An Egg Yolk Immunoglobulin (RVP6-IgY) Specific for a Constructed Rotavirus VP6 Antigen (rVP6) Inhibited Rotavirus Replication in vitro

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Group A rotaviral diarrhea continues to be highly prevalent worldwide among children younger than 5 years of age, as well as among pre-weaning piglets. The middle capsid of rotavirus, VP6, is highly immunogenic and conserved among mammalian species, making it an ideal immunogen candidate. We developed a construct using the partial segment (nucleotides 8-1194) of the VP6 gene from Rotavirus strain OSU, subcloned into the expression vector pET 21b and expressed in Escherichia coli BL21 (DE3) to produce RVP6 that is ~45 kDa in size. Purification of RVP6 using a Ni-NTA column produced 3-4 mg L⁻¹ of transformed E. coli culture after induction with 1 mM isopropyl beta-D-thiogalactoside (IPTG). RVP6 was then orally administered to mice to establish the characteristic immune response produced in serum and fecal samples. Likewise, RVP6 was also given intramuscularly to laying hens to recover RVP6-specific antibodies (RVP6-IgY) in yolk. RVP6-IgY was then tested for its ability to inhibit rotavirus replication in vitro. Three oral doses of RVP6 induced a characteristic systemic immune response as shown by increased serum IgG titer along with a complementary increase in fecal IgA titer suggestive of an induced mucosal response. It also mounted increased serum titers in laying hens, eventually recovering RVP6-IgY from yolk optimally at 6-weeks post immunization. The yolks with high titers were then selected for partial purification. Partially purified RVP6-IgY was shown to be specific to RVP6 immunogen (dot-blot assay) suggesting its potential for use in diagnostics. Replication was inhibited in vitro when RVP6-IgY was added before virus infection and when co-incubated with the virus at 100μg/ml concentration, suggesting its promise for prophylactic use. However, it was not able to inhibit replication when added post-infection. Our results provided basis to describe the potential of RVP6 and RVP6-IgY; therefore, efficacy studies in piglets are encouraged to confirm its potential.

Key words: E. coli BL21 (D3), Immunogen, Immunoglobulin Y (IgY), Inhibition of Virus Replication, Rotavirus VP6

INTRODUCTION

In order to gain food security for all, raising animals such as swine has been important, whether the rearing may be from a simple backyard farming practice or a commercialized husbandry. However, lapses during animal care practices, such as improper housing, feeding and management, can expose the pig population to several infectious disease-causing agents such as those causing...
viral gastroenteritis (Malik et al. 2014), particularly in developing countries. Group A rotaviruses (RVAs) are the most significant group in piglets not only because of their economic impact in the livestock sector, in terms of mortality and morbidity, but also as a potential source of heterologous rotavirus (RV) infection in humans and many other animal species (Tate et al. 2009). In fact, group A rotavirus-associated gastroenteritis remains to be a global public health concern primarily in children aged <5 years (Vega et al. 2011; Aiyegbo et al. 2013; Papp et al. 2013) despite being a vaccine-preventable disease.

Currently, the licensed vaccine contains two serotypes of porcine group A rotavirus given orally and intramuscularly to pregnant swine or orally to nursing piglets. This is believed to boost colostrum and milk antibodies, providing increased lactogenic immunity to nursing pigs (Saif and Vlasova 2004). However, limited controlled studies on the efficiency of this vaccine for boosting rotaviral antibodies in colostrum and milk or for preventing rotavirus-associated diarrhea in nursing pigs are available. Safe and cheaper animal vaccine alternatives are therefore needed to utilize viral structural proteins that have been reported to be capable of inducing immune responses.

The complete viral particles of rotavirus have a triple-layered icosahedral protein capsid surrounding the genome of 11 segments of double-stranded RNA, encoding six structural and six non-structural proteins. The outer capsid contains VP4 and VP7 followed by the intermediate layer formed by VP6, and the inner layer, consisting of VP2 enclosing two other proteins VP1 and VP3. Among these segments, VP4 and VP7 have been highly exploited as possible vaccine candidates because they are the only proteins known to induce virus-neutralizing antibodies. However, recent developments concerning the cloning of VP6 into virus-like particles (VLPs) (Choi et al. 2000) and a DNA vaccine expressing bovine VP6 (Lee et al. 2010) with protective immunity in mice against rotaviral shedding suggests the potential of VP6 as a candidate immunogen.

