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Evaluation of monoclonal antibody–based direct, rapid immunohistochemical test for rabies diagnosis

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ABSTRACT

Rabies is a major public health problem in developing countries in Asia and Africa. Although a number of laboratory diagnoses can be used for rabies control, the WHO and OIE recommended gold standard for rabies diagnosis is the direct fluorescent antibody test (FAT). However, FAT is not widely used in developing countries because of deficient financial sources to procure fluorescent microscope. Recently the direct rapid immunohistochemical test (dRIT) has been developed and has a worldwide promising application, particularly in developing countries, since its result can be read by inexpensive light microscopy, in addition to be consistent with that of FAT. However, no commercial conjugated antibody is available to meet the laboratory demand. We describe here the production of a monoclonal antibody (MAb) against rabies virus (RABV) N protein and its use as a biotinylated conjugate in a dRIT. Tested against a batch of 107 brain specimens representing a wide phylogenetic diversity of RABV collected from different animal species with multiple geographical origins in China, results showed that the dRIT had 100% specificity (95% CI 0.93–1.00) and 96.49% sensitivity (95% CI 0.88–1.00) as compared with the gold standard FAT. It therefore provides a simple, economical alternative to FAT, particularly for use in rabies diagnosis in developing countries.

1. Introduction

Rabies is a fatal zoonotic disease of humans and other animals, causing severe disfunction of the central nervous system (Hemachudha et al., 2002). The 99% human rabies cases occur in developing countries, mainly in Asia and Africa (Knobel et al., 2005). Nearly all the human rabies cases are caused by rabies viruses (RABVs) (WHO, 2004). In order to eliminate dog-mediated human rabies by 2030 as proposed by WHO, animal rabies surveillance, especially in less developed countries and regions should be strengthened (Tan et al. 2017). Validated diagnostic tests that confirm the presence of variant RABVs have been the foundation of rabies control strategies (Duong et al., 2016; Mani and Madhusudana, 2013). The WHO and OIE recommend the direct fluorescent antibody test (FAT) as the gold standard of rabies diagnosis, This test allows the visualization of RABV antigens by fluorescent microscopy (Dean et al., 1996). However, fluorescent microscopes are expensive and not widely used in resource-limited developing countries, and therefore opportunities to conduct animal rabies surveillance and diagnosis are severely constrained. A direct rapid immunohistochemical test (dRIT) was developed to detect RABVs using an immunoperoxidase technique by the Centers for Disease Control and Prevention (CDC) of USA. This method uses highly concentrated and purified biotinylated MAbs against RABV and shows high sensitivity and specificity when testing good quality brain samples as compared with the gold standard FAT (Lembo et al., 2006). Moreover, an important advantage of the dRIT is the use of a light microscope rather than an expensive fluorescence one, and the test is therefore within the reach of laboratories in developing countries. Unfortunately, the necessary biotinylated MAb conjugates have not been available commercially to meet the laboratory demand for rabies diagnosis (Duong et al., 2016). Here we report the development of a biotinylated MAb conjugate against RABV N protein, and have established use of it

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in a dRIT. The high consistency with FAT so obtained supports its application as an alternative to FAT in rabies diagnostic laboratories.

2. Materials and methods

2.1. Cell lines and virus

Mouse SP2/0 myeloma cells (ATCC CRL-1581) were maintained in RPMI-1640 medium (Gibco BRL Life Technologies, Grand Island, NY) and supplemented with 20% fetal bovine serum (FBS; Gibco), 0.2 mM L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin, Sigma-Aldrich, St. Louis, USA) at 37 °C, in a 5% CO_2 atmosphere. BHK-21 cells (ATCC CCL-10) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Ham F12 (DMEM F12; Gibco) with 10% FBS and antibiotics at 37 °C, in a 5% CO_2 atmosphere. Avirulent RABV strain SRV9 was grown in BHK-21 cells to reach a titer of 10^{-7} $TCID_{50}$/ml after propagation in DMEM containing 5% FBS (Hou et al., 1995), then was inactivated by heating at 56 °C for 30 min prior to use.

