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Poly (D,L-lactide-co-glycolide) nanoparticle-entrapped vaccine induces a protective immune response against porcine epidemic diarrhea virus infection in piglets

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ABSTRACT

Porcine epidemic diarrhea (PED) causes 80–100% mortality in neonatal piglets, and its causative agent, the porcine epidemic diarrhea virus (PEDV), poses an important threat to the swine industry worldwide. In this study, we prepared biodegradable poly (D,L-lactide-co-glycolide) (PLGA) nanoparticle–entrapped PEDV killed vaccine antigens (KAg) (PLGA-KAg). Late-term pregnant sows were intranasally inoculated with PLGA-KAg, and the mortality resulting from challenge with highly virulent PEDV was investigated in their passively immunized suckling piglets. PEDV-specific IgG and IgA antibody titers were enhanced in pregnant sows immunized with PLGA-KAg relative to the titers in sows inoculated with KAg. Similar results were seen in the passively immunized suckling piglets of these sows. Improved lymphocyte proliferation responses and IFN-γ levels were induced in pregnant sows immunized with PLGA-KAg compared with those vaccinated with KAg or with Montanide™ ISA 201 VG emulsified killed PEDV vaccine (201-KAg). Importantly, there was less piglet mortality in the group vaccinated with PLGA-KAg than in the groups vaccinated with KAg or 201-KAg. These results demonstrate that PLGA-KAg is a promising candidate vaccine that can provide protective immunity against PEDV infection in suckling piglets.

1. Introduction

Porcine epidemic diarrhea (PED), which is caused by the porcine epidemic diarrhea virus (PEDV), manifests clinically as severe watery diarrhea with subsequent dehydration in swine of all ages; however, it is most severe in suckling pigs [1]. PEDV is a member of the genus *Alphacoronavirus* of the family *Coronaviridae*. It is an enveloped virus with a 28 kb genome that encodes four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [2]. PEDV was first isolated in China in 1984 [3]. In October of 2010, a highly pathogenic variant PEDV strain was identified in China. In 2013, this variant strain struck the United States and subsequently spread to Canada and Mexico, causing high mortality rates in newborn piglets and significant economic losses [4–13].

PEDV infects pigs mainly via the fecal-oral route, therefore, protective mucosal immunity is essential for preventing infection. Immunization of sows with PEDV vaccines at 20–30 days before farrowing has been reported to provide substantial passive immunity to their newborn piglets [14,15]. Biodegradable and biocompatible PLGA nanoparticles (NPs) can be used as a particle-mediated delivery system for vaccines. The use of NPs protects the entrapped vaccine from proteases mediated degradation at mucosal surfaces, and leads to stimulation of the underlying mucosal immune cells [16–18]. NPs prepared from PLGA containing inactivated PRRSV delivered to mucosal sites in pigs have been shown to generate a protective mucosal immune response [19–21].

In this study, we evaluated the immune responses of pregnant sows and suckling piglets induced by inoculation with KAg, Montanide™ ISA 201 VG emulsified killed PEDV vaccine (201-KAg), or PLGA-KAg. Our results indicate that PLGA-KAg induced systemic and mucosal immunity in pregnant sows and suckling piglets that was significantly enhanced compared with the immunity induced

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by KAg or 201-KAg. In addition, the PLGA-KAg efficiently protected suckling piglets against challenge with PEDV.

2. Materials and methods

2.1. Cells and viruses

The Vero-81 (ATCC No. CCL-81) cell line was used for PEDV propagation. Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Life Technologies), penicillin (100 units/mL), streptomycin (100 mg/mL), and fungizone (0.25 mg/mL). PEDV AH2012/12 strain (GenBank Accession No. KU646831) was isolated and maintained in our laboratory, as previously described [28,31–33].

2.2. Preparation of the vaccine antigens and two formulations of inactivated PEDV vaccine

A Vero-81 cell-monolayer was infected with the PEDV AH2012/12 strain and maintained in DMEM containing 10 μg/mL trypsin at 37 °C in a 5% CO_2 atmosphere until a cytopathic effect became evident. The infected cells were then lysed using the freeze-thaw method and centrifuged at 2000*g* for 10 min. The supernatant was tested and adjusted to $10^{7.0}$ $TCID_{50}$/mL, and inactivated with 0.1% formaldehyde at 37 °C for 48 h. The killed antigen was stored at −70 °C.

201-KAg was prepared by mixing the inactivated PEDV vaccine antigen in an equal volume of ISA201 adjuvant (Seppic, France). PLGA (Sigma Aldrich, USA) nanoparticle–entrapped killed PEDV vaccine (PLGA-KAg) was prepared using a standard double emulsion solvent evaporation technique [34]. Briefly, 15% (w/v) PLGA (75/25; 300 mg) was dissolved in 2 mg of killed PEDV antigens, which were dissolved in dichloromethane, homogenized at 6000 rpm for 90 s, then added to an aqueous solution of 10% polyvinyl alcohol and homogenized. Finally, the preparation was stirred overnight and the washed nanoparticles were freeze-dried and stored at 4 °C. The amount of entrapped PEDV protein in the nanoparticles was determined as described previously [34]. The size and shape of the nanoparticles was determined using scanning electron microscopy (JSM-5610LV).

