

Molecular Identification of the Vaccine Strain from the Inactivated Rabies Vaccine

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Since 1994, several different inactivated rabies vaccines have been used to immunize domestic animals such as dogs, cats, and cattle in South Korea. The Korean Veterinary Authority has conducted safety and efficacy tests of inactivated vaccines using laboratory animals. In this study, we applied a molecular method to investigate the genetic characterization of the rabies virus (RABV) genes in six commercial inactivated rabies vaccines, and determined the efficiency of two extraction reagents (i.e., sodium citrate or isopropyl myristate) to separate the vaccine antigens from the antigen/adjuvant complexes. Six partial nucleocapsid (N: 181 bp) and five partial glycoprotein (G: 306 bp) genes were successfully amplified with specific primer sets, which demonstrated that sodium citrate is more efficient than isopropyl myristate in extracting viral RNA from inactivated gel vaccines. In addition, we identified the viral strain of the vaccine by analyzing the nucleotide sequences of the *N* and the *G* genes. The nucleotide similarity of the partial *N* and *G* genes ranged from 97.1 to 99.4% and from 91.8 to 100% among rabies vaccine strains, respectively, indicating that each manufacturer used different rabies virus strains to produce their vaccines. The molecular method used in this study could also be used to identify viral strains in other inactivated vaccines.

Key Words: Identification of vaccine strain, Inactivated rabies vaccine, Sequence

INTRODUCTION

Rabies is one of the most important infectious diseases as it is almost invariably fatal in animals and humans (1). According to the World Health Organization (WHO), rabies infections result in approximately 55,000 human deaths worldwide every year (2). In South Korea, since the first case of rabies was reported in 1907, a number of additional rabies cases have been reported (3~5). Two types of

inactivated rabies vaccines were manufactured in 1945 and 1959. The first was made from infected rabbit brain or spinal cord tissue after then the rabies virus (RABV) was inactivated with phenol or merthiolate. The second type was manufactured using calf brain and spinal cord tissue that was infected with wild RABV. Unfortunately, these inactivated vaccines provided short-acting immunity as they contained the small amount of RABV, and had many adverse effects due to tissue debris present in vaccines (6, 7). The first live rabies vaccine, which was used during the 1960~1980s, was made from a chicken embryo infected with the Flury-LEP viral strain. The live virus also had several drawbacks including a complicated production process, high production costs and severe unintended side effects. The second live vaccine, which used the ERA (Evelyn-Rokitnicki-Abelseth) strain, was introduced from Canada in 1974; it was cloned by several limit dilutions and by propagating the ERA strain

Received: November 18, 2010/ Revised: February 7, 2011

Accepted: February 14, 2011

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**This work was supported financially by a grant from the National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Table 1. List of the oligonucleotide primers used for RT-PCR of the rabies vaccine

Primer	Nucleotide sequences (5'-3')	Nucleotide position ^a	Sense	RABV gene	Size of amplicon (bp)
RVNDF	GRA ATT GGG CTT TGA CTG GA	353~372	+	<i>N</i>	181
RFNDR	AAA GGG GCT GTC TCG AAA AT	514~533	-		
RVGDF	CCC GAG AAT CCG AGA CTA	595~612	+	<i>G</i>	306
RFGDR	CTC TCT CTT CCT GAC CAA CTC	880~900	-		

^aThe positions of primers are based on the ERA strain (GenBank accession no. AF406695, AF406693).

