

## Short Communication

# Detection of Rabies-Specific Antigens by Egg Yolk Antibody (IgY) to the Recombinant Rabies Virus Proteins Produced in *Escherichia coli*

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**SUMMARY:** We obtained rabies-specific egg yolk antibodies (IgY) by immunizing hens with recombinant His-tagged nucleoprotein and phosphoprotein (rN, rP) of the rabies virus (CVS-11 strain) expressed in *Escherichia coli*. The anti-rN and rP IgY were shown to bind specifically to the respective proteins of the CVS-11 strain of rabies virus by Western blotting, immune fluorescent assay and immunohistochemistry, indicating that IgY to rabies recombinant proteins could serve as a reagent for diagnosis of rabies virus infection.

Rabies is a serious public health problem in developing countries, especially in Asia. Approximately 35,000 to 50,000 human deaths occur due to rabies each year (1). Administration of rabies vaccine along with anti-rabies immunoglobulin is known to prevent development of rabies; however, prompt and precise diagnosis is essential. For rabies diagnosis, direct immunofluorescence detection of the antigens of the rabies virus has been used worldwide as the most rapid and reliable method.

Most rabies-specific antibodies used for diagnosis are made from sera of immunized mammals such as mice, rabbits and goats. However, producing a large amount of specific antibodies from these animals is time-consuming and labor-intensive. Additionally, the preparation of rabies antigen is required for immunization of animals. There is a concern that handling live and large amounts of rabies virus to produce antigen may pose a potential risk of infection to laboratory personnel.

Recent advances in molecular biology together with a newly invented method of producing antigen-specific antibodies in egg yolk (IgY) have created new opportunities to develop a safe, convenient and inexpensive way of manufacturing various immunodiagnoses (2, 3). These methods have already led to the development of orally administered agents for the prevention of enteric colibacillosis (4), dental caries (5) and human rotavirus infection (6). The method of producing IgY antibody has some advantages over the production of antibodies from mammals in that (i) there is no need to bleed the animals, (ii) it is easy to purify a large amount of antibody; and (iii) it is feasible to produce a specific antibody to a small amount of antigen that is poorly immunogenic in mammalian hosts (2,7,8).

In this report, we have developed a method to produce a large amount of rabies virus-specific antibodies by immunizing layer hens with recombinant rabies virus internal

proteins expressed in *Escherichia coli*.

Preparation of the recombinant nucleoprotein (rN) of rabies virus was carried out according to the protocol reported by Inoue et al. (3). The recombinant P protein (rP) was prepared similarly. The primers used for amplification of the P gene were RabiesPV-P5-SalI (designed to include a *SalI* site [CCG TCG ACA TGA GCA AGA TCT TTG]) and RabiesPV-P3-PstI (designed to incorporate *PstI* site [TGG ACT GCA GCG GTT AGC AAG ATG TAT]). The PCR amplicon was inserted into the pQE-9 vector plasmid (QIAGEN, Valencia, Calif., USA) so that six consecutive histidine residues (His-Tag) could be attached. The rN and rP were expressed in *E. coli* DH5 $\alpha$  after transformation by plasmid DNAs. The recombinant proteins were purified using a nickel-nitrilotriacetic acid column (QIAGEN), and the expected sizes of purified rN and rP were confirmed by SDS-PAGE.

Immunization of hens was performed by intramuscularly injecting Goto's MOMIJI laying hens with 0.36 mg each of antigen, rN or rP, emulsified in 1 ml of Freund's complete adjuvant. Booster shots were also given intramuscularly at 2-week intervals after the first injection with the half dose of the antigen emulsified in 0.5 ml Freund's incomplete adjuvant. Eggs were collected every day and egg yolks were isolated. Purification of IgY from egg yolk was carried out according to the protocol of the  $\lambda$ -carrageenan method described by Hatta et al. (9). In brief, the stored egg yolks (200 g) were mixed with 600 ml of 0.5% NaCl and homogenized. The homogenate was mixed with 400 ml of 0.4%  $\lambda$ -carrageenan solution. The mixture was left for 1 h at room temperature followed by centrifugation at 7,000  $\times$  g for 30 min. The supernatant was filtered, precipitated by 15% (w/v) sodium sulfate three times and dialyzed against 10 mM disodium hydrogenphosphate. Purified IgY fractions were stored at  $-30^{\circ}\text{C}$  until use.

