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Comparison of Anti-rabies Virus Nucleoprotein IgY Prepared by DNA Immunization and Protein Immunization

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Immunization of egg-laying hens with viral antigens efficiently produces large amounts of virus-specific IgY antibodies from egg yolks. A supply of practical and economical antibodies against the rabies virus is being desired worldwide. We immunized hens with the antigen gene DNA of the rabies virus, purified specific IgY antibodies from the egg yolk, and characterized the immuno-protein chemistry for use as a diagnosis. To prepare specific IgY antibodies against rabies virus nucleoprotein (RV-N) by DNA immunization, laying hens were pre-injected with λ-carrageenan or Freund's complete adjuvant to increase local immune activity (pre-immune stimulation), and then immunized with RV-N recombinant plasmid DNA. RV-N-specific IgY antibodies were prepared from egg yolks of immunized hens. For comparison, conventional protein antigen immunization was also used to induce the production of RV-N-specific IgY antibodies. Laying hens were immunized with an RV-N protein antigen and RV-N-specific IgY was purified from egg yolks. The binding activity against RV-N antigens was examined using IgY samples prepared by DNA (with pre-immune stimulation) and protein immunization. Immunohistochemical staining showed that IgY antibodies prepared by protein immunization strongly detected viral antigens in the brain sections of dogs infected with the virus, whereas IgY antibodies prepared by DNA immunization did not. Enzyme-linked immunosorbent assay was performed using a commercially available rabies vaccine (inactivated virus) treated with 10% formalin and heating (60°C, 30 min and 90°C, 5 min). IgY prepared by DNA immunization had weaker reactivity with denatured antigens and lower antigen concentrations than IgY prepared by protein immunization. These results suggest that it is necessary to develop a DNA immunization method for inducing IgY antibodies against the rabies virus that strongly bind to native and denatured antigens to prepare specific IgYs that can be used for antigen detection in clinical tests.

Key words: DNA immunization, FCA, IgY, IHC, λ-carrageenan, Rabies virus

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Introduction

Rabies is a zoonotic disease that kills approximately 60,000 people annually (Hemachudha *et al*., 2002; Rupprecht *et al*., 2002; Hemachudha *et al*., 2013; Fooks *et al*., 2017; WHO, 2018a). In case of exposure to the body fluids of animals, mainly dogs, suspected of having a rabies viral infection, the World Health Organization recommends an injection of purified immunoglobulin (Ig)-containing virus-neutralizing antibodies in addition to the rabies virus (RV) vaccine (WHO, 2018b). However, the purified Igs required for treatment are in short supply worldwide (Wilde *et al*., 2002). In addition, the supply of specific antibodies for immunohistochemical (IHC) staining, which is used to diagnose rabies in animals (Stein *et al*., 2010), is insufficient (Madhusudana *et al*., 2012).

We previously succeeded in developing chicken IgY antibodies against the RV using a conventional method with purified recombinant proteins as antigens. IgYs can be a promising substitute for purified Igs for treating rabies viral infections and can be used as a primary antibody in IHC (Motoi *et al*., 2005a; 2005b). We also reported that RV nucleoprotein (RV-N)-specific antibodies can be prepared by DNA immunization combined

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with various adjuvant injections (Kubo *et al*., 2022a). We further developed and reported an immunization method using intramuscular injection of an adjuvant before immunization of laying hens to increase antibody activity in DNA immunization (Kubo *et al*., 2022b).

Recently, DNA immunization using recombinant plasmid DNA as an immune source has been used for the development of DNA vaccines (Liu, 2003; Lim *et al*., 2020) and the preparation of specific antibodies against antigens encoded by genes (Chambers and Johnston, 2003; Bates *et al*., 2006). DNA immunization does not require the additional steps for the expression or purification of recombinant proteins using *Escherichia coli*. If an IgY antibody could be prepared by DNA immunization for IHC detection, DNA immunization could be a simpler method for preparing antibodies for Ravies diagnosis. In this study, chicken IgY antibodies against RV-N prepared by both DNA immunization and conventional protein immunization into lying hens as described in our previous study (Kubo *et al*., 2022b) were used to investigate these characteristics by applying IHC against rabies-positive dog brain samples and performing enzyme-linked immunosorbent assay (ELISA) using undenatured or denatured RV-N as the coated antigen.

Materials and methods

Animal care

This study was approved by the Animal Care and Use Committee of Kyoto Women's University (2020-12). All procedures involving animals and their care conformed to the Guidelines for the 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). Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were purchased from Becton Dickinson and Company (BD) (Franklin Lakes, NJ, USA). The rabies TC Vaccine "KMB" (inactivated tissue culture rabies vaccine) was obtained from KM Biologics (Kumamoto, Japan). All other chemicals used were of special grade.

