Evaluation of a rapid immunodiagnostic test kit for rabies virus

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Abstract

A rapid immunodiagnostic test kit for rabies virus detection was evaluated using 51 clinical samples and 4 isolates of rabies virus. The quick detection of rabies virus under field conditions may be helpful in determining if post-exposure prophylaxis is needed, thereby avoiding unnecessary treatments, as well as undue economic burden. There are several widely used diagnostic methods for rabies, including fluorescent antibody tests, reverse transcription polymerase chain reaction, and electron microscopy; however, these methods include time-consuming, intricate, and costly procedures. The rapid immunodiagnostic test was able to detect rabies virus in clinical samples, including brain tissue and saliva, in addition to 10^{3.2} 50% lethal dose (LD50)/mL cell-adapted rabies virus. The assay was not cross-reactive with non-rabies virus microbes. When the performance of the rapid immunodiagnostic test was compared to a fluorescent antibody test, the rapid immunodiagnostic test had a sensitivity of 91.7% and specificity of 100% (95.8% CI).

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Keywords: Rapid immunodiagnostic test; Rabies; Sensitivity; Specificity

1. Introduction

Rabies is a viral disease of mammals, and is transmitted by the bite of a rabid animal. Rabies virus (RABV) infects the central nervous system, causing encephalopathy and ultimately death (Baer et al., 1996; Timoney et al., 1988). The virus is a single stranded RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae (Timoney et al., 1988; Wunner et al., 1995).

Since 1907 the occurrence of rabies in South Korea has varied: before 1945, the number of rabies-infected dogs ranged from 200 to 800 heads reported every year; in the early 1980s the number diminished to 3–91 infected heads per year; and from 1985 to 1992 there were no rabies outbreaks reported. In 1993, a rabies-infected dog near the demilitarized zone in Chollwon, Kangwon-do was identified, and since then, 7–61 infected heads have been reported every year. The majority of rabies cases reported each year since 1996 have occurred in domestic animals, including dogs and cattle; raccoons might be the principal host in South Korea (Eun et al., 2003; Lee, 2005; Shin et al., 2004; Rupprecht and Smith, 1994; Winkler and Bogel, 1992).

For rabies diagnosis, the direct fluorescent antibody test most frequently used and the gold-standard test approved by both WHO and OIE for rabies diagnosis (Dean et al., 1996; Meslin et al., 1996; Warner et al., 1997; WHO technical report series; 931). This test is performed on brain tissue from animals suspected of being rabid. The test can only be performed post-mortem. Other methods for diagnosis and research, such as electron microscopy, histologic examination, immunohistochemistry, reverse transcription polymerase chain reaction (RT-PCR), and isolation in cell culture are used for studying rabies virus structure, histopathology, molecular typing, and virulence (Anthony and Werner, 1994; Baer et al., 1996; Dean et al., 1996; Loza-Rubio et al., 2005; Meslin et al., 1996; Warner et al., 1996).
1997). However, these methods have limited utility under field conditions because they are time-consuming and need highly technical skill and equipment. Rapid and accurate laboratory diagnosis of rabies in humans and other animals is essential for timely administration of post-exposure prophylaxis (Fishbein et al., 1991; Helmick, 1983). If the animal is not rabid, prompt diagnosis may save a patient from unnecessary physical and psychological trauma, as well as financial burden (Helmick, 1983).

The objective of this study was to evaluate a rapid immunodiagnostic test (RIDT) kit for rapid detection of RABV in clinical samples under nonlaboratory conditions. The lower limit of detection of the RIDT was conducted by comparing its performance to that of RT-PCR (Al-Yousif et al., 2002; Lee, 2005; Tsuguto et al., 2004; Cross-reactivity of the RIDT was evaluated using several bacteria, including: Escherichia coli, Clostridium perfringens, Salmonella suis; and viruses: canine distemper virus, pseudorabies virus, infectious bovine rhinotracheitis virus, porcine encephalomyocarditis virus, and Japanese encephalitis virus. Sensitivity and specificity of the RIDT were evaluated by comparing its performance to that of an indirect fluorescent antibody test (IFAT) (Al-Yousif et al., 2002; Lee, 2005; Tsuguto et al., 2004; Kuroiwa et al., 2004).

