FULL PAPER *Virology*

Immunoprophylactic Effect of Chicken Egg Yolk Immunoglobulin (Ig Y) against Porcine Epidemic Diarrhea Virus (PEDV) in Piglets

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ABSTRACT. Porcine epidemic diarrhea virus (PEDV) is the causative agent of neonatal diarrhea in piglets, which causes high mortality rates. In this study, the immunoprophylactic effects of chicken egg yolk immunoglobulin (Ig Y) against PEDV were investigated in neonatal pigs. Ig Y was found to reduce the mortality in piglets after challenge exposures. The field application of Ig Y also revealed significant differences in survival rates of piglets given Ig Y, as compared with placebo or control. The results in this study indicated that Ig Y against PEDV could be an alternative way of supplementing prophylactic measures like colostral antibodies from sows.

KEY WORDS: chicken egg yolk immunoglobulin (Ig Y), immunoprophylactic effect, porcine epidemic diarrhea virus.

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Porcine epidemic diarrhea virus (PEDV), a porcine coronavirus, is the etiological agent of entero-pathogenic diarrhea in swine [3, 17]. Although the clinical symptoms of PEDV infection are similar to transmissible gastroenteritis virus (TGEV) infection, PEDV is antigenically different from TGEV with high mortality rate in neonatal pigs [12].

Since swine are born without immunoglobulins, immunoprotection for newborn piglets mainly consists of passive immunity through colostral immunoglobulins from the immunized dam [2, 9, 13]. In this respect, passive immunity from colostrum is of primary importance in piglets for protection against infectious enteric diseases.

Recently, egg yolk immunoglobulin G (Ig Y) from immunized chickens has been discovered to be a convenient source for specific antibodies on a large scale [6]. Ig Y has been shown to be effective, safe and protective, especially against infectious intestinal infection, indicating similar biological activities to colostral antibodies in newborn piglets [10, 15, 16].

In this study, we produced Ig Y against attenuated PEDV strain and investigated its immunoprophylaxis in neonatal pig.

MATERIALS AND METHODS

Cell culture and virus: Vero cells (CCL81, U.S.A.) were regularly maintained in alpha-MEM, supplemented with 5% fetal bovine serum, penicillin (100 unit/m*l*), streptomycin (100 unit/m*l*) and amphotericin (0.25 g/m*l*). A strain of attenuated PEDV was plaque-purified once at the passage level of 81, and further cloned through limiting dilution method three times in Vero cells [7]. The growth of cloned PEDV, designated KPEDV-91842, was trypsin independent and produced cytopathic effects even in the presence of serum, and thus was proven compatible with serum neutralization assay in Vero cells. For the preparation of the antigen, the virus-inoculated cells were maintained in alpha-MEM with 0.02% yeast extract, 0.3% tryptose phosphate broth (TPB) and 1–2 μg of trypsin as described previously [12]. The infected cells and culture supernatant were frozen-thawed once and harvested after centrifugation at 3,000 $\times$ g for 20 min. The collected supernatant was then concentrated with polyethylene glycol (PEG, M.W. 6000, Serva, Germany) by the procedures described before [1]. The PEG-treated viral solution was resuspended at 1/200 of original volume with TEN buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 7.4). The concentrated virus was then used for the immunization of chickens and ELISA.

Immunization of chickens: White Leghorn hens of 10 weeks old were immunized with concentrated PEDV as described above. Primary immunizations were conducted by intramuscular injection with 1 m*l* (0.5 mg/m*l*) of virus emulsified with an equal volume of aluminium hydroxide gels. Second immunizations were conducted at a 2 week interval after the first inoculation. Second inoculations were given through the same route with 0.5 m*l* of virus emulsified with two volumes of oil adjuvants (Montanide ISA25, France). After the second immunization, booster inoculations were conducted at 4–8 week intervals with oil adjuvants. Ig Y was extracted from the egg yolk by using 0.1 % λ carrageenan solution (Sigma Chemical, Type IV, St Louis, U.S.A.) and concentrated by the procedures described previously [4, 6]. The extracted Ig Y was then filtered through 0.2 μm membrane filter and stored at –20°C before further experimentation.