2.2. Production of monoclonal antibodies

Two female BALB/c mice of age group 4–6 weeks (16–20 g) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. were injected with 0.1 mL inactivated SRV9 strain in an equal volume of Complete Freund's Adjuvant (Sigma–Aldrich) at day 0 and boosted twice in Incomplete Freund's Adjuvant (Sigma–Aldrich) at days 15 and 22 (Harlow and Lane, 1988). Tail bleeds of mice were collected and tested for antibody titers against the virus by ELISA 7–10 days after each injection. ELISA was established by coated with inactivated SRV9 in 96-well plates, and then incubated with different dilutions of the mouse sera. The mouse IgG Fc-specific HRP-conjugated goat-anti-mouse (Pierce, IL, USA) was used as secondary antibodies. When the highest endpoint dilution of antibody titers ≥1:50,000, the mice were euthanized and their spleens were collected aseptically. Cultures of splenocytes were then fused with SP2/0 myeloma cells following the procedure manual of the ClonaCell-HY Hybridoma Cloning Kit (StemCell Technologies, Vancouver, Canada). Culture supernatants from individual hybridoma clones were then screened by ELISA. The clones demonstrated reactivity to SRV9 (OD > 0.5) and were further screened in an immunofluorescence assay. BHK-21 cells were infected with SRV9 at 10^4 $TCID_{50}$ virus/well for immunofluorescence assay. After 48 h infection, cells were fixed with 80% acetone and reacted with supernatants collected from hybridoma cultures. Goat anti-mouse IgG labeled with Alexa Fluor 555 (Invitrogen, Carlsbad, CA, USA) was used as the secondary antibody. The selected positive antibodies were purified by a protein G column to test their reactivity with SRV9.

2.3. Identification of MAbs

To identify MAbs recognizing RABVs, western blotting was performed after running 200 ng SRV9 on SDS-PAGE. SRV9 was purified by sucrose density gradient centrifugation method in advance. After transfer of the gel to a PVDF membrane (Bio-Rad, CA, USA), RABV proteins on the membrane were probed with each of the clones (1 μg/ml), followed by goat anti-mouse antibodies conjugated with peroxidase (1:2000) (Promega, Madison, WI, USA). Protein bands were visualized in a Dual color infrared laser imaging system (Odyssey, USA).

2.4. Biotinylation of MAb

MAb selected as above was dialyzed against PBS overnight at 4 °C and then biotinylated using an EZ-Link Sulfo-NHS Biotinylation Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, the biotinylation process was performed by mixing 2 mg clarified MAb preparation with 30 μL reconstituted Sulfo-NHS biotin compound (10 mM). The reaction mixture was incubated at room temperature for 1 h and then desalted by passage through a dextran desalting column (Thermo Scientific, USA).

2.5. Establishment of dRIT

The dRIT diagnostic assay was developed according to a method of Lembo et al., 2006. Glass slides with brain tissue impressions were fixed in 10% buffered formalin and then immersed in 3% hydrogen peroxide for 10 min. To optimize the concentration of reagents, different dilutions of the above biotinylated MAb and commercial peroxidase-labeled streptavidin at 0.5 mg/ml (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) were tested using 10 weakly FAT-positive brain tissue smears. A stock AEC solution was prepared by dissolving 20 mg 3-amino-9-ethylcarbazole (Sigma, Germany) in 4 mL *N*, *N*-dimethylformamide (Sigma, Germany). The slides were incubated with a working dilution of AEC (1 mL AEC stock solution in 14 mL 0.1 mol/L acetate buffer and 0.15 mL 3% hydrogen peroxide) and then counterstained with Gill's hematoxylin (Solarbio, China). Finally, the impression smears were mounted with coverslips using a water-soluble mounting medium (BioMeda Corp., Foster City, CA, USA) and examined by light microscopy.

