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# Efficacy of orally administered porcine epidemic diarrhea vaccine-loaded hydroxypropyl methylcellulose phthalate microspheres and RANKL-secreting *L. lactis*

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ABSTRACT

Here, we examined the efficacy of are combinant subunit antigen-based oral vaccine for preventing porcine epidemic diarrhea virus (PEDV). First, we generated a soluble recombinant partial spike S1 protein (aP2) from PEDV in *E. coli* and then evaluated the utility of aP2 subunit vaccine-loaded hydroxypropyl methylcellulose phthalate microspheres (HPMCP) and RANKL-secreting *L. lactis* (LLRANKL) as a candidate oral vaccine in pregnant sows. Pregnant sows were vaccinated twice (with a 2 week interval between doses) at 4 weeks before farrowing. Titers of virus-specific IgA antibodies in colostrum, and neutralizing antibodies in serum, of sows vaccinated with HPMCP (aP2) plus LL RANKL increased significantly at 4 weeks post-first vaccination. Furthermore, the survival rate of newborn suckling piglets delivered by sows vaccinated with HPMCP (aP2) plus LL RANKL was similar to that of piglets delivered by sows vaccinated with a commercial killed porcine epidemic diarrhea virus (PED) vaccine. The South Korean government promotes a PED vaccine program (live-killed-killed) to increase the titers of IgA and IgG antibodies in pregnant sows and prevent PEDV. The oral vaccine strategy described herein, which is based on a safe and efficient recombinant subunit antigen, is an alternative PED vaccination strategy that could replace the traditional strategy, which relies on attenuated live oral vaccines or artificial infection with virulent PEDV.

## 1. Introduction

Porcine epidemic diarrhea (PED) is a highly infectious and contagious enteric disease of swine (Song and Park, 2012). The disease is characterized by severe diarrhea, vomiting, dehydration, and death, and has a mortality rate among suckling piglets of more than 90 % (Lee, 2015). Porcine epidemic diarrhea virus (PEDV), the causative agent of PED, is a RNA virus belonging to the alpha genus of the coronavirus family (Wang et al., 2013). The spike(S) protein is the primary target for development of subunit vaccines against PEDV. The S protein of PEDV, which comprises two domains(S1 (amino acids (aa) 1–789) and S2 (aa 790–1383)), is a transmembrane glycoprotein localized on the virion surface (Sun et al., 2007). The S protein plays a pivotal role in binding to cellular receptors, fusion with the target cell membrane, and most importantly, induction of neutralizing antibodies (Song and Park, 2012). Subsequently, a novel neutralizing epitope, S1D (aa 636–789), was identified on the S1 domain; this is the epitope responsible for induction of neutralizing antibodies against PEDV (Sun et al., 2007). To date, a number of expression systems have been used to generate the S protein of PEDV; these include mammalian cells, transgenic plants, yeast, and *E. coli* (Sun et al., 2007; Kang et al., 2004; Oh et al., 2014; Park et al., 2007). Despite the absence of post-translational modifications (i.e., phosphorylation, acetylation, and glycosylation), expression of recombinant proteins in *E. coli* has marked advantages over other expression systems in terms of cost, ease-of-use, and scale. However, over expressing recombinant proteins in *E. coli* often leads to the misfolding of the protein of interest into biologically inactive aggregates known as inclusion bodies (IBs) (Yamaguchi and Miyazaki, 2014).

IgA titers in colostrum and milk correlate with PEDV neutralizing antibody titers, which provide protective immunity against PEDV.

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Optimal vaccine regimens induce high levels of both types of antibody in the lactogenic secretions of vaccinated sows (Lee, 2015; Langel et al., 2016; Song et al., 2016). Both synthetic and natural polymers, including enteric coated polymers, are being evaluated as vehicles to deliver proteins to the gastrointestinal (GI) tract. Hydroxypropyl methylcellulose phthalate (HPMCP) is an example of an enteric coating excipient used widely by the pharmaceutical industry (Singh et al., 2008). The RANKL/RANK system plays an important role in development and regulation of the immune system; for example, it is involved in lymph-node organogenesis, lymphocyte differentiation, dendritic cell survival, and T-cell activation, and it promotes the functions of antigen-presenting cells (Maharjan et al., 2016). Therefore, increasing the number of microfold cells (M cells) by delivering RANKL may be a promising biomimetic strategy to increase the efficacy of oral vaccination (Kim et al., 2015). Oral vaccination of pregnant sows to induce lactogenic immunity is a fundamental strategy used to protect suckling pigs from infection with PEDV (Langel et al., 2016). The extent of this protection depends on the presence of virus-specific IgA antibodies in the milk of immunized sows.

Here, we used an *E.coli* expression system to generate a soluble aP2 protein expressing the neutralizing epitope (S1D) of the PEDV S1 domain. The aP2 protein is the expressed S1D epitope (aa 636–789) that elicit formation of neutralization antibodies of the PEDV (CV777 strain) S1 domain (Sun et al., 2007). Next, we developed aP2-loaded HPMCP microspheres and tested their efficacy as an oral vaccine candidate for prevention of PED. Moreover, we used RANKL ligand-secreting *L. lactis* as an oral adjuvant. This combined oral vaccination strategy was evaluated in pregnant sows. In addition, the protective efficacy of the vaccine was evaluated by challenging newborn suckling piglets with a Korean virulent field PEDV strain.