Immunological assay: The titers of Ig Y were initially

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screened by indirect enzyme-linked immunosorbent assay (ELISA) and further tested for virus neutralization (VN) in microneutralization assay. The procedures for ELISA were basically the same as the previous method except for the addition of O-phenylenediamine [7]. Briefly, Ig Y diluted to 1:1,000 in PBST containing 10% skim milk was added to the microwell coated with PEDV and incubated for 1 hr at 37°C. After incubation, the plates were washed and then 100 μl of horseradish peroxidase (HRP), labelled anti-chicken IgG (KPL, Maryland, U.S.A.) and diluted 1:3,000 in PBST containing 10% skim milk, was added to each well for 1 hr at 37°C. The plates were washed three times with PBST and added to 2, 2'-azino-3 ethyl-benzthiazoline-6-sulfonate (ABTS) substrate solution (KPL, Maryland, U.S.A.). After 30 min incubation at room temperature, the reaction was stopped by adding 0.1% sodium dodecyl sulfate (SDS) solution and optical density (O.D) was measured at 405 nm. In order to assay neutralization activities, two-fold serial dilutions of samples in alpha-MEM were mixed with an equal volume of virus containing 200 $TCID_{50}$/m*l*. After incubation for 1 hr at 37°C, 100 μl of mixture was dispensed in duplicate into 96 well microplate before adding the same volume of Vero cells (20,000 cells/well). The plates were incubated for 7 days at 37°C in a humidified CO_2 incubator, and neutralization titers were determined to be the last dilution of sample that inhibited the cytopathic effects by 50% compared with positive control wells.

Animal experiment (Prophylactic efficacy of Ig Y): In order to avoid potential effects from maternal immunity, four seronegative pregnant swine were initially screened by ELISA from a farm without a history of PEDV outbreak. The prophylactic effect of Ig Y was tested with different challenge doses of PEDV. Four litters of newborn pigs (36 piglets) of 3 days old were transferred to an isolated room and fed with dairy milk. Nineteen piglets, half of three litters, and 2 more pigs from a fourth litter, because of difficulty in marking, were randomly selected and orally administered with 2–3 m*l* of Ig Y (32–64 SN titer) by syringe-attached silicone tube three times for one day before challenge exposure. Seventeen of the piglets in the same litter remained as control. The challenge virus was prepared from ground intestines of neonatal pig that had died from PEDV. All pigs were uniformly fed with a dilution of a 5 LD 50/m*l* dose of wild PEDV, as described before [7]. After challenge exposures, all piglets except the controls were kept with oral administration of Ig Y throughout the experiment. Clinical signs and the mortality of the piglets were observed for two weeks. Histological examinations were conducted by the procedures described previously two weeks after the challenge experiment [3].

Field application: The field experiment was conducted in farms having PEDV outbreaks. Briefly, fecal samples from piglets were first examined by reverse transcription polymerase chain reaction (RT-PCR) on PEDV as described [8].

Three farms that showed positive results were chosen for the application of Ig Y. Piglets were orally administered with 2–4 m*l* of Ig Y twice per day. At each farm, treatment of Ig Y was also conducted within the same litters of piglets for monitoring during the same period of application. The number of deaths and survival of piglets were recorded every day for a week, and the mortality was calculated one week after administration of Ig Y under field veterinarians' observation.