2.3. Pigs and inoculations

Twelve commercial healthy sows were randomly divided into four groups (n = 3 per group) and were housed in four separate rooms. These sows were confirmed virologically negative for PEDV, TGEV, and porcine deltacoronavirus by RT-PCR and serologically negative for PEDV and TGEV antibodies by indirect ELISA.

The pigs in the four groups were intranasally immunized either with PBS, KAg, 201-KAg, or PLGA-KAg at 28 and 14 days prior to farrowing. Every dose of PEDV vaccine (KAg, 201-KAg, or PLGA-KAg) contained the same inactivated virus. Serum samples were collected at 14 and 28 days after the first immunization and colostrum was collected after parturition for detection of N protein-specific antibodies and PEDV-neutralizing antibodies as has been previously described [15]. The colostrum samples were also tested for the presence of IgA antibodies against PEDV using commercial ELISA kits (Anigen, Korea). On the day of farrowing, fresh peripheral blood mononuclear cells (PBMCs) were collected from the sows for assessment of lymphocyte proliferation and for IFN-γ release assay.

The piglets from each sow were housed with their mothers with no artificial supply of colostrum or milk. Five suckling piglets from each sow were chosen randomly at 5 days of age and orally challenged with 100 LD_{50} PEDV AH2012/12. Before challenge, serum samples were collected from the piglets for detection of PEDV-specific IgG antibodies and neutralizing antibodies. The challenged piglets were then monitored for signs of PED.

2.4. ELISA

PEDV N protein specific antibodies in serum and colostrum were analyzed by ELISA. Briefly, ELISA plates were coated with purified PEDV N protein (0.8 μg/mL) in carbonate-bicarbonate buffer (pH 9.6) washed and blocked (5% skimmed milk +0.1% Tween 20 in PBS). Twofold serial diluted serum samples (started with 1:8) were added and incubated for 1 h at 37 °C. PEDV N protein specific antibody was detected using anti-pig IgG secondary antibodies conjugated with HRP (KPL). Plates were developed using the chromogen TMB and read at 450 nm. The titers were expressed as the reciprocal of the highest dilution of sera producing ratio values of 2.1. Data are presented as the means ± S.E.M. PEDV specific IgA antibodies in the colostrum were analyzed using commercial ELISA kits (Anigen, Korea), as instructed by the manufacturer. The reaction is stopped by addition of the stop solution and colorimetric reading will be performed by using a spectrophotometer at 450 nm and 620 nm. Data are presented as the means of $OD_{450\text{-}620}$ ± S.E.M. for three sows per group.

2.5. Serum neutralization (SN) test

The SN test was performed using a previously published method, with some modifications [15]. Briefly, swine sera and colostrum were inactivated at 56 °C for 30 min, and stored at −20 °C until use. After twofold dilution, serum or colostrum was mixed with PEDV (200 $TCID_{50}$/0.1 mL) at an equal volume, and incubated for 1 h at 37 °C. Subsequently, 0.1 mL of each mixture was transferred to Vero cell monolayers of a 96-well tissue culture plate washed twice with PBS. After adsorption for 1 h at 37 °C, inocula were discarded, and washed twice with PBS. Next, maintenance medium containing trypsin (10 μg/mL) was added to each well, and the plate incubated for 5 days at 37 °C. The neutralization titers were expressed as the reciprocal of the highest serum or colostrum dilution resulting in complete neutralization. Each sample was run in triplicate.

2.6. Lymphocytes proliferation assay

Lymphocyte proliferation assay was performed using peripheral blood mononuclear cells (PBMCs) of pregnant sows by density centrifugation. The PBMCs were collected and stimulated with or without 20 μg/mL of PEDV proteins, which were prepared by ultracentrifugating the PEDV-infected Vero-81 cells at 80,000*g* for 2 h. Lymphocyte proliferation assays were performed as described previously [35]. Briefly, PBMCs were added to wells of a 96 well plate, and stimulated with or without 20 μg/mL of PEDV proteins. Each sample included three repetition wells. The 96-well cell culture plate was incubated in a 5% CO_2 incubator at 37 °C for about 72 h, and then 10 μL (5 mg/mL) MTT (Zhuyan, Nanjing, China) solution was added to each well, before incubating for 3–5 h in a 5% CO_2 incubator at 37 °C. Then 20 μL of DMSO (Zhuyan, Nanjing, China) were added to each well, and an ELISA microplate reader measured the absorbance at 450 nm. T-cell stimulation was expressed as stimulation index (SI), which was calculated as the ratio of the average OD value of antigen-stimulated wells to that of unstimulated wells.

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