Letters in
Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

A rapid one-step immunochromatographic test strip for rabies detection using canine serum samples

C. Tao[1,2] and G. Li[1]

1 State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing Scientific Observation and Experiment Station for Veterinary Drug and Veterinary Biotechnology, Ministry of Agriculture, Beijing, China
2 Guangxi Center for Disease Prevention and Control, Nanning, China

Significance and Impact of the Study: Simple and cheap techniques to detect rabies virus or monitor immunity against it are central in maintaining epidemiological control over the disease, particularly in endemic developing countries. While many techniques meet this requirement, they are confined to this usage as they are time-consuming and demand expensive instrumentation. Our immunochromatographic test strip can detect rabies antibody with high specificity and sensitivity; the output can be measured with naked eye. It allows safe and quick detection that will be of value in the surveillance of the immunization status of potential targets in rabies-endemic regions and will aid disease control.

Keywords
colloidal gold, ELISA, immunochromatographic test strip, rabies, staphylococcal protein A.

Correspondence
Gang Li, State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing Scientific Observation and Experiment Station for Veterinary Drug and Veterinary Biotechnology, Ministry of Agriculture, Beijing 100193, China.
E-mail: gli358@gmail.com

2014/0029: received 7 January 2014, revised 17 April 2014 and accepted 7 May 2014

doi:10.1111/lam.12282

Abstract

We developed an immunochromatographic test strip using colloidal gold-coated staphylococcal protein A (SPA) for the detection of rabies antibody in canine serum samples. The recombinantly expressed rabies virus phosphoprotein (RV-P) and the anti-staphylococcal protein A (anti-SPA) polyclonal antibody were coated on the test (T) and control (C) lines on a nitrocellulose membrane, respectively. This layout is designed such that the polyclonal antibody in canine serum is captured by the colloidal gold–SPA conjugates, before the rabies antibody complex is specifically selected by the RV-P deposited on the T line, forming a ‘sandwich’ pattern. Unbound excess colloidal SPA then proceeds to the control line where SPA specifically interacts with the anti-SPA antibody, producing a red precipitation at the C line, indicating the validity of the strip. We tested 165 canine serum samples with the strips, and the results were compared with those obtained using ELISA. The specificity and sensitivity of ICTS were found to be 93·1 and 92·2%, respectively. As a rapid technique, not demanding expensive instrumentation, the strip offers potential in disease monitoring, especially in rabies-endemic developing countries.

Introduction

Rabies is a neurotropic and infectious zoonosis with a high fatality rate (Jackson 2013). Among several species carrying rabies, dogs act as the predominant reservoir for the virus. Rabid dogs transmit the virus through infected saliva by penetrating the skin of humans or animals through bites (Schupbach *et al.* 2012) and pose potential threat to humans. Rabies detection has been extensively studied for decades. The fluorescent antibody test (FAT) was first applied as a technical recommendation by the World Health Organization (WHO) in the 1950s and is still widely used for the detection of the rabies virus antigen in the cerebral tissue of infected animals (Whitfield *et al.* 2001; Wunner and Briggs 2010). FAT, together with the rapid tissue culture infection test (RTCIT) and the mouse inoculation test (MIT), serves as the golden standard for rabies detection. While these techniques have good reproducibility and high accuracy (Kasempimolporn *et al.* 2011), they require well-trained personnel to perform the characteristic stringent protocols, thus limiting their application.

Serological detection methods have also been developed for the detection of rabies antibody; these include methods such as ELISA (Welch *et al.* 2009) and rapid neutralizing antibody detection test (Li *et al.* 2012). To assess the immunization status of the infected or immunized animal or the effectiveness of the vaccine, it is recommended that the animal serum be monitored after infection or immunization (De Benedictis *et al.* 2012). New antigen detection-based assays have recently become available for rabies diagnosis, including the recently developed immunochromatographic tests (Wang *et al.* 2010) that provide a one-step, rapid and low-cost tool for naked eye detection. Among these methods, ELISA is the one prescribed in international trade, wherein the evaluation of vaccine responses in canines and felines prior to international movement is mandatory (Servat and Cliquet 2006).

We have previously developed a highly sensitive and specific staphylococcal protein A (SPA)-based ELISA for the detection of the rabies (Fan *et al.* 2010a,b). Despite high specificity and sensitivity, this method required several washing steps and other procedures that further complicate the process. In addition, the equipment necessary to read the results in such assays is expensive, limiting their application outside the laboratory. The objective of this study was to develop a simple, portable, rapid and one-step technology for the detection of rabies antibody in canine serum, so as to facilitate early serological surveys of dogs postimmunization.

