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Expression of bovine rotavirus VP8 and preparation of IgY antibodies against recombinant VP8

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Summary. – Group A rotavirus is the leading cause of acute gastroenteritis in cattle and swine. Although, vaccination against this virus is an effective strategy for prevention, additional strategy to control disease is necessary. Egg yolk immunoglobulin (IgY)-based passive immunization could be a better option in preventing this disease. Bovine rotavirus (BRV) is group A rotavirus and possesses a genome of 11 segments of double-stranded RNA. The outer layer of capsid is composed of two proteins (VP7 and VP4), which induce virus neutralizing antibodies. Trypsin cleavage of VP4 produces VP8 (28 kDa) and VP5 (60 kDa) fragments. Since a number of studies have demonstrated the induction of neutralizing antibodies using VP8 subunit vaccines, we have produced IgY against the recombinant VP8. The cDNA spanning the VP8 subunit was amplified from bovine rotavirus-infected cells and cloned into pET21d(+) expression vector to generate recombinant VP8. The resulting carboxy-terminal His-tagged VP8 proteins were expressed in *Escherichia coli* strain BL21(DE3) by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The recombinant proteins were purified using Ni-NTA agarose beads, and the purified protein was used as the immunizing agent to produce polyclonal antibodies in chicken. The resulting polyclonal antisera specifically recognized VP8 in Western blot assay and were able to neutralize BRV replication in cell cultures. These results demonstrate that IgY can be used in immunological assays and, in addition, in passive immunization of newborn calves against BRV.

Keywords: bovine rotavirus; egg yolk immunoglobulin (IgY); passive immunization; VP8

Introduction

Neonatal calf diarrhea is one of the most common and economically devastating diseases of the cattle industry. This disease can be caused by viruses, bacteria and protozoa. Since the discovery of bovine rotavirus in feces of calves suffering from diarrhea, rotavirus has been shown to be ubiquitous in most mammalian species including calves, sheep, swine and poultry (Bendali *et al.*, 1999; Dhama *et al.*, 2009; Estes and Kapikian, 2007). Group A rotaviruses are the leading cause of acute gastroenteritis in mammalian species including infants worldwide. Increase in morbidity and mortality caused by these viral infections leads to substantial economic losses (Papp *et al.*, 2013; Saif *et al.*, 1994). It is therefore important to develop control measures for the prevention of rotaviral disease.

Rotaviruses belong to the *Reoviridae* family and possess a genome of 11 segments of double-stranded RNA enclosed in a triple-layered icosahedral capsid (Shaw *et al.*, 1996). They are classified into 7 serogroups (A to G). The intermediate-layer capsid VP6 contributes to the group and subgroup specificity. Group A bovine rotaviruses are divided into G (glycoprotein) and P (protease-sensitive) genotypes. The outer-layer proteins VP7 and VP4 define two independent serological specificities of G and P genotype, respectively. To date, 27 G and 37 P genotypes have been identified in humans and animals. Several studies have shown that G6, G8, and G10 together with P[1], P[5], P[11], P[15], and

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Abbreviations: BRV = bovine rotavirus; IgY = egg yolk immunoglobulin

P[21] predominate in calve infections (Estes and Kapikian, 2007). VP4 is the spike protein of the virus and is associated with hemagglutination, neutralization and infectivity. Trypsin cleavage of VP4 produces VP8 (28 kDa) and VP5 (60 kDa). Studies with neutralizing monoclonal antibodies have identified eight neutralization epitopes on VP4. Five of them are located in the VP8 subunit of VP4 (Favacho *et al.*, 2006; Fernandez *et al.*, 1998; Grennberg *et al.*, 1983). VP8 of BRV can be a useful subunit vaccine candidate (Angel *et al.*, 2007; Rathi *et al.*, 2007).

