Production of Egg Yolk Antibody (IgY) against Recombinant Porcine Epidemic Diarrhea Virus COE Protein

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ABSTRACT

Background: Porcine epidemic diarrhea (PED) is a highly contagious disease of pigs, and is characterized by a series of clinical symptoms, such as severe diarrhea, vomiting and dehydration. Partial protective antigen gene (COE gene) of S protein possessing the main B cell epitope, is able to encode proteins with reactogenicity to induce the production of neutralizing antibodies. IgY was found to reduce the mortality in piglets after challenge exposures. Anti-COE IgY antibody has never been reported before, here it is described a method for the production of anti-COE IgY, which could be applied in the treatment for the porcine epidemic diarrhea virus (PEDV) infection.

Materials, Methods & Results: A PEDV strain was isolated from a clinical sample. The COE ORF (Open reading frame, ORF) was amplified by PCR and inserted into the pMD18-T clone vector. The isolated was defined as Porcine epidemic diarrhea virus strain JS-HZ2012 subtype by sequencing, the clinical sample was defined as the nucleic acid sequence has a 99.5% homology with that of PEDV CV777 strain. And then the COE ORF was subcloned into pET-32a by T4 DNA ligase and introduced into the E.coli Bal21 (DE3). COE protein was produced by the induction of the E.coli Bal21 containing pET-32a-COE with isopropyl-β-D-1-thiogalactopyrannoside (IPTG). Expression of the recombinant COE protein (rCOE) fused with His-tag was analyzed by SDS-PAGE and detected by western-blotting using anti-His monoclonal antibody. The rCOE was purified by Ni⁺ affinity purification chromatography under denature condition and dialyzed against PBS. The concentration of the rCOE was determined by BCA method. After immunizing the chickens with rCOE, All animal handling procedures were performed under veterinary supervision and following the recommendations of the local laws and regulations on Animal Experimentation. Anti-COE IgY was isolated by chloroform extraction method and analyzed by SDS-PAGE. The activity and specificity of the IgY antibody were analyzed by indirect ELISA and western-blotting. Furthermore the neutralization activity of the IgY antibody was analyzed by virus-neutralization test. SDS-PAGE analysis showed that the rCOE fused with His-tag was a molecular of 37 kDa, and the rCOE could be recognized by the anti-His monoclonal antibody. The IgY antibody isolated by chloroform extraction method and analyzed by SDS-PAGE showed the IgY mainly contained two parts 22 kDa and 66 kDa, which corresponded to light and heavy chain, respectively. In additional, some lower bands around 40 kDa were presented on the gel. The anti-COE IgY reached to 1:12800 after the third immunization, and the antibody levels could last for a long time. The anti-COE-IgY could recognize the COE fraction indicated that it possess the satisfied immunogenicity. The anti-COE-IgY could neutralize porcine epidemic diarrhea virus partly, and the neutralization titer reached to 1:12, and the control was less than 1:2.

Discussion: The COE protein were expressing with high efficacy, and the anti-COE IgY could recognize the COE fraction dictated that it possess the satisfied immunogenicity. The anti-COE-IgY could neutralize porcine epidemic diarrhea virus partly, and the neutralization titer reached to 1:12. The result in this study indicated that anti-COE IgY against PEDV could be an alternative way of supplementing prophylactic measures like colostral antibodies from sows. Also, it could be significant at preparation of the products of preventing the porcine epidemic diarrhea.

Keywords: porcine epidemic diarrhea virus (PEDV), COE protein, egg yolk antibody(IgY), neutralization test.
INTRODUCTION

Porcine epidemic diarrhea (PED) is a highly contagious disease of pigs. It is caused by porcine epidemic diarrhea virus (PEDV) [15]. PEDV is highly contagious enteric virus affecting mainly neonatal pigs and is one of the most economically important viral causes of diarrhea in piglets [3,9,18]. In Asia (China, Japan and Korea), mortality in sucking piglets infected with PEDV can be very high (30%-80%) [16,17]. PEDV S protein in the virus particles on the surface of the fiber glycoprotein, and it plays an important biological function of mediating neutralization antibodies produced in the infected hosts. In recent years, researchers have studied the PEDV S gene. Chang [1] estimates the 499-638aa of the PEDV S gene is an important antigen epitops, and named it the COE gene.

Since swine are born without immunoglobulins, immunoprotection for newborn piglets mainly consists of passive immunity through colostral immunoglobulins from the immunized dam [4,12]. In this respect, passive immunity from colostrum is of primary importance in piglets for protection against infectious enteric diseases. Recently, egg yolk immunoglobulin G (IgY) from immunized chickens has been discovered to be a convenient source for specific antibodies on a large scale [7]. IgY has been shown to be effective, safe and protective, especially against infectious intestinal infection, indicating similar biological activities to colostral antibodies in newborn piglets [6,14,22]. Here it is presented a simple and effective method to develop high qualified antibody based on IgY technology. Thus, the purpose of this study was to investigate the ability of the IgY to neutralize the PEDV.