## 2. Materials and methods

### 2.1. Generation and purification of recombinant aP2 antigen from IBs

A recombinant *E. coli* strain harboring the pG5-S1D expression vector (Piao et al., 2016a) was used to generate soluble aP2 antigen. The antigen comprised the S1D epitope (aa 636–789) of the S1 domain of the S protein of PEDV CV777 strain (GenBank Accession No. AF353511). The antigen was synthesized after codon-optimization using GenScript (Piscataway, NJ, USA). A seed culture was prepared by inoculation of a single colony of recombinant *E. coli* BL21 strain into a 50 ml sterile plastic tube containing 5 ml LB broth supplemented with 100 μg/ml ampicillin; the culture was then grown overnight. To express the recombinant protein, 1 % of the seed culture was routinely inoculated into 500 ml LB broth containing 100 μg/ml ampicillin. Cells were cultured at 37 ˚C with shaking (at 230 rpm). When the $OD_{600}$ of the culture reached about 0.6, recombinant proteins were induced by addition of 0.3 mM IPTG and cultured at 37 ˚C for 4–6 h. After induction, cells were harvested by centrifugation and stored at −20 ˚C until required. To purify IBs, cells from a 1 l culture of each recombinant strain were harvested by centrifugation for 5 min at 5000 *g*, washed twice in distilled water, and lysed in lysis buffer I (50 mM Tris-HCl, pH 8.0) containing 10 mg/ml lysozyme and 1 mg/ml DNase I. After 1 h, cells were disrupted by sonication for 3 min using a 2 s-on and 5 s-off cycle, and an amplitude of 30 %. The resulting suspension was centrifuged at 4 ˚C for 5 min at 32,000 *g*. Next, the pellet was washed three times in lysis buffer II (lysis buffer I containing 0.5 % Triton X-100). Purified IBs were collected by centrifugation for 5 min at 32,000 *g*. The concentration of purified IBs was determined by SDS-PAGE and densitometry using BSA as a standard.

### 2.2. Recovery of soluble aP2 from IBs

Soluble aP2 was recovered from IBs using the alkaline pH solubilization method, as described previously (Piao et al., 2016b). Briefly, aP2 IBs obtained from 1 l cell culture were solubilized in 40 ml alkaline pH buffer (10 mM Tris-HCl, pH 12.5). The supernatant was collected by centrifugation for 10 min at 32,000 *g* and 4 ˚C. Solubilized proteins were precipitated by adding four volumes of ice-cold acetone, followed by centrifugation for 15 min at 48,000 *g* and 4 ˚C. After removing the supernatant, the pellet was washed once in 20 ml of ice-cold acetone, followed by contrifugation for 15 min at 48,000 *g*. The acetone was evaporated by air-drying for 10–15 min at room temperature and the pellet was dissolved immediately in distilled water.

### 2.3. Preparation of aP2-loaded HPMCP microspheres and electron microscopy

HPMCP was obtained from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Soluble aP2 (50 mg) was dissolved in 1 ml distilled water and stabilized with Pluronic F-127 solution (1 wt - %) to form an internal aqueous phase (W1). Next, 0.2 ml W1 (10 mg aP2) was emulsified for 1 min with an organic phase (O), which comprised 200 mg HPMCP dissolved in 5 ml dichloromethane (DCM), using an ultrasonic processor (Vibra cells, USA; output of 2 W) to form a primary emulsion (W1/O). The prepared primary emulsion (W1/O) was added drop wise to 50 ml of 1 wt - % PVA solution (W2) to create an external aqueous phase. This was then homogenized (Ultra-Turrax™ Homogenizer T25, IKA) for 4 min at 27,000 *g* to form a W1/O/W2 emulsion, which was then stirred with a magnetic stirrer for 12 h at room temperature to allow the solvent to evaporate. The synthesized microparticles were collected, centrifuged at 8000 *g* for 10 min at 4 ˚C, and washed three times with distilled water. The microspheres were lyophilized and stored at −70 ˚C prior to characterization. The surface topography and average size of the microparticles were analyzed by field-emission scanning electron microscopy (FE-SEM) using a Supra 55 V P scanning electron microscope(Carl Zeiss, Oberkochen, Germany). Prior to examination, microparticles were mounted on metal stubs using thin adhesive tape and then coated with gold in a coating chamber (CT 1500 H F, Oxford Instruments Oxfordshire, UK) under vacuum.

### 2.4. Loading content and encapsulation efficiency

The amount of antigen encapsulated per unit weight of microparticles was determined as follows: 5 mg of dried microparticles were dissolved in 500 μl of 0.1 M NaOH solution (pH 12) containing 0.05 % SDS. Themixture was vortexed vigorously and incubated at room temperature for 1 h. After centrifuging at 32,000 g for 10 min, 200 μl aqueous solution was withdrawn and the aP2 content was calculated in a BCA protein assay or by SDS-PAGE densitometry using BSA as a standard. The encapsulation efficiency was expressed as the ratio of the actual amount of loaded antigen to the total amount of antigen used to prepare the microparticles. The encapsulation efficiency and antigen loading were calculated using the following equations:

$$\text{Loading efficiency (\%)} = \frac{\text{amount of protein in microspheres}}{\text{amount of protein initially used}} \text{X100\%}$$

$$\text{Loading content (\%)} = \frac{\text{amount of protein in microspheres}}{\text{amount of microspheres}} \text{X100\%}$$

### 2.5. In vitro release test

AP2-loaded HPMCP microspheres (10 mg) suspended in 1 ml small intestinal fluid (pH 7.37, 6.73, 6.83, and 6.83) or 1 ml gastrointestinal fluid (pH 2.08, 2.28, 2.26 and 2.93) of 4 pigs were agitated for up to 24 h at 37 ˚C with shaking at 100 rpm. Next, a 100 μl aliquot was withdrawn from the release medium at each time point and replaced by an equal volume of fluid. The amount of released protein was determined as cumulative release (wt. - %) against incubation times using a Micro BCA™ protein assay kit ThermoFisher scientific com, cat no. 23235.