In addition to vaccines, passive immunity thru antibody administration has been an accepted alternative therapy for viral diseases. One option is through the use of egg yolk antibodies or IgY. The IgY technology is a non-invasive technology that can offer several advantages over other methods of antibody (Ab) production (Schade et al. 2005) to include non-activation of mammalian complement and the inability to bind protein A or G (Sesarman et al. 2008). Since IgY is the only Ab isotype present in chicken egg yolk, its extraction is simple, and offers a faster and cheaper method for polyclonal Ab production from sources other than mammals (Tini et al. 2002).

This study describes the development of a construct using the partial segment (nucleotides 8-1194) of the VP6 gene from Rotavirus strain OSU and expression in Escherichia coli in vitro. RVP6 is then used to immunize mice to study the characteristic immune response it mounts. Furthermore, RVP6 is used to immunize laying hens to obtain RVP6-specific antibodies from yolk (RVP6-IgY) and test them for their ability to inhibit rotavirus replication in vitro.

MATERIALS AND METHODS

Amplification and Cloning of the VP6 gene

Porcine rotavirus strain OSU (KVCC-VR0000177) was propagated in MA104 cells (ATCC 2378.1) in the presence of trypsin at a final concentration of 3 μg ml⁻¹ (w/v). Total viral RNA was extracted and the corresponding copy DNA (cDNA) was synthesized using commercial kits and the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), respectively, according to the manufacturer’s instructions.

The sense primer (VP6-1) 5'-GGCTTTTAAACGAAGTCTTC and antisense primer (VP6-2) 5'-GGTCACATCTCCTCTCACA were designed based on the published Simian Rotavirus A VP6 gene (GenBank accession number: NC_011509.2) to amplify the desired gene. Next, the following components of the reaction were mixed: 1X ExTaq buffer; 0.2 μM each of forward and reverse primers; 200 μM dNTP mix; 1.25 units ExTaq polymerase (Takara Bio, Shiga Japan); and PCR water in a total reaction volume of 50 μl. Tubes were then placed in a thermocycler optimized for the following conditions: 94 °C for 5 min; 30 cycles of 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 1 min; and 72 °C for 10 min. The PCR product of approximately 1.3 kb was purified and subjected to DNA sequencing. Purified amplicons were then introduced into the T&A cloning vector (RBC, Taiwan) by direct ligation, and the resulting plasmid was used to transform E. coli HST08 for use in the succeeding steps upon sequencing confirmation.

Construction of recombinant plasmids

To construct an expression vector bearing the VP6 gene, the forward primer 5'-AAA CAT ATG GAG GTT CTG TAC TCA TGG-3' (VP6NdelF) and reverse primer 5'-AAA CTC GAG CTT AAT CAA CAT GCT TCT AA-3' (VP6XhoIR) were designed and used to re-amplify the partial segment of the VP6 gene (nucleotide 8-1194; GenBank Accession number: KR052771.1) using the obtained amplicons cloned in the T&A vector as template. The resulting PCR product was purified and then ligated into the prokaryotic expression vector pET21b (Novagen, Madison, WI, USA) to generate the recombinant plasmid pET21-VP6.
E. coli BL 21(DE3) was transformed by introducing the pET21-VP6 plasmid and protein expression was induced using isopropyl beta-D-thiogalactoside (IPTG) at a final concentration of 1 mM. Bacterial culture containing an empty plasmid was used as the control. The expressed protein, designated as RVP6, was subjected to the nickel-nitrilotriacetic acid (Ni-NTA) system-purification (Qiagen, Valencia, CA, USA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and renaturation by dialysis according to a previously reported protocol (Zhu et al. 2013).

Animals
All procedures involving the experimental use of animals were approved by the Dankook University Institutional Animal Care and Use Committee (13-047) and were carried out in an SPF-grade animal facility. Animal-specific food and water were given ad libitum. Room temperature was controlled at 24°C, and humidity at 50% during the entire study.

