Front. Agric. China 2009, 3(1): 104–108
DOI 10.1007/s11703-008-0080-9

RESEARCH ARTICLE

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Prophylactic and therapeutic effects of egg yolk immunoglobulin against porcine transmissible gastroenteritis virus in piglets

Abstract Porcine transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets that provokes high mortality rates in affected farms. In this study, specific immunoglobulin from egg yolk against TGEV was produced by immunization of White leghorn hens. Enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test revealed that the specific antibody titer started to increase on the tenth day post-immunization, reached its peak on the eighth week, and remained at a high level until the last week that we tested. The prophylactic and therapeutic effects of egg yolk immunoglobulin (IgY) was investigated in piglets. IgY was found effective to increase piglets survival rate significantly after challenge exposures in prophylactic efficacy analysis. The therapeutic effects test revealed that the mortality was dramatically reduced by orally administered IgY. All these results in our study indicated that IgY specific to TGEV could be an alternative prophylactic method like colostral antibodies against TGEV in piglets.

Keywords egg yolk immunoglobulin, immunoprophylactic effect, porcine transmissible gastroenteritis virus

1 Introduction

Transmissible gastroenteritis virus (TGEV) is a pleomorphic enveloped RNA virus with single-stranded, positive-sense genome. This virus belongs to the family Coronaviridae (Siddell et al., 1983) and it causes a highly contagious enteric infection in swine of all ages (Saif and Wesley, 1999). The disease is especially severe in newborn animals less than 2 weeks old in which mortality approaches 100% (Yan et al., 2007; Yang et al., 2005). Although there are several commercially available TGEV vaccines, either inactivated or attenuated, these do not fully protect piglets (Tuboly and Nagy, 2001; Liu et al., 2007; Lu et al., 2007). Immunoprotection for newborn piglets mainly consists of passive immunity through colostral immunoglobulin from the immunized dam (Kweon et al., 2000; Li, 2003). It suggests that passively derived antibodies are important in piglets for protection against infectious enteric disease. The results of previous studies have shown that passive immunization upon oral administration of antibodies can be effective in preventing intestinal infection (Carlander et al., 2000; Sarker et al., 2001; Song et al., 2003). However, oral administration of antibodies is prohibitively expensive when large amounts of antibodies are required (Shin et al., 2002; Song et al., 2003).

Laying hens transfer large amounts of immunoglobulin from serum to egg yolk of their eggs (Kariyawasam et al., 2004). An average egg may contain 100–150 mg yolk immunoglobulin, and substantial amounts of specific antibodies may be collected and purified from the eggs of immunized hens (Akita and Nakai., 1993; Li et al., 2006). Therefore, IgY from immunized chickens has been considered to be an inexpensive, convenient source for specific antibodies on a large scale (Kweon et al., 2000; Wang et al., 2004). And its therapeutic application has been assessed by passive immunization therapy through oral ingestion, as in fortified food products for prevention or control of intestinal infection, such as those caused by enterotoxigenic *Escherichia coli* (Hennig-Pauka et al., 2003; Ding et al., 2008), *Salmonella enterica* serovar typhimurium (Sunwoo et al., 1996), and rotavirus (Sarker et al., 2001; Li et al., 2006). Other studies also showed that IgY was effective, safe and protective, especially against intestinal infection, indicating similar biological activities to

Received July 17, 2008; accepted August 7, 2008

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colostral antibodies in neonatal pigs (Yokoyama et al., 1993; Li, 2003). These studies provide a potential advantage of using IgY specifically to TGEV for preventing and controlling porcine transmissible gastroenteritis. The purpose of our study was to produce IgY against TGEV and investigate its immunoprophylactic effect in neonatal pigs.

2 Materials and methods

2.1 Virus propagation and purification

The HB06 strain of TGEV which was used for this study had been isolated in piglets in our previous study (Fan et al., 2007). HB06 was propagated in the pig kidney cell lines PK15. A stock of 1 mg of purified virion was prepared as described elsewhere (Laude et al., 1986), divided into aliquots, and stored at −70°C and used throughout the experiment.

2.2 Immunization of hens

White leghorn hens (obtained from a local breeder, 25 weeks old) were immunized intramuscularly with 0.5 mL of TGEV (1 $mg \cdot mL^{-1}$) emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories). Three booster injections of 500 μg antigen, mixed with incomplete Freund's adjuvant, were given (through the same route) at 2-week intervals. The eggs were collected daily for up to 3 months and stored at 4°C.

2.3 Separation and purification of IgY

The crude antibody from yolk was extracted by the water-soluble fraction as described by Akita and Nakai (1993) with some modifications. The Egg yolk was separated from the white, and the yolk preparation was diluted by distilled water with a ratio of 1 to 9 at pH 5.0. The mixtures were kept overnight at 4°C. After centrifugation at 10000×g at 4°C for 30 min, the water-soluble fraction (WSF) was carefully collected. The antibody was further purified using ammonium sulphate precipitation (Ko and Ahn, 2007). Briefly, WSF was twice precipitated with 40% (wt/vol) ammonium sulphate and resuspended with phosphate-buffered saline (PBS). Residual salts were removed by buffer exchange with PBS. Purified IgY was filtered through 0.2 μm membrane filter and stored at −20°C for the following tests.