2.6. Evaluation of dRIT

To evaluate whether the selected MAb was suitable for dRIT, 107 animal brain tissue samples were assayed. The samples had been collected between 2005–2016 and tested initially by FAT with FITC Anti-rabies monoclonal globulin (Fujerebio Diagnostic Inc., Malvern, PA, USA) (Dean et al., 1996). Of the total, 57 were RABV positive and other 50 were negative (Table 1). Information of the animal samples (species, sex, clinical signs, domestic or feral, collection sites and date) were recorded. Sequencing and phylogenetic analysis of the N gene of RABV-positive samples showed that they contained strains of Asian 1, Asian 2, Arctic-related and Cosmopolitan clades (Feng et al., 2016). All samples were blind tested by two separate persons. For any sample that gave a contradictory result with FAT, the dRIT was repeated a further three times. Mouse brain tissue samples experimentally infected by CVS-11 and stored in a 50% glycerol solution in 0.01 M phosphate-buffered saline were used to test the shelf life of the biotinylated antibody.

2.7. Data analysis

Normalized data were used for statistical analysis with SAS 9.1. The kappa test and McNemar chi-square tests were used for statistical comparison of the diagnostic tests (SAS command proc freq; table/agree). The confidence interval was calculated by assuming a binomial distribution.

Table 1
Animal brain tissue species and their diagnostic results based on FAT.

Species	Total Number	Positive	Negative
dog	75	35	40
sheep	7	5	2
cattle	8	7	1
camel	5	4	1
donkey	1	1	0
wild fox	2	2	0
mouse	9	3[a]	6
total	**107**	**57**	**50**

[a] Experimentally infected.

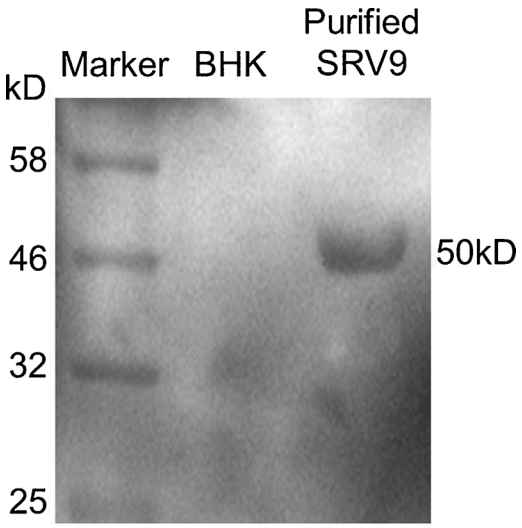

Fig. 1. Western blotting was performed by using MAb 5H7 against SRV9 on SDS-PAGE to identify MAb recognizing linear epitope of the RABV nuclear protein.

3. Results

3.1. Generation of monoclonal antibodies

Splenocytes of one of the SRV9-immunized mice with a serum antibody titer ≥1:50,000 by ELISA assay were fused with SP2/0 cells to generate hybridomas. In total, 32 monoclonal hybridomas were subjected to ELISA screening with 20 clones having OD values > 0.5 and showing fluorescent foci in RABV-infected BHK-21 cell monolayers. Among these positive clones, 7 hybridomas having the highest endpoint dilution (> 1:50,000) in ELISA were selected as candidates for MAb production.

3.2. Western blotting analysis of anti-RABV monoclonal antibodies

Among the positive hybridomas, only MAb from hybridoma 5H7 was found to recognize N protein of RABV in Western blotting, while other hybridomas did not recognize any protein of RABV (Fig. 1). Therefore MAb 5H7 was used to prepare the conjugate.

