Title page

Humoral immunity is the primary means of heterologous *Propionibacterium acnes* vaccination against *Actinobacillus pleuropneumoniae* infection in piglets

Linxi Li[1], Xin Feng[1], Fang Xie[1], Jingmin Gu[1], Yu Gao[1], Liancheng Lei[1*§], Wenyu Han[1§]

[1]College of Animal Science and Veterinary Medicine, Jinlin University, Jilin 130062, China

Correspondent Footnote:

Prof. Liancheng Lei, College of Animal Science and Veterinary Medicine, Jilin University, Changchun, China. Phone: +86-431-87836173; Fax: +86-431-87836173; Email: leilc@jlu.edu.cn

§These authors contributed equally to this work

*Corresponding author

Abstract

Background. *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) is the causative agent of swine pleuropneumonia. *Propionibacterium acnes* (*P. acnes*) could provide immunoprotection to mice and pigs against the infection of *A. pleuropneumoniae*. **Methods.** To analyze the immunoprotection of pigs by heterologous *P. acnes* vaccination against *A. pleuropneumoniae* infection, 12 five-week-old healthy pigs were randomly divided into 2 groups, of which 1 group (n=6) was vaccinated twice with living *P. acnes* and the other group served as a non-vaccinated control (n=6). Both groups were experimentally infected with *A.*

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pleuropneumoniae (serotype 1, $5*10^7$ CFU, CCVC 259) then the clinical symptoms and pathological changes were monitored. **Results.** Compared to the control group, the vaccination group showed significantly reduced clinical symptoms and pathological changes. The *P. acnes*-vaccinated group showed a significant increase in the levels of specific serum antibody titer against *A. pleuropneumoniae* soluble antigen and serum IL-2 and IL-4 concentration. Therefore, immunization with heterologous *P. acnes* enabled an effective humoral immune response against *A. pleuropneumoniae* infection in pigs.

Key words: *Actinobacillus pleuropneumoniae*; immunoprotection; *Propionibacterium acnes*; heterologous; pigs; vaccine

35 **Introduction**

36 Swine pleuropneumonia is one kind of highly contagious and lethal swine respiratory diseases caused by
37 *A.pleuropneumoniae* (Pattison, Howell et al. 1957), which has widely prevailed all over the whole world
38 since 1980's. This disease has the morbidity of up to 40-80% and mortality rates of 6-20% (Woeste and
39 Grosse Beilage 2007), leading to large annual economic losses worldwide in swine industry (Losinger
40 2005). So far, two biotypes and 15 serotypes of *A.pleuropneumoniae* have been identified (Blackall,
41 Klaasen et al. 2002). The studies on the vaccines against *A.pleuropneumoniae* focus mainly on the
42 development and commercialization of inactivated or ghost vaccines, subunit vaccines and attenuated
43 vaccines (Ramjeet et al 2008). However, current *A.pleuropneumoniae* vaccines could not provide
44 satisfactory immune protection against multiple serotypes of *A.pleuropneumoniae* infection, which severely
45 restricts the development of vaccines for *A.pleuropneumoniae* (Bei, He et al. 2005). Therefore, the
46 development of new *A.pleuropneumoniae* vaccines is especially urgent.
47
48 *P.acnes* is one kind of microorganism usually seen in nature, which is related to the inflammation in acne in
49 human skins (Thiboutot 2008). *P.acnes* could enhance the resistance of animals to a variety of pathogens
50 such as parasites (Clark, Cox et al. 1977; Abel, Chen et al. 2009), bacteria (Miyata, Nomoto et al. 1980;
51 Dinsmore, Cattell et al. 1995) and viruses (Kirchner, Hirt et al. 1977; Megid, Cremonini et al. 2002), by
52 improving the non-specific immunity of animals. In our previous work, we found that several potential
53 vaccine candidate genes of *A.pleuropneumoniae* showed remarkable simiariities to those of the genes
54 serotypes of *P.acnes* (Lei, Sun et al. 2008). ELISA assays showed that there was a cross-immune response
55 between *A.pleuropneumoniae* and *P.acnes*, suggesting that *P. acne* could offer heterologous immunization
56 protection against *A.pleuropneumoniae*. This was confirmed by the further experiments that a significant
57 immunoprotection against *A.pleuropneumoniae* was observed after the vaccination in mice (serotype 1,
58 CCVC 295 and serotype 5, CCVC 263) and pigs (*A.pleuropneumoniae* serotype 1, CCVC 259) with
59 *P.acnes*. Furthermore, we treated mice with the swine anti-*P.acnes* serum and then challenged them with
60 *A.pleuropneumoniae* serotype 1 and 5, all the mice (n=10) continued to live for 7 days after
61 challenge(Yang, Lei et al. 2009).
62
63 This study was aimed to further analyze the immunoprotection of weanling piglets by *P.acnes* against
64 *A.pleuropneumoniae* infection. 7 isolated strains of *P.acnes* were vaccinated to mice challenged with