The recovery rate of immunoreactive IgY was calculated using the formula OD_{492nm} (A)/OD_{492nm} (B) ×100, where A is OD_{492nm} in the crude extract (WSF) or ammonium sulphate purified product at a given dilu-tion and B, OD_{492nm} in the egg yolk pooled at the same dilution (Li et al., 2006).

2.4 Immunological assay

The liters of IgY were measured by enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) assay. ELISA was carried out in 96 well flat bottom microtiter plates (COSTAR). Each well was coated overnight at 4°C with purified TGEV antigen which was diluted in coating buffer. After 3 cycles of washing with PBS solution containing 0.05% Tween-20 (PBS-T), 250 μL volumes of blocking buffer (0.5% poly vinyl alcohol in PBS) were added, and the plates were incubated for 2 h at 37°C. After washing for three times, 100 μL volumes of appropriately diluted IgY preparations were added to the wells. The plates were washed as described above after incubation for 2 h at 37°C. One hundred μL of HRP-conjugated goat anti-chicken IgG (invitrogen) were added. After an incubation period of 1 hour at 37°C followed by another 3 cycles of washing, 100 μL of the substrate solution (0.04% 3, 3′, 5, 5′- tetramethylbenzidine in phosphate-citrate buffer (pH5.0) containing 0.02% H_2O_2) was pipetted into the wells, and the plates were allowed to stand for 10 min at room temperature. The reactions were stopped with 50 μL of 2 $mol \cdot L^{-1}$ H_2SO_4 per well and the absorbance at 492 nm was read with an Immunoreader BIO RAD 680. The antibody titer was expressed as *P/N* values, where *P* represents the OD_{492} nm of IgY from immunized hens at a given dilution and *N*, the OD_{492nm} of IgY from non-immunized hens at the same dilution.

The neutralizing activity of IgY was determined by assaying the PK15 cell protection activity as previously described. Briefly, IgY underwent serial two-fold dilution in alpha-MEM and mixed with an equal volume of TGEV suspension (200 $TCID_{50} \cdot mL^{-1}$). After being incubated for 1 h at 37°C, 100 μL of the mixture was dispensed in duplicate into PK15 cells which cultured in 96-well flat bottom microtiter plates. The plates were incubated at 37°C for 7 days. The virus neutralization titer (NT) was expressed as the reciprocal of the highest dilution of antibody that protected the cells from showing cytopathic effects by 50% compared with the positive control wells.

2.5 Prophylactic efficacy of IgY

Two litters of newborn Seghers piglets (15 neonatal male pigs) that are 3 days old and that have no maternal antibody against TGEV were selected for this study. Part of the two litters' groups were randomly selected and orally administrated with 3 mL of IgY (64 NT) three times a day before challenge exposure. The other piglets in the same litters remained as control. All piglets were uniformly fed and orally challenged with a dose of 5 $LD_{50} \cdot mL^{-1}$ TGEV. After challenge exposures, all piglets except the controls were kept with oral administration of IgY throughout the experiment. Clinical symptoms and the mortality of the piglets were be observed during the following two weeks.

2.6 Therapeutic efficacy of IgY

The therapeutic efficacy of IgY was tested in pig farms having outbreaks of diarrhea. Fecal samples from infected piglets were used for bacteriological and virology examination by polymerase chain reaction. Only the farms that have no aetiology pathogen but TGEV were selected for the experiment.

Two farms that showed TGEV positive results were chosen for the test. Part of the piglets from the same litters were orally administered with 3 mL IgY twice daily. The other piglets were treated as control. The amount of death and survival of piglets were recorded every day for a week, and the mortality was calculated one week after administration of IgY.

2.7 Statistical analysis

The sum of survivors for each group of pigs was analyzed by χ^2 test. Differences at the level of $P \leqslant 0.05$ will be considered to be significant.

3 Results

3.1 Specific antibody production

Eggs were produced up to three months after the first inoculation of hens with TGEV. The IgY was extracted by the water-soluble fraction (WSF) and purified by ammonium sulphate precipitation. The recovery rate of IgY was 79.3% in the crude extract of the pooled egg yolks and 67% upon further purification with 40% ammonium sulphate precipitation of the crude extract (Fig. 1). The titer of IgY was examined by ELISA and virus neutralization assay. ELISA test showed that the specific antibody started to increase in the egg yolk on the 10th day and got higher after each boost. The IgY reached its peak on the eighth week and maintained at a high level until the last week in our test (Fig. 2).