For the immunofluorescent assay (IFA), the confluent MNA cells in a 96-well plate were infected with  $2 \times 10^2$  FFU of rabies virus, then incubated for 48 h at  $35^{\circ}\text{C}$ . After fixation with 70% cold acetone, the anti-rN IgY or anti-rP IgY was added. After incubation for 30 min at room temperature, the cells were washed and further incubated with FITC-conju-

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gated anti-chicken IgG (whole molecule) (SIGMA, St. Louis, Mo., USA) for 30 min. The presence of rabies-specific antigen was observed using a fluorescence microscope.

For the Western blotting analysis, the CVS-11 strain of rabies virus ( $10^7$  FFU/ml) in 20  $\mu$ l of SDS gel-loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 3.75% 2-Mercaptoethanol) was subjected to SDS-PAGE using 12% gel after boiling for 10 min. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. The membranes were immersed in 5% skim milk solution in PBS(-) for 30 min to block nonspecific reaction and then incubated for 45 min with 3  $\mu$ l of 1 mg/ml of purified anti-rN IgY or 0.2  $\mu$ g/ml of anti-rP IgY. The membranes were washed four times in PBS(-) containing 0.05% Tween20 and incubated for 30 min with 3 ml of 1:1,000 diluted alkaline phosphatase-conjugated anti-chicken IgG (SIGMA). After washing, the immune complex was detected by staining with Western Blue (Promega, Madison, Wis., USA).

To ascertain that both IgY could detect rabies virus antigen in the neurons of infected mice, immunohistochemical analysis of formalin-fixed mouse tissue infected with CVS-11 was performed according to the procedure described by Inoue et al. (3), with the exception that anti-rN or rP IgY and horseradish peroxidase-conjugated anti-chicken IgG (ICN/CAPPEL, Aurora, Ohio, USA) were used as the primary and secondary anti-bodies, respectively.

The rN and rP purified from *E. coli* lysates using the affinity columns was subjected to SDS-PAGE analysis (Fig. 1). The rN was detected as a single band of 52 kDa molecular mass. The rP migrated slightly slower than the P protein derived from virus particles, probably due to the presence of His-tag (Figs. 1, 4). This result indicated that both proteins were successfully purified.

We immunized layer chickens with the respective recombinant proteins, then collected the egg yolks and determined the antibody titers by IFA. As shown in Fig. 2, the anti-rN IFA titer increased and reached a peak (1:4,000) at 4 weeks after immunization, then declined and remained at a titer of 1:500 for 4 weeks. On the other hand, the anti-rP IgY titer increased to 1:4,000 at the end of the second week and reached its peak (1:32,000) at the end of the third week. The titer remained high (1:32,000) for 3 weeks thereafter, then gradually declined. Representative results of the five IFAs performed

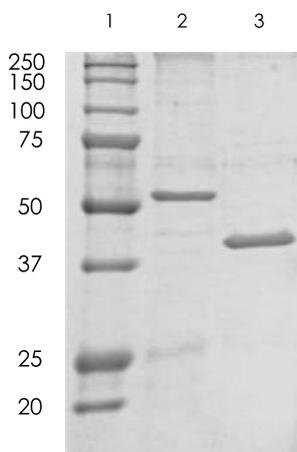


Fig. 1. SDS-PAGE analysis of rN and rP protein expressed in *E. coli*. The 12.5% polyacrylamid gel and tris-Tricine buffer were used for electrophoresis. Lane 1, molecular weight marker; lane 2, purified rN; lane 3, purified rP.

using purified IgY obtained from egg yolks of the immunized chickens are shown in Fig. 3. The fluorescent antigens were detected in the cytoplasm of infected cells.