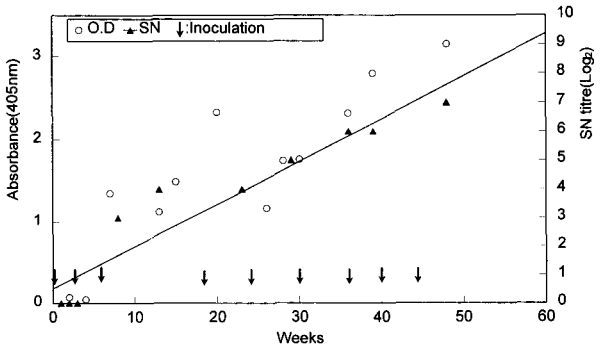

Fig. 1. Immunological titers of Ig Y by ELISA (A) and SN (B) after series of vaccination with attenuated PEDV (KPEDV-91842) strain. Each titer was the reciprocal of 1:1000 dilution (ELISA) and initial volume of extracted Ig Y, respectively. Immunization at 0, 2, 6, 18, 24, 30, 40 and 44 weeks are arrow indicated.

Statistical analysis: The sum of survival numbers for each group of pigs was analyzed by χ^2 test. Differences with $P<0.05$ were considered to be significant.

RESULTS

Production of Ig Y on PEDV: Eggs were produced up to 47 weeks after the first inoculation of chicken with PEDV. Although Ig Y titers were increasing after the second inoculation with PEDV, titers became generally higher two weeks after each boosting throughout the experiment. The time course pattern of Ig Y titers measured by ELISA and microneutralization assay was illustrated in Fig. 1. After screening of titers at one-week intervals, each week's Ig Y was collected and stored at –20°C. In general, one egg normally produced 150 to 200 m*l* in volume after extraction, and the bulks of low titers were concentrated to 32–64 SN titer before the experiment.

Immunoprophylactic efficacy after challenge exposure: Protective efficacy of Ig Y was examined in piglets that had daily antibody treatment after the challenge exposure with wild PEDV. Since piglets were kept under the same environment except their being supplied with Ig Y, signs of diarrhea, vomit and emaciation were evident within 2 days after challenge exposure. Clinically, nearly all pigs, regardless of Ig Y administration, developed signs of yellow diarrhea that usually lasted 2 to 4 days, followed by the onset of dehydration. However, 17 out of 19 treated animals survived, whereas the mortality of the control segment reached up to more than half (9 out of 17) within one week after the PEDV challenge exposure (Table 1). The cause of death of the pigs was basically dehydration due to diarrhea, and all the remaining animals became visibly gaunt in the weeks after

Table 1. Prophylactic effect of Ig Y 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	12	14
Control	17	17/17	14/17	10/17	8/17	7/17	7/17	7/17	7/17
Treatment	19	19/19	19/19	17/19	17/19	17/19	16/19	14/19	14/19

*Surviving pigs after challenge exposure with field isolate before cell adaptation.

Table 2. Survival of piglets after administration of Ig Y within litters

Farms designated	No. of survival*/Head of piglet		
	Treated	Control	No. litter
A	7/8	5/10	2
B	8/8	5/12	2
C	10/27	4/27	5
Sum	25/43	14/49	9

*One week after oral administration.

the challenge exposure. Although there were no visible signs of diarrhea after one week, one pig from the control group and two pigs from the Ig Y treated group did not recover from artificial challenge with PEDV by two weeks post challenge experiment (Table 1). Since signs of retardation of weight gain were clinically evident in all surviving pigs, pigs were euthanized for pathological examinations. When the intestines from surviving pigs were histologically examined, the villi of jejunum and ileum were detectably shortened, and villus to crypt ratio was reduced both in control and treated animals, compared with normal intestines of pigs in the same age as expected (data not shown here).

Prophylactic efficacy of Ig Y in field: The prophylactic efficacy of Ig Y was tested in farms having a positive diagnosis of PEDV. The piglets showed signs of diarrhea in the two or three days after delivery was orally supplied every day for a week. In application of Ig Y within litters, a total of 58.14% survival rate was observed compared with 28.57% of the control from 9 litters in three separate farms (Table 2 and Fig. 2, A). Although there were variations in the survival rates in the three farms, it was detected that the rates from piglets treated with Ig Y turned out to be higher than the rates from untreated control (Table 2). In general, analysis also indicated that the survival rate increased significantly in pigs from treated groups over those in control groups in both applications. When a total survival rate from 49 treated litters was compared with control (30 litters), the sum of three farms was 49.24% from Ig Y treated group and 33.71% from control (Fig. 2, B).

