Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea

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It is essential to rapidly and precisely diagnose rabies. In this study, we evaluated four diagnostic methods, indirect fluorescent antibody test (FAT), virus isolation (VI), reverse transcriptase polymerase chain reaction (RT-PCR), and rapid immunodiagnostic assay (RIDA), to detect rabies in animal brain homogenates. Out of the 110 animal brain samples tested, 20 (18.2%) were positive for rabies according to the FAT. Compared to the FAT, the sensitivities of VI, RT-PCR, and RIDA were 100, 100, and 95%, respectively. The specificities of VI, RT-PCR and RIDA were found to be 100, 100, and 98.9%, respectively. Rabies viruses circulating in Korea were isolated and propagated in murine neuroblastoma (NG108-15) cells with titers ranging from $10^{1.5}$ to $10^{4.5}$ TCID50/mL. Although the RIDA findings did not completely coincide with results obtained from FAT, VI, and RT-PCR, RIDA appears to be a fast and reliable assay that can be used to analyze brain samples. In summary, the results from our study showed that VI, RT-PCR, and RIDA can be used as supplementary diagnostic tools for detecting rabies viruses in both laboratory and field settings.

Keywords: immunodiagnostic assay, rabies, RT-PCR, virus isolation

Introduction

Rabies is one of the most important viral diseases in animals and can impact human beings. It is difficult to accurately diagnose rabies in animals based on clinical symptoms such as frenzy, extreme tremors, salivation and paresis to distinguish this disease from encephalitic conditions caused by canine distemper virus or acute trauma [17]. Specific histopathologic changes in the central nervous system called Negri bodies have provided a basis for diagnosing rabies for about 100 years [17]. However, this pathological diagnostic method may be no longer suitable for providing guidance for post-exposure prophylaxis (PEP) as new diagnostic methods have been developed [10,17]. In Korea, a number of rabies cases have been recently reported in only two provinces and indirect fluorescent antibody test (FAT) for the detection of rabies has been used to analyze brain samples [4,5,11]. Recent technical advances can also provide more definitive evidence of rabies virus (RABV) infection and detect the presence of the entire virion, RABV proteins, and viral genes in infected tissue [1,2,18]. These techniques include direct visualization by electron microscopy, direct or indirect FAT, virus cultivation in cell lines, mouse inoculation test (MIT), immunohistochemistry, enzyme immunoassay, molecular hybridization, reverse transcript polymerase chain reaction (RT-PCR) including conventional, nested and real-time PCR; and demonstration of specific antibodies in cerebral fluid [1,2,6,13-15,18].

The most confirmatory test among these several diagnostic methods is virus isolation (VI) by inoculating cell cultures with brain homogenates suspected to harbor RABV. Neuroblastoma cells have been used to isolate RABV because these cells can better propagate wild RABV than other cells such as BHK21 or primary porcine kidney cells [12]. An advantage of VI is the availability of cultivated virus for further characterization by antigenic analysis. RT-PCR has been used to diagnose RABV worldwide due to its sensitivity and immense versatility, and can even be useful for examining paraffin-fixed archival and decomposed samples [3]. The nucleoprotein
(N) gene of RABV is targeted for diagnosing and analyzing genetic characteristics and antigenic properties since this gene is highly conserved and associated with encapsidation of genomic RNA [20]. A rapid immunodiagnostic assay (RIDA) kit using specific monoclonal antibody against RABV can also be used for identifying RABV in brain or saliva samples from infected animals [8,9]. The RIDA is rapid and simple, and does not require any special equipment or technical expertise. In this study, we compared the ability of four diagnostic methods (FAT, VI, RT-PCR, and RIDA) to detect RABV circulating in Korea.

Materials and Methods

Collection of samples
One hundred and ten brain samples including Ammon’s horn were collected from 84 cattle, 12 raccoon dogs (Nyctereutes procyonoides koreensis), and 14 dogs between October 2008 and December 2010. The samples were obtained from Gyeonggi-do and Gangwon-do provinces (Korea) where recent rabies cases had been reported according to the National Animal Disease Database (KAHIS, Korea). Approximately 1 g of brain sample was homogenized with sand (Sigma, USA) in 10 mL of alpha-minimum essential medium (α-MEM; Gibco BRL, USA), and was centrifuged at 8,000 × g for 5 min. All homogenate samples were stored at −70°C until used. In addition, the Ammon’s horns samples were frozen until analyzed by the FAT.

Fluorescent antibody test
The FAT was performed according to the procedure described by the Office International des Épizooties (OIE) and World Health Organization (WHO) [10,19]. In brief, frozen thin sections of Ammon’s horn tissue on slides were fixed in cold acetone (−20°C) for 20 min. After three successive wash with phosphate buffer saline (PBS, pH 7.2), the slides were incubated with a monoclonal antibody (JenoBiotech, Korea) against rabies for 45 min and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgA, IgG and IgM (KPL, USA). After rinsing in PBS, the cells were examined by fluorescent microscopy (Nikon, Japan). Titration of wild-type RABV isolates propagated in NG108-15 cells was also done by indirect fluorescent assay (IFA) mentioned above. Viral titers were determined by 50% tissue culture infectious dose per mL (TCID₅₀/mL).

