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Use of IgY antibody to recombinant avian reovirus σC protein in the virus diagnostics

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Summary. – Avian reovirus (ARV) is an important agent of several diseases causing considerable losses in poultry farming. An outer capsid protein (σC) of ARV, is known as a virus-cell attachment protein essential for virus infectivity. In this study, the σC gene of ARV was cloned and expressed in *Escherichia coli*. The expressed recombinant protein was used as immunogen for raising a specific IgY antibody in laying hens. At 14 weeks post immunization, the antibody titers in serum and egg yolk reached 302,000 and 355,000, respectively. The IgY antibody was capable to neutralize ARV in BHK-21 cells and it strongly reacted in ELISA with ARV but not with heterologous viruses. The IgY antibody detected ARV in field samples of infected animal tissues in dot blot assay. These results suggest that an efficient, economic and rapid diagnostics of ARV can be performed routinely using the IgY antibody against a recombinant ARV σC protein.

Keywords: avian reovirus; σC protein; IgY antibody

Introduction

Avian reovirus (ARV) belongs to the *Orthoreovirus* genus, one of the 12 genera of the *Reoviridae* family. ARV is an important cause of disease in poultry, especially in broiler breeds. Reovirus induced viral arthritis/tenosynovitis is associated with enteric problems, such as cloacal pasting, chronic respiratory syndrome, ulcerative enteritis, inclusion body hepatitis, increased malabsorption syndrome and mortality, which result in extensive economic losses to the poultry industry. Most birds encounter infection via the fecal-oral route, but also evidence of infection via the respiratory tract and egg transmission has been reported (Dobson *et al*., 1992; Jones, 2000; Olson *et al*., 1978; Van, 2000).

Orthoreoviruses contain 10 dsRNA genome segments which are packaged within a non-enveloped double protein capsid of about 70–80 nm in diameter. The genome segments are divided into three size classes L (large), M (medium), S (small), by polyacrylamide gel electrophoresis (Nibert *et al*., 1996). Amongst the S-class segments of ARV, the S1 contains three open reading frames that are translated into P10, P17, and σC proteins (Liu *et al*., 1997). The σC protein, also known as σ3, which is encoded by the third open reading frame (ORF) of the ARV S1 genome segment, has been identified as the cell-attachment protein. This protein is a minor component of the outer capsid (Schnitzer *et al*., 1982) and is associated with neutralization of virus infectivity (Wickramasinghe *et al*., 1993). Protein σC is involved in main roles of avian reovirus infection and pathogenesis (Grande *et al*., 2002).

Several methods have been used to detect antibodies to ARV, including agar gel immunodiffusion, virus neutralization test, indirect immunofluorescence, and RT-PCR (Jones, 2000). Although these methods are useful for the detection of ARV infection, most of them are laborious and time consuming (Van *et al*., 1976; Wood *et al*., 1986; Meanger *et al*., 1995). Recently, ELISA is much studied for the ARV diagnosis (Hsu *et al*., 2006; Xie *et al*., 2010; Yang *et al*., 2010). ELISA is sensitive, reproducible and easily automatized (Xie *et al*., 2010). But where ELISA results are equivocal, sera can be re-tested

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Abbreviations: ARV = avian reovirus; σC = capsid protein

by western blot analysis or indirect immunofluorescence (Jones, 2000). Development of antisera used in ELISA has a high production cost, and it is difficult to achieve high and stable antibody titers (Kuroki, 1999).

IgY antibody is recognized as a convenient source of polyclonal antibodies, with a comparatively simple and inexpensive method, as well as a large-scale production advantage, compared with mammalian antibodies (Kobayashi *et al*., 2004).

Molecular approaches to detection of ARV in infected tissues have been described by several authors. These include dot-blot hybridization (Liu *et al*., 1996; Yin *et al*., 1998). This method is relatively rapid and sensitive (Jones, 2000).

The aim of this study was to raise a specific IgY antibody against recombinant ARV σC protein and to use it for diagnostics of ARV in field samples by dot-blot immunoassay.