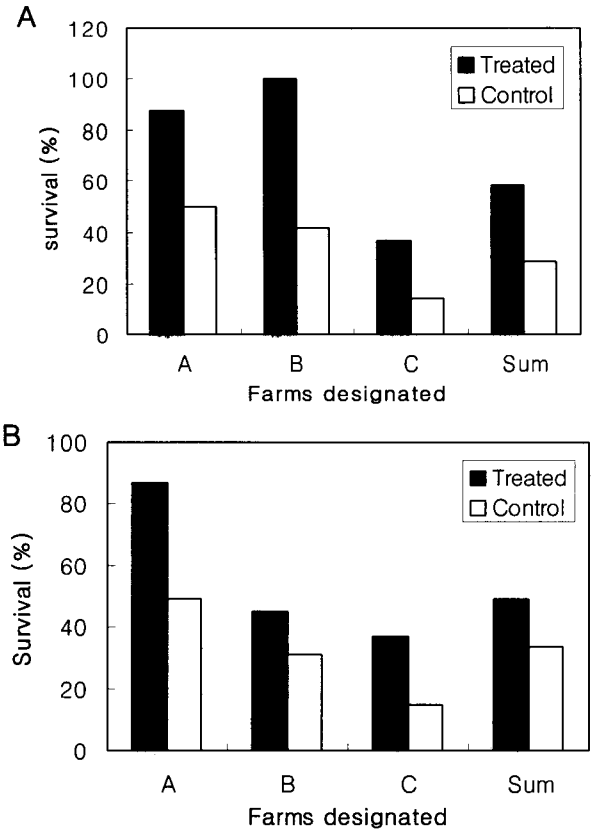

Fig. 2. Comparison of survival rate between Ig Y administered piglets and control either within litter (A) or groups of piglets (B) from three different farms having outbreak of PEDV.

DISCUSSION

Since viral diseases like PEDV and TGEV frequently occur in farms with highly dense breeding systems without proper vaccination, preventive measures must be immediate and effective against further spread of diseases after outbreaks. Although it is well known that PEDV infection, like TGEV, causes high mortality rates (50–100%) in neonatal pigs under 7 days of age, epidemiological observations have also indicated that the spread of disease seems to be slower, but rather persistent, compared with TGEV outbreak [11, 12, 14]. In this situation, Ig Y can be an alternative method for providing passive protection, because PEDV replicates mainly in the villi of the small intestines [3]. When we tested the prophylactic efficacy of Ig Y in piglets after challenge exposures, although it is not completely protective, the administration of Ig Y could, within a week, significantly reduce the mortality of piglets compared with the control, indicating that the application of Ig Y can partially block the virus from invading the small intestines. In addition, it was possible to detect that the death of piglets supplied with Ig Y was delayed (by at least two to four days) compared with control piglets. However, it should be noted that the surviving pigs showed an equally retarded condition

Table 3. Evaluation of Ig Y against PEDV as immunoprophylactic application in field

Farms designated	No. of survival*/No. of head (litter size)	
	Treated	Control
A	40/46 (7)	24/49 (6)
B	145/323 (37)	32/102 (19)
C	10/27 (5)	4/27 (5)
Sum	195/396 (49)	60/178 (30)

*One week after oral administration.

after the challenge exposures due to the damage to intestinal villi, suggesting the practical limitation of Ig Y against viral disease. Nevertheless, this result was rather expected, because, theoretically, one pig infected with PEDV can produce anywhere from 10^7–10^8 infectious doses of virus [11].