To be sure that these antibodies were directed to rabies virus proteins, Western blotting analysis was carried out. As

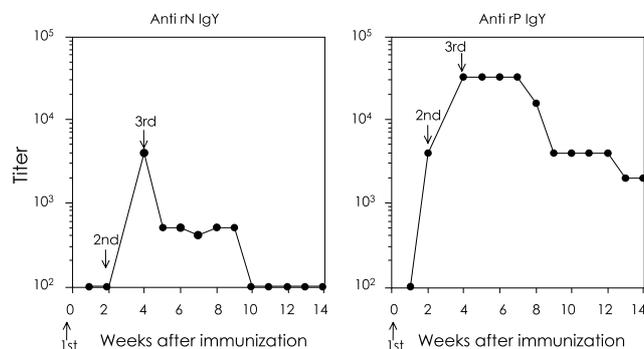


Fig. 2. Antibody titer of IgY obtained from a hen at various days after immunization with rN or rP protein. Antibody titers were measured by immunofluorescence assay. Booster inoculations were given at 2 and 4 weeks after the primary inoculation.

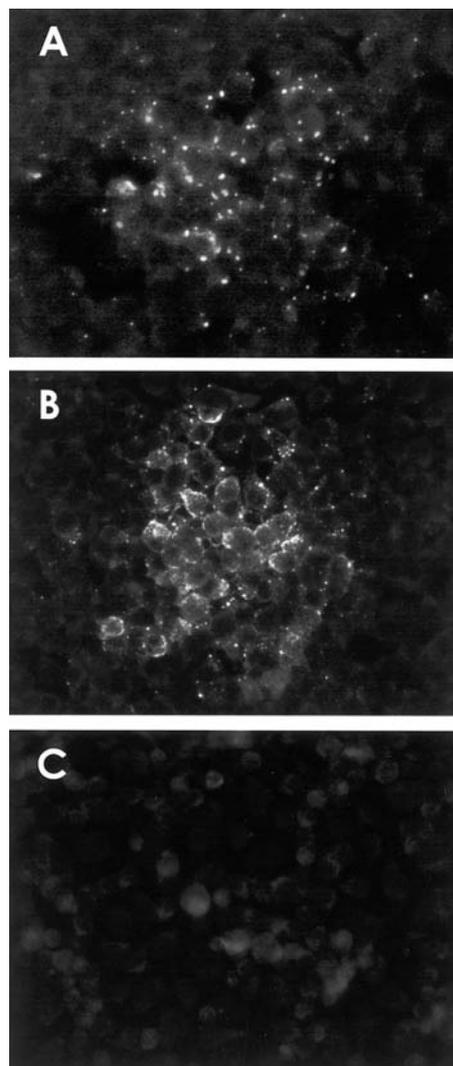


Fig. 3. Detection of viral antigen by immunofluorescence assay. MNA cells infected with CVS-11 were stained with anti-rN IgY, 10  $\mu$ g/ml (A), anti-rP IgY, 2.5  $\mu$ g/ml (B), non-immunized IgY, 10  $\mu$ g/ml (C) followed by FITC-labeled anti chicken IgG.

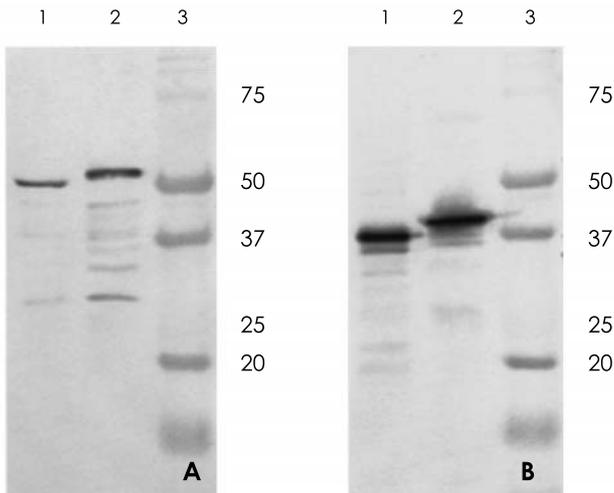


Fig. 4. Western blot detection of the CVS-11 virus protein and recombinant protein with anti-rN IgY (A) or anti-rP IgY (B). (A) Lane 1, CVS-11 virus protein; lane 2, rN protein; lane 3, molecular weight marker. (B) Lane 1, CVS-11 virus protein; lane 2, rP protein; lane 3, molecular weight marker.

shown in Fig. 4, anti-rN IgY and anti-rP IgY specifically bound to the proteins with molecular weights corresponding to the N or P proteins of rabies virus, respectively.