**Table 1**
Vaccine safety and number of offspring produced by pregnant sows.

| Group | Type of vaccine | Sow | Period of pregnancy (days) | No. of piglets | No. of live piglets | No. of dead piglets | No. of abortions | Average weight of live piglets | Clinical signs or side effects after inoculation |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Mock | M12-7 | 114 | 10 | 9 | 1 | 0 | 1.83 ± 0.24 | No |
| | | M17-2 | 117 | 16 | 14 | 2 | 0 | 1.58 ± 0.17 | No |
| 2 | Commercial killed PED | K32-6 | 116 | 14 | 10 | 4 | 0 | 1.87 ± 0.26 | No |
| | (IM) | K52-8 | 118 | 10 | 8 | 2 | 0 | 1.66 ± 0.19 | No |
| 3 | HPMCP-aP2 | HI8-2 | 116 | 13 | 11 | 2 | 0 | 1.74 ± 0.18 | No |
| | (IM) | HI6-4 | 117 | 15 | 14 | 1 | 0 | 1.65 ± 0.23 | No |
| 4 | HPMCP-aP2 | HO4-4 | 114 | 11 | 11 | 0 | 0 | 1.71 ± 0.15 | No |
| | (Oral) | HO7-1 | 116 | 11 | 8 | 3 | 0 | 1.68 ± 0.21 | No |
| 5 | HPMCP-P2 + LL RANKL | HL3-3 | 115 | 12 | 12 | 0 | 0 | 1.64 ± 0.13 | No |
| | (Oral) | HL9-6 | 117 | 12 | 12 | 0 | 0 | 1.58 ± 0.18 | No |

### 2.6. Vaccination of pregnant sow and challenge for offspring with PED

Before 4 weeks of parturition, ten pregnant sows (serologically negative for PEDV neutralizing antibodies) were vaccinated twice (with a 2 week interval between doses) with HPMCP microspheres containing 10 mg aP2 via the oral (group 4) or intramuscular (IM) (group 3) routes. Another group of pigs received oral vaccination with $10^{12}$ CFU RANKL-secreting *L. lactis* (group 5). A control group (group 2) received IM vaccination with a commercial PED vaccine, a commercial killed vaccine (SM98 strain), using the same regimen. Finally, another control group (group 1) was mock-vaccinated (Table 1). Serum samples were collected from pregnant sows before and at 4 weeks post-vaccination (at the time of farrowing), and colostrum samples were collected from lactating sows at the time of delivery. Whey was separated from colostrum and tested in a virus neutralization assay. Offspring suckling piglets were housed with their sows and received no artificial colostrum or milk. Three-day-old piglets were selected at random from each farrowing sow in the vaccinated and control groups, and challenged with virulent PEDV. Briefly, 76 sucking piglets were transported from the farm to an environmentally controlled facility at the Animal and Plant Quarantine Agency (APQA), where they received artificial milk four times daily. The suckling piglets (aged 5 days) were challenged orally with 10 ml/dose ($10^{2.5}TCID_{50}$/ml) of wild-type virulent PEDV (K14JB01 strain, GenBank Accession No. KJ623926). Clinical signs of diarrhea, morbidity, and mortality were monitored three times daily up to 14 days post challenge (dpc). Rectal swabs were collected and body weight was checked every day (from Day 0 (pre-challenge) to 14 dpc). Diarrhea was scored daily for the first 14 dpc and then every other day thereafter. Fecal consistency was scored as follows: 0, solid; 1, pasty; 2, semi-liquid; and 3, liquid.

### 2.7. Real-time PCR (qRT-PCR) of fecal samples from piglets

QRT-PCR was performed to detect the PED antigen copy number in fecal swab samples to assess PEDV shedding after challenge. The AnyQ PEDV qRT-PCR (MEDIANDiagnostic Co. Cat No. PS105, Korea), which uses TaqMan probes to detect the PEDV spike region, was used according to the manufacturer's instructions. The RT-PCR program comprised the following steps: cDNA synthesis (50 ˚C, 30 min) and initial inactivation (95 ˚C, 15 min), followed by a two-step PCR comprising 42 cycles of denaturation (95 ˚C, 10 s) and annealing/extension (60 ˚C, 60 s).

### 2.8. Detection of PED IgA antibodies and histopathological examination

IgA levels in the colostrum were tested using the PED IgA ELSIA kit (Bionote co., Cat No. EB4410PO, South Korea). The mean $OD_{450}$ of the standard negative (NCx) and standard positive (PCx) controls were ≤ 0.200 and ≥ 0.500, respectively. Interpretation of the results is based on a cut-off value calculated as follows: cut-off value = [0.35 + NCx]. The VH/CD ratio in the jejunum and ileum were measured in six villi per intestinal section. Moribund piglets that showed signs of anorexia and dehydration over a period of 24 h were euthanized on the instruction of institutional veterinarians.

### 2.9. Ethics statement and statistical analysis

The study was approved by the Animal Care and Use Committee of the APQA. All animal experiments were performed in accordance with the guidelines of the APQA Animal Care and Use Committee. All statistical analyses were performed using GraphPad Prism software, version 6.0, for Windows. Data were analyzed using one-way analysis of variance, followed by Tukey's multiple-comparison test. In the Tables, groups showing significant differences ($p < 0.05$) at the same time point are indicated by different letters.

## 3. Results

### 3.1. Electron microscopy image analysis and loading efficiency of aP2-loaded HPMCP microspheres

Scanning electron microscopy (SEM) images of aP2-loaded HPMCP microspheres revealed that the microspheres were 1–10 μm diameter size of the spheres (Fig. 1A). AP2-loaded HPMCP microspheres (5 mg) were dissolved in 0.1 M NaOH (pH 12) buffer containing 0.05 % SDS and the amount of released aP2 protein was determined in a BCA assay. The optimal loading content and efficiency were 6.3 % and 42.5 %, respectively. The band intensities of the aP2 protein were similar to those of the BSA standard (Fig. 1B).