IgY is the major antibody produced by laying hens. After immunization, IgY is secreted into the blood and transported to the egg yolk (Dias da Silver and Tambourgi, 2010; Hädge and Ambrosius, 1984). IgM and IgA are undetectable in egg yolk, but IgY levels are quite high with concentrations being 50–100 mg/egg yolk. One hen can produce 20 eggs per month and it is possible to obtain >20 g of IgY/year, as hens continually produce eggs for at least 10 months. IgY production has several advantages over conventional mammalian antibody production: chickens produce larger amounts of antibodies, IgY isolation is fast and simple, and egg collection is non-invasive (Jensenius *et al.*, 1981; Rose *et al.*, 1974). The use of IgY may help lower costs of immunological testing. IgY has been used in several studies for diagnostics and therapy (Chalbhoumi *et al.*, 2009; Dai *et al.*, 2013; Spillner *et al.*, 2012; Vega *et al.*, 2011). Decreased mortality and morbidity have already been reported during the feeding of calves with colostrum from vaccinated dams (Parreno *et al.*, 2010). As the administration of antibodies to newborn calves can be an alternative to the use of conventional vaccines, antibody-based passive immunization will reduce the incidence of diarrhea.

In this study, we produced IgY against the recombinant VP8 for immunological assay and prevention of BRV infection. This IgY VP8 can be used as a milk supplement within the immediate postnatal period for passive protection of newborn calves against BRV or it can provide valuable immune reagent for rotavirus diagnostics and epidemiologic surveys.

Materials and Methods

Cells and viruses. A fetal monkey kidney cell line, MA-104, was grown in monolayer in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. Bovine rotavirus (KVCC-VR9200179; 10^3 PFU/100 µl) was treated with 20 µg/ml trypsin for 1 h at 37°C and inoculated to the 1 x 10^6 MA-104 cells. After washing with Dulbecco's phosphate-buffered saline, the cells were maintained in serum free medium supplemented with 2 µg/ml trypsin. When infected cells showed 80% of the cytopathic effect (CPE), the cell supernatant was harvested and clarified by low speed centrifugation at 4°C and served as stock of the virus. The stock of bovine rotavirus was used for amplification of VP8 and neutralization assay.

PCR amplification. When complete cytopathic effect had been reached, total viral RNA was extracted from cell supernatant using the Trizol reagent according to the manufacturer's protocol (Invitrogen, USA). RNA was solubilized in 30 µl of diethylpyrocarbonate-treated water and stored at -20°C. cDNA was synthesized in a total volume of 20 µl using a Maxime RT premix kit (Intron Biotechnology, Korea). The VP8 forward primer, 5'-CCA TGG CTT CAC TCA TTT ATA GA-3', containing *Nco*I restriction site (underlined) and the VP8 reverse primer, 5'-CCC TCG AGG TCT TGA TTA GGT TGT GCT CT-3', containing *Xho*I restriction site (underlined), were used for amplification of VP8. The PCR was carried out in total volume of 20 µl containing: 1 µl of synthesized total cDNA, 1 µl of 10 pmol of each primer and 17 µl of 2 x EF-Taq premix (SolGent, Korea). The PCR cycles were: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, and final extension time of 5 min at 72°C. VP8 PCR product was electrophoresed in 1% agarose gel. The amplicon was cloned into pGEM-T Easy vector (Promega, USA) by T4 DNA ligase. The resulting plasmid, named pGEM-VP8, was used for transformation of competent *E. coli* DH5α cells.

Expression plasmid construction. The expression plasmid was constructed by inserting the *Nco*I and *Xho*I digested VP8 fragment into pET21d(+) vector (Novagen, Germany). New plasmid, designated as pET21d(+)-VP8, contained a hexa-histidine tag (6x His-Tag) in frame with the 3' end of the VP8 gene. It was used to transform *E. coli* strain BL21(DE3). The new recombinant strain was called BL21/pET21d(+)-VP8.