Materials and Methods

Virus. ARV (ATCC VR-826) samples were obtained from the American type culture collection (ATCC) and were stored at -70°C for use as positive controls.

Field ARV-infected tissue samples. Chickens at market age (ranging from 31 to 34-day-old), showing various clinical symptoms of infection, were sampled from broiler farm in South of Korea where a viral arthritis/tenosynovitis occurred in February and June 2012. The clinical signs were lameness, poor feathering and growth retardation. Aliquots of the tissue samples were stored at -70°C prior to analysis.

Cloning and expression of ARV σC protein in E. coli. Total RNA from infected tissue was extracted by using a Total RNA extraction kit (Intron Biotechnology, Korea) following the manufacturer's instructions. A primer for the specific detection of ARV was designed based on the alignment of the σC sequence of pathogens available in the GenBank database (Acc. No. AF330703). The ARV σC forward primer (5'-TAT GGATCC CTT GGG ATG GCG GGT CTC-3', *Bam*HI restriction site is underlined), the ARV σC reverse primer (5'-GTA AAGCTT CAC ACC TTA GGT GTC GAT GC-3', *Hind*III restriction site is underlined) was used to amplify the σC gene of ARV, respectively. Reverse transcription and subsequent PCR reactions were conducted using a Pro-DNi Termal Cycler (GnC Bio, Korea) with a DiaStar™ Onestep RT-PCR kit (Solgent, Korea). The reactions were incubated at 50°C for 30 min followed by 94°C for 15 min. Thermocycling was performed for 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 90 sec. Final polymerization was preformed at 72°C for 7 min. Amplification products were examined by electrophoresis in 1.5% agarose gel. The RT-PCR products were cloned into a T&A Plasmid vector (RBC, Taiwan) for recombinant plasmids construction. The extended σC insert was obtained from T&A/σC plasmid by digestion with *Bam*HI and *Hind*III restriction enzymes, and was then ligated overnight at 4°C with a standard T4 DNA ligase, into the *Bam*HI/*Hind*III-digested pQE-30 vector (QIAGEN, Netherlands). Construct contains his-tagged protein and the thrombin recognition site in frame with the 5'-end of the extened σC insert. Recombinant ARV σC were expressed in *E. coli* strain M15 (pREP4) by induction with 0.5 mmol/l IPTG at 37°C for 4 hr. Induced bacteria were pelleted by centrifugation at 10,000 x g for 10 min, and analyzed by SDS-PAGE. The inclusion bodies pellet was solubilized as in previously described procedure (He and Kwang, 2013). The unfolded ARV σC antigen was dialyzed against 2 l volume of 1x PBS for 12 hr at room temperature, using dialysis tubing (Sigma, Korea). The dialysis was repeated twice, followed by dialysis against 2 l volume of 1x PBS for 4 hr at room temperature. Refolded antigen was then subjected to protein concentration determination, SDS-PAGE analysis, western blot analysis, and ELISA to evaluate the antibody titer.

Western blot analysis. The σC protein was detected by monoclonal rabbit anti-his-tag antibody (1:2,000) (KOMA biotech, Korea), followed by AP-conjugated goat anti-rabbit IgG (1:10,000) (Sigma, Korea). Also σC protein was detected using a 1:10,000 dilution of egg yolk antibody obtained by immunization, followed by AP-conjugated rabbit anti-chicken IgY (1:10,000) (Bethyl, USA). Specific bands were visualized by NBT/BCIP.

Preparation of IgY antibody against ARV σC. Ten 48-week-old Hy-line Brown laying hens were kept in individual cages, and were provided with food and water *ad libitum*. They were immunized intramuscularly with the σC protein mixed with Freund's adjuvant at different sites of the pectoral muscle of each chicken. The dialyzed protein solution was then emulsified with an equal volume (500 µl) of complete Freund's adjuvant. The hens were boosted two weeks after the first immunization, with the second injection composed of incomplete Freund's adjuvant. The hens were boosted again two weeks after the second immunization. Blood samples were collected from wing veins and were stored at -20°C until analyzed. Eggs were collected daily, marked and stored at 4°C until analyzed. IgY antibody was extracted and purified from eggs using Kim's procedure, as previously described (Kim *et al*., 2000).