### 3.2. Time-dependent release of aP2 from HPMCP microspheres in gastrointestinal and small intestinal fluids

The *in vitro* time-dependent release of aP2 from HPMCP microspheres was assessed in small intestinal fluid (pH 7.37, 6.73, 6.83, and 6.83) or gastrointestinal fluid (pH 2.08, 2.28, 2.26 and 2.93) of 4 pigs; this was designed to the same environment in the GI of pig. We suspended 10 mg of aP2-loaded HPMCP microspheres in 1 ml small intestinal fluid (pH 7.37, 6.73, 6.83, and 6.83) or gastrointestinal fluid (pH 2.08, 2.28, 2.26 and 2.93), and agitated them (100 rpm) for 24 h at 37 ˚C. Almost 70 wt- % of aP2 was released from the microspheres within 1 h at pH 6.73–7.37; by contrast, less than 20 wt- % of aP2 was released within 1 h at pH 2.08–2.93 (Fig. 2).

### 3.3. Parturition and antibodies after vaccination of sows

The titer of serum neutralizing antibodies in all vaccinated sows increased from 2 to 4 ($\log_2$) to 5–7 ($\log_2$) at 4 weeks after the first vaccination (Fig. 3A). Neutralizing antibody titers in colostrum and whey samples from vaccinated sows were higher (6–8 $\log_2$) than those

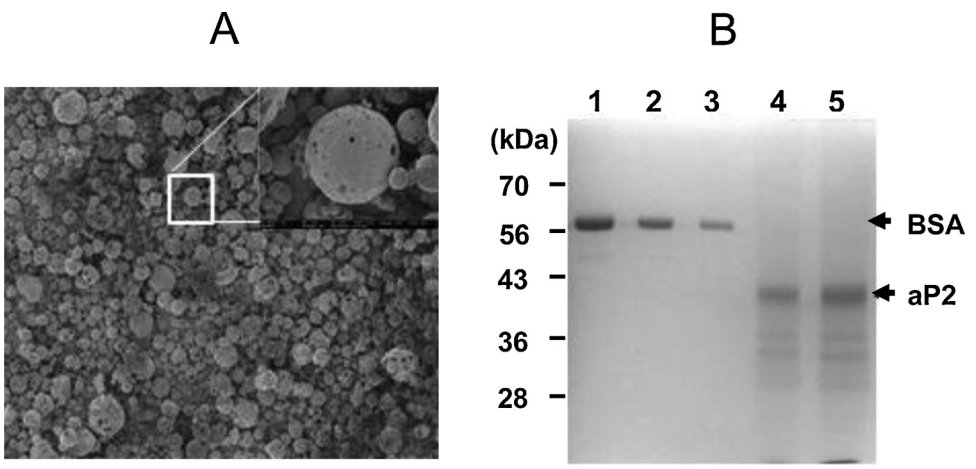


**Fig. 1.** Scanning electron microscope (SEM) images of aP2-loaded HPMCP microspheres (A). The microspheres were 1–10 μm in size and spherical. SDS-PAGE analysis of aP2 released from HPMCP microparticles (B). A total of 2.5 (lane 4) and 5 μg (lane 5) of released aP2 were loaded, and the intensities of aP2 protein bands were compared with those of a BSA standard (lanes 1–3).

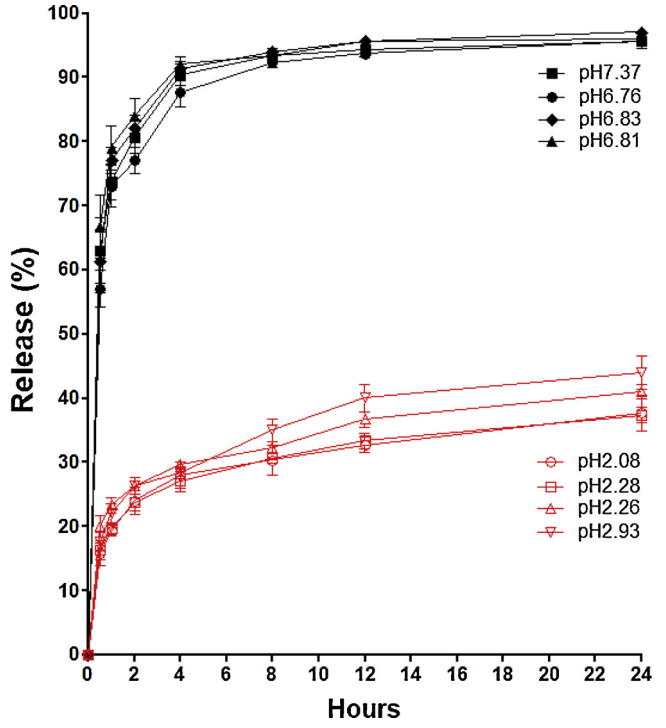


**Fig. 2.** Profiles of aP2 antigen release from the HPMCP microspheres during 24 h incubation at pH 2.0 and pH 7.0. Data are expressed as the mean ± S.D.

from sows in control group 1 (1–3 log $_2$) (Fig. 3A). The mean gestational period was 116 days, and the average number of piglets was 12.4 (Table 1). After birth, the number of surviving piglets ranged from 9 to 14, with an average of 10.9; the average number of still-born piglets was 1.5. The body weight of live piglets from each sow varied (Table 1). None of the pregnant sows showed clinical signs or side effects after vaccination. The cut-off value for the PED IgA ELISA was 0.47 (OD). The OD value of colostrum from sows in group 1 was negative (0.365 ± 0.085), while that from sows in group 2 (inoculated with killed PED) was positive (0.68 ± 0.17) (Fig. 3B). IgA levels in colostrum and whey samples from sows inoculated orally with the HPMCP(aP2) plus LL RANKL vaccine (group 5) were significantly higher (0.975 ± 0.255 and 0.99 ± 0.17, respectively) than those in samples from mock-vaccinated sows ($p < 0.05$) (Fig. 3B).