in primary porcine kidney cells in 1980 (8). Until recently, this is the vaccine that was used to immunize dogs. Although live attenuated vaccines are considered safe in several countries, including South Korea, the use of inactivated cell culture vaccines is increasing around the world. In addition, the WHO does not recommend live vaccines for parenteral use in animals (9). As a result, in 1994, the Korean Veterinary Authority permitted the use of seven inactivated rabies vaccines for immunizing pets and other species of animals such as dogs, cats, cattle, goats, and fox. Laboratory animals have been used to monitor the efficacy of the vaccines. However, it is difficult to measure the quantity of vaccine antigen without conducting immunogenicity studies with animals, because the vaccine strain is killed by inactivating agents such as formalin and binary ethylenimine (BEI) during the manufacturing process. In addition, identification of antigen in the inactivated rabies vaccine is difficult as the antigen is mixed with adjuvant and has no special features like hemagglutination activity. To overcome these difficulties in identification of the RABV antigen, we used a molecular method to amplify the target gene of the viral RNA extracted from six commercially available inactivated rabies vaccines. This study also investigated the extraction condition of the antigen from commercially available inactivated gel-type rabies vaccines and compared the genetic characterization of rabies vaccine strains using generalized reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequence analysis.

MATERIALS AND METHODS

Vaccines and pretreatments

Inactivated rabies vaccines from six different manu-

facturers were used. Two methods were used to recover RABV antigen from the gel vaccines. First, 200 μ l of the inactivated vaccine were mixed thoroughly with 800 μ l isopropyl myristate (Sigma, Steinbeim, Germany) for 5 min. The mixture was centrifuged at $3,000 \times g$ for 10 min, and the water phase was collected and used for the extraction of RNA. Second, 1 ml of inactivated vaccine was mixed with 0.1 g of sodium citrate (Sigma) on a rotator at 37°C for 12 hrs. The mixtures were frozen and thawed three times and centrifuged at $7,000 \times g$ for 5 min, and the supernatant was then collected and used for the extraction of RNA.

Extraction of viral RNA and RT-PCR

Viral RNA was extracted from the six inactivated vaccines using an RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was diluted in 50 μ l of RNase- and DNase-free water. RT-PCR was performed using specific primer sets (RVNF, RVNR, RVGF, and RVGR) that amplify the nucleocapsid (*N*) and glycoprotein (*G*) gene of RABV (Table 1). The RT-PCR was performed in a reaction mixture containing 2 μ l of denatured RNA, 1 μ l of each primer (50 pmol), 10 μ l of 5 \times buffer (12.5 mM MgCl₂), 2 μ l of dNTP mix, 2 μ l of enzyme mix (reverse transcriptase and *Taq* polymerase), and 32 μ l of distilled water (Qiagen, Hilden, Germany). The cycling profile consisted of cDNA synthesis at 42°C for 30 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were visualized using electrophoresis on 1.8% agarose gels containing ethidium bromide.

Cloning and sequencing

All PCR products that were purified using the gel extrac-

tion kit were ligated with pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Plasmid DNA was isolated from amplified *Escherichia coli* (DH5 α), and recombinant plasmids were identified using *EcoRI* enzyme digestion (Bioneer, Daejeon, Korea). The sequences of the purified plasmids were analyzed using an MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM BigDyeTM Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems, USA) according to the manufacturer's instructions. Single-pass sequencing was performed for each template using universal primer sets (e.g., SP6 and T7). The fluorescent-labeled fragments were purified from the unincorporated terminators using an ethanol precipitation protocol. The samples were resuspended in distilled water and then subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, USA). Both DNA strands were sequenced to verify the sequences.

Phylogenetic analysis

Nucleotide sequence similarities were calculated using the DNASIS (Hitachi Software, Japan) software. Individual sequences were initially aligned using BioEdit and Clustal X 1.81. Phylogenetic reconstructions were generated using the neighbor-joining (NJ) method by the computer program PHYLIP 3.572c. Phylogenetic trees were reconstructed on aligned nucleotide sequences using ClustalW (version 2.0.12, UK). The robustness of the phylogenetic analysis was determined by bootstrap analysis with 1,000 replications. Graphic output was produced by TreeView (version 1.6.6, UK). Sequences used for phylogenetic analysis in this study were obtained from the GenBank database, and their strains (accession number) were as follows: KRVR0801 (GU937039, GU937028), KRVR0802 (GU937040, GU937029), KRVR0803 (GU937038, GU937027), KRVR0804 (GU937037, GU937026), KRVR0901 (GU937036, GU937025), KRVB0902 (GU9370345, GU937034), KRVB0903 (GU937044, GU937033), KRVB0904 (GU937043, GU937032), KRBB0905 (GU937042, GU937031), KRVR0906 (GU937035, GU937024), KRVB0907 (GU937041, GU937030), SKRDG9901GY (DQ076122, DQ076110),