ELISA. Antibody titers in sera and egg yolk were determined by an indirect ELISA method, as previously described (Kim *et al*., 2000). In the same method, antibody specificity in egg yolk was determined using canine rotavirus, canine adenovirus, white spot syndrome virus, bovine coronavirus, and bovine rotavirus.

Virus neutralization test. BHK-21 cells (Korean cell line bank, KCLB) were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. BHK-21 cells were seeded in a 6-well plate (6 x 10^5cells/well) and were incubated at 37°C in a 5% CO_2 incubator. After 24 hr incubation, the growth medium was discarded and the cells were washed with PBS. 2 ml of DMEM was then added to the cells. Neutralization titer of the egg yolk antibody against σC antigen was analyzed by microneutralization assay as previously described (He and Kwang, 2013). Briefly, undiluted, 1:10 diluted, and 1:100 diluted IgY antibodies were applied onto BHK-21 cells grown in a 6-well plate. Simultaneously, the cells were infected with avian reovirus (ATCC VR-826). After 48 hr, the cytopathic effect was observed by microscope (Nikon, Japan).

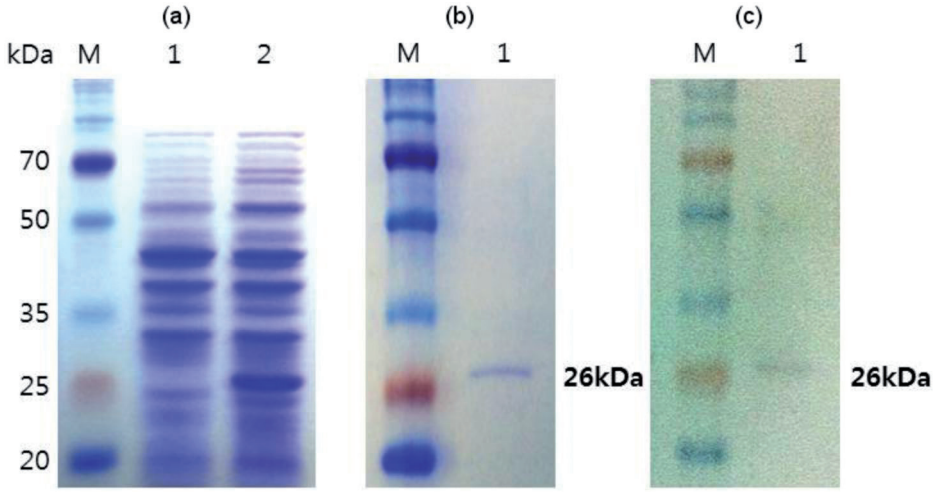

Fig. 1
Expression of recombinant ARV σC protein
(a) SDS-PAGE of extracts from *E. coli* transformed with the recombinant (lane 2) and empty plasmid (lane 1), respectively. (b) SDS-PAGE of solubilized σC (lane 1). (c) Western blot analysis of solubilized σC (lane 1). Protein size marker (lanes M).