### 3.4. Villous atrophy and mortality of piglets

After challenge with PEDV, 16 piglets in group 1 (mock control) died by 8 dpc, and all piglets (16/16) in group 3 (inoculated IM with the HPMCP(aP2) vaccine alone) died between 5 and 8 dpc (Fig. 4A). One of the fourteen piglets in group 4 (inoculated orally with the HPMCP(aP2) vaccine alone) survived, whereas the remainder died between 5 and 8 dpc (Fig. 4A). Half (8/16) of the piglets born to sows in group 2 (inoculated with the commercial killed PED vaccine) survived (Fig. 4A) as did half (8/16) of the piglets born to sows inoculated with the HPMCP(aP2) plus LL RANKL vaccine (Group 5) (Fig. 4A). The severity of villous atrophy in infected pigs was quantified by measuring the ratio between villus height and crypt depth (the VH:CD ratio). Villous atrophy in the ileum of piglets born to sows vaccinated with the HPMCP(aP2) plus LL RANKL vaccine was significantly milder than that in mock group piglets ($P < 0.05$) (Fig. 4B). Villous atrophy in the jejunum of piglets born to sows vaccinated with the commercial killed vaccine (SM98 strain) or the oral HPMCP(aP2) plus LL RANKL was significantly milder than that in mock group piglets ($P < 0.05$) (Fig. 4B).

### 3.5. Diarrhea score for offspring piglets

Sixteen piglets born to sows in group 1 experienced diarrhea from 2 dpc; the average diarrhea scores (ADS) were 1.00 (2 dpc), 1.93 (3 dpc), 2.87 (4 dpc), 2.80 (5 dpc), 2.80 (6 dpc), 2.60 (7 dpc), and 3.00 (8 dpc) ($P < 0.05$) (Fig. 5A). Piglets in group 2 (commercial killed PED vaccine) began having diarrhea at 4 dpc, but the ADS were lower than those of group 1: 0.66 (4 dpc), 1.16 (5 dpc), 1.66 (6 dpc), 2.16 (7 dpc), 2.40 (8 dpc), 1.66 (9 dpc), 1.00 (10 dpc), 0.66 (11 dpc), and 0 (12–14 dpc) (Fig. 5A). The 16 piglets in group 5 (sows inoculated with the oral HPMCP(aP2) plus LL RANKL vaccine) also showed low and long-lasting ADS: 0.37 (2 dpc), 0.87 (3 dpc), 1.18 (4 dpc), 1.87 (5 dpc), 2.12 (6 dpc), 1.80 (7 dpc), 1.61 (8 dpc), 0.12 (9 dpc), and 0.13 (10 dpc) (Fig. 5A).

### 3.6. Body weight changes in offspring piglets

The piglets in groups 1 and 3 showed significant body weight loss; 16 suckling piglets born to mock sows lost a mean 30.6 % of body weight at 8 dpc, whereas 16 piglets born to sows inoculated with oral HPMCP(aP2) lost a mean 28.8 % body weight at 8 dpc ($P < 0.05$) (Fig. 5B). By contrast, piglets born to sows inoculated with the commercial killed vaccine lost only 19.3 % body weight at 8 dpc; however, by 14 DPC their body weight recovered to 94.4 % of that prior to PEDV challenge (Fig. 5B). The 16 piglets born to sows inoculated with the oral HPMCP(aP2) plus LL RANKL vaccine (group 5) also lost 13.9 % body weight at 8 dpc; however, the surviving piglets regained 100.1 % of the lost weight by 4 dpc (Fig. 5B).