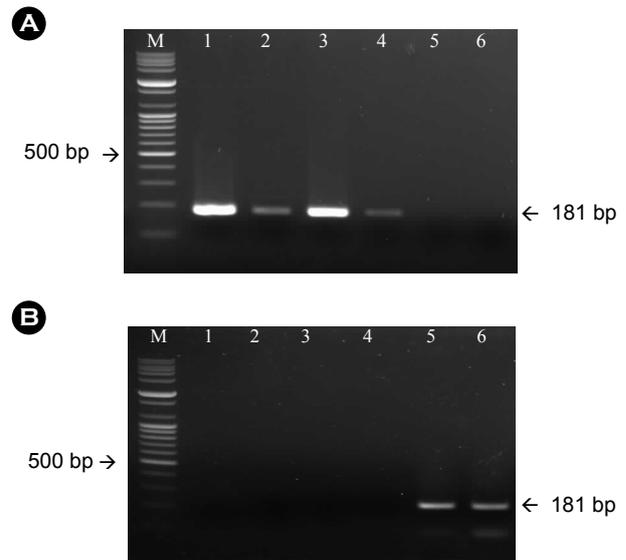


Figure 1. Amplification of the *N* gene from the rabies virus using RNA extracted from sodium citrate-treated antigen (A) and isopropyl myristate-treated antigen (B) with specific primer sets for the *N* gene. The expected size was 181 bp. M: 100-bp DNA ladder; lane 1-6: company A, B, C, D, E, F, respectively.

SKRBV0403CW (DQ076129, DQ076095), SKRBV-0404HC (DQ076130, DQ076097), Flury HEP (GU992325, GU565704), Flury LEP (GU992324, GU565703), SAD-B19 (EU877069, M31046), SAG2 (EF206719), ERA (M38452), GuizhouAI7 (DQ666293), GuizhouAI0 (EU-2627745), N99RF (EF025114), RC-HL (AB009663), PV11 (AF233275), CVS (GU992321, AF406694).

RESULTS

RT-PCR according to pretreatments

Before extracting the viral RNA from the inactivated vaccines, two reagents (sodium citrate or isopropyl myristate) were used to separate RABV antigens from the antigen/adjuvant complex in the vaccine. Four *N* genes specific positive reactions were detected when sodium citrate was used to recover RABV antigens, while two positive reactions were amplified when isopropyl myristate was used (Fig. 1A and B). A 181 bp fragment of *N* gene from all six inactivated rabies vaccines was successfully amplified with the *N*-gene primer set used in this study. When antigens were pretreated, four *G*-gene specific reactions were detected



Figure 2. Amplification of the *G* gene from the rabies virus using RNA extracted from sodium citrate-treated antigen (A) and isopropyl myristate-treated antigen (B) with specific primer sets for the *G* gene. The expected size was 181 bp. M: 100-bp DNA ladder; lane 1: company A; lane 2: company C; lane 3: company B; lane 4~6: D, E and F, respectively.