A.pleuropneumoniae, and then one of the *P.acnes* strain with the best immune protection was chosen for the next experiment. The challenge of the piglets with living *A. pleuropneumoniae* used for monitoring the survival status and the development of clinical symptoms. The anatomy study on the lungs of pigs was carried out for the lesion score, and the lung tissue slices of pigs were also made for the microscopic observation of pathological changes after the challenge. The specific antibody titer of both groups was measured and calculated. Moreover, the serum interleukin four (IL-4) and interleukin two (IL-2) concentration were compared between *P.acnes* vaccinated and non-vaccinated pigs before the challenge with *A.pleuropneumoniae*.

Materials and method

1. The strains, growth condition and living cell preparation. 7 *P.acnes* strains (S1-S7) were isolated from acne lesion or normal skin surface in human. *A.pleuropneumoniae* serotype 1 strain CCVC 259 (from China Institute of Veterinary Drug Control, Beijing, P.R. China) was used in this study, under the storage condition the same as that of *P.acnes*.

P. acnes was cultured in the brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) containing 1% glucose (Schlecht, Freudenberg et al. 1997) and incubated at 37℃ for 72 h. The whole incubation was finished in an anaerobic cabinet (MiniMACS; Don Whitley Scientific, West Yorkshire, UK) at an atmosphere containing 80% N_2, 10% CO_2 and 10% H_2. The brain heart infusion broth was also used for the growth of *A.pleuropneumoniae* strain containing NAD (10μg/ml; Sigma Chemical Co., St. Louis, MO, USA). The *A.pleuropneumoniae* cells were incubated at 37℃ for 6 h in a water bath shaker with 150 rpm.

2. The animals. 80 ICR strain mice were purchased from Experiment Animal Center, School of Basic Medical Science, Jilin University (age, 5 – 6 weeks, female:male ratio, 1:1; body weight,18 – 22 g). The mice then randomly divided into 8 groups (n=10) designated as Group 1 to Group 8. Groups 1 to 7 were for vaccination, Group 8 was for control. 12 of five-week-old female Duroc piglets were purchased from *A.pleuropneumoniae*-free Swine Breeding farm in Jilin University, with the average body weight of 10.3 kg. All the Piglets in this study were tested sero-negative for *A.pleuropneumoniae* antigen and antibody with PCR and ELISA assay described in previous study (Lei, Sun et al. 2008). The healthy status of all the

piglets were monitored for one week before immunization. The care and treatment of mice and pigs in our study were conducted in accordance with the guidelines on animal experimentation of Jilin University.

3. Vaccination and challenge. The *P.acnes* cells were centrifuged at 5000 rpm for 3 min, then washed for 3 times with the sterile saline (0.9% NaCl solution) and diluted to 2.5×10^9 cell/ml for vaccination. The *A.pleuropneumoniae* cells were washed for 3 times with sterile saline and then diluted to 2.5×10^7 cell/ml for experimental infection.

Mice in Group 1 to Group 7 were injected *P.acnes* strain S1 to S7 respectively by intraperitoneally route, 5×10^8CFU for each mouse. Mice in Group 8 was injected with 0.9% saline for negative control. *A.pleuropneumoniae* serotype 1 was challenged for 17 days after vaccination, 8×10^7CFU for each mouse, and observed for 7 days.