Expression and purification of VP8 in E. coli. The BL21/pET21d(+)-VP8 strain was cultured in fresh LB broth at 37°C. When absorbance of 600 nm reached the value of 0.8, expression of the 6x His-Tag-VP8 was induced by adding IPTG to a final concentration of 1 mmol/l. A sample of induced bacterial cells was taken every hour, and the cells were pelleted by centrifugation at 13,000 rpm for 1 min. The cells were resuspended in 50 µl of 1x SDS loading buffer and boiled for 5 min. The degree of expression was evaluated by SDS-PAGE. At 4 h post induction, the cells were harvested for protein extraction by centrifugation at 10,000 x g for 20 min at 4°C. The pelleted cells were resuspended in lysis buffer supplemented with 1% Triton X-100, 40 µg/ml phenylmethylsulfonyl fluoride (PMSF) and incubated at room temperature for 20 min. The suspension was freeze-thawed twice and centrifuged at 10,000 x g for 30 min at 4°C to obtain the supernatant and the pellet. Both, the soluble and the insoluble fractions (the inclusion bodies) were analyzed by SDS-PAGE. To solubilize the inclusion bodies, the pellet was washed with wash buffer A (0.01 mol/l Tris-HCl, 0.1 mol/l sodium phosphate buffer, and 2 mol/l urea, pH 8.0) and solubilized in denaturing buffer (0.01 mol/l Tris-HCl, 0.1 mol/l sodium phosphate buffer, and 8 mol/l urea, pH 8.0). After centrifugation, the supernatant and pellet were analyzed by SDS-PAGE. The supernatant containing 6x His-Tag-VP8 was purified using Ni-NTA agarose beads (Qiagen, Germany) as described previously (Jung *et al.*, 2014).

Immunization of chickens. One hundred μg of purified recombinant VP8 protein were used as an immunogen and 28 weeks old ISA-brown chickens were immunized three times in week 0, 2 and 4. For the first immunization, antigen was emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, Germany). Freund's incomplete adjuvant (Gibco/Thermo Fisher, USA) was similarly used for the subsequent boosting immunizations. Eggs were collected 28 days after initial immunization.

ID-ELISA. To determine specificity and the suitable dilutions of IgY, ID-ELISA was performed using serial dilutions (1/2000~1/32000) of IgY. Purified VP8 antigen was serially diluted to a concentration of 0~2 μg/ml. One hundred μl of each antigen in the coating buffer were added to each well of a 96-well microplate and incubated overnight at 4°C. Plates were blocked with 1x phosphate-buffered saline-0.05% Tween-20 (PBS-T) containing 5% skim milk powder for 1 h at 37°C. After washing with PBS-T, serial dilutions (1/2000~1/32000) of VP8 specific-IgY were added and incubated for 1 h. The bound IgY was detected with AP-conjugated rabbit anti-chicken IgY (Sigma-Aldrich). After 1 h incubation at 37°C, the plate was washed four times with PBS-T. Next, p-nitrophenyl phosphate disodium salt substrate (pNPP) (Thermo Scientific) was added and incubated for 15 min at 37°C. The absorbance value of the developed color was measured at 405 nm.

Characterization of IgY by Western blot. To confirm the specificity of IgY to VP8, Western blot was performed as previously described (Jung *et al.*, 2014). Briefly, purified VP8 was mixed with loading buffer, boiled for 5 min, and the samples were separated on 12% SDS-PAGE gels. The proteins were transferred onto nitrocellulose membranes by running at 80V for 2 h. The membranes were blocked in PBS-T containing 5% skim milk at 4°C overnight. VP8 protein was detected using IgY and a specific goat antibody for rotavirus (Goat polyclonal anti-bovine rotavirus antibody against NCDV(Nebraska calf diarrhea virus), ProSci, USA) at RT for 2 h. Alkaline phospatase-conjugated rabbit anti-chicken and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma-Aldrich) were used as secondary antibodies, respectively. Membranes were washed with 1x PBS-T and then visualized using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrates (Roche, Switzerland).

Neutralization assay. The neutralizing property of IgY produced in chickens against recombinant VP8 was determined on MA-104 cells in 6-well plate. Viruses (10^6 PFU/100 μl and 10^5PFU/100 μl) with antibody and trypsin (20 μg/ml) were mixed and activated for 60 min at 37°C. MA-104 cells were washed with PBS and activated mixtures were added to each well for infection, and then incubated for 2 h at 37°C. The plates were washed once, and DMEM (without serum) with 1.5% agarose overlay containing 5 μg/ml of trypsin and 0.3% bicarbonate phosphate was added to each well. After fixing with 3.7% formaldehyde, the overlay medium was removed and cells were stained with second overlay containing 1% crystal violet. To obtain neutralizing antibody titers, plaques were counted on the following day.