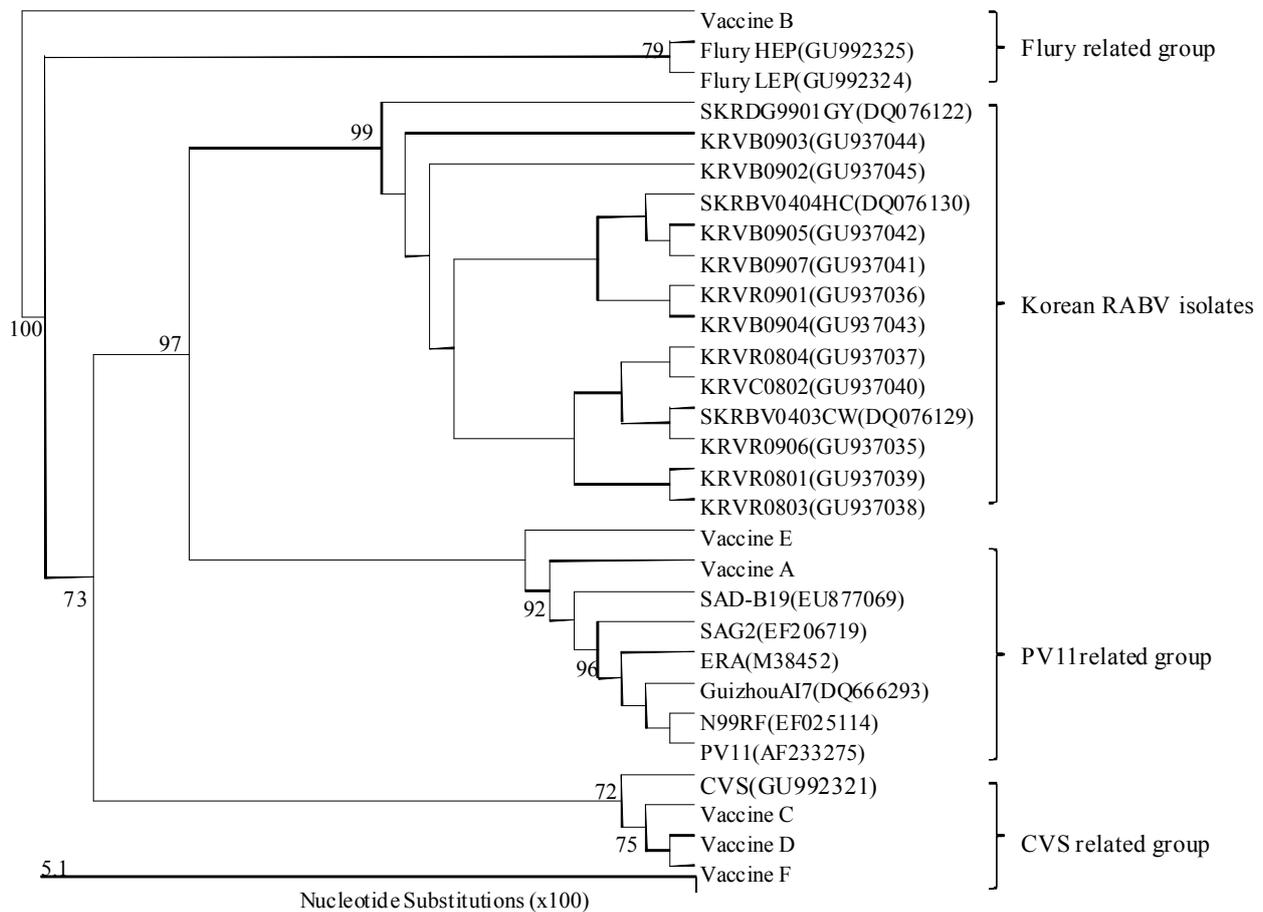


Figure 3. Phylogenetic analysis based on the partial *N*-gene nucleotide sequences of the vaccine strains and other sequences obtained from the GenBank database. Numbers at each key node indicate the degree of bootstrap support and only those with >70% support are shown.

with sodium citrate, while one *G* gene specific reaction was amplified with isopropyl myristate (Fig. 2A and B). Overall, a 306-bp fragment was found in five of six inactivated rabies vaccines that were amplified with the *G* gene primer set.