MATERIALS AND METHODS

PEDV strain was isolated from feces and intestinal tissues of diarrhea pigs in the Li Shui pig farm. Feces and intestinal tissues were grinded with normal saline (NS, pH 7.0) containing double resistant (streptomycin 100 µg/mL, penicillin 100,000 IU/L). DNA clone of the COE gene

The full of COE ORF was amplified by PCR. The primers (F:5′-CG GGATCC ATGGAAGGAACTGCCATTAC-3′;R:5′-AGG GAATTCCTCGAGAGATGGGACATAGC-CAATAC-3′) including BamHI and EcoRI restriction site represented underline were designed using primer 5.0 based on the PEDV reference strain (GenBank Accession No.AF353511), and custom-synthesized by the Sangon Biotech1. The optimization of PCR was denatured at 95°C for 5 min, followed by 30 cycles of 94°C for 40 s, 56°C for 30 s, and 72°C for 45 s and final extension at 72°C for 10min.

The amplified fragments were purified and ligated into pMD18-T plasmid vector2 by T-A clone. The resulted plasmid was designated to pMD18-T-COE and introduced into E.coli DH5a. The positive colony was further confirmed by restriction enzyme digestion and sequencing.

Expression of the His-tagged recombinant PEDV-COE protein

The plasmids pMD18-T-COE and pET-32a were digested with BamHI and EcoRI respectively, eluted by gel purification, and then ligated with T4 DNA ligase to yield pET32a-COE recombinant plasmid. And then the plasmid was transferred into E.coli BL21(DE3), inoculated into LB agar medium containing ampicillin (100 µg/mL) and grown 12-18h until the OD 600 reached 0.6-0.8, and then induced by adding IPTG at a final concentration of 0.8 mmol/L for 5 h. After harvesting the cell by centrifugation (5,000 g, 15 min, 20°C), the pellets were lysed by sonication (20 min) in iced water bath. After centrifugation (12000 g, 15 min), the precipitation and supernatant fluid were collected, respectively. Both of the precipitation and supernatant fluid were analyzed on 12% SDS-PAGE followed by Western blotting to detected 6 His-tagged recombinant proteins. Diluted mouse anti-His monoclonal antibody3 was used as primary antibody (1:500,v/v), and HRP conjugated rabbit anti-mouse antibody3 was used as secondary antibody (1:4000,v/v).

Purification of the PEDV-COE fusion protein

Expressed fusion protein existed in the form of inclusion body. After dissolution of inclusion bodies in binding buffer containing 8 M Urea, the fusion protein was purified with Ni+ column1 to obtain high purity protein. The washing buffer containing 0.5 M NaCl, 8 M Urea, 20 mM imidazole, pH 8.0. And the eluting buffer cotaining 0.5 M NaCl, 2 M Urea, 250 mM imidazole, pH 8.0. SDS-PAGE (12%) was used to analyze the purification efficiency of COE protein followed by Comassie blue staining.
Immunization

Fifteen Twenty-week-old Hy-line Brown hens were kept in individual cages with food and water ad libitum. Three days before the first to collect eggs as control group. Every chicken was immunized with 100 µg COE protein emulsified in an equal volume of complete Freund adjuvant at different sites of the breast, then was boosted three times with an emulsion of 150 µg COE protein and incomplete Freund adjuvant at 2-week intervals.

IgY antibody purification

Yolk antibody was purified through ammonium sulfate gradient sedimentation. The eggs surface were disinfected used Bromogeramine disinfectant. Under aseptic conditions, egg whites were poured out and absorbed using the bilubus paper as much as possible. Egg yolks was added into the sterile beaker and joined PBS in volume 1:9, and stirred for 20 min at 4°C. IgY antibody were purified according to the previous study [11]. Briefly, the egg yolk diluent adjusted pH value to 5.11 and let it sit overnight at 4°C followed by centrifugation (10,000 g, 20 min). The supernatant was collected and added to a final concentration of 0.6 mol/L of Na2SO4, and adjusted the pH to 7.5. The solution was mixed with an equal volume of PBS, added saturated ammonium sulfate to 40% (w/v) of the final concentration at 4°C. After stewing at 4°C for 1 h followed by centrifugation(10,000 g, 30min, 4°C). The precipition was collected and suspended with the original volume of PBS. Saturated ammonium sulfate was added to a final concentration of 35% (w/v) at 4°C. The mixture was centrifuged again under the above condition after gentle shaking at 4°C for 1 h. The precipition was suspended with the half of the original sample volume of PBS. Finally, the solition was dialyzed against bulks of PBS over night at 4°C. The purity of the isolated IgY antibody was determined by 12% SDS-PAGE.

