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Preparation of Anti-Rabies Virus N Protein IgYs by DNA Immunization of Hens Using Different Types of Adjuvants

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DNA immunization has been used to study vaccination methods and for production of specific antibodies. The present study aimed to apply DNA immunization to prepare specific IgYs, which react against rabies virus N protein (RV-N) and can be used to research and diagnose rabies virus. The DNA sequence of RV-N was ligated into a pcDNA 3.1 plasmid for constructing pcDNA-N. Eight hens were divided into four groups. Group 1 comprised the control group (non-immunized). In Groups 2, 3, and 4, hens were injected intramuscularly with pcDNA-N (400 μg/hen). Eight injections were administered every other week. From the 4th week, an adjuvant was injected in addition to pcDNA-N. Freund's complete adjuvant (FCA) and λ-carrageenan were administered to Groups 3 and 4, respectively. Eggs were collected daily, and the specific antibody activities of egg yolks were measured by ELISA. IgYs were purified from pooled egg yolks at 16−19 weeks post-administration in each group. The detection sensitivities of the RV-N were compared using purified IgY as the primary antibody for ELISA, dot blotting, and western blotting. Egg yolks from one of the two hens in Group 2 (pcDNA-N alone) and all hens in Groups 3 (pcDNA-N + FCA) and 4 (pcDNA-N + λCarra) had increased ELISA values. The combined use of λ-carrageen in DNA immunization resulted in an adjuvant effect comparable to that of FCA. Each purified specific IgY detected RV-N in the ELISA, western blotting, and dot blotting; however, the detection sensitivity differed. Higher detection sensitivity of the +λCarra IgY was observed by ELISA, whereas there was higher detection sensitivity of +FCA IgY in western blotting and dot blotting. In summary, anti-rabies virus N protein IgY was prepared through DNA immunization of hens using FCA or λ-carrageenan as adjuvants and can be used as a primary antibody to detect rabies viruses.

Key words: adjuvant, DNA immunization, IgY, rabies virus, λ-carrageenan

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Introduction

DNA immunization has been studied as a vaccination method and for antibody production. Plasmid DNA that contains a foreign gene encoding a protein antigen with its promoter/terminator is used for DNA immunization. Such injected plasmid expresses a foreign protein in immunized animals, and the protein antigen stimulates humoral immunity. Specific antibodies against the antigen are then generated. The safety of DNA immunization have been confirmed in animal and clinical studies (Wolff *et al*., 1992; MacGregor *et al*., 1998; Le *et al*., 2000; Zhao *et al*., 2013). Recently, a vaccine candidate for COVID-19 has been used for DNA immunization (Smith *et al*., 2020). Some studies in hens have shown that specific IgYs against protein antigens coded by viral (Lu *et al*., 2008; Witkowski *et al*., 2009; Sawant *et al*., 2011), bacterial (Brujeni and Gharibi, 2012; Cho *et al*., 2004, Niederstadt *et al*., 2012), and mammalian (Brujeni *et al*., 2011) genes were induced by the DNA immunization method.

Rabies is a zoonotic disease caused by infection with rabies virus (in the family *Rhabdoviridae*, genus *Lyssavirus*), which is transmitted via the saliva of infected animals, especially dogs (Fooks *et al*., 2017). This infectious disease remains a significant public health problem, especially in most developing countries. Treatment guidelines for preventing rabies indicate the importance of serum therapy, in addition to vaccination (WHO, 2018). However, the horse antiserum required

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for treatment has been insufficient in recent years (Wilde *et al*., 2002). The active component of the antiserum is a specific antibody. Therefore, IgY antibodies against rabies pathogens can be a promising substitute for horse antiserum against rabies virus, because a large amount of specific IgY antibodies can be prepared from egg yolks. In addition, rabies diagnosis has been conducted with polyclonal and monoclonal antibodies targeting viral antigens (Rupprecht *et al*., 2002) to detect antigenically stable nucleoproteins of the rabies virus. Currently, there is a need to ensure a constant and uninterrupted supply of low-cost antibodies as a reagent for rabies diagnosis (Madhusudana *et al*., 2012).