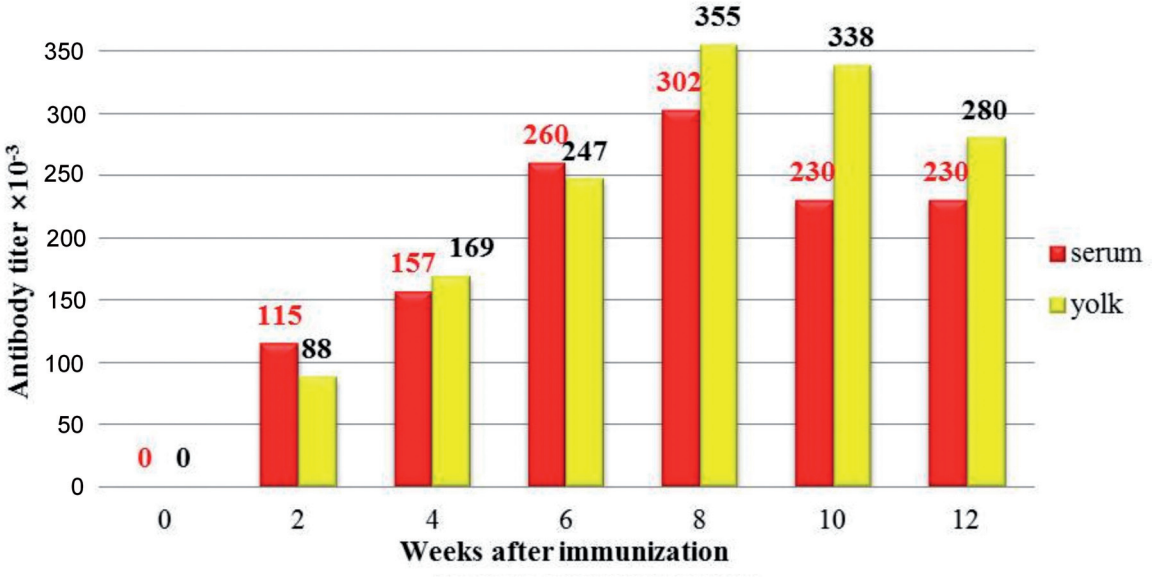

Fig. 2
Titers of IgY antibodies against recombinant ARV σC (ELISA)

Dot-blot assay of ARV in field samples. For the dot-blot assay, a nitrocellulose membrane (0.45 μm pore size; Amersham, UK) was cut into pieces. Antigen absorption was carried out by spotting 1 μl/dot of protein extracted from tissue by M-PER reagent (Pierce, USA). The membrane was air-dried and soaked in the blocking solution (5% skimmed milk in PBS-Tween 20) with gentle shaking for 15 min. After washing two times, by shaking for 1 min during each wash in PBS-Tween 20, the membrane was allowed to react with specific anti-ARV σC IgY antibodies (1:100,000) for 15 min. The membrane was washed again and incubated with rabbit anti-chicken immunoglobulins (1:10,000) (Bethyl, USA) for 15 min and finally washed to remove excess conjugate. The membrane was allowed to react with substrate solution for 5 min. After the appearance of the color, the membrane was washed with distilled water for several times. The development of well-defined blue dots on the nitrocellulose membrane was considered as positive.

RT-PCR. The total RNA from infected tissue was extracted by using a Total RNA Extraction kit (Intron Biotechnology, Korea). The primers S4 P4, S4 P5 (Bruhn *et al*., 2005) were used to detect ARV. RT-PCR reactions were incubated at 50°C for 30 min followed by 94°C for 15 min. Thermocycling was performed for 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1min and final polymerization was performed at 72°C for 7 min. Amplification products were examined by electrophoresis in 1.5% agarose gel.

Results and Discussion

Preparation of recombinant ARV σC

ARV σC was cloned from genomic RNA extracted from ARV infected tissue samples by RT-PCR, and the sequence was confirmed using an automated DNA sequencing system (GenBank Acc. No. AF330703). The amplified fragments were inserted between the *BamH*I and *Hind*III sites in pQE-30 plasmids to produce N-terminal his-tagged σC fusion protein. The resulting plasmids were designated as pQE-30/σC. ARV σC band with a molecular mass of 26 kDa was visible on SDS-PAGE in IPTG-induced pQE-30-ARV-σC-transformed bacteria but not in pQE-30-transformed control bacteria (Fig. 1a). Only shorter variant σC, (Mw 26 kDa) instead of full-length σC, (Mw 34 kDa) was expressed. Nevertheless the N-terminal region of σC expressed is responsible for variation and antigenicity between various isolates (Liu *et al*., 1997).

Recombinant his-tagged ARV σC was solubilized from the insoluble bacteria fractions by earlier reported method (Favacho *et al*., 2006). To use as immunogen, the solubilized protein was dialyzed using dialysis tubing. The resulting solution containing the refolded antigen was then determined using SDS-PAGE and western blot analysis (Fig. 1b and 1c). The protein concentration of the dialyzed protein was determined by BCA method, revealing a concentration of 600 μg/ml**.** The dialyzed product was used to generate IgY antibody in chickens.