Results and discussion

Characterization of colloidal gold and colloidal gold–SPA

TEM images of colloidal gold and SPA-conjugated colloidal gold particles are shown in Figure 1a,b, respectively. The colloidal gold particles were observed to be spherical in shape, with an estimated diameter of 20 nm. The particles were well dispersed after conjugation with SPA, and no aggregates were observed in the solution, offering as an indicator for successful modification (Duy *et al.* 2010).

Optimization and evaluation of the strip

The effect of three different concentrations of RV-P on T line coloration was investigated to determine the optimal RV-P concentration to be used. Rabies-positive canine serum was used in these experiments. The T line colour density consistently increased up to a RV-P concentration of 2·4 mg ml^{-1}, after which it decreased. The optimal concentration of the P protein, deduced from these observations, was determined to be 1·6 mg ml^{-1}.

Three blocking reagents, including 1% BSA, 10% FBS and 3% gelatin, were also investigated to achieve optimal line colour density. Strips blocked with 10% FBS produced the weakest colour at both T and C lines, while strips blocked with 1% BSA produced the strongest precipitation line. Thus, 1% BSA was chosen as the best blocking reagent in all subsequent experiments.

Rabies-negative serum and serum with nonrabies antiviral antibodies were used to test for specificity. Only rabies-positive serum produced a red line at the T line, while neither rabies-negative serum nor any other antibody-positive serum elicited a visible line at the T line (Fig. 2a). These results indicate high specificity for rabies. For a sensitivity assessment, positive dog serum was diluted to 1·5, 1·0, 0·5 and 0·1 IU ml^{-1} and tested by the strip. The colour density was found to correlate well with the antibody titre in serum (Fig. 2b). The T line coloration was clearly distinguishable up to 0·5 IU ml^{-1}, while the line produced by the 0·1 IU ml^{-1} sample was difficult to discriminate against the background. These results set the detection limit of the strips to 0·5 IU ml^{-1}.

The stability of the strips was evaluated by testing the strips postfabrication, on the same day as fabrication and then at 2-, 4-, 8- and 10-week intervals during which the strips were stored at 4°C. The red lines showed no significant loss at 8 weeks, but slight intensity loss was observed

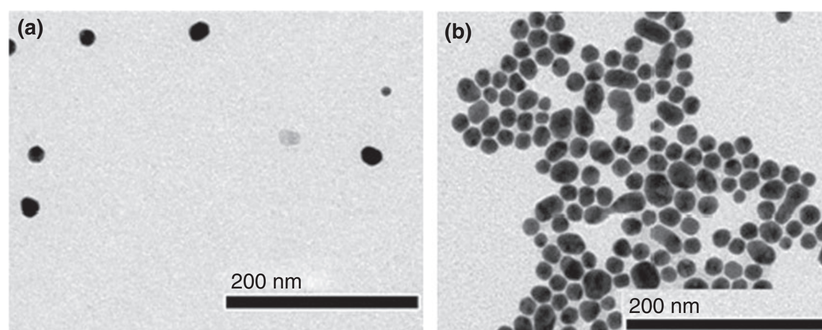

Figure 1 (a) TEM images of colloidal gold and (b) colloidal gold–SPA-conjugated particles. Scale bars indicate 200 nm.

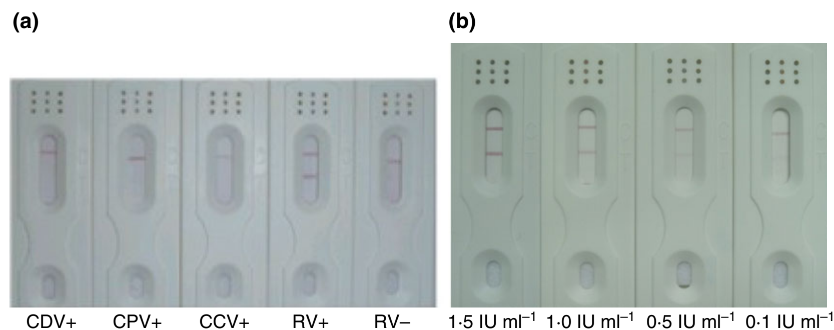

Figure 2 Evaluation of the (a) specificity and (b) sensitivity of the strip.

at 10 weeks, revealing strip stability for at least 8 weeks, provided they were stored at 4°C.