We previously succeeded in developing chicken antibodies (IgYs) against rabies virus nucleoprotein (RV-N), phosphoprotein (RV-P), and glycoprotein (RV-G) through a conventional method using purified recombinant proteins as antigens, which were obtained after being expressed in *E. coli* (Motoi *et al*., 2005a, 2005b). Moreover, we have reported that anti-RV-N IgY and anti-RV-G IgY can detect the virus and prevent infection as a substitute for antiserum, respectively.

The purpose of this study was to prepare an anti-RV-N IgY, which can be used for rabies research and diagnosis, through DNA immunization of hens followed by IgY purification from immune egg yolks. Moreover, our previous study showed that λ-carrageenan, a water-soluble polysaccharide (a food additive), had an adjuvant effect on hens injected with formalin-killed *S. aureus* antigen (Kubo *et al*., 2021). Therefore, this present study also investigated the effect of λ-carrageenan as an adjuvant in the DNA immunization method in comparison with that of Freund's complete adjuvant (FCA).

Method

Animal Care

This study was approved by the Animal Care and Use Committee of Kyoto Women's University (2020-13). All procedures involving animals and their care conformed to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Materials

Food-ingredient-grade λ-carrageenan was obtained from Taiyo Kagaku Co. Ltd. (Mie, Japan). FCA was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). DNA purification NucleoBond Xtra Midi was obtained from Macherey-Nagel (Düren, Germany). Rabies TC Vaccine "KMB" (inactivated tissue culture rabies vaccine) was obtained from KM Biologics (Kumamoto, Japan). The other chemicals used were of special grade.

Preparation of Recombinant Plasmid

The N protein gene derived from a challenge-virus-standard (CVS) strain of rabies virus was ligated into a pcDNA 3.1 plasmid (Thermo Fisher Scientific, Waltham, MA, USA) (Fig. 1). The recombinant plasmid DNA (pcDNA-N) was amplified in *E. coli* DH5α competent cells (Takara, Kusatsu, Japan) and purified using the NucleoBond Xtra Midi plasmid DNA purification kit (Takara) according to the manufacturer's protocol. The purified recombinant plasmid was adjusted to a concentration of 1 mg/mL in 10 mM phosphate Buffer +150 mM NaCl solution (PBS, pH 7.4) and stored at −30℃.

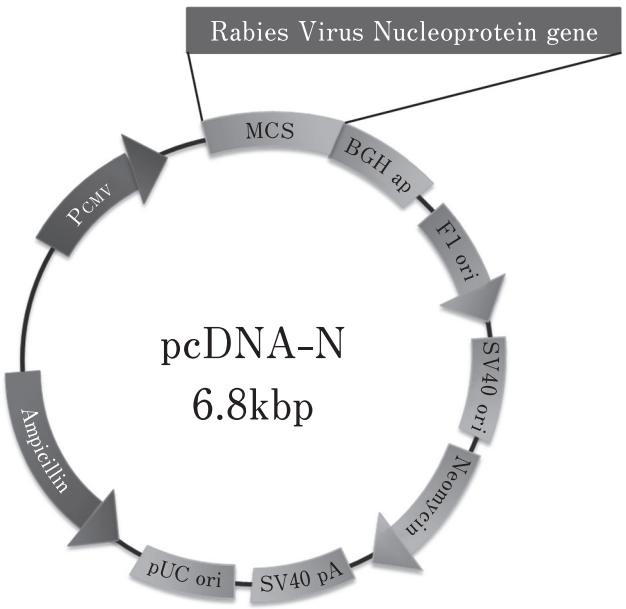

Fig. 1. **DNA map of pcDNA3.1 containing a rabies virus N protein gene (pcDNA-N).** The rabies virus nucleoprotein (RV-N) gene amplified from rabies challenge-virus-standard (CVS) was inserted into the pcDNA3.1 vector, which has a human cytomegalovirus immediate-early (CMV) promoter, to create the recombinant plasmid, pcDNA-N. MCS: Multiple cloning site

