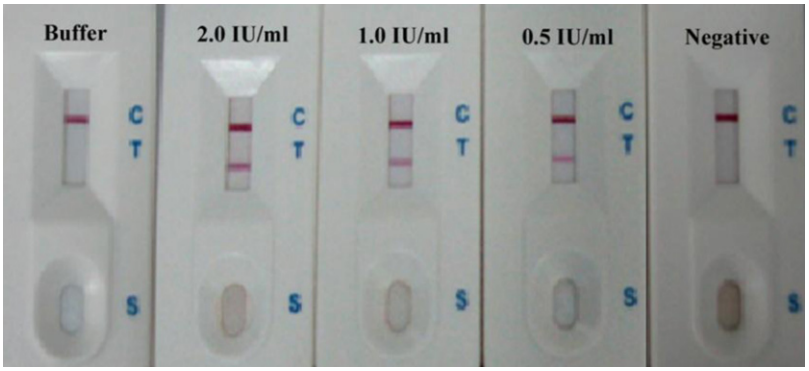

Fig. 4. ICTS to detect neutralizing antibody against rabies virus. Strip 1: only buffer (20 mM phosphate buffer containing 0.5% Tween-20 and 0.05% sodium azide, pH 7.4) is applied to the strip, a band is formed on the Control line. Strip 5: negative sera or sera with virus neutralizing antibody titer < 0.5 IU/ml produces only one band on the Control line. Strip 2, 3 and 4: serum sample with VNA titer ≥ 0.5 IU/ml, forms 2 bands on test line and control line.

Table 3
Results of antibody detection in clinical serum samples using the ICTS and the FAVN test[a].

		FAVN test			
		≥0.5 IU/ml	<0.5 IU/ml	Negative	Total
ICTS	Positive	331	7	0	338
	Negative	35	245	138	418
	Total	366	252	138	756

[a] Relative to FAVN test, specificity of ICTS: (245 + 138)/(252 + 138) = 98.2%; sensitivity of ICTS: 331/(331 + 35) = 90.4%; Po = (383 + 331)/756 = 94.4%; Pe = [(366/756) × (338/756) + (390/756) × (418/756)] = 0.502; kappa = (Po − Pe)/(1 − Pe) = 0.4424/0.498 = 0.888.

compared with those obtained by FAVN tests (Table 3). Of the total 756 serum samples, 138 negative samples as determined by FAVN tests were also confirmed to be negative by ICTS. Of 252 sera with virus neutralizing antibody titers < 0.5 IU/ml, 245 sera were negative by the ICTS with a consistency of 97.2%, and all 7 sera with conflicting results between the two tests had FAVN titers ranging from 0.45 IU/ml to 0.49 IU/ml. Of 366 sera with virus neutralizing antibody titers ≥ 0.5 IU/ml, 331 sera were positive by the ICTS with a consistency of 90.4%. There was an excellent agreement (kappa = 0.888) between ICTS and FAVN tests.

3.6. Storage life and reproducibility

Immunochromatographic strips packaged in a plastic bag with a desiccant stored at 4 °C or at ambient temperature for varying times up to 24 months were tested for specificity and sensitivity. All test results were identical by using the strips stored from 3 to 12 months, with all known serapositive samples being positive and all known VNA-negative sera being negative. After 15 months of storage at ambient temperature, the sensitivity of the strips decreased slightly as the known positives with titers between 0.50 and 1.0 IU/ml produced negative results (15.8%). However, sera with VNA titer > 1.0 IU/ml or < 0.5 IU/ml, or negative sera were still tested consistently with the FAVN tests. The strips stored at 4 °C for 18 months gave consistent results except that 12.6% of those in the range 0.50–1.0 IU/ml were tested as negative. All samples were tested on strips from three separate batches, showing that there was complete reproducibility between batches. After 24 months, most of the ICTS stored at either 4 °C or at ambient temperature showed the same appearance as before, only 1.8% had not been dehumidified properly. Therefore, the results showed that sealed strips could be stored for at least 15 months at 4 °C or 12 months at ambient temperature with only a slight loss of sensitivity.

4. Discussion

The present study describes a new approach for determining the virus-neutralizing antibodies against rabies virus by ICTS, a technique that has been used for detecting many other antigens and virus-specific antibodies. The cut-off value of the ICTS was 0.5 IU/ml, and with the FAVN test used as a reference test, the ICTS had high specificity (98.2%) and sensitivity (90.4%). There was an excellent agreement between the results obtained by FAVN test and ICTS (kappa = 0.888) for clinical samples.