The 12 pigs were randomly divided into two groups. One group (n=6) was for the *P.acnes* vaccination and the other non-vaccinated group (n=6) was for the control. The vaccination was carried out via intranasal routes. The total volume of vaccination was 2 ml each dose, of which 1 ml was sprayed in each nostril. After 21 days, these pigs were vaccinated again in the same way as above. The peripheral blood was drawn from both groups of pigs 21 days after the second immunization of *P.acnes* vaccinated group, followed by the preparation of serum. Each group was infected 2 hours after blood drawing, and the infection method was similar to vaccination one

The healthy status and living activities of all the pigs was monitored throuthout the entire study. Clinical signs of respiratory disorders such as increased dyspnoea, coughing and nostrils effluence were recorded. The recta temperature was tested daily within the following 7 days after the infection. All the survived pigs in both groups were euthanized for anatomy in the 8^{th} day after the infection. The severity and extent of lung lesions were assessed by a method described by Hannan (Hannan, Bhogal et al. 1982) . And the extent of lung individual tissue damage was also evaluated and recorded in a scoring diagram sheet. Lung tissues were collected from each pig for pathogenicity test of *A.pleuropneumoniae*.

4. The specific antibody reaction and pathogen test. In each group, peripheral blood serum of pigs was prepared and Anti-*P.acnes* and -*A. pleuropneumoniae* serotype I titers were determined by means of enzyme-linked immunosorbent assay (ELISA). In briefly, ultrasonicationed suspensions of *P.acnes* and *A.pleuropneumoniae* cell pellets were 100-fold diluted and utilized to coat at 96-well microtitration plate at a concentration of 10ug/ml as the antigen. 5 ml of peripheral blood samples were collected from each pig by the way of blood inferior vena cava on Day 28 after the first and the second vaccination, then 100-fold diluted by PBS as the antibody.. The *P. acnes* and *A. pleuropneumoniae* cells were sonicated and centrifuged at 5000 rpm for 5 min. The supernatant was used for the soluble antigen and incubated in ELISA plates at 4℃ overnight. Serum antibodies were tested in all the pigs in each group.

Two pieces of swine lung slices were collected from each pig immediately after humane euthanasia of pigs. Of both, one slice was used for Gram staining analysis under 1000 x optical microscope. And *A.pleuropneumoniae* was isolated from this slice and cultured on BHI medium contain 1.5% of agar. The conventional PCR was carried out for detection of *A.pleuropneumoniae* in each lung sample as described by (Lei, Sun et al. 2008). The samples were washed for three times with sterile saline, and ground in 1ml sterile water for 5 min in a sterile mortar. And then this tissue lysate was frozen and thawn for three times and centrifuged at 3000 rpm for 2 min. The supernatant was boiled for 10 min and then sit immediately in the ice for 5 min for the PCR templates.

5. Determination of IL-4 and IL-2. The serum levels of IL-2 and IL-4 in the peripheral blood which collected at 21days post second vaccine were determined with the ELISA kit (Rapidbio, US), rendering the standard curves.

6. Histopathology observation. Visceral tissue specimens from both groups were fixed in 4% buffered formalin, and then embedded in paraffin wax using routine protocols and sliced to section. Slides were stained using the combination of haematoxylin-eosin with Alcian blue. Tissue sections were observed in the optical microscope for the comparison of differences in the pathological changes between the *P. acnes* vaccinated group and non-vaccinated group.