We next investigated whether these antibodies could be applied to immunohistochemical detection of rabies-specific antigens. For this purpose, the trigeminal ganglion obtained from a rabid mouse was subjected to immunohistochemical staining using anti-rN IgY (10  $\mu$ g/ml) and anti-rP IgY (5  $\mu$ g/ml). Paraffin sections of formalin-fixed tissues were deparaffinized and incubated with IgY antibodies. As shown in Fig. 5, viral antigens were detected in the cytoplasm of neurons by either antibody, indicating that anti-rN IgY and anti-rP IgY recognized the authentic N and P proteins of rabies virus, respectively.

Production of rabies virus-specific antibodies requires immunization of animals with inactivated rabies virus. Preparation of inactivated virus must be conducted in a specially designed facility where highly pathogenic viruses can be handled safely. It would thus be ideal if antigens could be prepared without propagating infectious virus. The expression of viral proteins in bacterial cells not only can provide a large amount of antigens for immunization but also can be achieved very cheaply. We observed that the N protein expressed in *E. coli* migrated as the 52 kDa protein in the SDS-polyacrylamide gel slightly more quickly than the N protein from virus particles of the CVS-11 (Figs. 1, 4). The rP also migrated slightly more slowly than the authentic P protein. The difference in mobility in the gel between the recombinant and authentic proteins was probably due to the presence of the His-tag in the recombinant proteins. Antibodies prepared against the rN and rP reacted with the respective viral proteins synthesized in infected cells by either IFA or immunohistochemistry.

Using the  $\lambda$ -carrageenan purification method, we obtained about 1.2 g of purified IgY with a purity of 95% from about 180 g of egg yolk, which corresponded to approximately 14 eggs. It has been reported that about 1.4 g of specific antibody could be obtained from an immunized rabbit (6). In the present study, therefore, we showed that it is possible to obtain a nearly equivalent amount of specific antibody,

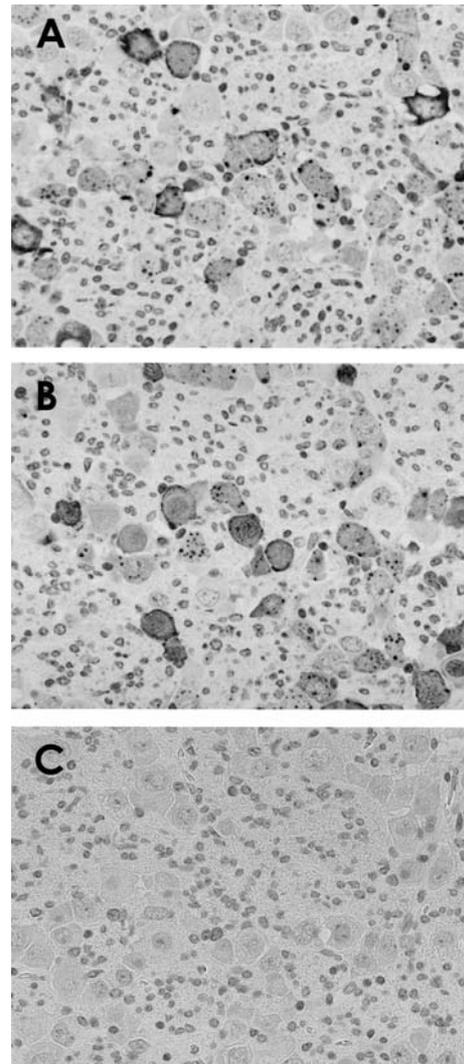


Fig. 5. Immunohistochemical detection of rabies virus in mouse tissue by anti-rN IgY or anti-rP IgY. The trigeminal ganglion of CVS-11 infected mouse was stained using anti-rN IgY (A) or anti-rP IgY (B), non-immunized IgY (C). Representative results of two independent experiments are shown.

without euthanizing animals, by immunization of a hen instead of a rabbit. In this way, by combining the expression of rabies N or P protein and the immunization of hens, it will be possible to supply a plentiful amount of antibodies for easy and inexpensive diagnosis of rabies.

The method reported here will provide rabies-endemic nations or developing countries with an opportunity to produce rabies-specific antibodies for the diagnosis of rabies.

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