3.3. Establishment of the dRIT

The lowest concentrations of biotinylated MAb 5H7 (5 μg/ml) and peroxidase-labelled streptavidin (2.5 μg/ml) that detected positives were determined by testing 10 weak FAT-positive brain tissue samples against a range of dilutions. Results were considered positive based on the presence of magenta inclusions visible on a blue neuronal background (Fig. 2A), while negative results were taken as showing only the blue neuronal background (Fig. 2B).

3.4. Evaluation of the dRIT

To validate the use of biotinylated MAb 5H7 conjugate, 107 animal brain tissue samples were tested, 55 of them being found dRIT positive, with the remaining 52 dRIT negative. These samples had previously tested by FAT (Feng et al., 2014; Feng et al., 2016) resulting in 57 positives and 50 negatives. All 50 FAT negative samples were also negative by dRIT, showing 100% specificity (95% CI 0.93–1.00) (Table 2), while 55 of the 57 FAT positive samples were positive also by dRIT, showing 96.25% agreement (95% CI 91.12–1.00) and 96.49% sensitivity (95% CI 0.88–1.00). As shown in Fig. 3 the conjugate could detect the RABV from brain tissues of all animal species tested, showing no difference in comparison with FAT. In addition, the conjugate could also detect the virus in CVS-11 infected mouse brain tissues stored in a 50% glycerol solution for at least 8 months, indicating that long-time storage of infected brain tissues in 50% glycerol did not impact the result.

4. Discussion

The dRIT was established as an alternative means to FAT, which was suitable in low income countries (Lembo et al., 2006). But one potential hindrance associated with the widespread application of the dRIT is the lack of commercial supply of the biotinylated MAb or a dRIT kit (Fooks

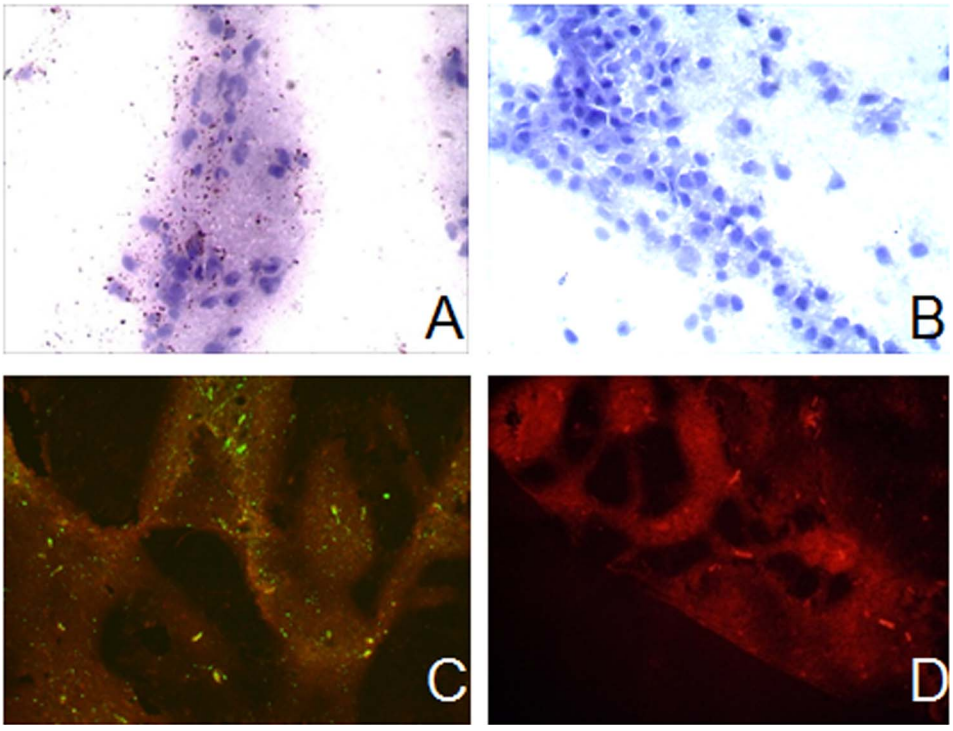

Fig. 2. Impressions of rabid and healthy dog brain tissues tested by FAT and dRIT. Magenta inclusions are visible on the blue neuronal background of the rabid dog brain tissue processed by dRIT (A), while no magenta inclusion is visible on negative brain impression (B). Magnification, 100×. Apple-green fluorescent inclusions are visible on the reddish counterstained neuronal background of the rabid dog brain tissue processed by FAT (C), while no apple-green fluorescent inclusion is visible on negative brain impression (D). Magnification, 200×.

