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Establishment of multiplex RT-PCR for differentiation between rabies virus with and that without mutation at position 333 of glycoprotein

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ABSTRACT

Rabid raccoon dogs (*Nyctereutes procyonoides koreensis*) have been responsible for animal rabies in South Korea since the 1990s. A recombinant rabies vaccine strain, designated as ERAGS, was constructed for use as a bait vaccine. Therefore, new means of differentiating ERAGS from other rabies virus (RABV) strains will be required in biological manufacturing and diagnostic service centers. In this study, we designed two specific primer sets for differentiation between ERAGS and other RABVs based on mutation in the RABV glycoprotein gene. Polymerase chain reaction analysis of the glycoprotein gene revealed two DNA bands of 383 bp and 583 bp in the ERAGS strain but a single DNA band of 383 bp in the field strains. The detection limits of multiplex reverse transcription polymerase chain reaction (RT-PCR) were 80 and 8 $FAID_{50}$/reaction for the ERAGS and Evelyn-Rokitnicki-Abelseth strains, respectively. No cross-reactions were detected in the non-RABV reference viruses, including canine distemper virus, parvovirus, canine adenovirus type 1 and 2, and parainfluenza virus. The results of multiplex RT-PCR were 100% consistent with those of the fluorescent antibody test. Therefore, one-step multiplex RT-PCR is likely useful for differentiation between RABVs with and those without mutation at position 333 of the RABV glycoprotein gene.

Keywords: Rabies virus; RT-PCR; detection

INTRODUCTION

Based on antigenic properties and the results of phylogenetic analyses, the genus *Lyssavirus* has been subdivided into two phylogroups. Phylogroup 1 contains 10 viruses: Aravan virus, Australian bat lyssavirus, Bokeloh bat lyssavirus, Duvenhage virus, European bat lyssavirus type 1, European bat lyssavirus type 2, Gannoruwa bat lyssavirus, Khujand virus, Irkut virus, and rabies virus (RABV). Phylogroup 2 consists of three viruses: Lagos bat virus, Mokola virus, and Shimoni bat virus [1]. Among the 13 lyssaviruses belonging to phylogroups 1 and 2, RABV causes a highly fatal disease in warm-blooded animals. RABV contains a single-stranded negative RNA genome, which encodes five structural genes designated as nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA polymerase (L) [2]. Among the five structural proteins, the G protein is related to

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pathogenicity, immunogenicity, and neurovirulence [3,4]. The amino acid at position 333 of the G protein (arginine [Arg] or lysine [Lys]) has been shown to be a determinant of virulence in adult mice [5].

Based on pathogenicity and passage in the laboratory, RABVs can also be classified into two categories: street and fixed strains [6]. The fixed strain has been attenuated through a series of passages in experimental animals or cells. The representative RABVs classified as fixed strains are Evelyn-Rokitnicki-Abelseth (ERA), Flurry low egg passage, high egg passage (HEP), and Challenge virus standard (CVS)-11 strains [7,8]. Most nonpathogenic strains, such as Street-Alabama-Gif (SAG2), SAD B19, SPBNGA, and recombinant high egg passage (rHEP), are classified as fixed strains. These strains were constructed after two successive mutations of the Arg at position 333 with monoclonal antibodies against G protein [9] or by replacing the amino acid residue by reverse genetics [5,8]. The street and field strains are RABV isolates obtained from naturally infected animals. Most RABV street isolates have an Arg or Lys at position 333 of the RABV G protein [10]. Consequently, the fixed and street strains differ in viral replication capacity, virulence, and incubation period [11]. In addition, most nonpathogenic strains do not produce any clinical symptoms in adult mice inoculated intracranially.