Results

Cloning of rotavirus VP8 subunit from VP4

MA-104 cells were cultivated in 6-well plates and infected with BRV. When infected cells showed 80% of CPE (Fig. 1), RNA was extracted from the culture supernatant to produce cDNA. The expected 769 bp cDNA encoding VP8 was amplified by RT-PCR and the sequence was confirmed using an automated DNA sequencing system. BRV strains showed high amino acid identities (97%) to the G5P[1] rotaviruses, from which novel G genotype was previously reported in cattle in Korea (Park *et al.*, 2006)

Expression of the VP8 protein in E. coli

The amplified fragment was inserted between the *Nco*I and *Xho*I sites in pET-21d(+) to produce a C-terminal His-tagged VP8 fusion protein. The resulting plasmid was designated as pET-21d(+)-VP8. SDS-PAGE revealed that the recombinant VP8 band with a molecular mass of 30 kDa was expressed successfully in IPTG-induced pET-21d(+)-VP8-transformed bacteria but not in the pET-21d(+)-transformed control bacteria. A 30 kDa protein band could be detected 1 h after induction and maximum expression was found after 4 h (Fig. 2).

Recombinant His-tagged VP8 was accumulated in inclusion bodies. To disrupt the inclusion bodies, 8 M urea was used. The soluble bacteria fractions were applied to

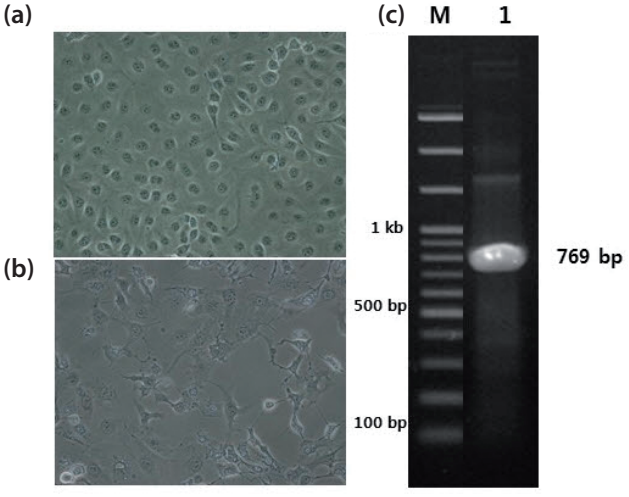

Fig. 1

Cloning of VP8 subunit from BRV-infected MA-104 cells
(a) Non-infected MA-104 cells, **(b)** BRV-infected MA-104 cells showing an 80% cytopathic effect, **(c)** RT-PCR product of VP8. The 769 bp fragment was amplified with VP8-specific set of primers. Lane M, molecular size marker (100 bp ladder); lane 1, PCR product of VP8.