Preparation and characterization of IgY antibody against ARV σC

The titers of specific avidity of anti-σC IgY antibody were determined both in sera and yolk of laying hens during the whole experimental period of immunization by ELISA. Ten weeks after injection, the titers of antibody in sera and yolk reached the peak of 302,000 and 355,000, respectively (Fig. 2). From that time on, the antibody titers decreased to about 200,000 to 300,000, respectively. It had been reported that antigen dose of 10–1,000 μg may more efficiently induce the antibody response (Erhard *et al*., 2000). The chickens were first immunized with recombinant σC protein at a dose of 300 μg per immunization. This dose of the antigen was most effective for the production of antibodies in chickens.

Specificity and virus neutralization

According to the results of the immuno-specificity test using ELISA, the IgY antibody against recombinant σC antigen strongly reacted with ARV (Table 1). These results suggest that the recombinant σC could be used as an immunogen to produce specific antibody (IgY). In order to investigate the neutralization titer of IgY antibody, a neutralization test was performed with reference to the paper of He and Kwang, 2013, using avian reovirus (Fashy-Crawley clone-1, ATCC VR-826) and BHK-21 cells. ARV reacted with the recombinant σC IgY antibody, followed by inoculation into BHK-21 cells. The degree of virus neutralization was determined by observation of the cytophatic effect 48 hrs after inoculation. Undiluted IgY antibody, strongly inhibited the infection (Fig. 3c) while the 10-fold diluted IgY antibody inhibition was weaker (Fig. 3d). The 100 fold diluted IgY antibody could not neutralize the virus (Fig. 3e). The IgY antibody blocked the interaction between viral particles and the cellular receptors by recognition of neutralizing epitope of ARV, and blocking the viral attachment.

Detection of ARV in field samples

The dot-blot assay procedure using IgY antibody was performed to test the presence of ARV in field samples. A total of ten samples were collected from poultry farm in Asan provinces of Korea in 2012. We used the joint tissues in this experiment. According to previous work, isolation of reovirus from the joints may be considered as diagnostic, because isolation from gut tissue or feces may be meaningless in view of the widespread nature of reovirus infection (Jones, 2000).

Joint tissue samples from chickens with ARV gave deep blue color dots, in comparison to healthy one (Fig. 4). To

Table 1. Specificity of the IgY antibody against ARV σC protein

Antigen	Dilution	
	<1:100,000	<1:150,000
Canine rotavirus	–	–
Canine adenovirus	–	–
White spot syndrome virus	+	–
Bovine coronavirus	–	–
Bovine rotavirus	–	–
Avian reovirus	+++	+++

(+++) titer > 100,000; (++) 100,000 > titer > 50,000; (+) 50,000 > titer > 10,000; (–) titer < 1,000.