Empirical detection and comparison of clinical samples

The results of 165 clinical samples assayed using ELISA and the immunochromatographic test strip (ICTS) are displayed in Table 1. Using the results of an ELISA test on the same samples as those used in the ICTS test, we calculated the specificity and sensitivity of the ICTS measurements to be 93·1 and 92·2%, respectively.

The predominant application of serological tests for rabies is to determine the response to vaccination. Serological testing is a convenient way for assessing the presence of the protective antibody following vaccination and very useful for epidemiological studies. It is critical to vaccinate rabies, particularly given the absence of any obvious clinical symptoms in dogs with latent-phase rabies. Determination of vaccination efficiency relies heavily on laboratory testing of the antibody level in blood and is necessary for the prevention and control of the disease (Wacharapluesadee *et al.* 2012).

Virus neutralization test (VN) is recommended to specifically assess antibody protection (Servat *et al.* 2007; Mani and Madhusudana 2013). According to the World Health Organization (WHO) guidelines, an antibody titre of 0·5 IU ml^{-1} is the minimum measurable antibody titre considered to represent a level of immunity in humans capable of rendering protection against rabies infection. The same measurement is used in dogs and cats to confirm a satisfactory response to vaccination. The titre of rabies-neutralizing antibody is not the only parameter of immune response but is the most reliable indicator of immune protection (Liu *et al.* 2012). Our immunochromatographic test strip offers detection at a titre of 0·5 IU ml^{-1} in a time-efficient manner compared with other techniques, thus making it suitable for on-site diagnosis.

We have developed a one-step rapid immunochromatographic test strip that only takes 15 min to determine the presence of the rabies antibody at concentrations as low as 0·5 IU ml^{-1} in dog serum. The method is rapid, very sensitive and does not require special equipment for detection of the results as they can be read off the strip by the naked eye. The strip offers great promise for rapid and simple analysis of the rabies antibody and may be used as a complementary tool to strengthen epidemiological surveillance programmes, particularly in rabies-endemic developing countries.

Table 1 ICTS specificity and sensitivity measurements

	ELISA		
	Positive	Negative	Total
ICTS			
Positive	71	6	77
Negative	7	81	88
Total	78	87	165

The specificity measured is as follows: true negative/(true negative + false positive) = (81)/(81 + 6) = 93·1%; the sensitivity is as follows: true positive/(true positive + false negative) = 71/(71 + 7) = 92·2%.

Note that ELISA result for each corresponding sample used on the ICTS was used to judge the 'correctness' of the result obtained with ICTS. The serum samples were tested in triplicate.

Materials and methods

Apparatus and reagents

Images were captured using a JEOL-1230 transmission electron microscope (TEM) (Japan). The UV-visible absorption spectra of colloidal gold, colloidal gold–SPA and ELISA were measured using a DU800 UV-visible spectrophotometer (Beckman Coulter, Brea, CA, USA). Staphylococcal protein A (SPA) and anti-SPA polyclonal antibody were purchased from Sigma. Glass fibre membranes, nitrocellulose membranes and cellulose absorbent pads were Millipore products (Billerica, MA). The presence of rabies antibody in canine serum was confirmed by a fluorescent antibody virus neutralization test (FAVN) and pET-RV-P recombinant plasmid stored in our laboratory.

Synthesis and characterization of colloidal gold–SPA conjugate

The 20-nm colloidal gold particle solution was prepared according to previous studies, with minor modifications (Mirkin *et al.* 1996; Ray *et al.* 2011). Fabrication was carried out by the citric acid-based reduction of $HAuCl_4$. The final concentration of the gold colloidal solution was 1·5 nmol l^{-1} (based on the molar absorption coefficient) (Georganopoulou *et al.* 2005).

The colloidal gold–SPA conjugate was synthesized according to our previous study (Fan *et al.* 2010a,b). Briefly, 100 μl of SPA (1 mg ml^{-1}) was added to 10 ml of dispersed colloidal gold solution (pH 5·3) and stirred for 30 min, followed by incubation at 37°C for 15 min. Next, 3% PEG-20000 was used to block the non-specific binding site. The colloidal gold was collected by centrifugation at 12000 rev min $^{-1}$ for 15 min at 4°C. The precipitant was resuspended in 50 mmol l^{-1} PBS containing 1% BSA, 0·05% PEG-20000, 2·5% sucrose and 0·5% Tween-20, incubated at 37°C for 30 min, and then unconjugated SPA was removed by centrifugation. The pellet was resuspended in 1 ml of the above resuspension buffer and stored at 4°C for use. The morphology and dispersity of colloidal gold and colloidal gold–SPA were characterized by TEM.