2. Materials and methods

2.1. Clinical samples, field isolates and diagnosis

A total of 51 samples collected during the years between 1999 and 2004 were examined: 44 specimens of brain tissue were collected from dogs (n = 13), cattle (n = 10), and raccoon dogs (n = 21), and 7 salivary specimens were collected from dogs (n = 4) and cattle (n = 3). Detailed information about the animals was not available. Four isolates of rabies virus from raccoon dogs and dogs had been diagnosed to be positive using IFAT method at the National Veterinary Research and Quarantine Services (NVRQS, Anyang, Korea). All clinical samples were kindly donated by the Gangwon Veterinary Service Laboratory (GVSL, Chuncheon, Korea). RT-PCR, IFAT, and the RIDT kit (Animal Genetics, Inc. Suwon, Korea) were used to diagnose rabies from the samples.

2.2. Virus and cells

Cell-adapted rabies virus (ERA vaccine strain used in Korea), supplied by Green Cross Veterinary Products. Co. Ltd. (Yongin, Korea) was propagated in baby hamster kidney epithelial cell line (BHK-21, ATCC CCL-10). The cells were grown with Earle’s minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) at 37°C. Confluent cells were inoculated with the rabies virus (RABV) and cultured for 4 days with Earle’s MEM supplemented with 2 mM L-glutamine and 2% FBS. The cell culture supernatant was harvested after 4 days after infection.

2.3. RIDT

2.3.1. Test principle

The RIDT was based on the principles of immunochromatography using gold conjugated detector antibodies as illustrated in Fig. 1. Antibodies were attached to two different zones on a nitrocellulose membrane (Catalog No. HF 13504; Millipore, USA). Purified monoclonal antibody against nucleoprotein (anti-N Mab) that had been identified as IgG2a subtype by using Mab isotyping kit (Pierce) was attached to the test zone at the concentration of 0.75 μg/strip, and purified goat anti-mouse IgG (Catalog No. ABGAR0500; Arista Biologicals) was attached to the control zone at the concentration of 0.70 μg/strip. The 30 nm colloidal gold conjugated antibodies were dried on the glass fiber (Fig. 1). The test strip was assembled in the order shown in Fig. 1: sample pad, gold pad, nitrocellulose paper, and absorption pad (cellulose paper). All pads overlapped to enable migration of samples along the test strip.

2.3.2. Application of the RIDT

Brain tissue specimens were vigorously homogenized, and 20% suspensions of the homogenate were made in PBS and
Fig. 2. Rapid immunodiagnostic test negative (middle) and positive (low) results.

stored at $-20^\circ C$ until testing by the RIDT kit. The other samples including saliva, cell cultured viruses and bacteria directly applied for the test without treatment. The prepared sample was diluted with a same volume of test buffer (50 mM Tris, 0.1% triton X-100, 0.02% sodium azide, pH 8.0), and 100 $\mu$L of the diluted sample was added to the sample hole of the kit. The final results are available maximum 10 min after application of the sample. A sample is positive for rabies if two lines appear, one in the test zone and one in the control zone. However, the sample is considered negative if only one line appears, in the control zone of the membrane (Fig. 2).

2.5. RT-PCR

Rabies virus genomic RNA was extracted from infected porcine kidney cells and clinical specimens using the Rneasy Mini Kit (Qiagen), according to the manufacturer’s instructions. Extracted RNA was resuspended in 30 $\mu$L of diethyl-pyrocarbonate (DEPC; Sigma Chemical Co., St. Louis, MO)-treated deionized water. The sequences of the primers were 5'-GCAGATAGGATAGGCAAAT-3' (sense) and 5'-AAAGTGAATGAGATTGAACA-3' (antisense) (Lee, 2005). Reverse transcription was carried out with the antisense primer: a mixture of 1 $\mu$L of resuspended RNA in DEPC-treated water plus 20 pmol of antisense primer was denatured by heating at 70 $^\circ C$ for 5 min immediately followed by placing the reaction mixture at $-20^\circ C$. Amplification reactions were conducted in 20 $\mu$L of reaction mixture containing 3 $\mu$L of the RNA-antisense primer mixture, 20 pmol of sense primer, 15 $\mu$L of DEPC-treated water in commercial RT-PreMix (Bioneer, Inc., Korea). The reaction was carried out with a reaction profile of 42 $^\circ C$ for 60 min for cDNA synthesis, followed by 35 cycles of 94 $^\circ C$ for 1 min, 55 $^\circ C$ for 1 min, and 72 $^\circ C$ for 1 min for amplification. In the first cycle of amplification, the denaturation step was prolonged to 5 min at 94 $^\circ C$, and in the last cycle, the extension step was prolonged to 10 min at 72 $^\circ C$. The expected amplicon is 447 bp.