In South Korea, many RABV strains have been used in the biotechnology industry since the 1980s. The ERA strain introduced from Canada in the 1970s has been used to produce live rabies vaccine [12] and the CVS-11 strain has been used to measure rabies antibodies in fluorescent antibody virus neutralization testing [13]. As rabid raccoon dogs (*Nyctereutes procyonoides koreensis*) were identified as rabies reservoirs, a new oral rabies vaccine candidate designated as the ERAGS strain has been developed and will be used as a bait vaccine in the future [14]. Methods are required to distinguish the ERAGS strain, which is nonpathogenic in 4-week-old mice, from attenuated ERA, CVS-11, and street strains. Diagnostic methods for rabies are divided into four categories: immunochemical identification of RABV antigen (fluorescent antibody test [FAT], immunochemistry, enzyme-linked immunosorbent assay [ELISA], and rapid immunodiagnostic tests [RIDT]), detection of RABV after inoculation (virus isolation, mouse inoculation test), molecular techniques (RT-PCR, PCR ELISA, real-time RT-PCR), and histological identification. However, these methods can only detect the presence of a RABV in samples [13] but cannot differentiate RAVB strains. Therefore, a method for differentiating the ERAGS strain from all other RABV stains without using experimental animals or nucleotide sequence analyses is required. In this study, we established a multiplex RT-PCR method for rapid and differential detection of RABV based on mutation at position 333 of the RABV G protein.

MATERIALS AND METHODS

Cells and viruses

A recombinant RABV designated as ERAGS was constructed for RABV bait vaccine and RABV vaccine strains [14]. The attenuated live vaccine strain ERA, introduced from Canada to South Korea in 1974, has been used for the prevention of rabies in animals [12]. The ERAGS and ERA strains were cultivated in Vero cells (ATCC CCL-1586), and CVS-11 was propagated in BHK21 cells (ATCC CCL-10) in medium containing antibiotics (100 IU/mL penicillin and 10 μg/mL streptomycin), an antifungal agent (0.25 μg/mL amphotericin B), and 10% heat-inactivated fetal bovine serum (Gibco BRL, USA). The ERAGS and ERA strains with a viral

titer of $10^{7.0}$ FAID$_{50}$/mL were used for the sensitivity test, and the ERAGS, ERA, and CVS-11 strains were used as positive controls in multiplex RT-PCR.

FAT

Direct FATs were carried out to detect RABV in 46 brain samples (from 3 dogs, 3 cattle, and 40 raccoon dogs) using the procedure described by the World Organization for Animal Health [13]. The dog and cattle brain samples were rabies samples from 2013, and the raccoon dog brain samples were obtained from Gangwon Wildlife Rescue Center from 2017 to 2018. Briefly, thin frozen sections of animal brains were placed on slides and fixed with cold acetone (−20℃) for 20 min. The fixed tissue sections were cut and incubated with a specific monoclonal antibody (Median Diagnostics, Korea) against RABV for 1 h. After washing with phosphate buffered saline, the slides were stained with fluorescent isothiocyanate-conjugated goat anti-mouse IgG+IgM (Median Diagnostics). Samples showing specific fluorescence in brain cells were deemed to have a positive diagnosis.

Design of primer sets and RNA/DNA extraction

Common and differential primer sets were designed based on the RABV G gene sequence of the ERAGS, ERA strain (GenBank accession no. EF206707), and Korean field RABV isolate KRVB1206 (GenBank accession no. KF709076) (**Fig. 1**). The position of the reverse primer, ERAGS-R, corresponded to amino acid 333, in which Arg has been substituted with glutamine. The location of the reverse primer, RABVcomR, corresponded to the nucleotide sequence of last part of the G gene. Primer sets RABVcomF/R and ERAGS-F/-R were expected to generate products of 383 bp and 583 bp, respectively. The sequences and targeted nucleotide positions of the primers are shown in **Table 1**.

Sensitivity of multiplex RT-PCR

Total RNA was extracted from the RABV strains (ERAGS and ERA) using an RNA extraction kit (Bioneer, Korea) in accordance with the manufacturer's instructions. The extracted RNA was eluted in 50 μL RNase- and DNase-free water and subjected to RT-PCR. Multiplex RT-PCR
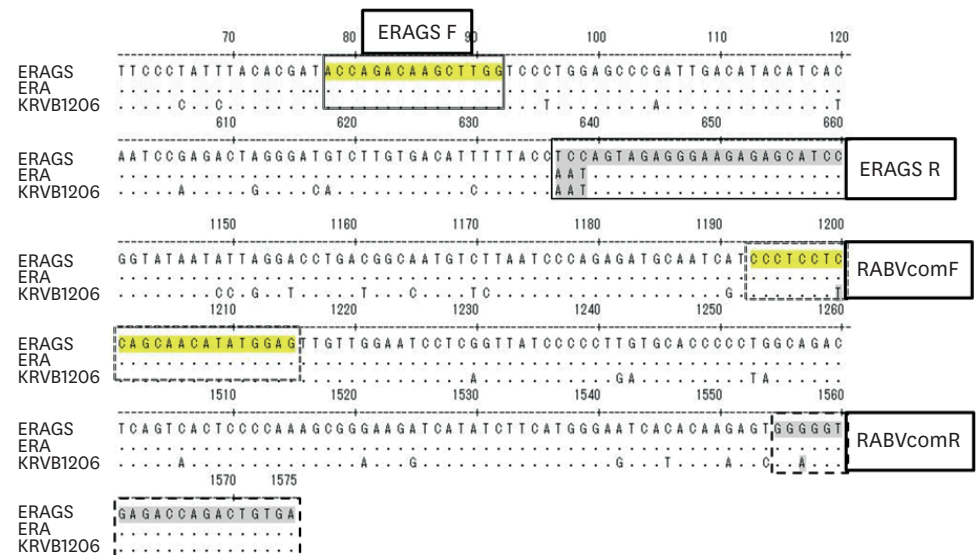