When we tested the immunoprophylactic effects in farms having outbreaks of PEDV, it was also demonstrated that the application of Ig Y significantly resulted in increased survival rates of piglets (49.24% compared with 33.71% of control), supporting the beneficial effect of Ig Y. However, even through overall reduced mortality of piglets was observed from Ig Y application in some farms, the efficacy of Ig Y was rather variable from farm to farm. At present, it is difficult to explain the exact efficacy of Ig Y. Some of the possible explanations for this result that are linked to variations in mortality can be the level of potential immune status, possible complications with other enteric pathogens like *E. coli* and the sanitary status of each farm.

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

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REFERENCES

1. Chu, H. J. and Zee, Y. C. 1984. Morphology of bovine viral diarrhea virus. *Am. J. Vet. Res.* 25: 103–107.
2. Cox, E., Pensaert, M, B. and Callebaut, P. 1993. Intestinal protection against challenge with transmissible gastroenteritis virus of pigs immune after infection with the porcine respiratory coronavirus. *Vaccine* 11: 267–272.
3. DeBouck, P. and Pensaert, M. 1980. Experimental infection of pigs with a new porcine enteric coronavirus CV 777. *Am. J. Vet. Res.* 41:219–223.
4. Hatta, H., Kim, M. and Yamamoto, T. 1990. A novel isolation method for hen egg yolk antibody, IgY. *Agric. Biol. Chem.* 54 : 2531–2535.
5. Hofmann, M. and Wyler, R. 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. *J. Clin. Microbiol.* 26: 2235–2239.
6. Jensenius, J. C., Anderson, I., Hau, J., Crone, M. and Koch, C. 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. *J. Immunol. Methods* 46: 63–68.
7. Kweon, C. H., Kwon, B. J., Lee, J. G., Kwon, G. O. and Kang, Y. B. 1999. Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate. *Vaccine* 17: 2546–2553.
8. Kweon, C. H., Lee, J. G., Han, M.G. and Kang, Y. B. 1997. Rapid diagnosis of porcine epidemic diarrhea virus infection by polymerase chain reaction. *J. Vet. Med. Sci.* 59: 231–232.
9. Lanza, I., Shoup, D. I. and Saif, L. J. 1995. Lactogenic immunity and milk antibody isotypes to transmissible gastroenteritis virus in sow exposed to porcine respiratory coronavirus during pregnancy. *Am. J. Vet. Res.* 56: 739–748.
10. Murata, H. and Namioka, S. 1977. The duration of colostral immunoglobulin uptake by the epithelium of the small intestine of neonatal piglets. *J. Comp. Pathol.* 87: 431–439.
11. Pensaert, M. 1999. Porcine epidemic diarrhea. pp. 179–185. *In*: Diseases of swine. 8th ed., Iowa State University Press. Ames, Iowa.
12. Pijpers, A., van Nieuwstadt, A. P., Terpstra, C. and Verheijden. J. H. M. 1993. Porcine epidemic diarrhea virus as a cause of persistent diarrhea in a herd of breeding and finishing pigs. *Vet. Rec.* 132: 129–131.
13. Roth, J. A. 1999. The immune system. pp. 799–819. *In*: Diseases of Swine, 8th ed., Iowa State University Press. Ames, Iowa.
14. Saif, L. J. and Wesley, R. D. 1999. Transmissible gastroenteritis and porcine respiratory coronavirus. pp. 295–323. *In:* Diseases of Swine, 8th ed., Iowa State University Press. Ames, Iowa.
15. Yokoyama, H., Peralta, R. C. and Diaz, R. 1992. Passive protection of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Infect. Immun.* 60: 998–1007.
16. Yokoyama, H., Peralta, R. C., Sendo, S., Ikemori, Y. and Kodama, Y. 1993. Detection of passage and absortion of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. *Am. J. Vet. Res.* 54: 867–872.
17. Wood, E. N. 1977. P An apparently new syndrome of porcine epidemic diarrhea. *Vet. Rec.* 100: 243–244.