Plasmid Injection to Hens

Hens (Boris Brown; 416 days old) were divided into four groups, with two hens per group. The control group did not receive any immunization (Group 1). For Groups 2 to 4, pcDNA-N (400 μg/hen) was injected into the pectoral muscle of each hen using a needle-free injector, Twin-Jector EzⅡ (JCR Pharmaceuticals, Hyogo, Japan). Injections were repeated eight times at two week intervals. From the 4th week, each hen from Groups 3 and 4 was additionally injected with adjuvants (FCA and λ-carrageenan, respectively) around the pcDNA-N injection site. Blood was collected from each hen via the wing vein before injection and centrifuged to prepare serum samples, which were then stored at −30℃. The eggs were collected daily. For the measurement of ELISA values, egg yolks obtained from individual hens every two weeks were diluted two-fold with 0.1% sodium azide solution and stored at 4℃. Other egg yolks were pooled together every two weeks for individual hens, homogenized, and frozen at −30℃ for IgY purification.

ELISA of Diluted Egg Yolk and Serum

ELISA was performed according to a modified method described by Kubo *et al*. (2021). Briefly, Rabies TC Vaccine "KMB" was diluted 10-fold with ELISA coating buffer (pH 9.6) and then used to coat 96 well ELISA plates (Sumitomo Bakelite, Tokyo, Japan). Egg yolk samples were further diluted with 20 mM Tris buffer containing 0.05% Tween20 (TBS-T, 1:4000) and added to the wells (50 μL/well). After

the plate was washed three times with TBS-T, alkaline phosphatase-conjugated rabbit anti-chicken IgY (H+L) antibody (Abnova, Taipei, Taiwan) was applied to each well as a secondary antibody. After washing, the plate was incubated with a substrate (1 mg/mL disodium *p*-nitrophenyl phosphate hexahydrate in substrate dilution buffer [pH 9.5]). Absorbance was then measured at 405 nm after stoped enzyme reaction by 2 M NaOH (50 µl/well). Each stored serum sample was also diluted with TBS-T (1:4000) and subjected to ELISA using the same protocol.

Purification of IgYs

Purification of IgYs from egg yolks were carried out according to the protocol of the λ-carrageenan method described by Hatta *et al*. (1990). Pooled egg yolks from hens at 16−19 weeks post the 1st immunization, which showed higher ELISA values in each group, were used for IgY purification. The concentrations of purified IgYs were estimated from the absorption at 280 nm using a value of 15.8, for a 1% solution in a light path of 1 cm (Gallagher and Voss, 1969). IgY purity was evaluated using HPLC gel filtration and SDS-PAGE.

ELISA of purified IgY

Each of the purified IgYs was subjected to ELISA according to the above protocol. The concentration of each purified IgY was adjusted to 0.1, 1.0, 10.0, and 100.0 µg/mL with TBS-T.

Purification of Recombinant RV-N

The whole gene of RV-N from a CVS strain was ligated into a pET-42a plasmid vector (Merck, Darmstadt, Germany) using In-Fusion HD Cloning (Takara). The recombinant vector was transformed into Rosetta-gami B (DE3) pLysS Competent Cells (Merck), and the synthesized RV-N was purified with HisTrap FF crude (Cytiva, Tokyo, Japan) under denaturing conditions using 8 M urea.