Analysis of the nucleotide sequences in *N* and *G* genes

The 181 *N* gene and 306 *G* gene nucleotide sequences obtained from six inactivated rabies vaccines were identified and compared with 23 RABVs retrieved from GenBank to

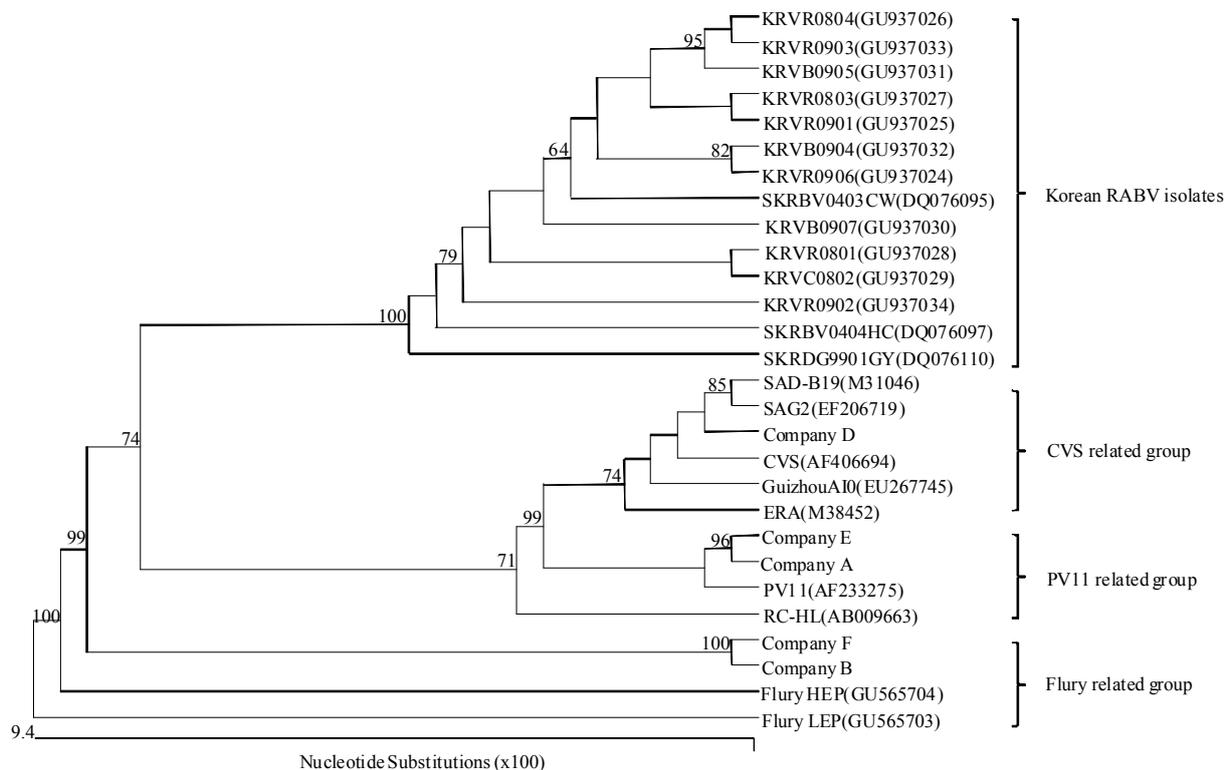


Figure 4. Phylogenetic analysis based on the partial *G* gene nucleotide sequences of the vaccine strains and other sequences obtained from the GenBank database. Numbers at each key node indicate the degree of bootstrap support and only those with >70% support are shown.

analyze the relationship of rabies vaccine strains. The phylogenetic tree based on the nucleotide sequence analysis of the *N* gene revealed that rabies vaccine strains were divided into three clusters (Fig. 3). The nucleotide similarity of the partial *N* gene ranged from 97.1 to 99.4% among the six rabies vaccine strains and 91.1 to 94.5% between the Korean RABV field isolates and the inactivated vaccine strains. The phylogenetic tree based on the nucleotide sequence of the partial *G* gene demonstrated that rabies vaccine strains also divided into three clusters (Fig. 4). The deduced amino acid analysis of the partial *G* gene showed that the PV11-related group had a total of 13 amino acid changes from the CVS related group and that the amino acid similarity of the partial *G* gene among five of the RABV vaccine strains ranged from 82.9 to 100%.