Enzyme-linked immuno sorbent assay(ELISA)

Specific activity of IgY antibody against VP2 protein was determined by ELISA. A 96- well microtiter plate was coated with 100 µL of COE antigen (10 µg/mL) in sodium bicarbonate buffer (pH 9.6), and incubated at 4°C overnight. After washing three times with PBS containing 0.05% (v/v) Tween 20 (PBST), the nonspecific binding sites were blocked with 5% (w/v) nonfat milk powder in PBST for 4 h at 37°C. After three times washing, 100 µL of a serially diluted IgY antibody in PBST was added to the wells as the primary antibody and then incubated for 2 h at 37°C. The plate was washed again and incubated with 100 µL of HRP labeled goat-anti-chicken IgG (1:5000) as the secondary antibody for 1 h at 37°C. Finally, the activity was detected with 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB, 1%, w/v) as substrate for 15 min at room temperature. The reaction was stopped by adding 50 µL of 2 mol/L H2SO4 to the wells. Optical density (OD) at 450 nm was read on microtiter plate reader. While OD_{Sample}/OD_{Negative} ≥ 2.1, the maximum dilution multiple of the sample was determined as IgY antibody titer.

Western blotting

The purified COE protein was separated using 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer western blotting apparatus. The unreacted sites were blocked with 5% (w/v) nonfat milk power in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 (v/v), (pH 8.0) buffer for 1 h and then incubated with anti-VP2 IgY antibody (1:1000) for 1 h at room temperature. After washing three times with TBST, HRP-conjugated goat-anti-chicken antibody diluted in TBST (1:5000) was added and incubated for 1 h at room temperature. The membrane was washed three times with TBST and the specific binding of IgY antibody to the COE protein was detected as described above.

Virus neutralization test

Determination of TCID50 of virus-infected cells. The PEDV virus stock was diluted by the concentration gradient 10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8}, Vero cells were inoculated with each dilution of a groups with five repeats. Incubated at 37°C, record 48-96 h lesion count and not the number of lesions, calculated according to Reed-Muech cells infected half the amount. Fixed virus diluted antibody per well 100 TCID50 viral load and antibody dilution after 1h at 37°C seeded cells, the cells were observed lesions. Record data to calculate the antibody titer.

Figure 1. SDS-PAGE analysis of recombinant COE protein expression with or without IPTG. Lane 1: pET-32a-COE without induction with IPTG; Lane 2: pET-32a-COE induction with IPTG; Lane M: Protein maker (Unstained Protein Molecular Weight Marker, Fermentas, Lithuania).

Figure 2. Western blotting analysis of recombinant COE protein using anti-His monoclonal antibody. Lane 1: pET-32a-COE induction with IPTG; Lane M: Protein marker (PageRuler Prestained Protein Ladder, Fermentas, Lithuania).

Figure 3. SDS-PAGE analysis of the isolated IgY antibody purified by saturated ammonium sulfate. Lane 1: Purified anti-COE IgY; Lane 2: Non-purified anti-COE IgY; Lane M: Protein marker (Unstained Protein Molecular Weight Marker, Fermentas, Lithuania).

Figure 4. Western blotting analysis of the binding activity of the anti-COE IgY. Lane 1: Protein marker (PageRuler Prestained Protein Ladder, Fermentas, Lithuania); Lane 2: pET-32a-COE induction with IPTG.
RESULTS

The coding region of the COE protein was amplified by PCR and inserted into the cloning vector to construct pMD18-T-COE. After restriction enzyme digestion analysis and sequencing, the clinical sample was defined as the nucleic acid sequence has a 99.5% homology with that of PEDV CV777 strain. After 6 h induction with IPTG, a prominent band with the expected molecular was observed in the insoluble fraction of the bacterial containing the pET32a-COE plasmid, while no proteins bands at the approximate size were observed in both none-IPTG induction and IPTG-treated culture containing pET32a only (Figure 1). Western blotting showed that the band around 37.5 kDa was recognized by mouse-anti-His monoclonal antibody (Figure 2).

Saturated ammonium sulfate precipitation was used for extracting the purpose protein. The purity of yolk antibody was above 90% (Figure 3). The SDS-PAGE results showed that the IgY is mainly made up with two chains. The molecular weight of heavy chain is about 66 kDa, while the light chain is about 22 kDa. Western blotting showed that the bands around 37.5 kDa was recognized by the anti-COE IgY (Figure 4).