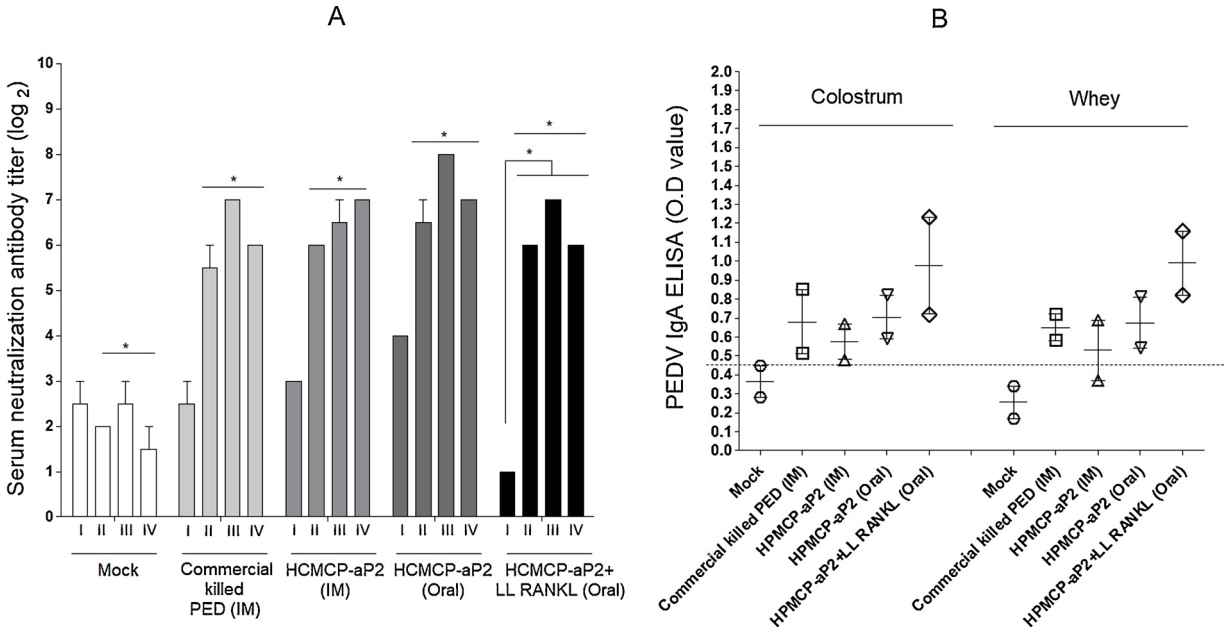


**Fig. 3.** Neutralizing antibodies and IgA antibodies in sows vaccinated with HPMCP (aP2) plus LL RANKL. (A) Neutralizing antibodies (log $_2$) in serum before vaccination (I) and in serum (II), colostrum (III), and whey (IV) at the time of farrowing. (B) Anti-PEDV-specific IgA levels in colostrum and whey, as detected by ELISA. The cut-off value (0.47 OD) in the PED IgA ELISA is marked by a dotted line. Bars represent the mean ± standard error (SE) (*$P < 0.05$).

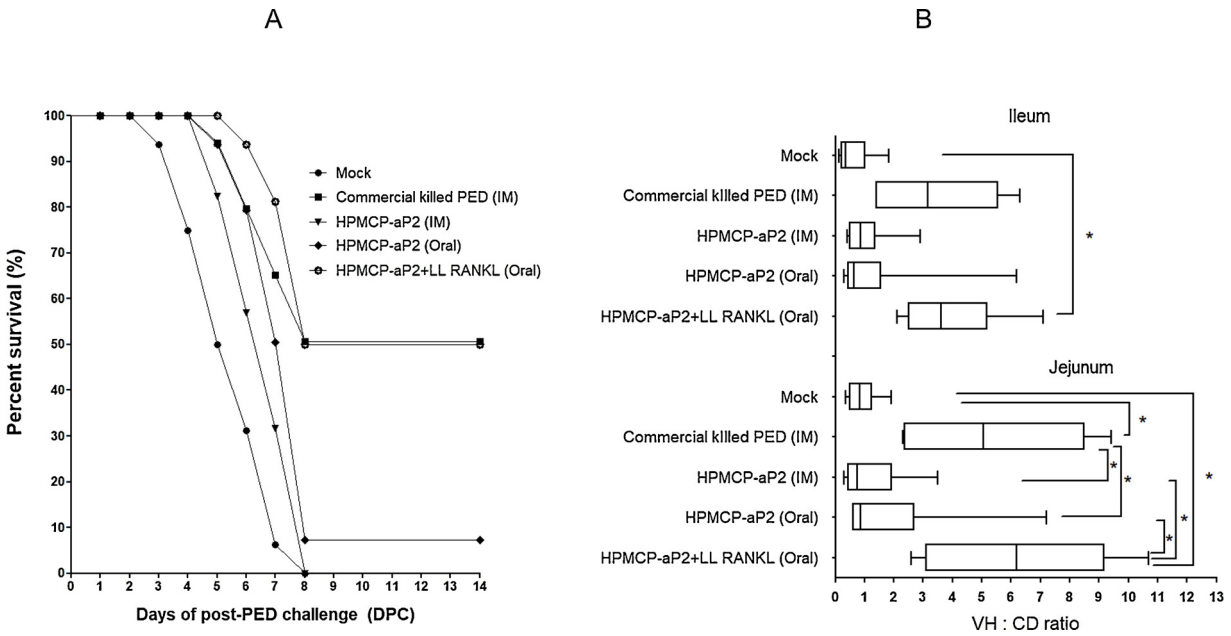


**Fig. 4.** HPMCP (aP2) plus LL RANKL protects 5-day-old piglets against virulent PEDV. (A) Survival of vaccinated piglets at 14 dpc. (B) VH/CD ratio. Six villi perintestinal section were examined. Groups showing significant differences (*$P < 0.05$) are indicated by different letters. Error bars indicate the S.D.

### *3.7. PEDV RNA copy number in the diarrhea of piglets*

The viral RNA copy number in feces from piglets in group 1 was 2.8 (log $_{10}$)/g at 2 dpc and 2.4 (log $_{10}$) at 8 dpc; copy number peaked at 7.18 (log $_{10}$) at 4 dpc (Fig. 6). The copy number in piglets vaccinated with the commercial killed vaccine was 2.45 (log $_{10}$) at 4 dpc and 0.77 (log $_{10}$) at 12 dpc, peaking at 5.33 (log $_{10}$) at 6 dpc (Fig. 6). Piglets vaccinated with the oral HPMCP(aP2) plus LL RANKL vaccine showed lower copy numbers than those in groups 1, 3, and 4. The copy numbers for piglets in group 5 were as follows: 2 dpc (0.94 log $_{10}$), 3 dpc (2.06), 4 dpc (2.91), 5 dpc (4.69), 6 dpc (5.00), 7 dpc (3.88), 8 dpc (2.76), 9 dpc (1.23), and 10 dpc (0.13) (Fig. 6).