Preparation of recombinant plasmid

Similar to our previous study (Kubo *et al*., 2022a), the RV-N gene (accession number: AB069973) derived from a challenge-virus-standard (CVS) strain of RV was ligated into a pcDNA 3.1 plasmid (Thermo Fisher Scientific, Waltham, MA, USA). The recombinant plasmid DNA (pcDNA-N) was amplified in *E. coli* DH5α competent cells (Takara, Japan) and purified using the NucleoBond Xtra Midi plasmid DNA purification kit (Takara, Japan), according to the manufacturer's protocol. The purified recombinant plasmid was adjusted to a concentration of 1 μg/μL in phosphate-buffered saline (PBS; 10 mM phosphate buffer + 150 mM NaCl, pH 7.4) and stored at −30 °C.

Purification of recombinant RV-N

Whole RV-N genes derived from the CVS strain were inserted into the pET-42a plasmid vector (Merck, Germany) using the In-Fusion HD Cloning kit (Takara). The recombinant vector was transformed into Rosetta-gami B (DE3) pLysS competent cells (Merck), and recombinant RV-N (rRV-N) was purified using His-trap FF crude (Cytiva, Japan) under denaturing conditions with 8 M urea. The urea was then removed by dialysis. However, when the urea concentration decreased, the recombinant protein became insoluble. Therefore, two recombinant RV-N antigen samples were prepared: an ultimate removal urea and insolubilized aggregate sample (rRV-N in 0 M Urea) and a solubilized state in a 4 M urea sample (rRV-N in 4 M Urea). Gene recombination experiments were approved by the Recombinant DNA Experiment Safety Committee of Kyoto Women's University (approval number 20-2-03).

Immune injection in hens

Five IgY samples were used in this experiment, including Control IgY, each with different immunizing antigens, adjuvant types, and use timing. Fig. 1 shows the immunization schedule used to immunize laying hens, as described previously (Kubo *et al*., 2022b). Fifteen hens (Boris Brown, 356 days old) were divided into five groups with three hens per group. The control group did not receive the immunization (Group 1). In Groups 2 and 3, in the week immediately before the first injection of pcDNA-N, hens were injected three times with 1 mL of an adjuvant (2% λ-carrageenan or FCA) around the pectoral muscle to induce inflammation (pre-immune stimulation). Subsequently, pcDNA-N (400 μg/hen) was injected into the inflamed area using a Twin-Jector® EZII needle-free injector (JCR Pharmaceuticals, Hyogo, Japan) on the first day at week 0. Additionally, 1 mL of each adjuvant (2% λ-carrageenan or FCA) was injected into the area around the pcDNA-N injection site. After the initial immunization, the immunization was repeated three times at two-week intervals. For Groups 4 and 5, 1 mL/hen of either rRV-N in 0 M Urea or rRV-N in 4 M Urea (0.5 mg/mL) was emulsified with an equal amount of FCA, and an immune injection was performed. Boost immunization was performed three times every two weeks by emulsifying each antigen solution (0.5 mL) with an equal amount of FIA.

Eggs were collected daily. Egg yolks obtained from individual hens every two weeks (after the first immunization) were diluted two-fold with 0.1% sodium azide solution and stored at 4 °C until the measurement of antibody activity. Other egg yolks were pooled every two weeks, homogenized, and frozen at −30 °C for IgY purification.

ELISA of diluted egg yolk

ELISA was performed according to a modified version of the method described by Kubo *et al*. (2022a). Samples were egg yolks from each hen, collected eight weeks after immunization. The RV vaccine was diluted 10-fold with ELISA-coated buffer (pH 9.6) and used to coat 96-well ELISA plates (Sumitomo Bakelite, Japan). The two-fold diluted egg yolks were further diluted 1,000-fold in Tris buffer (pH 7.4) containing 0.05% Tween 20, and added to each well as the primary antibody (50 μL/well). ALP-labeled anti-IgY (H + L) antibody (Abnova, Taiwan) was used as a secondary antibody at a dilution ratio of 4,000 times (100 μL/well). Disodium p-nitrophenyl phos-