DISCUSSION

At least 23 countries throughout the world produce rabies

vaccines for animals. These vaccines are classified into several types according to the materials used in the manufacturing process. Fourteen countries use cell culture with BHK21 and chicken embryo cells; seven use neural tissue-like infected brain or spinal cord; and six use embryonated eggs (9). Although modified live virus (MLV) rabies vaccines have proven to be safe and efficacious, the WHO does not recommend the use of MLV rabies vaccines for parenteral inoculation in animals. As a result, the use of MLV rabies vaccines has declined in several countries including South Korea (9). Twelve different inactivated rabies vaccines are used to immunize dogs and cats in the USA, and these vaccines were shown to protect 22 of 25 (88%) of vaccinated animals following challenge with pathogenic RABV at 90 days after vaccination (10). The Korean Veterinary Authority performed efficacy testing according to the Korean standard assay of veterinary biological products before officially approving the inactivated rabies vaccine (11). However, it is dangerous to challenge

animals with the pathogenic wild-type RABV to evaluate vaccine efficacy. Thus, the present study used molecular methods to identify the rabies vaccine antigen. Aluminum hydroxide, the most common adjuvant, is used to manufacture the inactivated rabies vaccine (12). We used two

kinds of reagents to separate vaccine antigens from the antigen/adjuvant complex present in the vaccines. The nucleocapsid protein is associated with encapsidation of the genomic RNA, and the glycoprotein plays an important role in the attachment of the virus to the host-cell surface (13,