Western Blotting and Dot Blotting

The His-tagged RV-N was subjected to SDS-PAGE using a slab-type vertical gel system (ATTO, Tokyo, Japan) with a 5−20% gradient gel (c-PAGEL HR, ATTO). After electrophoresis using the Laemmli method (Laemmli, 1970), proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was then washed with PBS and blocked with 5% skim milk solution (in PBS) for 1 h at room temperature. After washing with PBS, the membrane was cut into each lane, which was then incubated with 5 mL of 10 µg/mL purified IgY in 0.1% BSA/TBS-T. All membranes were washed with TBS-T and incubated in 5 mL of diluted (1:10,000) secondary antibody (horseradish peroxidase-conjugated anti-IgY rabbit IgG; Sigma-Aldrich, St. Louis, MO, USA) and washed in the same manner. Finally, the immune complexes were stained with Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan), and chemiluminescence was measured using LuminoGraph I (ATTO).

The same His-tagged RV-N was also subjected to dot blotting. Briefly, 0.25 mg/mL denatured His-tagged RV-N was diluted 2-fold serially (final concentrations: 0.250, 0.125, 0.063, 0.031, 0.015, and 0.007 mg/mL) and 2 µL of the diluted samples were dropped onto a PVDF membrane. Blocking of the membrane was performed with 10 mL of 3% skim milk in PBS, and the rest of the protocol was performed following the western blotting protocol described above.

Results

Changes in IgY Activities in Diluted Egg Yolks using ELISA

The ELISA values of egg yolks increased in only one of the two hens injected with pcDNA-N alone (Group 2). Adjuvant injection with FCA (Group 3) or λ-carrageenan (Group 4) contributed to an increase in the ELISA values. The IgY activities in Group 3 seemed to increase faster than those in Group 4; however, the peaks of IgY activities in Group 4 were higher than those in Group 3 (Fig. 2). The egg-laying rates in the three immunized groups did not decrease compared with those in Group 1 (data not shown).

IgY Purification

IgY purification was carried out using the λ-carrageenan method. The purified IgYs were: Cont IgY (control), pcDNA-N IgY, pcDNA-N + FCA IgY, and pcDNA-N + λCarra IgY. In the SDS-PAGE profile, each purified IgY had two major bands at around 70 kDa (heavy chain) and 20 kDa (light chain). There were also several faint bands between the heavy and light chains, indicating some impurities (data not shown). According to the HPLC measurement, a single peak was observed, and the purity of each IgY was more than 98.7% according to the area integration data (data not shown).

Reactivity of Purified IgYs by ELISA

Each purified IgY (except Cont IgY) detected RV-N in the rabies vaccine by ELISA. The IgY activities increased proportionally to the concentrations of each purified IgY except Cont IgY. At a concentration of 10 µg/mL, pcDNA-N + λCarra IgY had the highest ELISA value (approximately 1.1), followed by pcDNA-N + FCA IgY (approximately 0.8). The ELISA values of DNA-N IgY and Cont IgY were approximately 0.6 and 0.1, respectively (Fig. 3). At a concentration of 100 µg/mL, the ELISA value of Cont IgY increased; therefore, the appropriate IgY concentration as a primary antibody was considered to be between 1 and 10 µg/mL.

Western Blotting and Dot Blotting using Purified IgYs

From western blotting, Cont IgY did not detect His-tagged RV-N at any antigen concentration when subjected to SDS-PAGE. On the other hand, His-tagged RV-N was detected (even at the lowest RV-N concentration of 0.025 mg/mL) by pcDNA-N IgY and pcDNA-N + FCA IgY at approximately 57 kDa, which is similar to the calculated molecular weight of the antigen. For pcDNA-N + λCarra IgY, RV-N was detected only at RV-N concentrations of 0.05 and 0.10 mg/mL (Fig. 4A). The results of dot blotting showed that specific IgYs purified in this study detected His-tagged RV-N. In particular, pcDNA-N + FCA IgY detected RV-N with the highest sensitivity compared to the others (Fig. 4B).