Extraction of viral RNA and RT-PCR
Viral RNA was extracted from brain samples using an RNA extraction kit (Qiagen, Germany) according to the manufacturer’s instructions. The extracted RNA was eluted in 50 μL of RNase- and DNase-free water. RT-PCR was carried out to detect RABV genomic sequences using specific primer sets (RVNDF and RVNDR) that amplify the N gene of RABV. The primer set sequences are listed in Table 1. RT-PCR was performed in a reaction mixture containing 5 μL of denatured RNA, 1 μL of each primer (50 pmol), 5 μL of 5 × buffer (12.5 mM MgCl₂), 1 μL of dNTP mix, 1 μL of an enzyme mix (reverse transcriptase and Taq

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Table 1. Oligonucleotide primers used for RT-PCR to diagnose rabies

| Primer | Nucleotide sequences (5′-3′) | Nucleotide position* | Sense | Rabies gene | Size of amplicon (bp) |
|--------|-----------------------------|----------------------|-------|-------------|----------------------|
| RVNDF  | GRA ATT GGG CTT TGA CTG GA  | 353 ~ 372            | +     | N           | 181                  |
| RVNDR  | AAA GGG GCT GTC TCG AAA AT  | 514 ~ 533            | -     |             |                      |

*The positions of primers were based on the Evelyn-Rokitnicki-Abelseth strain (GenBank accession No. AF406695).
Table 2. Sensitivity and specificity of VI, RT-PCR, and RIDA relative to the FAT for detecting rabies virus in the field brain samples

| FAT | VI | RT-PCR | RIDA |
|-----|----|--------|------|
|     | P  | N      | Total| P  | N      | Total| P  | N      | Total|
|     | 20 | 0      | 20  | 20 | 0      | 20   | 19 | 1      | 20   |
| N   | 0  | 90     | 90  | 0  | 90     | 90   | 0  | 90     | 90   |
| Total| 20 | 90     | 110 | 20 | 90     | 110  | 19 | 91     | 110  |

| Sensitivity (%) | 100 | 100 | 95 |
| Specificity (%)  | 100 | 100 | 98.9 |

VI: virus isolation, RT-PCR: reverse transcriptase polymerase chain reaction, RIDA: rapid immunodiagnostic assay, FAT: fluorescent antibody test, P: positive, N: negative.

RIDA
A commercial RIDA kit was used according to the manufacturer’s instruction (Bionotes, Korea). Briefly, brain samples (about 10% of the brain homogenate materials) were individually prepared as described earlier and a swab supplied with the kit was dipped into the homogenate. The swab was transferred to the enclosed proprietary buffer designed for extraction of RABV. A 100 μL aliquot of the sample was transferred to the sample well. The final results were read 5 min after addition of the brain samples. The appearance of two lines (one was the test line and the other was the control line) was considered a positive result. The formation of one line was considered a negative result.

Calculation of sensitivity and specificity
Sensitivity was calculated with the formula \[ \text{Sensitivity} = \frac{TP}{TP + FN} \times 100 \] where TP was the number of samples with true-positive results as determined by the reference assay and FN was the number of samples with false-negative results. Specificity was defined as \[ \text{Specificity} = \frac{TN}{TN + FP} \times 100 \] where TN was the number of samples with true-negative results and FP was the number of samples with false-positive results.

Results
Comparison of the diagnostic methods was carried out with a total of 110 brain homogenates from 84 cattle, 14 dogs, and 12 raccoon dogs. When the 110 animal brain samples were tested, 20 were positive according to the FAT. These were designated as KRVR0801, KRVC0802, KRVR0803, KRVR0804, KRVR0901, KRVB0902, KRVB0903, KRVB0904, KRVB0905, KRVR0906, KRVB0907, KRVB0908, KRVB0909, KRVB0910, KRVB1001, KRVB1002, KRVC1003, KRVB1004, KRVC1005, and KRVB1006. The other 90 brain samples tested by the FAT were negative. Sensitivities of VI, RT-PCR, and RIDA were found to be 100, 100, and 95%, respectively. Specificities of VI, RT-PCR, and RIDA were found to be 100, 100, and 98.9%, respectively.
Table 3. Titers of Korean rabies virus isolates propagated in NG108-15 cells