Table 2
Test comparison of 107 animal brain tissues between FAT and dRIT.

n	FAT				dRIT				
	True positive	False positive	True negative	False negative	True positive	False positive	True negative	False negative	Correlation (%)
107	57	0	50	0	55	0	50	2	0.9625 (95% CI 91.12–1.00)

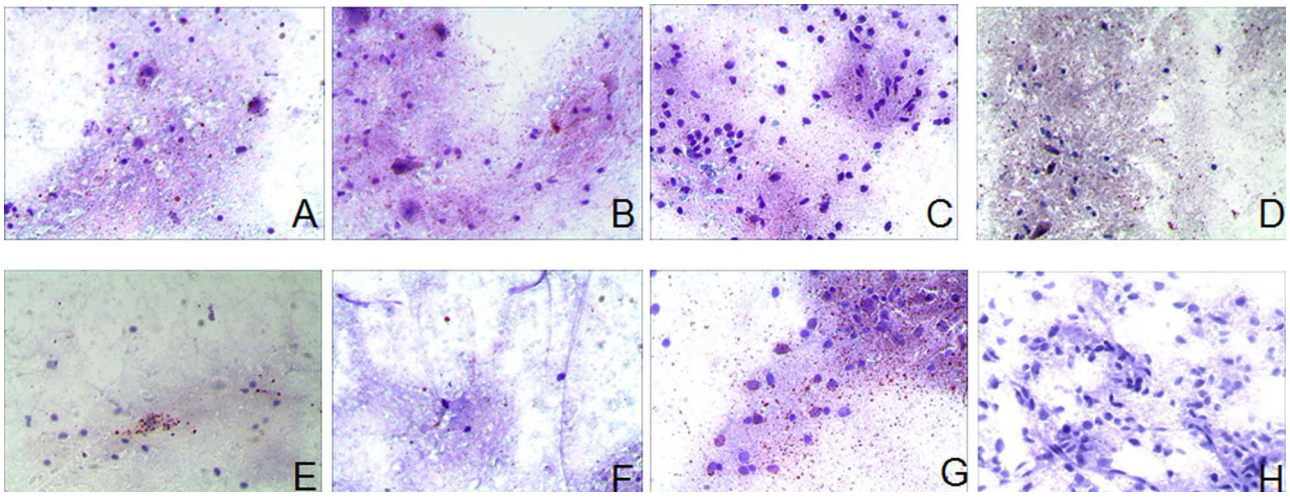

Fig. 3. The detection of brain tissues from rabid animals (A. Dog; B. Sheep; C. Donkey; D. Camel, E. Cattle, F. Wild fox, G. Experimental mouse) and a healthy dog (H) tested by dRIT. Rabies-positive results were based on the presence of magenta inclusions visible on the blue neuronal background in infected brain tissue (arrowed), while no magenta inclusion is visible in negative brain tissue. Magnification, 100×.