Discussion

We succeeded in preparing specific IgYs against rabies virus N protein (RV-N) by injecting recombinant plasmid DNA with adjuvant. Generally, specific IgY induction in hens without the use of adjuvants is rare. In the present study, however, the induction of a specific IgY antibodies was observed in one of two hens immunized with plasmid DNA

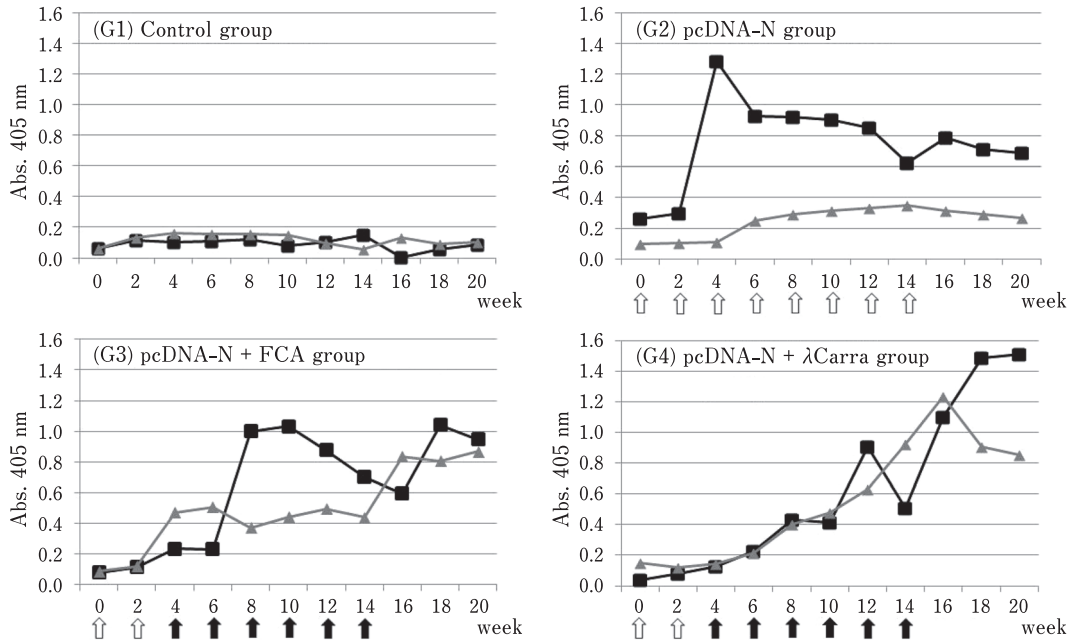

Fig. 2. **Changes in anti-RN-N IgY activities in egg yolks by ELISA.** Figures G1 to G4 show changes in IgY activities in the immune egg yolks of individual hens in Groups 1 to 4. Pooled egg yolks from 16−19 weeks post the 1st immunization from hens of each group (—■—) were used for IgY purification. Blank ⇧ and filled arrows ⬆ indicate pcDNA-N alone injection and adjuvant (FCA or λ-carrageenan) added injection, respectively.