8. Data statistical analysis. All data analysis was performed using the Student's *t*-test for the comparison

153 of the differences in clinical signs and other test results between both groups. $P \leq 0.05$ was taken as a
154 significant difference standard.
155
156 **Results**
157 **1. Immune protective of *P. acnes* isolate strains.** After 7-day challenge with *A.pleuropneumoniae*, as
158 shown in Table 1, some of the mice in Group 1 to Group 7 died and all the mice of the control group died
159 during 24 h after challenge. The survival rate of Group 4 (vaccine by *P.acnes* isolate strain S4) was 70%,
160 therefore the *P. acnes* strain S4 was selected for the next experiment.
161
162 **2. Healthy status and clinical symptoms of pigs after immunization and infection.** The average body
163 temperature showed no significant difference in *P. acnes* vaccinated group within 3 continuous days either
164 after the first vaccination or after the second vaccination. None of any clinical sign of diseases (depression,
165 loss of appetite and fever or cough) were observed in *P. acnes* vaccinated group after vaccination. Only tiny
166 local skin reactions were found in the site of administration, but disappeared in a few days. Neither
167 significant adverse reactions nor harsh stimulation were detected after treatment of pigs with *P.acnes.* The
168 above healthy status and clinical symptoms indicated that *P.acnes* could be applied to pigs to a certain
169 secure extent. Non-vaccinated group was in good health during this course.
170
171 The average body temperature of all the pigs started to increase after the next day after infection (Figure 1).
172 In *P.acnes* vaccinated group, the maximum of the average body temperature was 38.7℃ in the 7 days after
173 infection. In contrast, the average body temperature of non-vaccinated group was over 39℃ on and after
174 the third day after infection. Statistical analysis showed there was a significant difference between both
175 groups ($p < 0.05$).
176
177 In the non-vaccinated group, all the pigs showed continuous increase of body temperature after the *A.*
178 *pleuropneumoniae* infection. Especially, the two pigs showed more than 40 degrees of body temperature,
179 with typical clinical symptoms, such as dysponea, blood at nostril of which one died four days of after the
180 infection. Moreover, all the pigs showed the most marked severe pyretic reactions at the early phase.
181
182 In *P. acnes* vaccinated group, all the pigs showed no obvious clinical symptoms such as bad appetite or

abnormal activities after the infection, except that only two pigs showed phased recta temperature increase as shown in Table 2.

3. Antigen-antibody response. As shown in Table 3, non-vaccinated group had no detectable titer in the ELISA reaction against either the *P.acnes* or *A.pleuropneumoniae*, whereas *P. acnes* vaccinated group produced antibodies against *P.acnes* and *A.pleuropneumoniae*. *A.pleuropneumoniae* antibody titers were higher after the second vaccination than after the first vaccination, indicating an increasing trend of anti-*A.pleuropneumoniae* serotype I specific antibody after double vaccination with *P. acnes*.

Serum immunoglobulin concentrations were detected by ELISA kit in both groups (Table 3). The concentrations of serum IgG, IgA and IgM from *P.acnes* vaccinated group were statistically significantly higher than those of non-vaccinated group ($P<0.05$).

4.Serum IL-2 and IL-4 assay. As shown in Table 3, the concentrations of serum IL-4 from *P. acnes* vaccinated group were statistically significantly higher than those of non-vaccinated group ($P<0.05$). There was no significant difference of serum IL-2 ($P=0.294$) and lysozyme ($P=0.535$) concentrations between both groups on Day 21 after second vaccination.

5. Pathological observation. The necropsy observation of the lungs of all the euthanized pigs was carried out for the detection of pathological changes on Day 8 after infection. As shown in Figure 3a, the pink lungs surface lesions were observed in the pigs in *P. acnes* vaccinated group. Almost all the pigs showed, to some extent, partial lesions, handle soft and small area of diffuse bleeding, in addition to a big area of lung necrosis in one case. And then small-scale necrosis, focal hemorrhage was observed on the cut-open face of lungs (Figure 3b). The pigs had no visible lesions in other organs.

The lungs of non-vaccinated group were bigger than that of *P.acnes* vaccinated group. The non-vaccinated pigs showed bleeding points with about 5 mm of diameters, and large deep purple to black necrotic lesions (Figure 3C). The non-vaccinated group showed abscess or/and pulmonary interstitial lesions full of purulent bloody gel-like mobile liquids. No solid purulent with some aspect similar to the liver section and fully friability after lungs were cut open (Figure 3D). Lung lesions in both groups of pigs were measured

and calculated with the method of Hannan (Hannan, Bhogal et al. 1982), as shown in the Table 3. The average area of the lung lesions was 21% in the *P. acnes* vaccinated group and 75% in the non-vaccinated group. There was a significant difference in lung lesions between these two groups ($P<0.05$), as shown in the Figure 2, suggesting that the pigs increased the ability of against *A .pleuropneumoniae* after the *P. acnes* treatment. Only one pig in *P. acnes* vaccinated group showed more than 90% necrosis of the lungs, which may be caused by low levels of immunization induced by *P. acnes*.