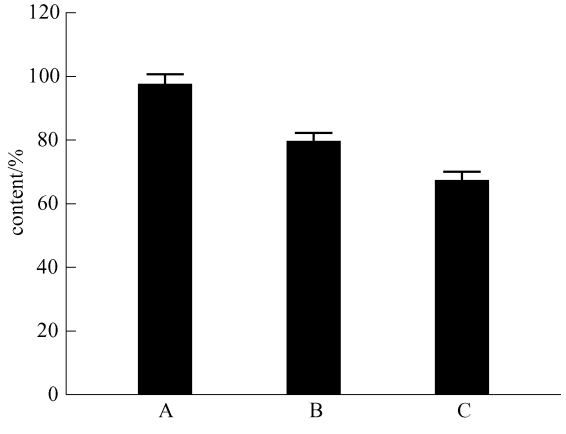

Fig. 1 Recovery of immunoreactive IgY
Note: A: Egg yolk pooled; B: Crude extract of IgY by water dilution; C: Purified IgY by 40% ammonium sulphate.

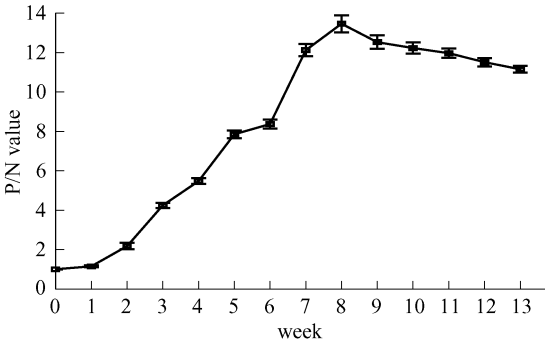

Fig. 2 Changes of IgY titers over time following immunization
Note: Immunization at 0, 2, 4, 6 weeks. Each date point represents the average of three determinations and the error bars, the standard error of means.

Changes of IgY virus neutralization titers over time following immunizations were consistent with the results of ELISA. On the eighth week, 256 diluted IgY solution can also protect the cells from showing cytopathic effects by 50%. The bulk of IgY was modulated to 64 NT before the animal experiment.

3.2 Prophylactic efficacy of IgY

After the challenge exposure with TGEV, piglets of the experiment group were orally administered with IgY daily. However, nearly all piglets deveopled clinical signs of yellow diarrhea within two days after the administration of TGEV. Two to four days later, dehydration symptom appeared. The mortality of the control group was more than 50% (4 out of 7) within one week, whereas the IgY treated group was 12.5% (1 out of 8) (Table 1).

3.3 Therapeutic efficacy of IgY

Two farms where TGEV was positive were chosen for the experiment of therapeutic efficacy. Piglets were orally administered with IgY twice a day for a week since the second day that they showed a symptom of diarrhea. The total surival rate was 82.35%, whereas the control was 36.84% (Table 2). Although there were differences in surival rate between the two farms, it indicated that the surival rate of the piglets treated with IgY was higher than that of the untreated control (Table 2). In general, the result showed that piglets were protected significantly by IgY.

4 Discussion

TGEV can induce diarrhea in all ages with a mortality of nearly 100% in piglets until the age of 2 weeks (Saif and

Table 1 Prophylactic effect of IgY in piglets after challenge exposure

treatment	no. of piglets	days after challenge exposure (no. of survival/head of piglet)						
		4	5	6	7	8	10	14
control	7	7/7	5/7	4/7	3/7	3/7	3/7	3/7
treatment	8	8/8	8/8	7/8	7/8	7/8	7/8	7/8

Table 2 Survival of piglets after administration of IgY within litters

designated farm	no. of survival/head of piglet	
	treated	control
A	8/9	4/10
B	6/8	3/9
sum	14/17	7/19

Wesley, 1999). Since there is no proper vaccination to prevent TGE outbreak, the disease occurs frequently in pig farms and TGE is of considerable economic importance to large swine –breeding units. In such cases, IgY can be an alternative method for providing passive protection, because TGEV replicates in villus epithelial cells of the small intestine (Cox et al., 1990).

Many studies have shown that egg yolk from an immunized hen has an antibody capable of specific recognition in an abundant quantity and is therefore economical (Verdolva et al., 2000; Li et al., 2006). IgY has a broad stability to pepsin and can penetrate the intestinal wall of neonatal piglets easily. Its biological activities were similar to colostral antibodies in neonatal pigs (Yang et al., 2007). Therefore, oral administration of IgY from chicken egg yolk has been used successfully by many researchers in preventing many intestinal diseases (Hennig-Pauka et al., 2003; Sarker et al., 2001; Ding et al., 2008). In our study, although IgY obtained from hens immunized with TGEV could not completely protect the piglets from death, it dramatically increased the piglets' surival rate which can be confirmed with the prophylactic efficacy analysis. When we tested the therapeutic efficacy of IgY in farms having outbreaks of TGEV, the application of IgY could significantly reduce the mortality of piglets. All the results of this study demonstrated a high preventive efficacy of IgY on TGEV outbreaks and its potential application as an alternative prophylactic method like colostral antibodies against this virus in piglets in the future.

Acknowledgements This work was supported by the National Science and Technology Infrastructure Fund of China (No. 2005DKA21205-5) and also funded by Agricultural University of Hebei, China. The authors are grateful to Dr. Rongyan Zhou for her assistance of data analysis.

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