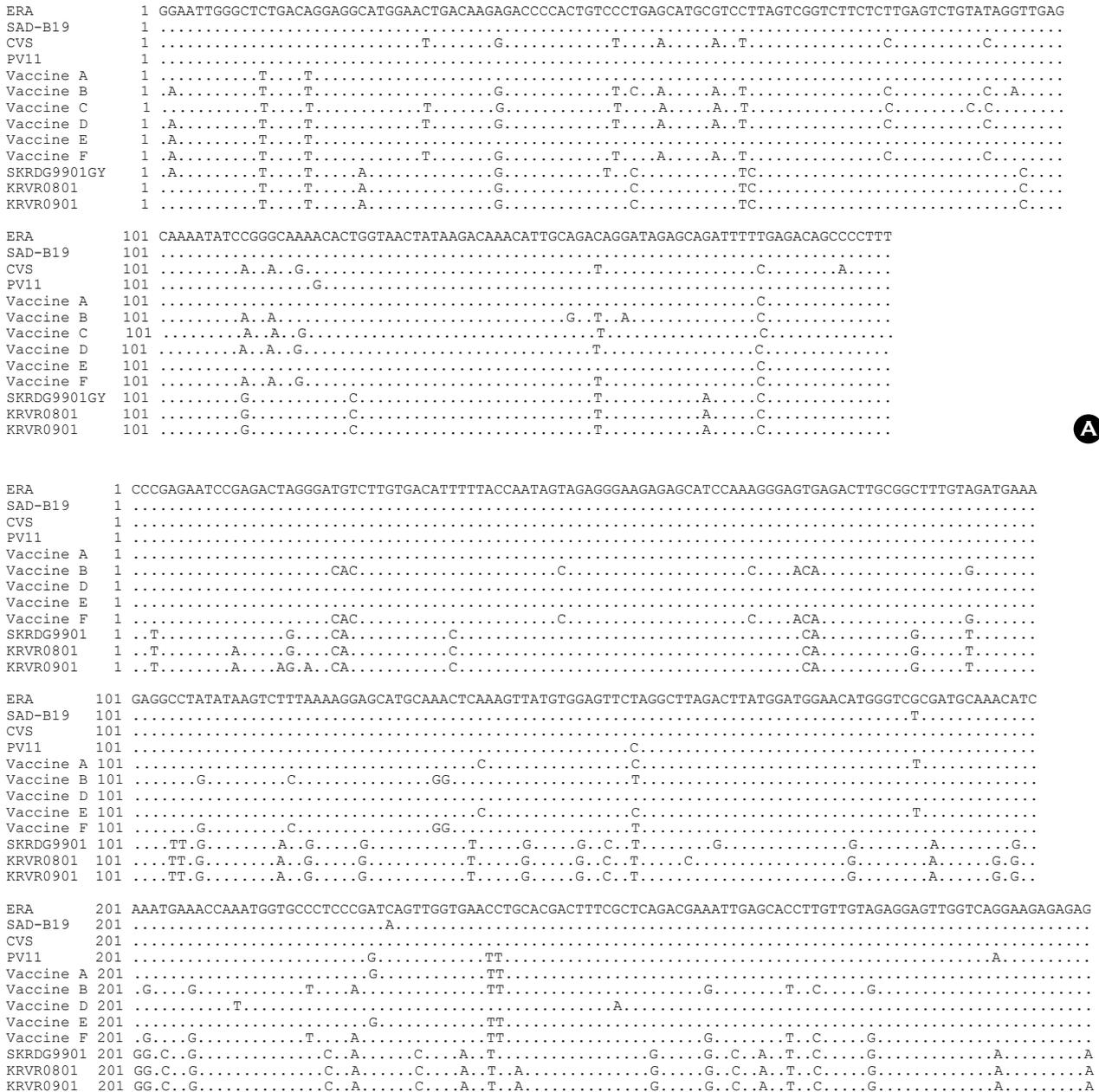


Figure 5. Comparison of the nucleotide sequences of nucleocapsid (A) and glycoprotein (B) genes of the vaccine strains and representative Korean RABV isolates. Dots indicate nucleotides agreeing with the first line sequence.

14, 15, 16). We focused on these two genes to identify the vaccine strain by RT-PCR. As shown in Figs. 1 and 2, sodium citrate-treated antigen was more easily detected by RT-PCR than isopropyl myristate-treated antigen, indicating that the vaccine antigen is rendered more accessible to RNA extraction by pretreatment with sodium citrate rather than isopropyl myristate. Although, Choi *et al.* (17) reported that extraction and amplification of target genes from all inactivated oil emulsion vaccines could be done using isopropyl myristate, several factors such as the vaccine virus, the method of virus inactivation, the valence of the vaccines, and the composition of the adjuvant are known to affect the ease of vaccine extraction (18).

The *N* and *G* gene sequences from the extracted vaccine antigens were compared with the gene sequences of RABV strains. As shown in Figs. 3 and 4, the phylogenetic trees based on the nucleotide sequences of the *N* and *G* genes revealed that the vaccine strains were divided into three clusters, PV11, Flury, and CVS related groups, and that the nucleotide similarity of the partial *N* gene (181 bp) ranged from 97.1 to 99.4% among the six vaccine strains, indicating that each vaccine manufacturer used different kinds of rabies virus strains for the inactivated rabies vaccine. However, a wider range in the nucleotide similarity was identified between the Korean RABV isolates and the inactivated vaccine strains.

Mutation of the nucleotide sequences in several positions can help to distinguish vaccine strains from other RABV strains. It is well known that RNA viruses have a high mutation rate during replication due to both the lack of proofreading and post-replication error correction by RNA polymerase (19). The RABV seed strain used to produce vaccines is regulated by the Korean Veterinary Authority, and changes of seed strains are not allowed without permission. Nucleotide sequences in the partial *N* and *G* region of rabies vaccine strains were compared with those of RABV strains (Fig. 5), and substitutions of nucleotides were identified in the vaccine strains. These changes in nucleotide sequence can be used as "markers" of the vaccine strains so that changes in the vaccine seed strain could be monitored.

We successfully amplified the *N*-gene of viral antigens extracted from six gel vaccines containing aluminum hydroxide adjuvant using two different reagents. However, the *G*-gene was amplified in only five of six extracted antigens using the two different reagents. Additional studies are needed to resolve this problem. The real-time RT-PCR method can be used to quantify the viral antigens in the inactivated vaccines. Although the amplified region targeted in this study is comparatively short, this method may be an efficient tool for identifying the rabies vaccine strains and possibly other types of inactivated vaccines as well.

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