Lung tissue sections stained by Hematoxylin-eosin were observed under 200 X optical microscope. Lungs tissues in *P .acnes* vaccinated group showed moderate bleeding, airway inflammatory cells around the stove infiltration, alveolar serous, exudation, inflammatory cell infiltration (Figure 4A). In non-vaccianted group, there were large amount of inflammatory cell infiltration, alveolar septal cell degeneration, necrosis, nuclear enrichment, the majority of alveolar collapse caused by consolidation, hemorrhage severe congestion, alveolar serous, fibrinous exudation and accompanied by a large number of shedding alveolar epithelial cells (Figure 4B).

6. The test for *A. pleuropneumoniae* in lung tissues

In the *P. acnes* vaccinated group, three pigs contained the pathogen *A.pleuropneumoniae* and the other three not. In the non-vaccinated group, all the pigs contained this pathogen. These results suggested the *P. acnes* vaccinated pigs increased the immunoprotecion against *A. pleuropneumoniae* infection, as compared with the non-vaccinated ones.

Discussion

The immuneprotection of the mice by *P. acnes* strains S1 to S7 against *A. pleuropneumoniae* infection significantly different, as compared with the control group. The immunoprotection ratios range from 30% to 70%, indicating that the immunoprotection antigen or component was varied between different *P. acnes* strains. Therefore, it should be necessary to isolate more *P. acnes* strains in order to select the most efficient immunoprotection strain against *A. pleuropneumoniae*. *P. acnes* strains isolated from invasive infection focuses have shown to form more produced biofilms than the strains isolated from human skin (Holmberg, Lood et al. 2009), and the biofilm production may be related to the pathogenic processes of *A.*

pleuropneumoniae (Kaplan and Mulks 2005). Thus, it is very necessary to isolate *P. acnes* especially from multiple sources such as deep infection focuss or post surgery infection exudates.

P. acnes has also been commercialized as non-specific immune-stimulating agents of the animals (Flaminio, Rush et al. 1998), and applied to the scientific research on the adjuvant (Nascimento, Costa et al. 1995; Vilela Mde, Gomes et al. 2007). *P. acnes* as is generally believed to be a low-virulence microorganism. The *P. acnes*-induced animal diseases are rarely reported, with only sporadic cases that they may be related to *P. acnes* (Lyons, Bemis et al. 2009), but not confirmed as a pathogenic disease. In our preliminary study, the immunization of pigs with live *P. acnes* showed neither obvious clinical symptoms nor body temperature changes. Although there is no biosafety experiments with *P. acnes* used in pigs reported, *P. acnes* is one of the common micro-organisms in the pigs (Salanitro, Blake et al. 1977), while *P.acnes* has not been involved in the pig disease reports as yet. Therefore, we believe that the development of *P .acnes* into vaccine candidates for *A.pleuropneumoniae* infection is absolutely safe.

Serum anti-*A.pleuropneumoniae* specific antibody titer was increased gradually after the first and second immunizations, indicating *P. acnes* could activate the *A. pleuropneumoniae* specific humoral immune responses of pigs. In the vaccinated group, the IL-4 levels were significantly higher than those in the control group. These results indicated that IL-4 production induced by *P. acnes* vaccination enhanced the humoral immune responses and induced the production of specific antibody. In our previous antibody transfer experiments, afer mice treated by swine anti-*P.acnes* serum were challenged with *A. pleuropneumoniae* serotype 1 (CCVC 259) and 5 (CCVC 264), the survival rates of both group were even 100% (n=5 for each group) (Yang, Lei et al. 2009). From above, we concluded that induction of the specific humoral immune response by *P. acnes* played an important role in the immnoprotection against *A.plruropneumonia* infection.

On day 7 after the second vaccination, the concentrations of IL-2 in each pig peripheral blood were measured. Even though the average concentration of vaccination group（260pg/ml） was higher than control group（213pg/ml）, there was no significant difference between both group.

In summary, the imunoprotection against *A. pleuropneumoniae* infection may be mainly completed by the

273 humoral immunity response.