ELISA results showed that IgY did not exist in the pre-immune chickens. Once first immunization last for one week, antibodies appeared in egg yolk. After the second immunization, the antibody level increased significantly, and reached the peak in the 5th week after the first immunization. The titer of purified yolk antibody came up to 1:106, and this state can last for a long period.

Neutralizing antibody responses showed, the titer of anti-PEDV COE antibodies was 1:12, with 1:2 for the control group, indicated manufactured yolk antibodies have a certain neutralizing activity (Figure 5).

DISCUSSION

Porcine epidemic diarrhea is one of the most serious infectious diseases in pigs, bringing heavy loss to pig industry. In a long period of time, the use of attenuated and inactivated vaccines has been reduced the economic loss significantly. But in 2006, the reports showed that porcine epidemic diarrhea became to be pandemic in the immune pigs [2]. The research of Sun showed that PED epidemic began to outbreak in more than 10 provinces of south China in October, 2010 and had led over one million pigs to death [19]. All these researches indicated, in some degree, the traditional vaccine has been unabled to play a good role in immune protection, and vaccination is not suffient to protect pigs from the infection of existing PEDV pandemic strain. Preliminary findings of this trial showed, as for the porcine epidemic diarrhea, passive immunization of piglets is mainly acquired from the sow’s milk, instead from blood with maternal antibodies, which may be due to the infection routes of PEDV. PEDV will first colonize in the intestine of piglets. When yolk antibody was taken in intestine by piglet, it will react with PEDV in the lumen and exert its immune protection, similar to the mechanism of colostrums [10]. Therefore, the development of yolk antibody against PEDV has played an extraordinary signification.
Yolk antibody, as an efficient biological agent, has showed a significant efficacy in prevention of infectious disease emergencies. There was research indicating that egg yolk antibody prepared by immunizing laying hens with PEDV was able to reduce the symptoms of diarrhea significantly [20]. Kweon [11] found that PEDV specific egg yolk antibodies, as well as breast milk, could protect the piglets from infection. When hyperimmune egg powder, prepared by Liu [13], was added into the feed of piglets, the occurrence of diarrhea had been reduced significantly. The egg yolk antibody mentioned above was all acquired from immunizing laying hens with PEDV attenuated or inactivated vaccines. The quality of vaccines produced by different factories is in different levels, and immunization dose has no uniform standard. Furthermore, the preparation procedure of vaccines is cumbersome and time-consuming. These factors all restrict the standardized production of egg yolk antibody. However, the use of proteins with virus neutralizing epitope as immunogen for acquiring anti-PED egg yolk antibody makes it possible to define the immunization dose. Moreover, the manufacture of immunogenic proteins is simple relatively, and its application can avoid the deficiency of using the vaccine as an immunogen. The research about neutralization titer of different yolk antibodies acquired with four different classical swine fever virus antigens showed that yolk antibody produced from recombinant proteins with neutralizing epitopes played a certain neutralizing role against classical swine fever virus [8]. Han [5] found the yolk antibodies acquired from recombinant VP2 protein exerted a good effect on preventing disease caused by canine parvovirus. Wang [21] constructed a recombinant protein, through genetic engineering techniques, to express the major subunit of ETEC K88ac and F18ac efficiently: FaeG and FedF. Then laying hens were immunized with the recombinant protein to acquire yolk antibodies. The results showed the yolk antibodies had played a very good effect on preventing piglet diarrhea.

In this study, the PEDV COE gene with neutralizing epitopes were expressed in prokaryotic cells, and laying hens the expressed fusion protein were immunized with the recombinant protein to produce yolk antibodies. Compared with the method of producing antibodies with vaccine, our method has several benefits, such as reducing the cost of preparing antigens, making the antigen source clear and making it possible to quantify. In addition, the following test of neutralization titer showed a certain neutralizing effect on PEDV. Therefore, further experiment with higher titers of IgY and its preventive effects on the spread of the virus, including the duration of the virus after application of IgY, may give more practical information for an alternative application of IgY in the future as a supplement to passive immunity in neonatal pigs against an economically important viral disease. Also, all of our researches have established a foundation for further development of PEDV egg yolk antibodies as a kind of feed additive.

**CONCLUSION**

In general, the preliminary results of the present study indicated the potential application of the anti-COE IgY as an alternative prophylactic method against PEDV infection. Therefore, further experiment with different titers of IgY and its preventive effects on the spread of the virus, including the duration of the virus after application of IgY, may give more practical information for an alternative application of IgY in the further as a supplement to passive immunity in neonatal pigs against an economically important viral disease.

**SOURCES AND MANUFACTURERS**

1. Sangon Biotech, Shanghai, China.
2. TaKaRa, Dalian, China.
3. Jackson ImmunoResearch, West Grove, PA, USA.
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REFERENCES