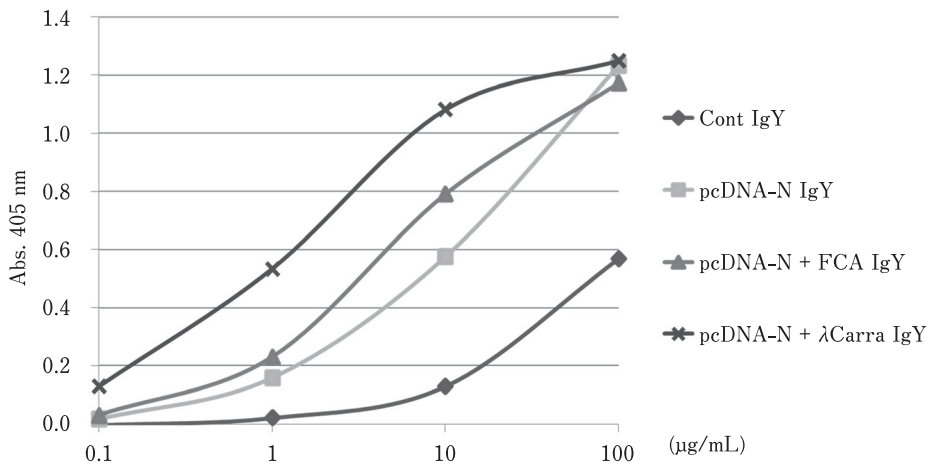

Fig. 3. **Reactivity of each purified IgY against rabies virus vaccine (by ELISA).** Each purified IgY was used as a primary antibody at concentrations between 0.1 and 100 μg/mL.

containing the RV-N gene (pcDNA-N) alone (Group 2). The ELISA values of egg yolks from the hen reached maximum values in the fourth week. The serum ELISA values of the hens in Group 2 showed similar results (data not shown). It is unclear why only one of the two hens was stimulated to generate specific IgY activity without adjuvant. It is possible that pcDNA-N might enter not only muscle cells but also a hen's antigen-presenting cells following immune injection (Liu, 2003). Consequently, rapid and strong activation of humoral immunity may have occurred.

It was also demonstrated that use of λ-carrageenan or FCA together with pcDNA-N injection promoted specific IgY activities in egg yolks and sera in all hens of Groups 3 and 4. As for the adjuvant activity of λ-carrageenan in hens, we previously reported its adjuvant effect in hens immunized with formalin-killed bacteria (Kubo *et al*., 2021). To our knowledge, the present study is the first to show that λ-carrageenan can be used as an adjuvant in the DNA immu-