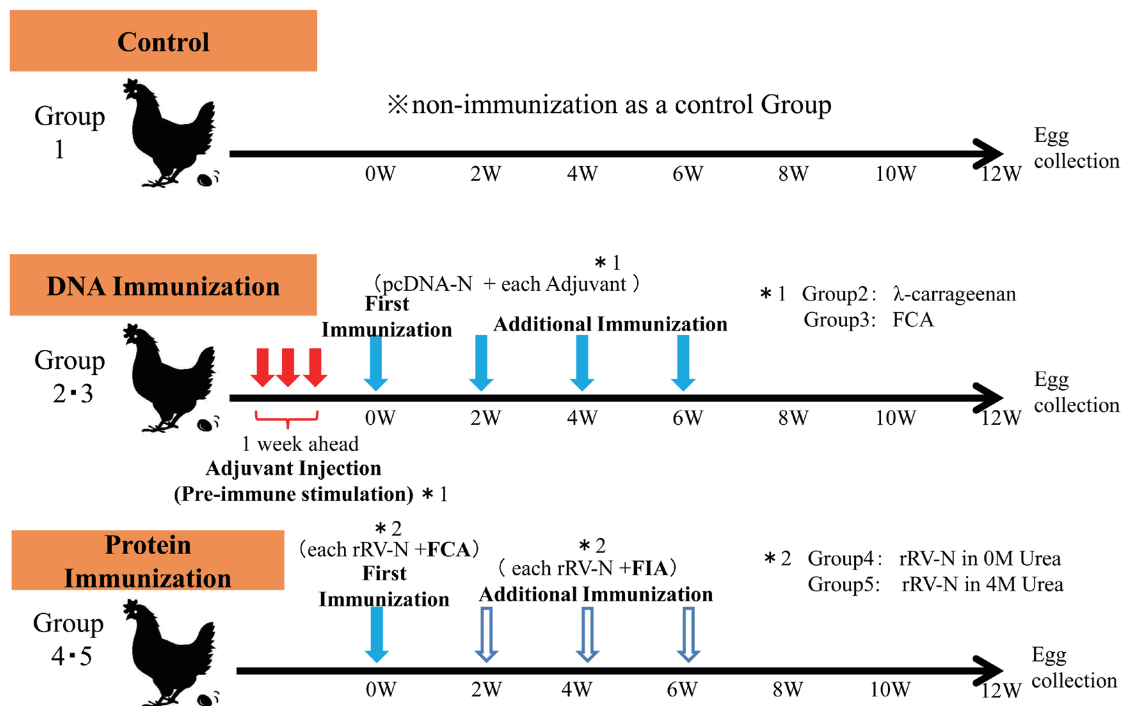

Fig. 1. **Immunization schedule**. FCA: Freund's complete adjuvant; FIA: Freund's incomplete adjuvant; W: week.

phate hexahydrate (pH 9.5, 1 mg/mL) was used as a substrate. The absorbance was measured at 405 nm (hereafter referred to as the ELISA value). The reaction temperature was 37 °C and the enzyme reaction time was 10 min.

Purification of IgYs

Purification of IgY from egg yolks was performed according to the protocol described by Hatta *et al*. (1990), with some modifications. Egg yolks showing higher ELISA values for one individual hen in Groups 2–5 at 6–12 weeks post-administration and egg yolks of one individual in Group 1 (control group) at 6–12 weeks post-administration were pooled together. These egg yolk samples were stored at −30 °C for more than two weeks to denature the egg yolk lipoprotein and then used for IgY purification. Purified IgY was pulverized after freeze-drying and stored at −30°C. The purity of the purified IgYs was determined using Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis method on a 5–20% polyacrylamide gel (c-PAJEL, ATTO, Japan). The gel was stained and photographed using Lumino Graph II (ATTO). The purity of each IgY antibody was determined as the ratio of heavy and light chains to the total protein fraction in the lane.

Antibody concentration and reactivity in ELISA

ELISA was performed to confirm the reactivity of each purified IgY antibody to the rabies vaccine. Each purified IgY antibody was diluted to a concentration of 0.1 to 100 µg/mL and subjected to ELISA as a primary antibody. A 10-fold diluted solution (50 µL/well) of the RV vaccine was used as the antigen and the other procedures were performed as described above. The enzyme reaction time was 10 min at 37 °C.