et al., 2009). Considering this, MAbs against RABV have been produced in this study. Among 7 MAbs recognized viral antigens in RABV-infected cells by immunofluorescent assay, only MAb 5H7 with linear epitope recognized RABV antigen. These results further confirmed that other 6 MAbs recognized conformational epitopes on RABV. While MAbs to linear epitopes were suitable for diagnosis as described previously (Dietzschold et al., 1987), MAb 5H7 was selected for developed dRIT. Previous studies showed that the dRIT had a sensitivity and specificity equivalent to those of the FAT in testing fresh samples in laboratories in China, India, Afghanistan, Iraq and Africa by using biotinylated MAb provided by the U.S. CDC (Tao et al., 2008; Madhusudana et al., 2012; Saturday et al., 2009; Durr et al., 2008). Coetzer et al. (2014) compared the biotinylated MAbs and polyclonal antibody (PAb) in both dRIT and FAT. The sensitivities of two individual MAbs, MAb cocktail and PAb were 83.50%, 90.78%, 91.75% and 100%. The specificities of two individual MAbs, MAb cocktail and PAb were 97.96%, 100%, 97.96% and 100%. In our study, 107 animal brain tissue samples were tested both by FAT and dRIT. Our MAb 5H7 in dRIT had a diagnostic sensitivity of 96.5% and a diagnostic specificity of 100%. However, the sensitivity of dRIT in this study was slightly lower than FAT in detection of poor quality samples (Durr et al., 2008), with kappa values of between 0.13 and 0.48. This is consistent with our results. In our study, two FAT positive samples tested negative by our dRIT. One was of a dog brain tissue in poor condition (decomposed, dried and hardened). Another was brain tissue of a rabid sheep, which tested positive 2/5 times by FAT, showing weak and sparse fluorescence. This sample was also tested five times by dRIT with every test negative. This result indicated that the sensitivity of our biotinylated MAb 5H7 was slightly lower than that of widely used Fujerebio FITC anti-rabies MAb conjugate. For this reason, fresh brain tissues are recommended for rabies diagnosis if using dRIT.

A glycerol saline solution is a suitable storage media for brain tissue samples in the absence of cold storage (Fooks et al., 2009). Viral antigen could be detected in glycerol preservative samples after considerable time periods (Lembo et al., 2006). In our study, the dRIT could detect the viral antigen of the brain tissues stored for a minimum of 8 months in 50% glycerol in room temperature after washing step (data not shown). The dRIT is therefore suitable for field rabies diagnosis or for resource-limited laboratories with a lack of cold storage (Duong et al., 2016). In addition to detect brain tissue smears, the dRIT established in our study could be used to detect RABV-infected cells as effectively as FAT in tissue culture.

To obtain optimal results in the dRIT, careful attention must be applied to the details. First, the impression smears of brain tissue should not be too thick because the overlapping nerve cells in brain tissue will interfere with the observation. Second, the protocol, and particularly the incubation times of each step, must be followed precisely.

In general, the dRIT, can be used in the field as an alternative to FAT, particularly in response to rabies outbreaks in remote areas, as it does not require refrigeration. The dRIT not only has a significant potential for diagnosing rabies, but also could be used for studies involving growth of RABV in cell cultures. To promote its wide application, the commercialization of the biotinylated MAb will be needed.

Compliance with ethics guidelines

The authors declared that they have no conflict of interest. The use of mice was approved by the Administrative Committee on Animal Welfare of the Institute of Military Veterinary, Academy of Military Medical Sciences, China (Laboratory Animal Care and Use Committee Authorization, permit number JSY-DW-2010-02).

All institutional and national guidelines for the care and use of laboratory animals were followed.

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References

Coetzer, A., Sabeta, C.T., Markotter, W., Rupprecht, C.E., Nel, L.H., 2014. Comparison of biotinylated monoclonal and polyclonal antibodies in an evaluation of a direct rapid immunohistochemical test for the routine diagnosis of rabies in Southern Africa. PLoS Negl. Trop. Dis. 8, e3189.

Dean, D.J., Abelseth, M.K., Atanasiu, P., 1996. The fluorescent antibody test. In: Meslin, F.X., Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies. World Health Organization, Geneva, pp. 88–95.