Fig. 1. RABV G gene sites targeted by the primers used for differential RT-PCR. The first three nucleotides (TCC) of the ERAGS reverse primer, corresponding to codon 333 of the G gene, were not identical to those (AAT) of the Evelyn-Rokitnicki-Abelseth strain or KRVB1206 isolate.

Table 1. Two specific primer sets for differentiation between ERAGS and fixed strains by multiplex reverse transcription polymerase chain reaction

Designation	Oligonucleotide	Expected size (bp)	Target gene	Remarks
RABVcomF	CCCTCCTY*CAGCAACATATGGAG	383	G	All RABV
RABVcomR	TCACAGTCTGGTCTCACCR*CC			
ERAGS-F	ACCAGACAAGCTTGGTCCCTGG	583	G	ERAGS only
ERAGS-R	GGA†TGCTCTCTTCCCTCTACTGGA			

*Y: C or T; R: A or G; †GGA: nucleotide sequence of glutamine at position 333 of the G protein.

was performed using OneStep RT-PCR reagent (Qiagen, Germany). The RT-PCR mixtures contained 2 μL denatured RNA, 1 μL each of the four primers (10 pmol), 5 μL 5× buffer (12.5 mM MgCl$_2$), 1 μL dNTP mix, 1 μL enzyme mix (reverse transcriptase and Taq polymerase), and 12 μL distilled water. The PCR profile consisted of cDNA synthesis at 50℃ for 30 min, followed by 40 cycles of 95℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 30 sec, with a final extension at 72℃ for 5 min. The PCR products were electrophoresed on a 2.0% agarose gel for 30 min and visualized using Redsafe™ Nucleic acid staining solution (iNtRON Biotechnology, Korea).

Specificity and application

Multiplex RT-PCR was performed to detect several RABV strains (CVS11, ERA, ERAGS, and field RABV strains). The three strains CVS11, ERA, and ERAGS were used as positive controls. Five canine viruses, canine distemper virus (CDV), canine parainfluenza virus (CPIV), canine adenovirus type 1 (CAV1), canine adenovirus type 2 (CAV2), and canine parvovirus (CPV), were used to test the specificity of the method. Genomic DNAs were extracted from CAV1, CAV2, and CPV and also added to the RT-PCR samples, instead of RNA. Multiplex RT-PCR was carried out under the same conditions as described above. A total of 46 brain samples collected from raccoon dogs, dogs, and cattle between 2013 and 2018 were tested by FAT. The brain samples were subjected to multiplex RT-PCR.

RESULTS

Primer design

The RABV common primer set was designed to detect the vaccine strains, fixed strains, and field RABV isolates, and the ERAGS-F and ERAGS-R primer set exclusively detects the recombinant RABV strain ERAGS by multiplex RT-PCR. The amplicon sizes amplified using the RABV common and ERAGS primer sets were expected to be 383 bp and 583 bp, respectively. As shown in **Fig. 1**, two primer sets were selected with sequences corresponding to the G gene, but two oligonucleotides in the RABVcomFR primer set were replaced with mixed bases, i.e., Y (C or T) and R (A or G). The sequence of the ERAGS reverse primer corresponded to a region of the G gene that is specifically mutated only in the ERAGS strain. It should be noted that the first three nucleotides of the ERAGS reverse primer, corresponding to position 333 of the G protein, were not identical to those for the other RABVs, as the reverse primer did not adhere to the starting site to amplify and then synthesis of cDNA did not start (**Table 1** and **Fig. 1**).