274

275 *P. acnes* is a widely-used adjuvant in animal immunization studies, the study on the non-specific immune
276 stimulation ability of *P. acnes* (formerly named Corynebacterium parvum) began in the 70's (Florman and
277 Holzman 1975), the following researches focused on the anti-tumor activity of *P. acnes* (Yuhas, Toya et al.
278 1975; Fisher, Rubin et al. 1976). At the end of last century, the commercialization agents based on *P. acnes*
279 began to be used for animal immune regulation (Flaminio, Rush et al. 1998). In general, *P. acnes* played a
280 role in non-specific immune protection by the study of immune stimulation, such as parasites (Iseki,
281 Takabatake et al. 2008; Abel, Chen et al. 2009) or viruses (Megid and Kaneno 2000). Our study for the
282 first time showed that *P. acnes* for specific heterologous protective immunity could develop into a possible
283 *A. pleuropneumonia* vaccine or adjuvant. So far, none of perfect *A. pleuropneumoniae* vaccines were
284 reported. This is mainly because of poor multiple serotypes protective effect of all the *A.pleuropneumoniae*
285 vaccine. And one subunit is difficult to be selected for vaccine or epitope, witch to provide multiple
286 serotype immunity protection (Ramjeet, Deslandes et al. 2008). In our previous study, vaccination of *P.*
287 *acnes* could protect mice against *A.pleuropneumoniae* serotype 1 and 5 infection(Yang, Lei et al. 2009).
288 Because the *A. pleuropneumoniae* vaccine was obstructed in the case of cross-serotype immunity study,
289 heterologous immunization by *P. acnes* may become a promising vaccine develop strategy.

290

291 In conclusion, the clinical symptoms and pathological changes in *P.acnes* vaccinated pigs were
292 significantly moderated after *A.pleuropneumoniae* infection. The humoral and mucosal immune responses
293 were stimulated in *P. acnes* vaccinated pigs. Our study confirmed that *P. acnes* could induce specific
294 immunity and provide effective protection against *A. pleuropneumoniae* infection. The determination of
295 elements from *P. acnes* inducing protection, such as the common antigen component, is further attractive in
296 our research. And the evaluation on the cross-protection of *P. acnes* against multiple serotype of
297 *A .pleuropneumoniae* is also required. All of the above may enable us to develop the promising *A.*
298 *pleuropneumoniae* vaccine from *P. acnes*.

299

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LEGENDS

Figure 1. Time-course of the average of body temperature（℃) recorded in 7 days after *P.acnes* treat in both groups.

Figure 2. The lung surface and deep cut picture of *P.acnes* vaccinated group and non-vaccinated group. A: The lung deep cut view of vaccination group pig,

Figure 3. Pathology of the pig lung. A: Lung slide of vaccination group pig, solid arrow indicate the bronchiolitis exudativa, hollow arrow indicate the alveolar wall thickness increased and capilliary hyperemia (hematoxylin and eosin stain). Magnification×100. B: Control group solid arrow indicate the severe bronchiolitis exudativa, hollow arrow indicate the alveolar wall thickness severe increased (hematoxylin and eosin stain). Magnification×100.

Table 1 Protective effect of *P.acnes* isolation strains against *A.pleuropneumoniae* challenge in mice.

Table 2 The recta temperature of every 24 hours after APP infection from each pig.

Table 3 Antibody titers after first and second challenge of vaccination group pigs and the levels of serum immunoglobulin, IL-2, IL-4, lysozyme and Necrosis score of each pig in both groups. The antibody titers was evaluated using soluble *A.pleuropneumoniae* and *P.acnes* antigen by ELISA assay, the coated antigen concentration was 25 μg/ml respectively. The antibody titers of both *A.pleuropneumoniae* and *P.acnes* were undetectable from control group pigs during the experiment period. The levels of serum immunoglobulin, IL-2, IL-4 and lysozyme were measured with ELISA kit.

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402 Table 1

Groups	No. of total mice	Vaccine strain	Challenge strain	No. of mice lived[a]	Rate of protection
1	10	P. acnes S1	CCVC259[b]	3	30%
2	10	P. acnes S2	CCVC259	4	40%
3	10	P. acnes S3	CCVC259	6	60%
4	10	P. acnes S4	CCVC259	7	70%
5	10	P. acnes S5	CCVC259	5	50%
6	10	P. acnes S6	CCVC259	5	50%
7	10	P. acnes S7	CCVC259	6	60%
8[c]	10	0.9% Saline	CCVC259	0	0

403 Note: a Record of 7 days post-challenge.

404 b The *A.pleuropneumoniae* serotype 1 (CCVC259) was used in challenge.