2.6. Detection limit of the RIDT

The concentration of the prepared ERA strain of rabies virus $(10^{3.8} \text{LD}_{50}/0.03 \text{mL})$ was measured by determining the 50% lethal dose $(\text{LD}_{50})$ per 0.03 mL in suckling mice, and two-fold serial dilutions of the virus in PBS were tested with the RIDT kit. In order to establish the lower limit of detection, the LD$_{50}$ was determined for the highest dilution of virus that tested positive by the RIDT. The detection limit of the kit was then compared to that of RT-PCR.

2.7. Cross-reactivity of the RIDT

Several organisms were used to assess cross-reactivity by the RIDT kit. The viruses and bacteria examined included: canine distemper virus $(10^{4.0} \text{EID}_{50}/\text{mL})$, pseudorabies virus $(10^{7.0} \text{TCID}_{50}/\text{mL})$, infectious bovine rhinotracheitis virus $(10^{5.5} \text{TCID}_{50}/\text{mL})$, porcine encephalomyocarditis virus $(10^{7.0} \text{TCID}_{50}/\text{mL})$, Japanese encephalitis virus $(10^{7.1} \text{TCID}_{50}/\text{mL})$, porcine parvovirus (29 HAU), porcine reproductive and respiratory syndrome virus $(10^{5.1} \text{TCID}_{50}/\text{mL})$, bovine viral diarrhea virus $(10^{4.5} \text{TCID}_{50}/\text{mL})$, E. coli $(2 \times 10^{8.0} \text{CFU/mL})$, C. perfringens $(2 \times 10^{8.0} \text{CFU/mL})$ and S. suis $(2 \times 10^{8.0} \text{CFU/mL})$. Each microbe was mixed with same volume of diluent, and 100 $\mu$L of the diluted solution was directly applied to the RIDT kit.
2.8. Sensitivity and specificity of the RIDT

IFAT and the RIDT kit were used to test 40 clinical samples and 4 isolates of rabies virus (SKRRD9903YG, SKRDG0203CW, SKRDG0204HC, and SKRRD0406CC). The sensitivity and specificity of the RIDT was determined using IFAT as the reference method.

3. Results

3.1. Virus detection in clinical samples

RT-PCR detected virus in 24 brain samples and 5 saliva samples, and did not detect virus in 20 brain samples and 2 saliva samples (Table 1). Around 3 min after the diluted samples were placed in the device of the RIDT kit, 18 of 44 brain tissue samples suspended in PBS, 4 of 7 saliva samples, and 4 rabies isolates tested positive for viral antigen, with the development of two colored lines. The intensities of the test lines varied between samples. PBS as a negative control, and 26 of the brain samples and 3 of saliva samples tested negative for antigen, with the appearance of one colored line.

3.2. Detection limit of the RIDT kit and RT-PCR

The infectivity of ERA strain virus from cell culture before dilution was $10^{3.8} \text{LD}_{50}/0.03 \text{mL}$. The detection limit of the RIDT was $10^{1.7} \text{LD}_{50}/0.03 \text{mL}$ (Fig. 3) and that of RT-PCR was $10^{0.5} \text{LD}_{50}/0.03 \text{mL}$ (data not shown). RT-PCR was 16-fold more sensitive than the RIDT; however, PCR method could not detect virus but only viral RNA.

3.3. Cross-reactivity of the RIDT kit

No positive-reactions were seen when several bacteria and viruses causing symptoms similar to rabies were tested by the RIDT. The only virus testing positive was the RABV.

3.4. Sensitivity and specificity of the RIDT kit

Forty-four brain samples and seven isolates of rabies virus were evaluated by comparing RIDT to IFAT (reference method). With IFAT, 20 brain samples tested positive and 24 brain samples were negative. With RIDT, 18 brain samples and 4 isolates were positive and 26 brain samples were negative (Table 2). Two samples were positive by IFAT and negative by the kit. With IFAT as the reference method, the results of 20 brain samples and 4 isolates indicated that the RIDT kit was 91.7% sensitive and 100% specific. These tests had already been performed twice at Animal Genetics in Suwon and GVSL in Chuncheon with same samples.