Sensitivity and specificity of multiplex RT-PCR

Serial 10-fold dilutions of the ERAGS and ERA strains were subjected to multiplex RT-PCR amplification. As shown in **Table 2** and **Fig. 2**, the detection limits of multiplex RT-PCR for ERAGS and ERA were 80 and 8 FAID$_{50}$/reaction, respectively. Five canine viruses, CDV, CPIV, CAV1, CAV2, and CPV, were subjected to multiplex RT-PCR using two primer sets to evaluate

Table 2. Infectivity titer equivalent of multiplex RT-PCR for detection of rabies virus

Virus dilution	Infectivity titer equivalent*	Multiplex RT-PCR result	
		ERAGS/ERA (583/383 bp)	ERA (583/383 bp)
10^0	80,000	+/+	−/+
10^{-1}	8,000	+/+	−/+
10^{-2}	800	+/+	−/+
10^{-3}	80	+/+	−/+
10^{-4}	8	−/+	−/+
10^{-5}	0.8	−/−	−/−
10^{-6}	0.08	−/−	−/−
10^{-7}	0.008	−/−	−/−

RT-PCR, reverse transcription polymerase chain reaction; ERA, Evelyn-Rokitnicki-Abelseth.
*$FAID_{50}$/2 µL one-step multiplex RT-PCR reaction. Aliquots of 200 µL of the ERAGS and ERA strains with a viral titer of $10^{7.0}$ $FAID_{50}$/mL were used for RNA extraction.
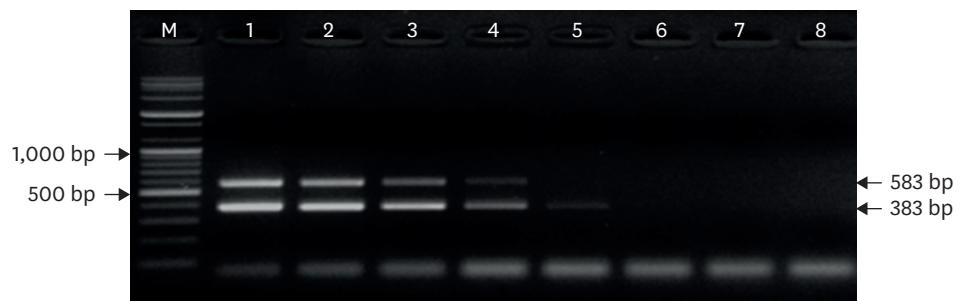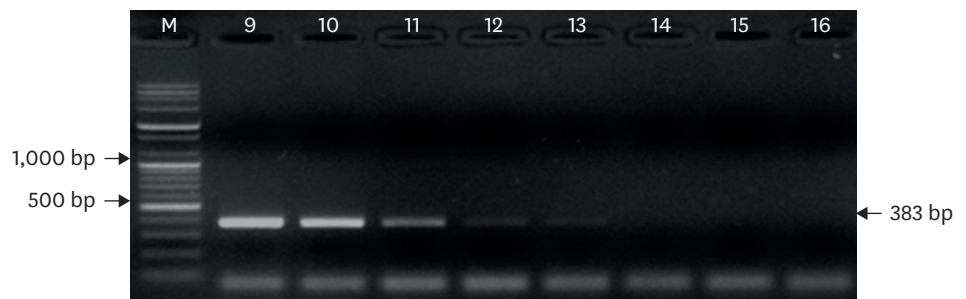
A

B

Fig. 2. Sensitivity of the specific primer sets used for differential detection of ERAGS and ERA strains. M, 100 bp DNA ladder; lanes 1–8, ERAGS strain 10^0–10^{-7} (A). M, 100 bp DNA ladder; lanes 9–16, ERA strain 10^0–10^{-7} (B). ERA, Evelyn-Rokitnicki-Abelseth.

the specificity of the reaction. As shown in **Fig. 3**, amplification of the ERA and CVS11 strains used as positive controls by both primer sets yielded only a product of 383 bp, whereas the ERAGS strain showed two products of 383 bp and 583 bp. No PCR products were obtained from any of the five canine viruses, indicating the specificity of the reaction.