IHC staining

Paraffin sections of dog brains confirmed to be positive for RV by the standard direct fluorescence antibody test for rabies (Rupprecht *et al*., 2002) were deparaffinized and rehydrated. Antigen retrieval was performed using a Histofine solution (pH 9.0) (Nichirei Biosciences, Tokyo, Japan) in a microwave (170 W). Endogenous peroxidase activity was quenched using 3% peroxidase in methanol (room temperature for 20 min). The sections were subsequently incubated with 10% goat serum blocking solution (Nichirei Biosciences) at room temperature for 60 min. The sections were then incubated with primary antibodies (diluted 500-fold antigen-specific IgY, 20 µg/mL) overnight at 4°C. After washing with PBS (pH 7.4), the samples were incubated with a secondary antibody [1000-fold diluted anti-chicken IgY (IgG); Sigma Aldrich, Japan] at room temperature for 30 min. After washing with PBS, the immunoreactions were visualized using a 3-3′-diaminobenzidine substrate kit (Nichirei, Japan). The samples were counterstained with hematoxylin. Finally, the sections were immersed in Clear Plus (Falma, Japan), sealed with ProLong® Gold anti-fading encapsulant (Molecular Probes, USA), and observed under an optical microscope (400× magnification).

ELISA using denatured antigen and purified IgY

The RV vaccine was denatured by formalin and heat treatments and coated onto ELISA plate wells to reproduce viral

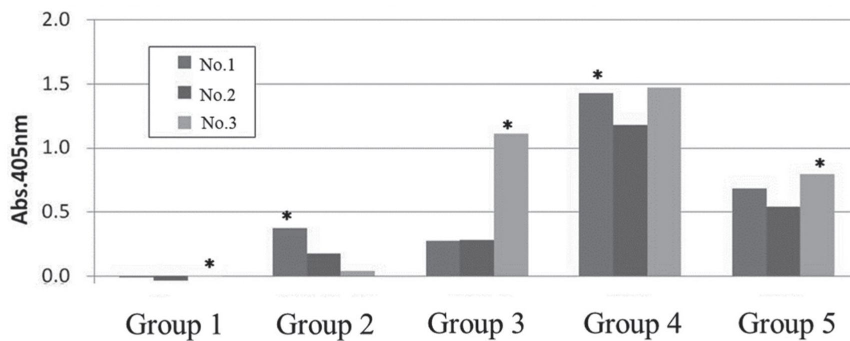

Fig. 2. **Comparison of IgY ELISA values (absorbance at 405 nm) in egg yolk**. The results of the ELISA performed using egg yolk collected at the 8th week are shown. ELISA was performed for 10 min at 37°C. Egg yolks from individual hens (indicated by *) were used for IgY purification. Group 1, control; Groups 2 and 3, DNA immunization (with pre-immune stimulation) using λ-carrageenan or Freund's complete adjuvant, respectively; Groups 4 and 5, conventional protein immunization with rRV-N in 0 M Uurea or rRV-N in 4 M Urea, respectively.

protein denaturation during IHC sample preparation. Then, the reactivity of purified IgY was examined. Four RV antigens were prepared: untreated (I: vaccine produced by KMB), 10% formalin treated (II: formalin-treated vaccine), 10% formalin-treated and heated at 60°C for 30 min (III: formalin + 60°C, 30 min vaccine), and 10% formalin-treated and heated at 60°C for 30 min followed by heating at 90°C for 5 min (IV: formalin + 60°C, 30 min + 90°C, 5 min vaccine). Each of these four antigens was diluted with a coating buffer (pH 9.6) to a 10-fold dilution with the stock solution (commercially available vaccine), and the 10-fold dilution was repeated twice to prepare 100- and 1,000-fold diluted solutions. These solutions (50 µL/well) were used to coat the wells of the plates for ELISA. The primary antibody concentration was 10 µg/mL and the other procedures were performed as described above. The enzymatic reaction time was 10 min at room temperature.

Statistical analyses

The ELISA values of various IgYs were analyzed using one-way analysis of variance, followed by the Tukey-Kramer post-hoc test. These analyses were performed using Bell Curve for Excel software (Social Survey Research Information, Tokyo, Japan).

Results

ELISA of the egg yolk of immunized hens

Fig. 2 shows the data of IgY antibody activity (ELISA values) obtained at the eighth week from egg yolks, as shown in a previous report (Kubo *et al.*, 2022b), to identify the five types of IgY-purified immune egg yolks used in this experiment. In the DNA immunization groups (Groups 2 and 3), ELISA values (absorbance at 405 nm) in the egg yolk of one hen in each group increased. In contrast, after protein immunization (Groups 4 and 5), the ELISA values of IgY in all hens increased.

IgY purification

The IgY antibody was purified from the pooled egg yolk of one hen each in Groups 1–5. Purified IgY included Control IgY, pcDNA-N with λ-carrageenan IgY, pcDNA-N with FCA IgY, rRV-N with 0 M Urea IgY, and rRV-N with 4 M Urea IgY. The yield of each IgY antibody per 100 g of pooled egg yolk ranged from 0.338 g to 0.682 g. The purity of each IgY antibody ranged from 86.7% to 90.1%.