4. Discussion

Rabies is one of the oldest diseases infecting animals, including humans, and it continues to infect animals in spite of the availability of effective vaccines and antiserum to prevent the disease (Baer et al., 1996; Eun et al., 2003; Helmick, 1983; Shin et al., 2004; Rupprecht and Smith, 1994; Rupprecht et al., 2004; Winkler and Bogel, 1992). The human infection is incidental to the reservoir of the disease in wild and domestic animals. Although some countries (UK, Australia) are rabies free because of vigorous control (Fishbein et al., 1991; Helmick, 1983; Rupprecht et al., 1995), rabies is endemic in wild animals in most parts of the world (Rupprecht and Smith, 1994; Rupprecht et al., 1995; Winkler and Bogel, 1992). The wild animal cycle constitutes the natural reservoir. Wild animals may bite and infect domestic animals (cattle, pigs, dogs, and cats), who in turn may infect humans, and occasionally wild animals infect humans directly. Rabies in dogs, cattle, and raccoon dogs has occurred endemically every year in South Korea (Eun et al., 2003; Lee,
2005; Shin et al., 2004), which results in the possibility of rabies transmission to humans. From 1926 to 1938, the number of humans in Korea bitten by rabid dogs was reported to be 15,929, out of which 282 had developed hydrophobia. Recent reports of human cases of rabies were 1 in 1993, 13 in 1994, and 11 in 1999. Since 2001, 1–2 human cases have been reported every year (http://dis.cdc.go.kr/eng_statistics/statistics.asp). Various routes of transmission have been documented, but the most common mode of rabies virus transmission is through the bite and virus-containing saliva of an infected host. Several factors may affect the outcome of rabies exposure. These include the viral variant, viral dose, infection route, location of exposure, as well as individual host factors such as age and host immune response.

Rapid, accurate diagnostic tools plus an effective vaccine or antiserum are necessary for efficient prevention and treatment (Eun et al., 2003; Fishbein et al., 1991; Winkler and Bogel, 1992). The direct FAT has been widely used in the standard protocol for rabies diagnosis in animals. All rabies laboratories in the United States perform this test (post-mortem) on animals suspected of having rabies (Anthony and Werner, 1994; Dean et al., 1996; Meslin et al., 1996; Warner et al., 1997). Even though the direct FAT is a sensitive and specific procedure used in the routine diagnosis of rabies, uncontrolled small variations in test procedures may have dramatic results on sensitivity and the necessity for all rabies diagnostic laboratories to follow one standard protocol had been proposed (Rudd et al., 2005). Recently, direct rapid immunohistochemical test (RIT) to detect rabies virus antigen has been developed and evaluated in the Rabies Section of the Centers for Disease Control and Prevention. The direct RIT showed a sensitivity and speci-
The RIDT kit detected the sample containing virus from cell culture, and all but one of the positive clinical samples. Using a limited panel of bacteria and viruses, we found that there was no cross-reactivity of the RIDT kit with several bacteria and non-rabies viruses. The detection limit of the RIDT kit was $10^{1.7}$ LD$_{50}$/0.03 mL, which was higher than that of RT-PCR, at $10^{0.5}$ LD$_{50}$/0.03 mL; however, the RIDT detection limit was sufficient for use under field conditions. According to Loza-Rubio et al. (2005), the detection limit of RT-PCR for the N gene of a vampire bat variant of rabies virus is $10^{0.5}$ LD$_{50}$/0.03 mL.

The RIDT kit had 91.7% sensitivity and 100% specificity compared to FAT results when both methods tested four brain samples and four isolates of rabies virus. Two of 20 positive brain samples showed negative results by RIDT. The reason for the false negative samples which were identified as positive by both RT-PCR and FAT, was thought to be that the clinical sample had viral content close to the limit of detection. Four of saliva samples (n = 7) from rabid animals were identified positive by the kit and five of seven saliva samples were identified positive by RT-PCR; the RIDT kit had 80% sensitivity and 100% specificity compared with RT-PCR results. This kit may prove to be useful and effective in diagnosing rabies by testing saliva and the effectiveness of the kit for testing saliva and brain tissues should be confirmed by testing more clinical samples. Until now, direct RIT is the most rapid antigen detection method that allows a diagnosis to be made in 50–60 min, however, RIDT takes only a 5–10 min (Leombo et al., 2006). Because the RIDT kit had a 8.3% false negative rate compared to IFAT and RT-PCR, careful interpretation and additional tests are required in cases where the kit yields negative results and rabies is suspected.

In conclusion, the rapid immunodiagnostic test kit is a useful method for quick detection of rabies virus under various conditions. To prevent rabies effectively, rapid diagnosis in the field is necessary. The advantages of the RIDT kit over other testing protocols include rapidity, simplicity, diversity of sample (including saliva) as opposed to FAT, which can only test brain tissue, and minimal training needs for personnel (Al-Yousif et al., 2002; Lee, 2005; Tsuguto et al., 2004; Kuroiwa et al., 2004). The RIDT kit can be used virtually anywhere, including laboratories, the office, or the field. The authors have found that the test can detect rabies virus and is easy to use.

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References