FAT and application of multiplex RT-PCR
FAT is a standard diagnostic method for rabies, and we applied it to 46 brain samples obtained from 3 cattle, 3 dogs, and 40 raccoon dogs. Six samples from the cattle and dogs were positive, whereas the other 40 brain samples from raccoon dogs tested negative by FAT. The 46 samples were also subjected to multiplex RT-PCR. As shown in **Fig. 4**, the FAT-positive samples and positive controls had positive results and the FAT-negative brain samples negative results by the multiplex RT-PCR.
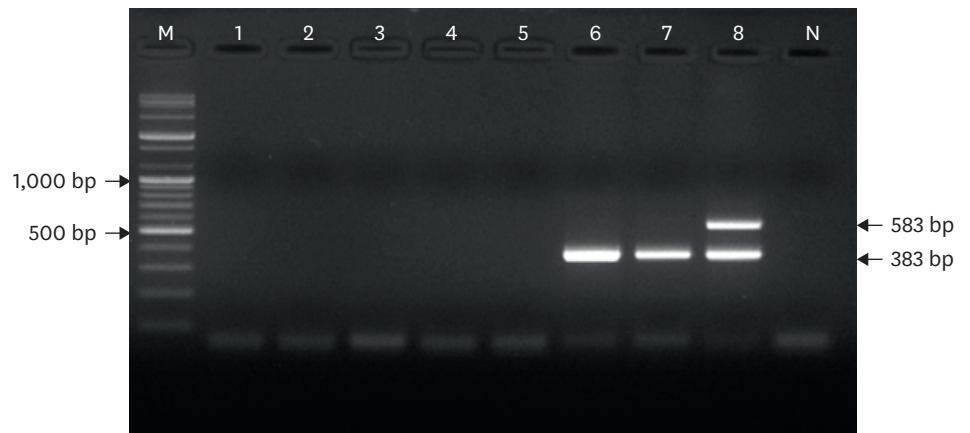
Fig. 3. Specificity of differential RABV RT-PCR using two primer sets. RT-PCR detected only RABVs, and no positive signals were obtained for the five canine viral pathogens. M, 100 bp DNA ladder; lane 1, canine distemper virus; lane 2, canine parainfluenza virus; lane 3, canine adenovirus type 1; lane 4, canine adenovirus type 2; lane 5, canine parvovirus; lane 6, CVS11; lane 7, Evelyn-Rokitnicki-Abelseth strain; lane 8, ERAGS strain; lane N, negative control (water only).
RT-PCR, reverse transcription polymerase chain reaction.
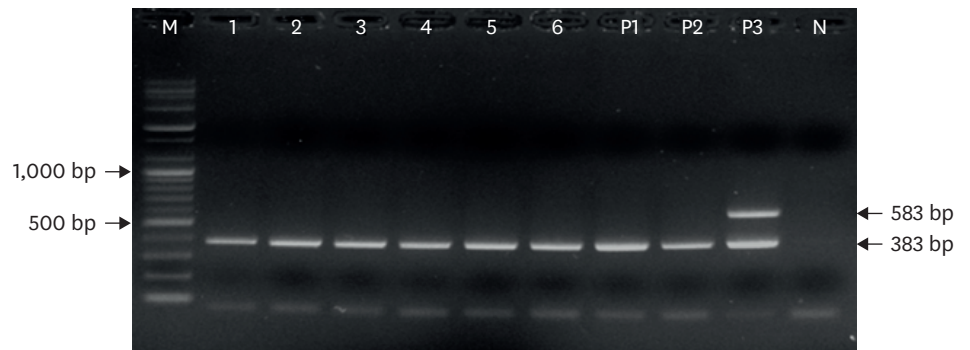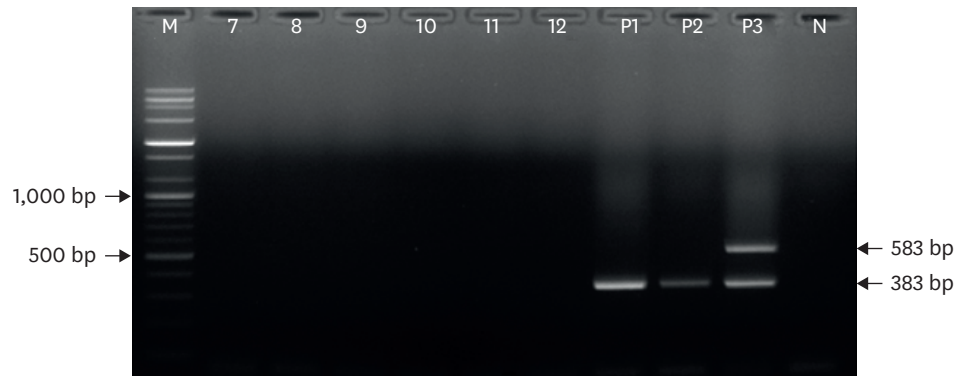