| Sample No. | Isolate   | Locality | Species   | Year of isolation | Virus titer (TCID_{50}/mL) |
|------------|-----------|----------|-----------|-------------------|-----------------------------|
| 1          | KRVR0801  | Sokcho   | Raccoon dog | 2008              | 3.5                         |
| 2          | KRVC0802  | Inje     | Dog*       | 2008              | 3.0                         |
| 3          | KRVR0803  | Sokcho   | Raccoon dog | 2008              | 3.0                         |
| 4          | KRVR0804  | Sokcho   | Raccoon dog | 2008              | 2.5                         |
| 5          | KRVR0901  | Goseong  | Raccoon dog | 2009              | 4.5                         |
| 6          | KRVB0902  | Goseong  | Cattle*    | 2009              | 3.5                         |
| 7          | KRVB0903  | Hongcheon| Cattle*    | 2009              | 2.0                         |
| 8          | KRVB0904  | Goseong  | Cattle*    | 2009              | 1.5                         |
| 9          | KRVB0905  | Inje     | Cattle*    | 2009              | 2.5                         |
| 10         | KRVR0906  | Goseong  | Raccoon dog | 2009              | 3.7                         |
| 11         | KRVB0907  | Inje     | Cattle*    | 2009              | 3.0                         |
| 12         | KRVB0908  | Goseong  | Cattle*    | 2009              | 2.5                         |
| 13         | KRVB0909  | Goseong  | Cattle*    | 2009              | 3.7                         |
| 14         | KRVB0910  | Goseong  | Cattle*    | 2009              | 4.0                         |
| 15         | KRVB1001  | Goseong  | Cattle*    | 2010              | 3.5                         |
| 16         | KRVB1002  | Goseong  | Cattle*    | 2010              | 3.5                         |
| 17         | KRVC1003  | Goseong  | Dog*       | 2010              | 2.5                         |
| 18         | KRVB1004  | Yangyang| Cattle*    | 2010              | 2.5                         |
| 19         | KRVC1005  | Goseong  | Dog*       | 2010              | 3.5                         |
| 20         | KRVB1006  | Goseong  | Cattle*    | 2010              | 2.7                         |

*These animals had contact with rabid raccoon dogs.

(Table 2). When testing NG108-15 cells with VI, 20 samples used to inoculate the cells induced rabies-specific fluorescence in the cytoplasm of the infected cells (Fig. 1). The samples that produced positive reactions in the FAT did not result in a distinctive CPE such as detachment in the NG108-15 cells. The wild-type RABV isolates propagated in the cells produced moderate virus titers ranging from $10^{1.5}$ to $10^{4.5}$ TCID_{50}/mL (Table 3).

Conventional RT-PCR using a primer set that amplified the N gene of RABV was able to detect RABV in 20 samples. Various levels of the amplified gene were observed among the samples (Fig. 2). However, RT-PCR could not detect the N gene in 90 samples without any non-specific reactions. Results of the RIDA revealed that 19 samples were positive. Intensity of the test lines in the positive samples was found to vary among different field samples (Fig. 3).

### Discussion

Since 1993, rabies in dogs, cattle, and raccoon dogs has been observed every year in Korea. A total of 417 rabies cases were reported until 2009 [5,7]. The FAT has been recommended by the OIE and WHO, and used for diagnosing rabies worldwide [10,19]. The main advantage
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Fig. 3. Result of the RIDA after applying the samples (ERA strain, KRVB0904, KRVC1003, and KRVB1004 isolates).

An advantage of VI is the availability of isolated virus for further characterization by genetic analysis as well as selection of inactivated vaccine candidates. Several cell lines such as BHK21 and Vero cells have been used to propagate fixed strains, including Evelyn-Rokitnicki-Abelseth and Pasteur vaccine strains, but they are not suitable for isolating wild-type RABV [12]. It has been reported that the NG108-15 cells possessing cell membranes with acetylcholine receptors and neurotransmitter synthetic enzymes are the most susceptible to wild RABV, and cytoplasmic inclusions appear in the infected cells [2,16]. In this study, NG108-15 cells were used to isolate wild-type RABV circulating in Korea from the brain samples. Fluorescence was specifically observed in the cytoplasm of the FAT is that the results of this procedure can be obtained within 3 ∼ 4 h. Therefore, all rabies laboratories, including those in Korea, have routinely performed the FAT on suspected animals. Even though the FAT is most the common rapid and sensitive diagnostic test for rabies, other supplementary diagnostic methods are required when a questionable FAT result is obtained in order to arrive at a define conclusion. Any false negative results may have a catastrophic impact and false positive results can lead to unnecessary PEP. The causes of FAT errors have been traced to inadequate sampling, unsatisfactory conjugate, and lack of experience in reading the slide [2].

In the present study, we analyzed a total of 110 brain homogenates from 84 cattle, 14 dogs, and 12 raccoon dogs from the Gyeonggi-do and Gangwon-do provinces of Korea which were submitted for rabies diagnosis between 2008 and 2010. We compared the results of four different diagnostic methods (FAT, VI, RT-PCR, and RIDA) on these suspected samples.

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In conclusion, our finding demonstrated that it is possible to use these diagnostic methods to help make rapid decisions for
detecting and controlling rabies.

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