405 c All the mice of Group 8 was died during the first 24h post-challenge.

406

407 Table 2

Pig No.	Recta temperature of certain hours after APP infection							
	0h (℃)	24h (℃)	48h (℃)	72h (℃)	96h (℃)	120h (℃)	144h(℃)	168h (℃)
1	38.4	38.4	38.7	38.8	38.6	38.4	38.6	38.5
2	38.6	38.6	38.6	38.5	39.5	39.2	38.4	38.4
3	38.4	38.4	38.4	38.7	38.5	38.4	38.5	38.3
4	38.3	38.9	38.7	38.6	38.6	38.3	39.1	39
5	38.3	38.3	38.5	39.5	39.4	39.5	39.2	39.1
6	38.5	38.5	38.4	38.5	38.4	38.5	38.1	38.2
7	38.5	38.5	38.9	39.1	39.3	39.1	39.3	39.2
8	38.4	38.6	38.7	38.7	38.7	38.8	39.2	39.1
9	38.5	38.8	38.9	38.8	39.3	39.1	39.2	38.9
10	38.4	38.4	39.1	39.2	38.4	39.2	38.9	39.2
11	38.5	38.5	39.2	39.3	39.5	40	Dead	Dead
12	38.3	38.3	38.8	39.3	39.4	39.5	39.1	40

408 Note: a Pigs No.1 to No.6 were from vaccination group and No.7 to No.12 were from control group.

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412 Table3

Pig NO.	Group	IL-2 (pg/ml)[a]	IL-4 (pg/ml)[b]	Necrosis score[c]	Anti-*A. pleuropneumoniae* After 1st vaccine[d]	Anti-*A. pleuropneumoniae* After 2nd vaccine[e]	Anti-*P. acnes* After 1st vaccine[d]	Anti-*P. acnes* After 2nd vaccine[e]
1	Vaccination	291	85	89.6	1:800	1:1600	1:1600	1:1600
2	Vaccination	236	81	14	1:1600	1:1600	1:3200	1:3200
3	Vaccination	228	87	11	1:1600	1:3200	1:3200	1:6400
4	Vaccination	257	83	3	1:1600	1:3200	1:3200	1:6400
5	Vaccination	282	80	3	1:3200	1:3200	1:6400	1:6400
6	Vaccination	267	90	6	1:1600	1:3200	1:3200	1:6400
7	Control	110	63	50	N/A	N/A	N/A	N/A
8	Control	127	32	66	N/A	N/A	N/A	N/A
9	Control	270	69	91.6	N/A	N/A	N/A	N/A
10	Control	285	68	64.2	N/A	N/A	N/A	N/A
11	Control	145	64	95.7	N/A	N/A	N/A	N/A
12	Control	340	34	85	N/A	N/A	N/A	N/A
Arithmetic Mean±SD	Vaccination	260±24.91	84±4.78	21.1±33.84				
	Control	213±97.15	55±17.20	75.4±18.03				

413

414 Note: a There was not significant difference of serum IL-2 concentration between the vaccination
415 group and control group (P=0.699).

416 b Serum IL-4 concentration was significant different between the vaccination group and control
417 group (P<0.01).