Assembly of the strip and the principle underlying detection

The components and the principle workflow of the strip are depicted in Figure 3. A typical strip is composed of an absorbent pad, a nitrocellulose membrane, a conjugate pad and a sample pad. The conjugate pad composed of a glass–fibre membrane is treated with a colloidal gold–SPA complex solution. The nitrocellulose membrane is incubated with recombinantly expressed rabies virus phosphoprotein (RV-P) at the T line and anti-SPA antibody at the C line (Fig. 3a). An XYZ3050 dispense workstation (BioDot, Irvine, CA, USA) is used to disperse the coating protein at a rate of 1 μl cm^{-1}. The protein-coated nitrocellulose membrane is then dried at 37°C. All these components are then adhered onto a backing plate in the appropriate order. The plate is then cut into 4 × 60 mm strips using a cutter machine, and the assembled strips are packaged in plastic cassette housing and stored at 4°C.

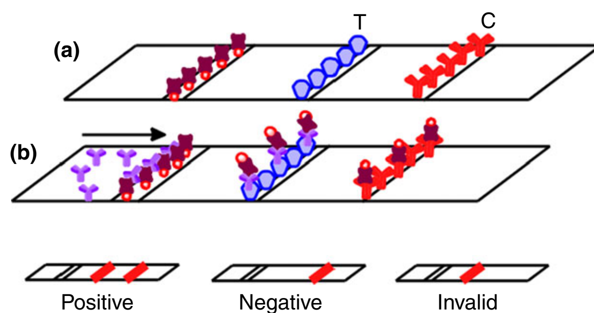

Figure 3 Schematic illustration of the components (a) and workflow (b) on the immunochromatographic test strip. (●) anti-P; (●) colloidal gold; (●) SPA; (●) RV-P and (●) anti-SPA.

Upon testing, the canine serum is dropped on the sample pad where the liquid flows towards the test (T) and control (C) lines by capillary action. When the liquid reaches the conjugate pad, the antibody is captured by colloidal gold particles. The captured antibody complex interacts with the rabies phosphoprotein coated on the T line, turning it red. The anti-SPA antibody at the control line then captures any unconjugated colloidal gold–SPA complexes (Fig. 3b). If the rabies antibody is present, both the T and C lines undergo colour change; otherwise, only the control line becomes visible. The appearance of the T line indicates an invalid result.

Optimization and evaluation of the strip

Optimization of RV-P concentration was performed by observing the colour density at the T line at three different concentrations of RV-P (2·4, 1·6, 1·2 mg ml^{-1}). To reduce non-specific adsorption, 1% bovine serum albumin (BSA), 10% foetal bovine serum (FBS) and 3% gelatin were separately tested as blocking reagents on the nitrocellulose membrane postincubation. The efficacy of each of these reagents was also evaluated based on colour density at the two lines on the strip.

The specificity of the strips was demonstrated by testing sera from dogs vaccinated with nonrabies pathogens (canine distempervirus, CDV; canine parvovirus, CPV; and canine coronavirus, CCV) and naive dogs that tested negative for rabies antibody. To test the sensitivity, the rabies-positive dog serum was diluted to 1·5, 1·0, 0·5 and 0·1 IU ml^{-1} and tested using the strip. The stability of the strips was evaluated by testing the strips on the same day fabricated and then at 2-, 4- and 8-week intervals postfabrication, during which they were stored at 4°C.

To test the efficacy of the strips, a total of 165 canine serum samples (78 vaccinated and 87 unvaccinated dogs) were examined.

Detection by ELISA

All serum samples were also examined by ELISA. Immuno-microplates were coated with RV-P overnight at 4°C. The plates were blocked at 37°C for 1 h with 1% BSA, followed by incubation for 1 h with dog sera diluted

1:200. Next, protein A-HRP diluted 1 : 3000 was added to the wells and incubated for 50 min. TMB indicator containing H_2O_2 was added for 15 min to generate colour. Finally, the reaction was terminated by adding 2M H_2SO_4, and the signal was measured with a spectrometer at 450 nm. At each interval, the plates were washed 3 times with PBS containing 0·01% Tween 20.

Acknowledgements

The authors would like to thank the National Science and technology support program of China (2013BAD12B05), Fundamental Research Funds for Central Commonweal Institutes (2014ywf-yb-6,14009), National Natural Science Foundation of China (NO. 31172342) and the National High Technology Research and Development Program of China (863) (NO. 2008AA10Z411) for providing support.