Reactivity of IgY concentration with antigen (ELISA)

Fig. 3 shows the ELISA results when the concentration of the purified IgY antibody ranged from 0.1 to 100 µg/mL. At 100 µg/mL, ELISA values (absorbances at 405 nm) were approximately 1.6–2.5 for each antigen-specific IgY antibody. However, at 10 µg/mL, the difference in ELISA values increased. The highest value was rRV-N in 0 M Urea IgY, followed by pcDNA-N with FCA IgY, rRV-N in 4 M Urea IgY, and pcDNA-N with λ-carrageenan IgY. At the 1 µg/mL concentration, the ELISA value of rRV-N in 0 M Urea IgY was approximately 0.7; however, there was almost no reaction with other IgYs (ELISA value of 0.3 or less).

Virus detection in RV-infected dog brain tissues by IHC

Fig. 4 shows representative results of IHC staining of brain sections (hippocampus) of dogs infected with RV. No antigen was detected in the brain section using two specific IgY antibodies (pcDNA-N with λ-carrageenan IgY and pcDNA-N with FCA IgY) prepared by DNA immunization (Fig. 4, II and III). By contrast, viral antigens were detected using two IgY antibodies (rRV-N in 0 M Urea IgY and rRV-N in 4 M Urea IgY) prepared by protein immunization (Fig. 4, IV and V). Better staining results were obtained for rRV-N in 0 M Urea IgY.

Reactivity of purified IgY antibody against denatured antigen by ELISA

Fig. 5 shows the reactivity of IgY antibodies with four differ-

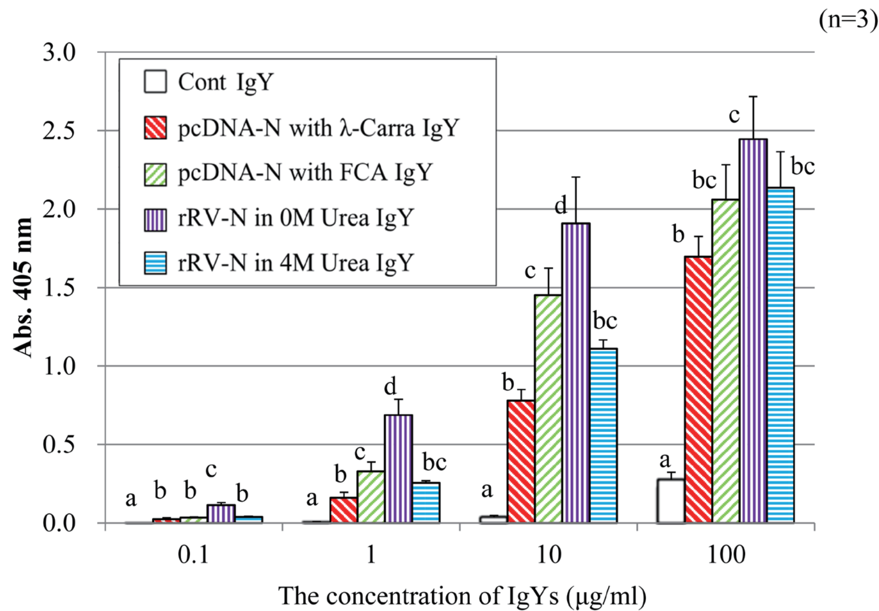

Fig. 3. **Reactivities of purified IgYs against RV-N vaccine (ELISA)**. The reactivity of each purified IgY was demonstrated when the rabies virus vaccine was used as the solid-phase antigen. Data represent mean ± SD (n = 3). Different lowercase letters above bars indicate statistically significant differences ($P < 0.05$) for each IgY dilution.

ent vaccines (I, undenatured vaccine; II, 10% formalin-treated vaccine; III, 60 °C, 30 min heat-treated vaccine after formalin treatment; and IV, 60 °C, 30 min and 90 °C, 5 min heat-treated vaccine after formalin treatment). When the untreated (undenatured) vaccine was used at a high concentration (F10) as the coating antigen, ELISA values did not show statistically significant differences between pcDNA-N with FCA IgY and IgY prepared by protein injection (Fig. 5, I). By contrast, when the vaccine was treated with formalin followed by 60 °C and 90 °C heating and used at a lower concentration (F100) as the coating antigen, the ELISA values of pcDNA-N with FCA IgY decreased significantly compared with those of IgYs made by protein injection (Fig. 5, IV).