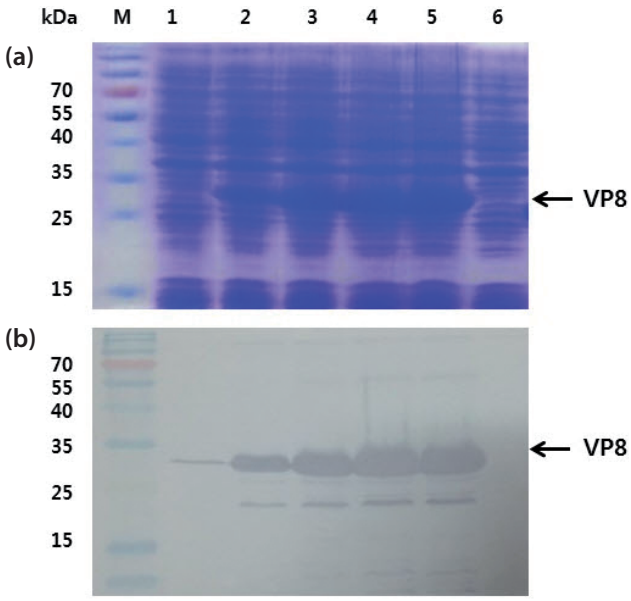

Fig. 2

Expression of the recombinant VP8 in *E. coli*

(a) Coomassie Blue stained 12% SDS-PAGE gel of the recombinant 6x His-Tag-VP8 (30 kDa), **(b)** Western blot of 30 kDa band of the recombinant VP8 was performed using monoclonal the anti-His-Tag antibody. Lane M, protein ladder; lane 1, whole cell lysate before induction; lane 2–5, 1, 2, 3, and 4 h after induction; lane 6, empty pET21d(+)vector.

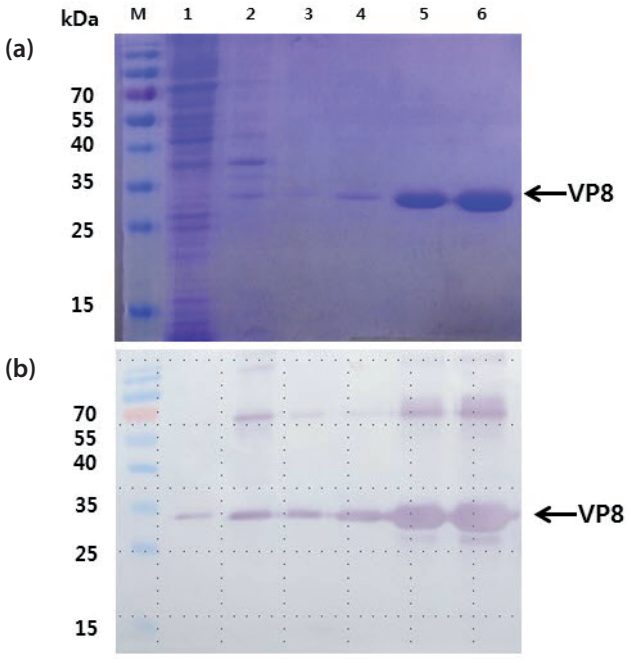

Fig. 3

Purification of the recombinant VP8 after solubilization of the inclusion bodies

(a) 12% SDS-PAGE and **(b)** Western blot using monoclonal anti-His-Tag antibody. Lane M, protein ladder; lane 1, soluble fraction after freezing & thawing lysis; lane 2, soluble fraction after washing; lane 3, column washing; lane 4–6, elution of recombinant VP8 from the column.

the Ni-NTA agarose column, and the recombinant protein was eluted with 300 mmol/l imidazole buffer. SDS-PAGE showed that the eluted fraction contained a major band of 30 kDa, corresponding to the recombinant protein (Fig. 3). The final purified protein with concentration of 1.03 mg/ml had high purity (>90%). After dialysis, recombinant protein was used to induce production of polyclonal antibodies in serum of chickens.

Characterization of anti-VP8 IgY antibody

The affinity of the IgY was determined by indirect ELISA in which various dilutions of IgY were tested with VP8. These IgY reacted strongly with VP8 (Fig. 4). To further confirm the specificity of the IgY, Western blot was performed. In agreement with ELISA results, VP8 could be detected at the dilution limit of 1: 8000 and a dose-dependent antibody response was observed (Fig. 5). Further, Fig. 5b shows that the same VP8 could be detected also by the goat anti-bovine rotavirus NCDV antibody. Our VP8 nucleotide sequence is highly homologous with bovine rotavirus NCDV. These results indicate that IgY could be used also as an immunodiagnostic reagent for the different bovine rotaviruses.