The sensitivity and specificity of ICTS is dependent largely on the antigen used in the test strip. Rabies virus glycoprotein is the only structural protein of the virus that induces the formation of virus-neutralizing antibodies, which confer immunity to animals. The protective activity was shown to be associated only with the G protein and not with the membrane associated protein M and P, although they contain residual traces of G protein (Cox et al., 1977). Competitive ELISA that utilizes a cell line stably expressing the rabies virus G protein as the coating antigen has higher specificity and sensitivity than that of ELISA based on whole rabies virus antigen (Bahloul et al., 2005; Servat and Cliquet, 2006). Also, there is an excellent agreement between the FAVN test and competitive ELISA. In the study presented here, the virus was cultured at 32 °C for 4 d, and this was sufficient to obtain more G protein than virus grown at 37 °C. In order to reduce the false-positive results, sucrose gradient centrifugation was used to purify the virus. Subsequently the G protein was dissociated from the purified virus by incubation with Triton X-100, which could have a higher biological and protective activity than that solubilized by deoxycholate or by Nonidet P-40 (Cox et al., 1977). The sensitivity and specificity of ICTS in this study did not produce any difference between serum samples derived from dogs vaccinated with inactivated Pasteur virus (PV) strain, inactivated Evelyn-Rokitnicki-Abelseth (ERA) strain, Flury-LEP vaccines and commercial vaccines (Intervet Nobivac) (Table 1), and there was also an excellent correlation with the FAVN test. This might be attributed to using the same strain of RABV (CVS-11) as an antigen in both of the ICTS and FAVN test.

Staphylococcal protein A (SPA) is bacterial proteins that bind with high specificity to mammalian immunoglobulins, and is used commonly to purify, immobilize or detect immunoglobulins. However, they have different affinities to immunoglobulin with different isotypes. Also, the binding capacity with immunoglobulin of SPA differs between mammalian species. In this study, SPA was immobilized on the membrane and used as the test line, and it showed high affinity to serum samples from dogs. In addition, the ICTS can also be used to detect serum samples positive for rabies virus derived from humans, cats, rabbits and mice, but the sensitivity varies (data not shown). The ICTS showed higher sensitivity when detecting sera from canine, human and rabbit, and a relatively lower sensitivity for detection of mouse and cat sera. Therefore, the ICTS may also be useful in serosurveillance with these species.

The ICTS has been validated by comparing the results of 756 clinical serum samples with those obtained using the FAVN test. False-negative results occurred with the ICTS test for samples possessing virus neutralizing antibodies ≥0.5 IU/ml, which implies that the sera did not have an adequate level of neutralizing antibody. The isotype of RABV-specific antibody from those serum samples was detected by ELISA, and it was shown that the isotype of those antibodies was IgM, but not IgG (data not shown). Since SPA has higher binding affinity with IgG than IgM, this may explain why those samples did not produce a positive result when tested by ICTS. Among 390 samples with virus neutralizing antibody titer < 0.5 IU/ml, only 7 samples were positive by ICTS. However, the virus neutralizing antibody titer of those samples ranged from 0.45 to 0.49 IU/ml. Sera derived from subjects which were unvaccinated or had received other vaccines such as CDV, CPV, CAV, CCV, CPIV did not show false-positive results when tested by ICTS. This result indicates that the ICTS is specific for sera against rabies virus.

To date, some ELISAs or competitive ELISAs (c-ELISAs) have been developed to test serum samples and compare the results with the RFFIT or FAVN test (Bahloul et al., 2005; Cliquet et al., 2004; Muhamuda et al., 2007; Servat and Cliquet, 2006; Servat et al., 2007; Zhang et al., 2009a,b). Those methods are simple and rapid for clinical use. However, they are still not suitable for use outside the research laboratory because of the requirement of special equipment such as a microplate reader or incubator. A RAPId Neutralizing Antibody (RAPINA) test has been described for quantitatively measuring neutralizing antibody against rabies virus (Shiota et al., 2009). In this method, serum is incubated with inactivated rabies virus, and then used for a RABV G detection kit based on the principle of immunochromatography as described previously (Nishizono et al., 2008). In comparison with ELISAs, the RAPINA has an advantage of convenience, safety and rapidity. No special equipment, cultured cells, live virus, or trained operators are needed,

and the results are easy to read. Sera with virus neutralizing antibody titers ≥ 0.5 IU/ml could neutralize the rabies virus and would not produce a test line; however, sera with titers < 0.5 IU/ml would not inhibit the band formation on the test line when detected by the RABV G detection kit. In the ICTS, 50 μl serum sample was applied to the test, two red-purple lines on the membrane indicate that the serum sample possesses a virus neutralizing antibody titer ≥ 0.5 IU/ml. If only one line appears at the control location, the serum sample is negative for rabies virus or has a virus neutralizing antibody titer < 0.5 IU/ml. Also, the stronger the test line appears, the more virus specific antibodies are present in the sample. It should be pointed out that one disadvantage of ICTS is that the intensity of the test lines could reach to a plateau when test the serum samples with titers higher than 8.0 IU/ml. This is because the amounts of colloidal gold-RABV G dispensed onto the fiberglass and Staphylococcal protein A immobilized on the membrane are constant, and even though more virus neutralizing antibodies are present in the sample, the colloidal gold-RABV G-RABV antibody complex trapped by the Staphylococcal protein A immobilized at the test line is limited, so the color cannot become stronger. In this case, the serum sample should be diluted further.

In conclusion, the ICTS is a useful method for quantitative analysis of virus neutralizing antibodies against RABV in serum and for differentiation of animals with adequate immunity from those without adequate immunity. It can be used virtually anywhere – laboratories, offices, or in the field. Also, it has been adapted for testing clinical samples, and could be used as a tool for surveillance of rabies in the field and vaccination efficacy testing. Since the binding capacity with immunoglobulin of Staphylococcal protein A differs between mammalian species, this method could be modified to detect sera derived from humans or other mammals.

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