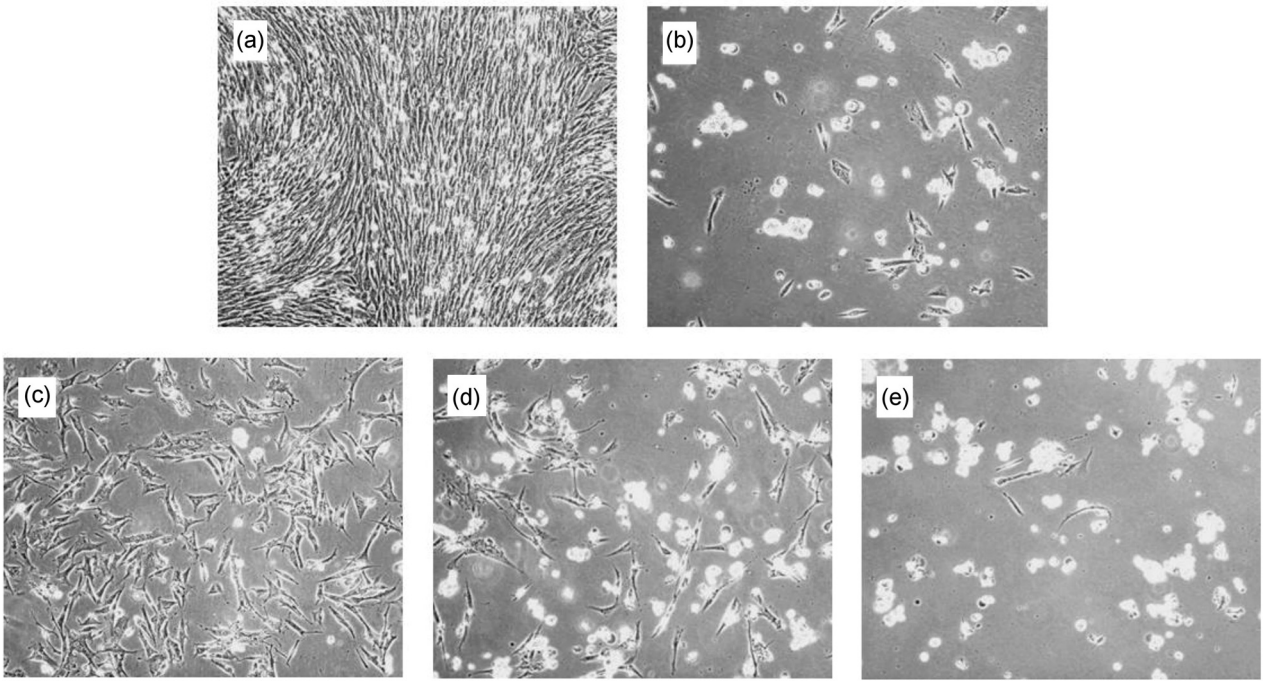

Fig. 3
Neutralization of ARV in BHK-21 cells with IgY antibody against recombinant ARV σC
Light microscopy 48 hr post infection Non-infected (a) and infected cells (b). Infected cells incubated with undiluted (c), 1:10 diluted (d), and 1:100 diluted (e) antibody.

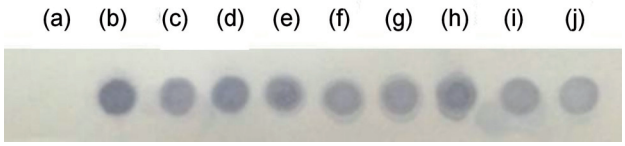

Fig. 4
Detection of ARV in field samples by dot-blot assay using the IgY antibody against recombinant ARV σC
Extracts from joint tissues of diseased chicken (b–j). Negative control (a).

Fig. 5
Detection of ARV in field samples by RT-PCR
Agarose gel electrophoresis of RT-PCR products. Extracts from joint tissues of diseased chickens (lane 2–10), negative control (lane 1), DNA size marker (lane M).

determine the suitable dilution of IgY antibody, various dilutions of IgY antibodies (1:1,000, 1:10,000, 1:100,000) were tested. The results suggested that the dilution of ARV IgY antibody at 1:100,000 worked well in dot-blot assay. Our established dot-blot method could be used to detect infections in chicken tissues.

The data obtained from the different tissues using two techniques (dot-blot assay and RT-PCR) are shown in Figs. 4 and 5. For each ARV detection technique, ten of different tissues from individual chickens were compared. Two techniques showed same results, but dot-blot assay using IgY antibody is more rapid and economic than RT-PCR.

In this study, ARV σC was expressed in *E. coli*, and the specific IgY antibody was produced in laying hens. The antibody production between chickens and mammals was compared, and a remarkable amount of antibody (about 17–35 g of total IgY/chicken/year) can be produced from one hen, almost 10% of which is antigen-specific (Shuzihong *et al.*, 2012). This study demonstrated that anti-σC IgY antibody is specific, able to neutralize ARV and could be applied to diagnostics for ARV infection.

In conclusion, ARV σC was expressed in *E. coli* and IgY antibody was produced. Using this antibody, dot-blot assay was established for the detection of avian reoviruses

in field samples. This method would be very economical and could be used to detect ARV rapidly in on-farm conditions.

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