## 4. Discussion

Newborn piglets rely on maternal lactogenic immunity supplied via colostrum and milk (Saif and Jackwood, 1990). Natural infection or oral immunization with live virus can generate sufficient mucosal or lactogenic immunity, whereas intramuscular administration induces a systemic immune response that provides little lactogenic immunity (Song et al., 2016; Chattha et al., 2015). In general, subunit oral vaccines show poor immunogenicity, and although some toxin adjuvants such as cholera toxin and *E. coli* heat-labile toxin can act as mucosal vaccine adjuvants, obvious safety concerns prevent their use in a clinical setting (Kim et al., 2015). Notably, a recent study demonstrated the importance of the receptor activator of NF-kB ligand (RANKL) for

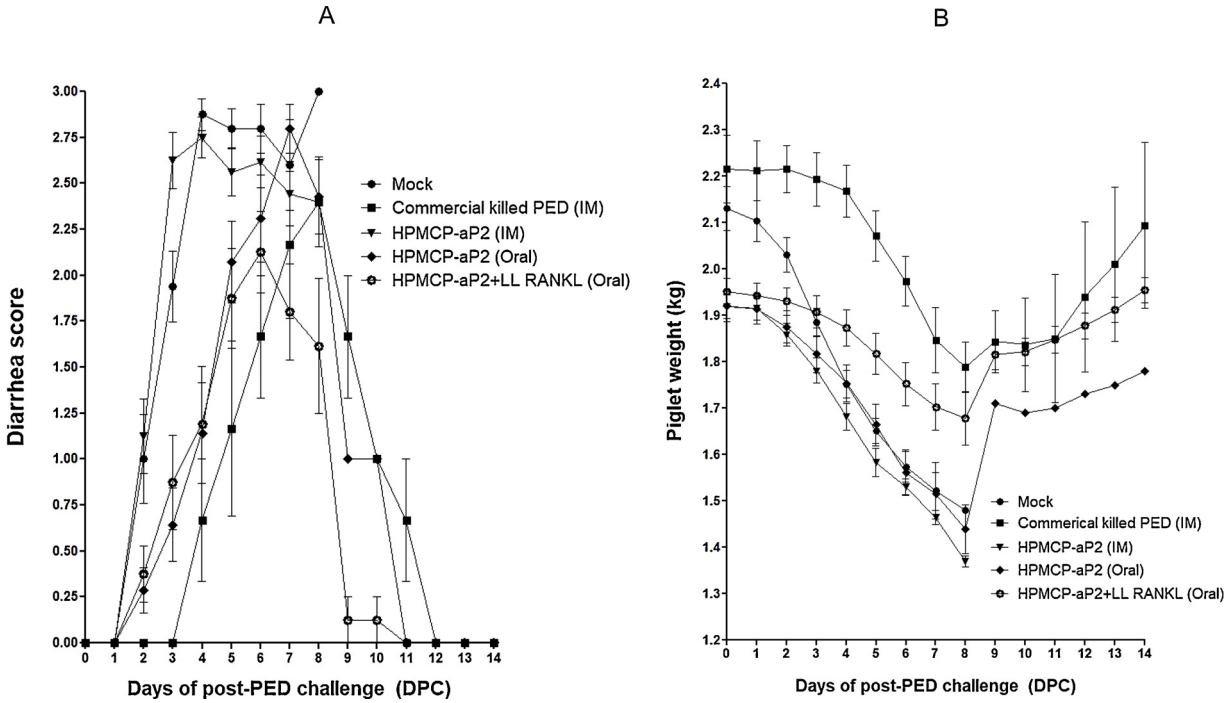


**Fig. 5.** Clinical signs and body weight changes in piglets post-challenge with PEDV. (A) Diarrhea score and (B) change in body weight. Data were expressed as mean ± S.D.

controlling M cell differentiation in Peyer's patches (Knoop et al., 2009).

Here, we generated a soluble aP2 protein containing PEDV S1D and loaded it into pH-sensitive HPMCP microspheres. SEM images revealed that the microspheres ranged from 1 to 10 μm in size and were spherical. The size of microspheres affects their uptake by APCs and M cells. Particles smaller than 5 μm may transfer to the draining lymph nodes and spleen, where they stimulate both mucosal and systemic immune responses, where as particle sizes measuring 5–10 μm are retained in the Peyer's patches, where they stimulate mainly mucosal immune responses. Particles measuring more than 10 μm are too large to be taken up by immune cells or M cells. The loading content and efficiency of aP2-loaded HPMCP microspheres were 6.3 % and 42.5 %, respectively. Moreover, an *in vitro* release test revealed that 70 % of aP2 was released from microspheres within 1 h at pH 7.0, but only 20 % were released at pH 2.0, suggesting that HPMCP (aP2) is suitable as an oral vaccine. HPMCP55 is a pH-sensitive polymer that dissolves at pH > 5.5. The observed rapid dissolution of aP2-loaded HPMCP microspheres may be due to the pH of PBS, which is far higher than 5.5. In the GI, the pH gradient from stomach to ileum is 2.0–7.4. Therefore, we assume

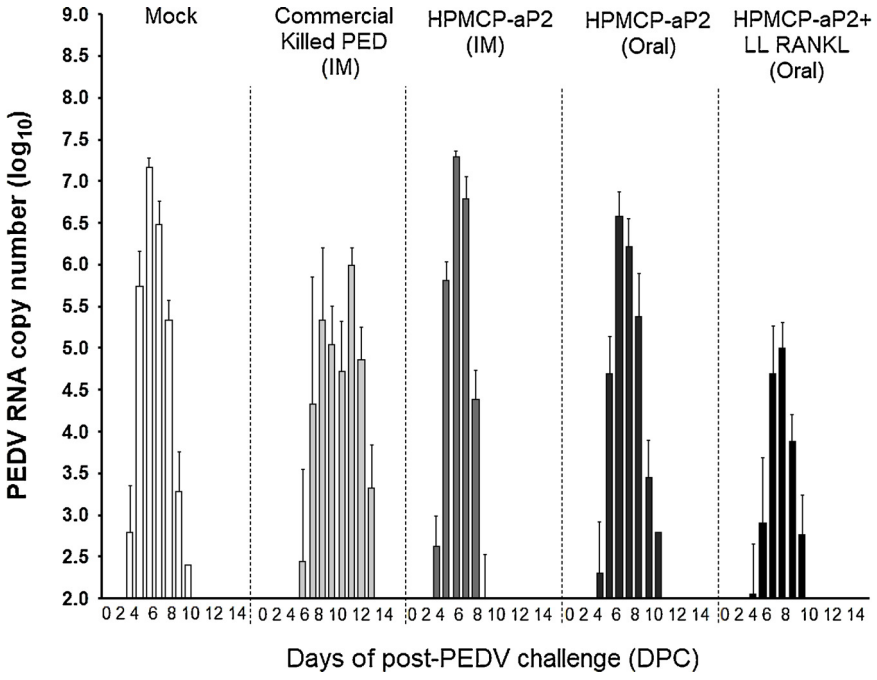


**Fig. 6.** Virus sheddings in the piglets post-challenge with PEDV. Viral RNA copy number detected from feces of piglets was shown as mean ± S.D.