Discussion

In this study, we compared the properties of specific IgYs against the RV-N protein using DNA immunization and conventional protein immunization methods. Conventional protein immunization appears to be more reliable and efficient for preparing specific IgY in egg yolks. However, DNA immunization with pre-immune stimulation using FCA was comparable to the conventional method for inducing equivalent IgY activity by ELISA. Changes in IgY activity and inflammation levels in each group have been described in detail in a previous report (Kubo *et al.*, 2022b).

The isolation of purified proteins as antigens for conventional protein immunization methods requires skillful techniques. In contrast, during DNA immunization, vector DNA is easily obtained by amplifying and purifying a plasmid containing the gene encoding the protein antigen in *E. coli*. As an additional technique for DNA immunization, pre-immune stimulation of laying hens with an adjuvant increases the activity of specific IgY antibodies. The effectiveness of pre-immune stimulation is due to the migration of various immune cells at the site of pre-immune stimulation due to inflammation.

We investigated the characteristics of each purified IgY sample using ELISA and IHC. The ELISA value for pcDNA-N IgY with FCA was equal to that of rRV-N with 4 M Urea IgY. Although it was expected that this pcDNA IgY could be used as a primary antibody in rabies testing by IHC, IgYs prepared by DNA immunization could not detect the Negri body. This was assumed to be due to denaturation by the protein antigen in the IHC samples. There are three steps in IHC procedures that could result in protein denaturation: (1) formalin fixation, (2) deparaffinization treatment (heating at 60°C for 30 min), and (3) antigenicity activation treatment by microwave (heating at 170 W for 10 min with a maximum temperature of 90 °C).

The causes of the non-detection of RV-N in IHC using IgYs prepared by DNA immunization were inspected by ELISA using non-treated or denatured RV-N. RV-N in the commercial vaccine (KM Biologics, 2018) is regarded as a non-denatured antigen protein. The treatments for coating the antigens imitated the three steps of IHC described above. In brief, ELISA was performed using the rabies vaccine as antigens, which was treated with for-

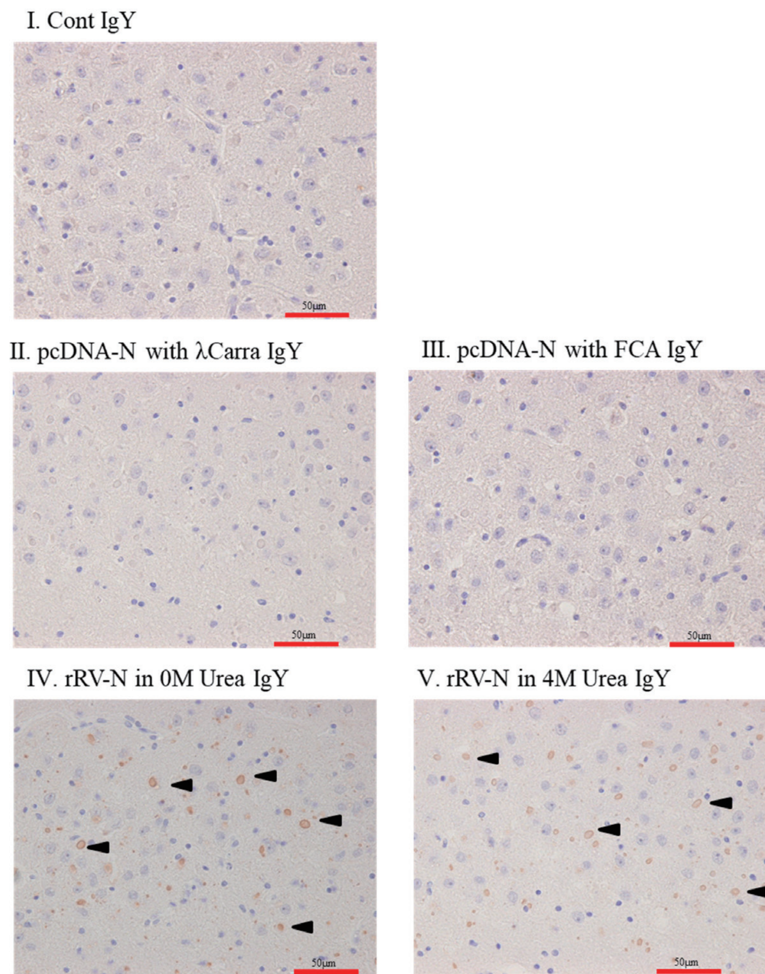

Fig. 4. **Immunohistochemical (IHC) staining**. Brain sections (hippocampus, formalin-treated) of rabies-infected dogs were used as samples for IHC and observed under an optical microscope (400×). Rabies virus inclusion bodies (Negri bodies) are stained red in IV and V (arrowheads).

malin and heating (60°C, 90°C), and the concentration of each antigen was changed.