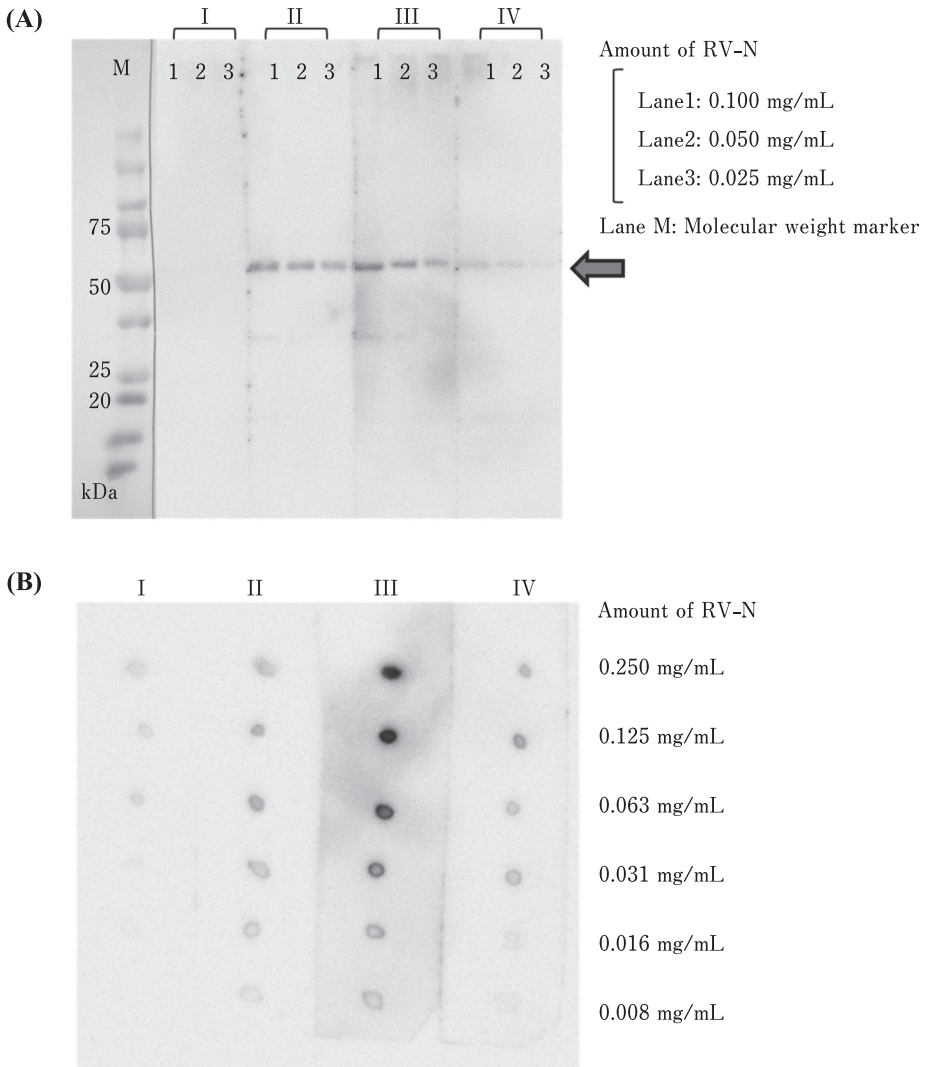

Fig. 4. **Detection of His-tagged rabies virus N protein by western blotting (A) and dot blotting (B).** Purified Cont IgY (I), pcDNA-N IgY (II), pcDNA-N + FCA IgY (III), and pcDNA-N + λCarra IgY (IV) were used as primary antibodies (10 μg/mL). Arrow in (A) indicates the calculated molecular weight of the rabies virus N protein (RV-N, 57 kDa).

nization of hens. We also reported that the egg-laying rate did not decline when λ-carrageenan was used as an adjuvant compared to FCA (Kubo *et al*., 2021). According to the egg-laying rate consistency, some stresses or pain from inflammation at the immunization site could be avoided by using λ-carrageenan as an adjuvant.

The different orders in reactivities of pcDNA-N + λCarra IgY and pcDNA-N + FCA IgY between ELISA and western blotting/dot blotting may be attributed to the different antigen-recognition sites induced by injection of different adjuvant types. In particular, in the ELISA protocol, undenatured RV-N in the rabies vaccine was coated as an antigen. Hence, pcDNA-N + λCarra IgY may contain more antibodies that strongly detect undenatured RV-N. On the other hand, His-tagged recombinant RV-N, which was denatured with 8 M urea and/or SDS + 2-mercaptoethanol, was used for western blotting and dot blotting. Therefore, pcDNA-N + FCA IgY may comprise of more IgY with high specificity for the denatured structure of the antigen.

In this study, intramuscular plasmid DNA immunization, including that of the RV-N gene together with adjuvants (FCA and λ-carrageenan), led to efficient humoral immunity in hens and induced anti-RV-N-specific IgYs. Each anti-RV-N IgY purified from an immunized hen's egg yolks was able to detect RV-N as a primary antibody. Although we have not yet confirmed whether the antibodies can detect the rabies virus in infected animal tissue samples using the same method of rabies diagnosis, the DNA immunization method followed by adjuvant injection could be beneficial for producing a substantial amount of specific IgYs. Moreover, this method of

specific IgY preparation might not only produce IgYs for diagnosis but also produce neutralizing IgY antibodies as a substitute for horse antisera against the rabies virus.

Regarding the preparation of viral proteins as antigens, DNA immunization does not require a time-consuming recombinant protein purification protocol necessary for conventional IgY preparation. Usually, in preparation for recombinant proteins, it is difficult to express sufficient amounts of some proteins, and many purification steps are needed to obtain highly pure target proteins for injection into animals. However, DNA immunization requires only preparing recombinant plasmids. This immunization method may be feasible in resource-limited situations. Therefore, the production of specific IgY antibodies in this study could be applied to prepare diagnostic reagents and antibodies that neutralize rabies virus, both of which are currently in short supply in developing countries.

Conflicts of Interest

The authors declare no conflict of interest.

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