that aP2 will be released slowly from HPMCP microspheres after passing through the stomach, and then more rapidly as it approaches the ileum. The colostrum neutralizing antibody titer of sows vaccinated intramuscularly with HPMCP(aP2) microspheres was 6–7 ($log_2$), suggesting that humoral immune responses were induce defficiently by injection of HPMCP(aP2) microspheres, and that virus-neutralizing IgG antibodies were transferred to the colostrum after farrowing. Sows vaccinated orally with HPMCP(aP2) microspheres alone or with microspheres plus the LL RANKL adjuvant showed higher serum and colostrum neutralizing antibody titers than mock-vaccinated sows, suggesting that both humoral and mucosal immune responses are induced efficiently.

Maternal L-K-K (attenuated live vaccine-killed vaccine-killed vaccine) vaccination is more efficacious than K-K vaccination in terms of absolute improvement in survival rates and significant mitigation of diarrhea, as well as the quantity and duration of viral shedding in stool (Jang et al., 2019). Oral vaccination of sows with HPMCP (aP2) plus LL RANKL induced high neutralizing antibody titers in serum, colostrum, and whey, and induced IgA antibodies in colostrum and whey. The mortality rate of piglets delivered by sows vaccinated with HPMCP (aP2) plus LL RANKL was the same (50 %) as that of piglets delivered by sows vaccinated with the commercial killed PED vaccine. Therefore, our current oral vaccine strategy based on a recombinant subunit antigen (aP2) in conjunction with the HPMCP carrier and LL RANKL adjuvant could be a novel alternative vaccination strategy for preventing PED. Lactogenic immunity is described as a continuous supply of passively acquired immunoglobulin (IgG, IgM, and sIgA) through ingestion of colostrum and milk (Klobasa and Butler, 1987). This is dependent on trafficking of pathogen-specific IgA + plasmablasts to the mammary gland (MG) and accumulation of secretory IgA (sIgA) antibodies in milk, defined as the gut-MG-sIgA axis (Bohl and Saif, 1975; Bohl et al., 1972; Fink, 2012). Maternal vaccination to increase the amount of protective antibodies (induced via the gut-MG-sIgA axis) passively transferred in milk is the strategy used to protect suckling piglets from PEDV immediately after birth (Langel et al., 2016; Chattha et al., 2015). The increased rate of protection is associated with high titers of IgA antibodies in colostrum and milk. We expect that if the suckling piglet challenge experiments were performed while the piglets were cohabitant with vaccinated sows (i.e., piglets received PED-sIgA-containing milk), then the survival rates after challenge with virulent virus would be higher. Because the milk of sows vaccinated with HPMCP (aP2) microspheres plus LL RANKL contained sIgA specific for PEDV, continuous milk intake would increase immunity to PEDV. This demonstrates that enteric viral infection stimulates the intestinal mucosa, thereby influencing lactogenic immunity via the gut-MG-sIgA axis (Langel et al., 2016; Chattha et al., 2015). Many swine researchers in South Korea promote a PED vaccine program (live-killed-killed) to increase IgA and IgG antibody production by pregnant sows to ensure effective prevention of PEDV. In Korea, the L-K-K vaccine program involves continuous inoculation during pregnancy to raise IgA and IgG antibody titers in pregnant sows and in the colostrum fed to their piglets. The L-K-K vaccine program involves vaccinating pregnant sows three times. The first vaccination is with a live attenuated PED vaccine at 7–8 weeks before delivery, followed by two vaccinations with killed vaccine at 5–6 weeks and 2–3 weeks before delivery. Optimal timing (at 58–65 days of gestation, the middle period of pregnancy) of oral vaccination increases maternal immunity and lactogenic immunity for at least 7–8 weeks. A previous study examined survival of piglets born to sows infected with PEDV during the early, middle, and terminal stages of pregnancy (Langel et al., 2019). All piglets born to sows infected during mid-gestation survived, compared with 75 % of those in the early gestation group and 25 % of those in the late gestation group (Langel et al., 2019). Thus, maternal immunity and lactogenic immunity were strongest when sows were exposed to PEDV during the mid-gestation period; in addition, the diarrhea scores and virus shedding by neonatal suckling piglets after PEDV challenge were low (Langel et al., 2019). Therefore, we propose that immunization with HPMCP (aP2) microspheres plus LL RANKL is likely to be most effective during the mid-pregnancy period.

In conclusion, IgA titers in maternal colostrum from sows vaccinated with HPMCP (aP2) microspheres plus LL RANKL play an important role in preventing virulent PEDV in suckling piglets. The survival rates of piglets delivered by this group of sows were the same as those of piglets born to sows vaccinated with a commercial PED killed vaccine. Existing L-K-K vaccine programs are very efficient; however, live attenuated strains have the potential for pathogenic reversal, and virulent PEDV used for artificial infection may propagate continuously in outdoor pig farms. We expect that the novel vaccine described herein will address these issues.

## Ethical approval and consent to participate

All animal experiments were approved by the Animal and Plant Quarantine Agency in Ministry of Agriculture Food and Rural affairs.

## Declaration of Competing Interest

The authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108604.

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