The results showed that IgY prepared by DNA immunization had weaker reactivity with denatured antigens and lower antigen concentrations than IgY prepared by protein immunization. Moreover, the result corresponded with the results of IHC (especially for vaccine IV at a concentration of F100). In contrast, the ELISA values of IgYs produced by protein immunization slightly increased after formalin treatment, especially for rRV-N in 4 M Urea IgY. This may be because the purified rRV-N was already denatured when injected into the hens. These differences in reactivity against denatured and low-concentration antigens may explain the IHC results.

It should be noted that antigen activation treatment was initially performed in IHC to remove the effect of formalin masking the epitope, but this does not entirely restore the three-dimensional structure of the epitope to its undenatured state (Ramos-Vara, 2005). Microwave heating, a common antigen activation method, did not positively affect antigen detection in IgY prepared by DNA immunization.

Specific IgY antibodies have been reported to be induced by DNA immunization, such as by introducing chicken cytokine genes into plasmid vectors (Cho *et al*., 2004; Sawant *et al*., 2011), modifying with gold particles (Witkowski *et al*., 2009), and injecting CpG oligodeoxynucleotides (Lu *et al*., 2008; 2009). However, there is currently no established method for preparing a specific IgY that can be practically applied as a test reagent or neutralizing antibody. The development of a DNA immunization method for preparing IgY antibodies with high binding activity is an opportunity for further consideration.

One characteristic of DNA immunization is that an antibody capable of recognizing the native three-dimensional structural