Neutralization of BRV in MA-104 cells

In order to investigate the neutralization activity of IgY against BRV, a neutralization test was performed using bovine rotavirus and MA-104 cells. BRV reacted with IgY, followed by inoculation into MA-104 cells. The degree of neutralization was determined by plaque reduction assay 72 h after inoculation. The undiluted IgY (1 mg/ml), strongly inhibited BRV replication in MA-104 cells (Fig. 6). As shown

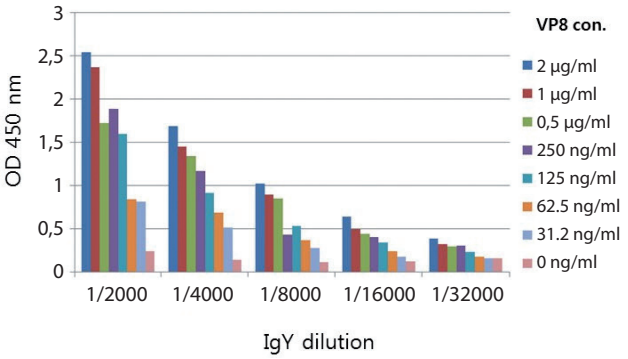

Fig. 4

Detection of the recombinant VP8 by ID-ELISA

Micro-titer plates (96-well) were coated with the serially diluted VP8 overnight at 4°C. Two-fold serial dilutions of IgY were incubated in pre-coated plate. Bound antibodies were detected by secondary rabbit anti-chicken IgY diluted 1:50,000 in PBS.

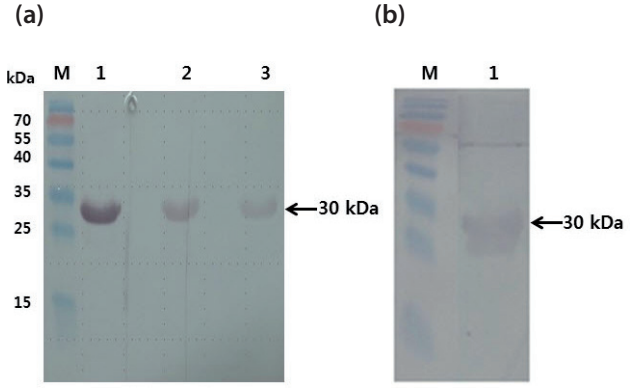

Fig. 5

Western blot of the recombinant VP8

To confirm the specificity of IgY to VP8, the recombinant VP8 proteins were separated by SDS-PAGE and then transferred onto a membrane. The membrane was incubated with either IgY **(a)** against recombinant VP8 or **(b)** goat anti-bovine rotavirus NCDV strain antibody. (a) lane M, protein ladder; lane 1, IgY diluted 1:2,000; lane 2, 1:4,000; lane 3, 1:8,000. (b) lane M, protein ladder; lane 1, IgY diluted 1:1,000.

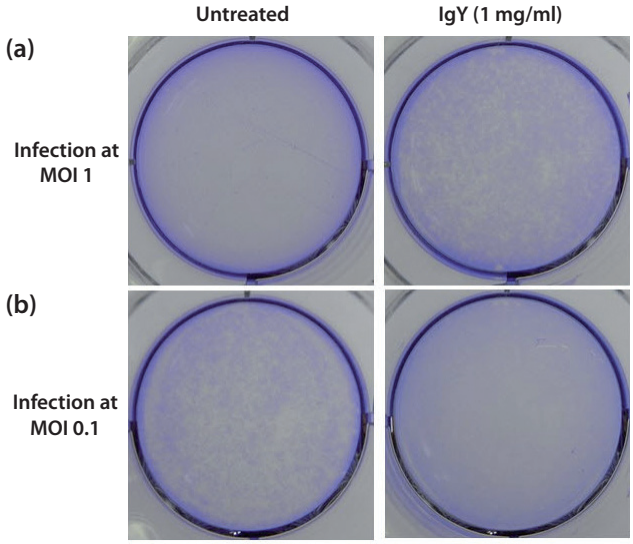

Fig. 6

Neutralization of BRV replication by IgY

(a) BRV at MOI 10 and **(b)** MOI 0.1 was preincubated with IgY and then inoculated with the MA-104 cells. Inhibition of BRV replication was quantified by the plaque reduction assay. Inhibition of BRV replication was observed in BRV pretreated with anti-VP8 IgY.

in Fig. 6b, a similar result was observed in the 10-fold dilution of BRV. The IgY is able to block the interaction between viral particles and the receptors on cells. It recognizes a neutralizing epitope present in BRV, thus leading to inhibition of viral infectivity.