Dietzschold, B., Wang, H.H., Rupprecht, C.E., Celis, E., Tollis, M., Ertl, H., Heber-Katz, E., Koprowski, H., 1987. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc. Natl. Acad. Sci. U. S. A. 84, 9165–9169.

Duong, V., Tarantola, A., Ong, S., Mey, C., Choeung, R., Ly, S., Bourhy, H., Dussart, P., Buchy, P., 2016. Laboratory diagnostics in dog-mediated rabies: an overview of performance and a proposed strategy for various settings. Int. J. Infect. Dis. 46, 107–114.

Durr, S., Naissengar, S., Mindekem, R., Diguimbye, C., Niezgoda, M., Kuzmin, I., Rupprecht, C.E., Zinsstag, J., 2008. Rabies diagnosis for developing countries. PLoS Negl. Trop. Dis. 2, e206.

Feng, Y., Shi, Y., Yu, M., Xu, W., Gong, W., Tu, Z., Ding, L., He, B., Guo, H., Tu, C., 2016. Livestock rabies outbreaks in Shanxi province, China. Arch. Virol. 161, 2851–2854.

Feng, Y., Wang, W., Guo, J., Alatengheli, Li, Y., Yang, G., Su, N., Zhang, L., Xu, W., Sheng, Z., Ma, L., Gui, J., Dejide, Lin, H., Tu, C., 2014. Disease outbreaks caused by steppe-type rabies viruses in China. Epidemiol. Infect. 5, 1–5.

Fooks, A.R., Johnson, N., Freuling, C.M., Wakeley, P.R., Banyard, A.C., McElhinney, L.M., Marston, D.A., Dastjerdi, A., Wright, E., Weiss, R.A., Muller, T., 2009. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. PLoS Negl. Trop. Dis. 3, 887–892.

Harlow, E., Lane, D., 1988. Antibodies: a Laboratory Manual. Cold Spring Harbor Laboratory, New York, pp. 148–242.

Hemachudha, T., Laothamatas, J., Rupprecht, C.E., 2002. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. Lancet Neurol. 1, 101–109.

Hou, S.K., Yue, J.M., Zhang, M.L., 1995. Development, identification and preclinical evaluation of an oral vaccine against rabies virus. Chin. J. Zoonoses 11, 145–148.

Knobel, D.L., Cleaveland, S., Coleman, P.G., Fevre, E.M., Meltzer, M.I., Miranda, M.E., Shaw, A., Zinsstag, J., Meslin, F.X., 2005. Re-evaluating the burden of rabies in Africa and Asia. Bull. World Health Org. 83, 360–368.

Lembo, T., Niezgoda, M., Velasco-Villa, A., Cleaveland, S., Ernest, E., Rupprecht, C.E., 2006. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg. Infect. Dis. 12, 310–313.

Mani, R.S., Madhusudana, S.N., 2013. Laboratory diagnosis of human rabies: recent advances. Sci. World J. 2013, 569712.

Madhusudana, S.N., Subha, S., Thankappan, U., Ashwin, Y.B., 2012. Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans. Virol. Sin. 27 (5), 299–302.

Saturday, G.A., King, R., Fuhrmann, L., 2009. Validation and operational application of a rapid method for rabies antigen detection. US Army Med. Dep. J. (Jane–March), 42–45.

Tan, J., Wang, R., Ji, S., Su, S., Zhou, J., 2017. One health strategies for rabies control in rural areas of China. Lancet Infect. Dis. 17 (4), 365–367.

Tao, X.Y., Niezgoda, M., Du, J.L., Li, H., Wang, X.G., Huang, Y., Jiao, Y., Cao, L., Tang, Q., Liang, G.D., 2008. The primary application of direct rapid immunohistochemical test to rabies diagnosis in China. Chin. J. Exp. Clin. Virol. 22 (3), 168–170 (in Chinese).

World Health Organization, 2004. Human and Animal Rabies. http://www.who.int/rabies/.