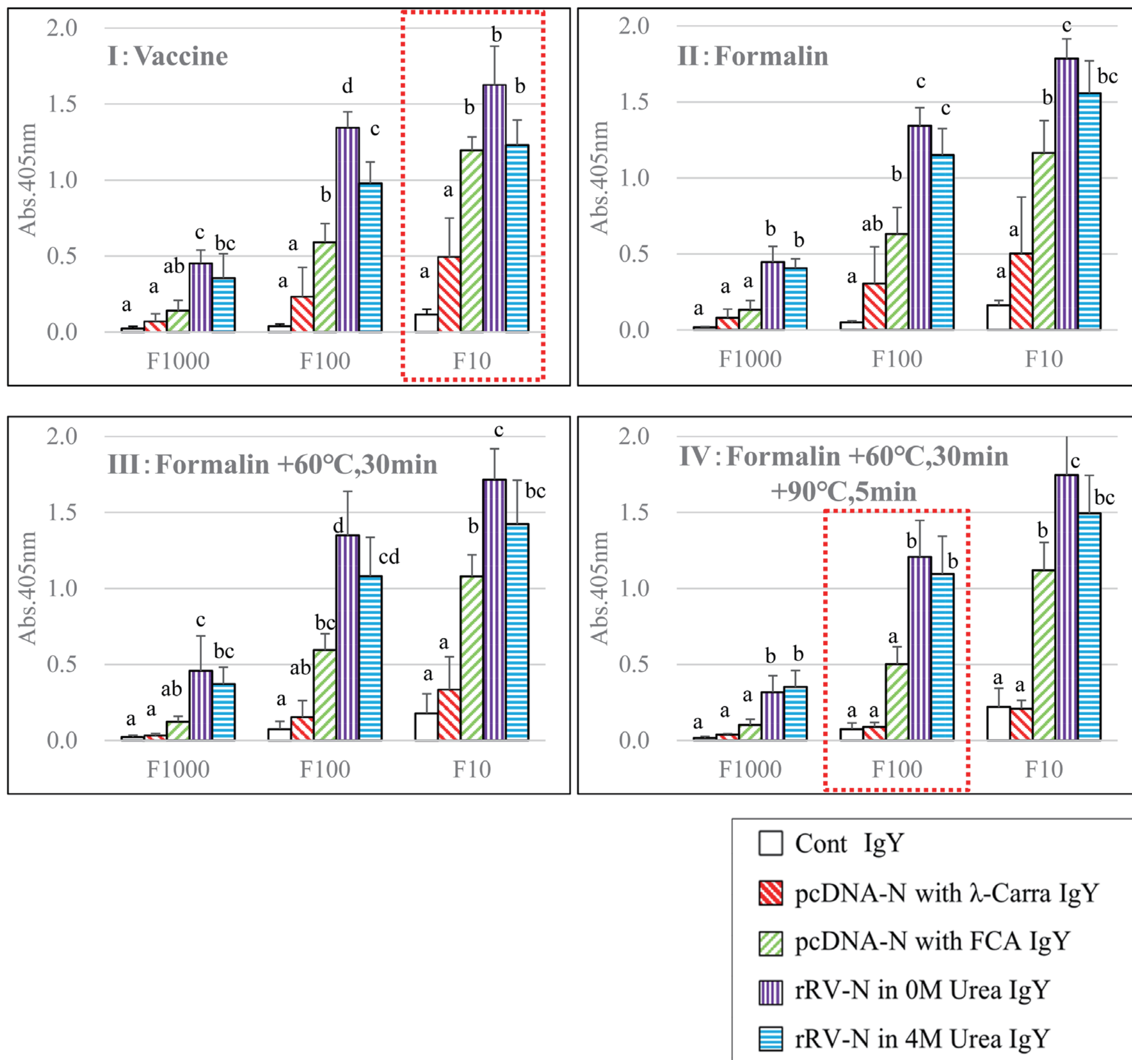

Fig. 5. **Evaluation of binding activity between denatured antigen and various IgY antibodies in ELISA**. Reactivities between various purified anti-RV-N IgY antibodies and untreated (I); 10% formalin-treated (II); 60°C, 30 min heat-treated after formalin treatment (III); and 60°C, 30 min and 90°C, 5 min heat-treated after formalin treatment (IV) antigens measured by ELISA. The horizontal axis shows the dilution ratio of the solid-phase antigen [10 (F10), 100 (F100), 1000 (F1000) times dilution from the vaccine stock solution]. Data represent mean ± SD (n = 3). Different lowercase letters above bars indicate statistically significant differences ($P < 0.05$) for each antigen dilution ratio.

epitope of a protein antigen expressed in animals can be obtained (Liu *et al.*, 2016). However, this study confirmed that reactivity against denatured antigens was slightly weaker. Therefore, even if an IgY showing high antibody activity against a native (undenatured) antigen can be prepared, it could be challenging to detect the denatured antigen using immunological methods such as IHC, in which the antigen is denatured by formalin and heat treatment. Therefore, these results suggest that attention should be paid to detection methods targeting native (undenatured) proteins and their use as antiviral infection-neutralizing antibodies.

In contrast, we detected viral antigens by IHC using IgY antibodies prepared by protein immunization. The reproducibility of the results from a previous study (Motoi *et al*. 2005b) was also confirmed. For rRV-N in 4 M Urea IgY, an immune injection was administered in a denatured state with 4 M Urea. The IgY antibody can stain antigen proteins using IHC. The reactivity to the viral antigen was lower than that of rRV-N in 0 M Urea IgY in both ELISA and IHC. Even though the antigen solution used for immune injection contained a high concentration of urea (4 M), the produced IgY showed high reactivity against the denatured antigens.

Protein immunization, which is a conventional method, re-

quires advanced techniques and considerable time, such as the expression and purification of recombinant proteins, to prepare antigens. However, this study demonstrates the possibility of inducing a specific antibody with a high probability that the IgY antibody prepared from the egg yolks of the immunized chicken has high antibody activity, and that a denatured antigen can be detected with high sensitivity. Intense antibody activity can be obtained with a higher probability compared to that obtained with the DNA immunization method because the immune injection is performed using more antigen proteins than the minimum amount required to elicit an immune response.

In this study, IgY antibodies with high antibody activity were prepared by combining DNA immunization with pre-immunization stimulation using adjuvants. However, these antibodies had weaker reactivity with denatured and lower-concentration antigens than IgY prepared by protein immunization. In addition, IHC showed difficulty in detecting viral antigens denatured by formalin and heat treatment. For DNA immunization, it is necessary to develop a method for inducing IgY antibodies that strongly bind to native and denatured antigens to prepare specific IgYs that can be used for antigen detection in clinical tests. It is also important to consider how to best utilize IgY antibodies prepared by DNA immunization to take advantage of their ability to recognize antigen proteins in their undenatured state, such as viral-neutralizing antibodies and antibodies used in flow cytometry.

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Author Contributions

Nanase Kubo: investigation and draft writing; Chun-ho Park: investigation of IHC; Satoshi Inoue: provision of resources; Hajime Hatta: supervision and draft review.

Conflicts of Interest

The authors declare no conflict of interest.

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