Fig. 4. Application of multiplex reverse transcription polymerase chain reaction to positive (A) and negative (B) samples. M, 100 bp DNA ladder; lanes 1–6, FAT-positive samples; lane P1, CVS11; lane P2, Evelyn-Rokitnicki-Abelseth strain; lane P3, ERAGS strain; lanes 7–12, FAT-negative samples; lane N, negative control (water only).
FAT, fluorescent antibody test.

DISCUSSION

The World Organization for Animal Health declared a goal to eradicate dog-mediated rabies in humans by 2030. However, the success of the goal is closely related to the absence of rabies in other animals [15]. Active rabies surveillance is an integral part of all of the activities in the rabies eradication program in most Asian countries, except rabies-free countries such as Japan, Brunei, and Singapore. Diagnostic tests available for detection of rabies in brain samples, such as FAT, RIDT, ELISA, RT-PCR, and histological examination, are accurate, sensitive, and reliable but are unable to differentiate RABV field strains from most nonpathogenic strains, such as SAG2 and recombinant RABV strains.

With the recent availability of reverse genetic systems, a variety of recombinant RABV strains for safer vaccine production have been reported [16,17]. Among the amino acids of the RABV G protein that affect pathogenicity, Arg at position 333 of the G protein determines virulence in adult mice [5,10,16]. Viral virulence is greatly reduced when the Arg at position 333 is substituted with other amino acids, such as glutamine, leucine, or glycine, which have a negative charge [4]. Previously, we reported that the ERAGS strain harboring mutations at positions 194 and 333 within the RABV G protein of the ERA strain was safe in 4-week-old mice [14]. Therefore, a diagnostic method for differentiation between RABVs with and those without mutations at position 333 is required in manufacturing and diagnostic laboratories. In this study, we designed two specific primer sets for the recombinant ERAGS and common RABV strains and established a multiplex RT-PCR method for simultaneous identification and differentiation of RABV in a single reaction.

Real-time RT-PCR has been reported as a sensitive and rapid method of detection of Lyssavirus species [18,19]. There are several critical factors involved in the development of multiplex RT-PCR, including the sensitivity and specificity of primer sets yielding PCR products of different sizes [20]. In this study, the reverse primer for the ERAGS strain was designed to target the mutation site in the G protein, because the difference in the first three nucleotides of the RABV reverse primer set did not allow multiplex RT-PCR amplification of RABV without mutation. This strategy for primer design made it possible to differentiate RABV with from that without mutation at position 333. To determine the sensitivity of the primer set, RABV RNA obtained from the ERAGS and ERA strains were subjected to multiplex RT-PCR. Although the sensitivity of the ERAGS primer set was 10 times lower than that of the RABVcomFR primer set, the detection limit of multiplex RT-PCR reached over 80 $FAID_{50}$/reaction. Variable multiplex RT-PCR detection limits for lyssaviruses have been reported [18]. The slight differences in sensitivity may be due to the selection of specific sites for differential amplification. In addition, multiplex RT-PCR showed good specificity, as no reaction products were found in the negative control samples, which comprised CDV, CPIV, CAV1, CAV2, and CPV assayed under the same experimental conditions. The results of multiplex RT-PCR were 100% consistent with the results of FAT in 46 brain samples, indicating that this method is applicable to field samples for diagnosis of rabies in South Korea. Although 3 fixed RABV strains and 46 brain samples were tested, further studies are required to determine the performance of the multiplex RT-PCR assay with non-Korean RABV isolates and to develop a real-time multiplex RT-PCR assay.

In conclusion, multiplex RT-PCR was shown to be a sensitive and specific assay for differentiating RABVs with from those without mutation at position 333 of the G protein. This

method is reliable for obtaining a confirmative diagnosis of RABV infection in suspected animals and for detecting the ERAGS strain in veterinary biological products and diagnostic laboratories.

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