Discussion

Although BRV-specific Ab from colostrum intake is key factor in protecting the neonates, it declines after calving. Another source of large amounts of antibody is necessary. It has been previously demonstrated that the administration of milk supplemented with IgY to newborn calves for the first 14 days of life induced a high degree of protection (Fernandez *et al.*, 1998; Parreno *et al.*, 2010). In addition, Vega *et al.* (2011) reported that IgY positively modulated the mucosal and systemic immune responses of newborn calves. Recent study showed that IgY could offer a potential antiviral strategy for prevention of human rotavirus, norovirus and influenza B infections (Dai *et al.*, 2013; Wen *et al.*, 2012). These results indicate that IgY have neutralizing activity in the intestinal tract as well as the respiratory tract and lungs.

In this study, we expressed the VP8 fragment of VP4 from BRV. In agreement with previous results, the recombinant protein was expressed in inclusion bodies. Maximum expression was obtained 4 h after IPTG induction at 37°C and the expression level of VP8 was higher than results obtained with VP8 from G6P6[1] bovine rotavirus strain C486 reported previously by others (Favacho *et al.*, 2006). Here, we used VP8 from G6P[1] bovine rotavirus KVCC-VR9200179. The BRV (KVCC-VR9200179) VP8 had 97% deduced amino acid identity to the C486. Liu *et al.*, (2012) classified rotaviruses into five P genogroups covering all 35 known P genotypes of rotaviruses using phylogenetic analysis. Since NCDV, C486, and bovine rotavirus (KVCC-VR9200179) are sharing both G6 serotype and P[1] genotype, these high sequence identities may lead to successful expression of VP8 consistent with previous results (Lee *et al.*, 1995; Liu *et al.*, 2012). The yield of VP8 was determined to be 20 mg/l of culture, which is about 5 to 10-fold higher protein yield compared to previously reported results for VP8 (Favacho *et al.*, 2006; Kovacs-Nolan *et al.*, 2001). The yield of human rotavirus Wa strain VP8 was 1.8 mg/l of culture and expression of bovine rotavirus C486 strain VP8 resulted in 4 mg/l of culture. Suitable vector and expression host may allow production of very high yields of VP8.

To test whether IgY could be used in an immunodiagnostic assay, we compared the specificity of IgY with IgG. Our results showed that IgY was able to recognize BRV VP8, suggesting that IgY could be used in an immunological detection assay. The virus neutralization activity of IgY was confirmed in a plaque reduction assay. IgY was able to inhibit viral infection by >50% compared to the control with MA-104 cells infected with bovine rotavirus. This partial block of BRV replication maybe derived from high MOI. In other studies, viruses at MOI 0.01 were used in a plaque reduction assay (Dai *et al.*, 2013; Wen *et al.*, 2012). To confirm further, the antiviral activity observed with plaque reduction assay, a focus reduction assay or immunofluorescent staining are necessary.

Previous studies have demonstrated that IgY could be used against BRV infection (Vega *et al.*, 2011,2015). They used purified BRV for immunization, but it was difficult to obtain enough VP8 in the native viral particles. To obtain large quantities of VP8 as immunogen for development of polyclonal antibodies, we tried to establish a bacterial system for overexpression of VP8. If purified BRV and overexpressed VP8 are used together as immunogens for production of IgY, significant increase of the IgY activity could be obtained.

In summary, we produced a large amount of specific IgY against BRV. This IgY was able to neutralize BRV replication in cell cultures and exhibited high specificity. These results suggest that polyclonal IgY can be used in diagnostics and as therapeutic treatment against BRV infection. In addition, it can be used as liquid or solid feed additive to protect newborn calves against BRV.

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