Conflict of interest

We declare that there is no conflict of interest.

References

De Benedictis, P., Mancin, M., Cattoli, G., Capua, I. and Terrregino, C. (2012) Serological methods used for rabies post vaccination surveys: an analysis. *Vaccine* **38**, 56–59.

Duy, J., Connell, L.B., Eck, W., Collins, S.D. and Smith, R.L. (2010) Preparation of surfactant-stabilized gold nanoparticle-peptide nucleic acid conjugates. *J Nanopart Res* **12**, 2363–2369.

Fan, X.J., Li, G., Qiu, W.Y. and Zhao, G. (2010a) Develoment of gold immunochromatograhic assay on detection of rabies virus IgG antibodies in dogs and cats. *Chin J Zoonoses*, **26**, 753–757. In Chinese.

Fan, X.J., Zeng, N. and Li, G. (2010b) Development of SPA-ELISA for detection of the antibodies against rabies virus in dogs and cats. *Chin Vet Sci*, **40**, 616–621. In Chinese.

Georganopoulou, D.G., Chang, L., Nam, J.M., Thaxton, C.S., Mufson, E.J., Klein, W.L. and Mirkin, C.A. (2005) Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease. *Proc Natl Acad Sci USA* **7**, 2273–2276.

Jackson, A.C. (2013) Current and future approaches to the therapy of human rabies. *Antiviral Res* **99**, 61–67.

Kasempimolporn, S., Saengseesom, W., Huadsakul, S., Boonchang, S. and Sitprija, V. (2011) Evaluation of a rapid immunochromatographic test strip for detection of Rabies virus in dog saliva samples. *J Vet Diagn Invest* **6**, 1197–1201.

Li, X., Li, G., Teng, Q., Yu, L., Wu, X. and Li, Z. (2012) Development of a blocking ELISA for detection of serum neutralizing antibodies against newly emerged duck Tembusu virus. *PLoS ONE* **12**, e53026.

Liu, Y., Zhang, S., Zhang, F. and Hu, R. (2012) A semi-quantitative serological method to assess the potency of inactivated rabies vaccine for veterinary use. *Virol Sin* **4**, 259–264.

Mani, R.S. and Madhusudana, S.N. (2013) Laboratory diagnosis of human rabies: recent advances. *Sci World J* doi: 10.1155/2013/569712.

Mirkin, C.A., Letsinger, R.L., Mucic, R.C. and Storhoff, J.J. (1996) A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature* **6592**, 607–609.

Ray, D., Aswal, V.K. and Kohlbrecher, J. (2011) Synthesis and characterization of high concentration block copolymer-mediated gold nanoparticles. *Langmuir* **7**, 4048–4056.

Schupbach, A., Zhang, L., Thomas, B., Ellerbroek, J. and Leone, D. (2012) Fatal human rabies infection confirmed with skin biopsy specimen. *J Am Acad Dermatol* **2**, 321–324.

Servat, A. and Cliquet, F. (2006) Collaborative study to evaluate a new ELISA test to monitor the effectiveness of rabies vaccination in domestic carnivores. *Virus Res* **2**, 17–27.

Servat, A., Feyssaguet, M., Blanchard, I., Morize, J.L., Schereffer, J.L., Boue, F. and Cliquet, F. (2007) A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. *J Immunol Methods* **1**, 1–10.

Wacharapluesadee, S., Tepsumethanon, V., Supavonwong, P., Kaewpom, T., Intarut, N. and Hemachudha, T. (2012) Detection of rabies viral RNA by TaqMan real-time RT-PCR using non-neural specimens from dogs infected with rabies virus. *J Virol Methods* **1**, 109–120.

Wang, H., Feng, N., Yang, S., Wang, C., Wang, T., Gao, Y., Su, J., Zheng, X. *et al.* (2010) A rapid immunochromatographic test strip for detecting rabies virus antibody. *J Virol Methods* **2**, 80–85.

Welch, R.J., Anderson, B.L. and Litwin, C.M. (2009) An evaluation of two commercially available ELISAs and one in-house reference laboratory ELISA for the determination of human anti-rabies virus antibodies. *J Med Microbiol* **6**, 806–810.

Whitfield, S.G., Fekadu, M., Shaddock, J.H., Niezgoda, M., Warner, C.K. and Messenger, S.L. (2001) A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *J Virol Methods* **2**, 145–151.

Wunner, W.H. and Briggs, D.J. (2010) Rabies in the 21 century. *PLoS Negl Trop Dis* **3**, 591.