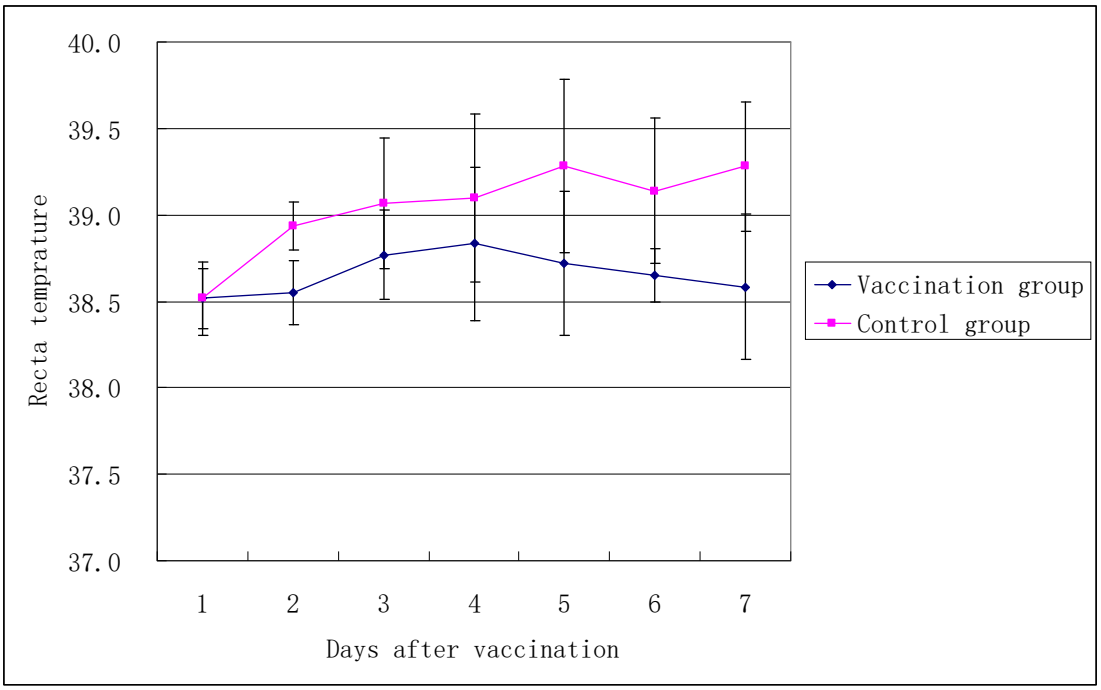
418 c The lung lesion score was significant differernt between the vaccination group and control group
419 (P<0.01).

420 d Antibody titer was evaluated on the 21st day after first vaccination.

421 e Antibody titer was evaluated on the 21st day after second vaccination before challenge.

422

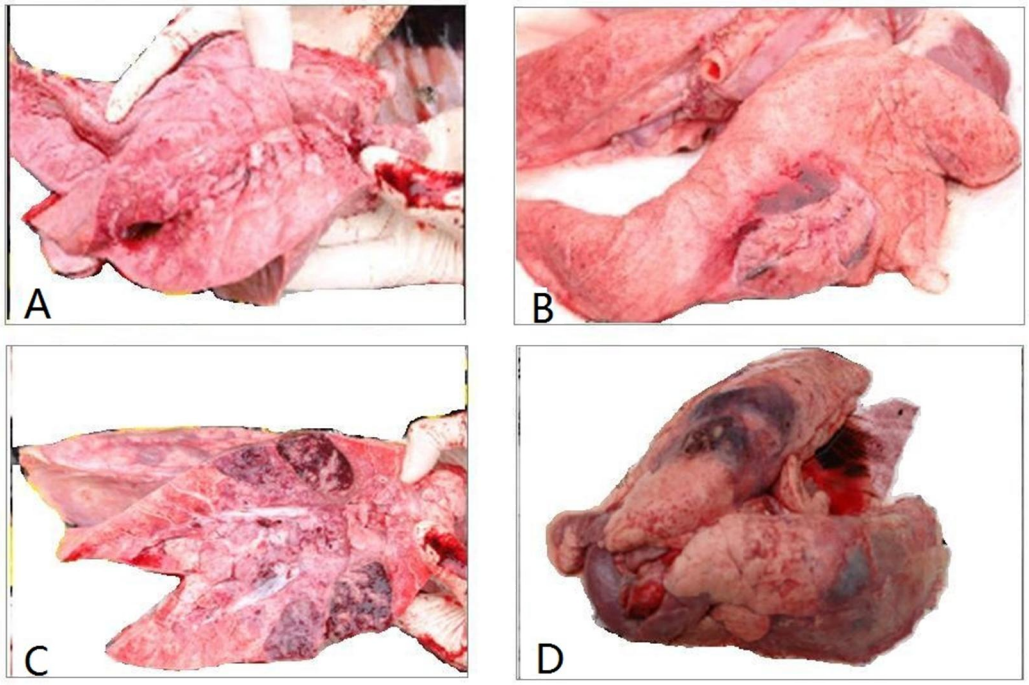
423 Figure 1



424

425

426 Figure 2



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429

Figure 3