Characteristic immune response in mice
To determine the characteristic immune response of RVP6 in mice, three inoculation cycles of three doses in 3 consecutive days spaced by 2-week intervals were conducted. Specific pathogen-free 6 to 8-week old, male BALB/c mice from Raon Bio (Seoul, Korea) were housed in plastic cages on a 12-h light/dark cycle and fed mouse specific food and water ad libitum. Room temperature was controlled at 24°C, and humidity at 50% during the entire study.

RVP6-IgY recovery from yolk
Ten laying hens about 20-week old (naive, hyaline brown) were used to recover RVP6-IgY through hyperimmunization. About 200 μg/ml previously prepared recombinant antigen (RVP6) was mixed at a ratio of 1:1 in Freund’s complete adjuvant (Sigma, St Louis, MO, USA) for the first dose and Freund’s incomplete adjuvant (Sigma) for the succeeding dose. Birds were immunized intramuscularly in the breast with 1 ml immunogen given at a 2-week interval for a total of five doses. Egg yolks from immunized birds were pooled, processed, and purified according to Akita and Nakai (1993). The titers for RVP6-IgY were determined using indirect ELISA at OD 405 nm.

Specificity of RVP6-IgY to RVP6
Specificity was analyzed using the standard dot-blot method. In this step, 1mg/ml of RVP6 and 25 μg RVP6-IgY were used. Briefly, 2 μl samples at a 1:2 dilution were dotted onto nitrocellulose paper and blocked with 5 μl 5% BSA in TBS-T. The blot was incubated with 5 μl primary antibody dissolved in BSA/TBS-T for 30 min at room temperature and washed three times with TBS-T (3 × 5 min). This was followed by incubation with conjugated secondary antibody (Goat anti-Chicken IgG (IgY)-heavy and light chain Antibody Alkaline Phosphatase Conjugated) for 30 min at room temperature in the dark. The reaction was stopped by washing with distilled water, followed by drying for the final color visualization. Purified egg yolks from non-immunized laying hens were used as the negative control.

RESULTS

Construction of recombinant plasmids
The expected ~1.2-kb cDNA encoding the partial segment of the VP6 gene was successfully ligated to pET21b and inserted into E. coli BL 21 (DE3) to express RVP6 recombinant protein (Figure 1). Sequence analysis of this new construct showed the expected sequence of interest.
and a six-His-tag that can be digested with NdeI/XhoI.

The maximum expression of RVP6 was attained 3–4 h after induction with 1 mM IPTG following optimization, with a yield of approximately 4 mg/L of bacterial culture. RVP6 remained as an inclusion body in the pellet after induction and was not recovered from the supernatant after sonication. RVP6 was then recovered from the inclusion bodies and dialyzed. The resulting product was used to immunize hens and mice to determine its systemic and mucosal response, respectively. SDS-PAGE confirmed the presence of RVP6, with a size of approximately 45 kDa as expected (Figure 2).

Characteristic immune response in mice

Similarly, the ability of the recombinant RVP6 immunogen following oral immunization of expressed immunogen induced a mucosal immune response in mice (Figure 3A and B). IgA levels increased following three doses of RVP6 immunization, compared with the non-immunized control, as early as the first dose and intensified until the day of termination.

RVP6-IgY recovery from yolk

A systemic response was induced in laying hens following RVP6 intramuscular immunization as shown by the increased serum titer of anti-RVP6 antibodies as early as the second week post immunization (wpi) as described in Figure 4A. The increased systemic response between weeks 2 and 6 showed a booster effect reflecting immunological memory following the successive administration of the RVP6 antigen. As expected, antibodies accumulated in the yolk and were detected by the fourth wpi (Figure 4B). Anti-RVP6 antibody titers were optimally produced 6 wpi and started to plateau by week 8 wpi.

Inhibition of rotavirus replication in vitro

Figure 5 (1) describes the ability of RVP6-IgY to inhibit virus replication indicative of the absence of CPE in virus cell culture. Results show that 100μg RVP6-IgY helped control virus replication as shown by the consistent degree of CPE after 48 hours when added prior to infection or when co-incubated with the virus. However, this concentration may have been too low to inhibit replication.
even after 24-hour post infection. It can be noted that the degree of CPE further increased after 48 hours. It can also be noted that the ability of RVP6-IgY to inhibit replication is dose-dependent. Doses lower than 100μg failed to inhibit replication whether added before, after, or even when co-incubated.

**Specificity of RVP6-IgY to RVP6**

The specific reactivity of the recovered RVP6-IgY to RVP6 antigen at varying titers was then analyzed by dot-blot assay. The assay confirmed the antigenic characteristic of RVP6 as shown by the distinct coloration on the blotting paper compared to the non-immunized control pools (Figure 5 (2)).

**DISCUSSION**

Rotavirus VP6 is an important structural component of rotavirus particles that plays an important role in replication and immunity. This study utilized a partial segment of the VP6 gene, constructed an immunogen and used this immunogen to immunize model animals and study their characteristic immune responses. Antibodies recovered from yolk with high titers were evaluated on their capacity to inhibit rotavirus replication in vitro.

Gene cloning and expression in heterologous hosts is one useful approach to obtain large quantities of individual proteins for such studies. The E. coli system offered low cost, high production, and manipulation convenience to express heterologous proteins. E. coli as an expression host was used for years to express proteins for use in diagnostics (de Goes et al. 2008; Zhu et al. 2013) or studying the VP6 protein structure and function (Smith et al. 1989; Ito et al. 1998). An important advancement has been the development of novel recombinant cloning approaches and protocols to express heterologous proteins. Previous studies have utilized protein expression and E. coli BL21 to produce high-quantity proteins of rotavirus VP5* (Dowling et al. 2000), VP1 proteins of Coxsackie virus B3 (Magsoudi et al. 2007), rotavirus VP8* (Favacho et al. 2006), and VP1 proteins of chicken anemia (Lee et al. 2011) for immunogen production.

Our study has shown that oral immunization of RVP6 induced both systemic and mucosal response in mice. This characteristic immunogenicity was also observed when rotavirus VP6 and maltose-binding protein (MBP) was co-expressed as a fusion protein (Choi et al. 2007), here MBP acted as an adjuvant for VP6 during intra-nasal administration in mice. Both experiments proved therefore that mucosal route, either oral or nasal, in delivering VP6 can effectively mount a desired effective response. Most
recent results used VP6-VLP (virus-like proteins) system to deliver the desired antigen (Li et al. 2014). Although promising results were described in their report using subcutaneous delivery, there was no correlation presented between serum antibody titer and protection elicited using the mouse model.

It was previously described that the immunization of a hen can lead to the transfer of specific antibodies from the serum into the egg yolk (Schade et al. 2005) to protect the young animal, as a systemic response, and this type of response can significantly block pathogen replication (Ramig 2004). It is shown in this study that it takes 6 weeks post immunization to recover RVP6-IgY from yolk and this showed inhibitory properties against rotavirus replication. Antibody titer kinetics in chicken after the first immunization often shows a transient increase in antibodies during the first phase and going towards the second phase (booster immunizations). This is characterized by an initial increase in the antibody titer within approximately 10 days (Schade et al. 2005) followed by a plateau 10 days after, leading to a decline thereafter. This was similarly noted in our birds following immunization with RVP6. Our results supported the ease of producing antibodies of significant utility from laying hens.

Recent antibody therapy initiatives using VP6 involved VP6-specific antibodies in llama, a member of the Camelidae family (Vega et al. 2013). The antibodies, termed as VHH, were given to piglets and protected them from challenge. They said that the performance of VHH is better than the recombinant IgY they produced or the IgG specific of Wa strain they have developed.

CONCLUSIONS AND RECOMMENDATIONS

Overall, the RVP6 construct developed was found to mount both systemic and mucosal immune response in mice. It would be interesting to discover the various applications of this construct not only as an immunogen but also in the field of diagnostics. The RVP6-IgY that accumulated and recovered from yolk with high titers showed inhibitory characteristics towards rotavirus replication before and during co-incubation of a virus infection. Although it was not shown to inhibit when added after infection, it will be interesting to explore whether increasing the concentration of the IgY will also favor its inhibitory potential. Therefore, the immunogenicity traits of RVP6 and the inhibitory property of RVP6-IgY merit its further evaluation with regard to its ability to protect animals during a virus challenge. This will be a good direction in the future and it would be interesting to verify protection and correlate them with the mounted immune response as well as make comparisons on how it would protect the animals in comparison with VHH.

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