CHAPTER 3
MATERIALS AND METHODS

3.1 Study population

The trial was conducted on a chronically infected farm that became PRRS positive in 1996. The PRRS status was unstable active, which meant that the herd had evidence of sow-to-sow (vertical transmission) or sow-to-piglet (horizontal transmission) transmission and endemic respiratory problems magnified in the nursery pigs, despite the use of antibiotics. It experienced recurrent episodes of porcine pleuropneumonia which resulting from *A. pleuropneumoniae* infection in the fattening pigs and replacement gilts. Therefore, *A. pleuropneumoniae* was a major secondary bacterial pneumonia infection in this farm.

The herd consisted of 600 sows located at the breeding site. This site-1 production housed all sows and unweaned pigs. All females were bred by artificial insemination using semen from a PRRS virus-naïve boar. Sows were housed in crates during the production period. Pigs were weaned at an average of 18 to 21 days of age and were moved to the nursery–finisher site (site-2 production) 10 kilometers away. This site-2 contained one nursery building with 11 nursery rooms and 10 finishing houses. Pigs from two nursery rooms were moved together into a single finishing house. Pigs were housed in the nursery for approximated 40 days and in the finishing house for 100 to 112 days. All rooms were all-in all-out. The replacement gilts were housed within the breeding site from nursery through finishing stages. Gilts were moved into the gilt pool at 5 to 6 months of age for serological testing and vaccination before mating.

3.2 Biological samples and sampling

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In site-1 production, all adult animals (~600 sows) in all stages of production and their offspring were studied using PRRS control and eradication intervention strategies. A cross-sectional serological profile of the herd was performed on a yearly basis during 2003 to 2006 for seroprevalence evaluation (Table 3.1).

In breeding sows and finishers, the sample size was calculated to provide a 95% level of confidence if the expected true prevalence was <20% or >80% with the population of 600 sows and 6000 pigs. Blood samples of finisher pigs were selected at random from pigs aged 4, 6, 8, 12, 16 and 20 weeks, and sows were selected at random from breeding and lactating sows over a full rang of parities and periods of gestation.

In replacement gilts, a monthly ELISA testing of 30 gilts per replacement stock were selected randomly using a sample size capable of detecting at least one positive pig at an estimated prevalence of ≥ 10% at a 95% confidence level.

At the end of the study, tissue samples (tonsil, retropharyngeal lymph node) were collected from 10 culled sows, using a sample size capable of detecting at least one positive pig at an estimate prevalence of ≥ 30% at a 95% confidence level, to detect PRRS and *A. pleuropneumoniae* carrier pigs by PCR assay.

Table 3.1: The number of sample animals for seroprevalence evaluation during 2003 through 2006.

Year	Sows	Finishing pigs	Gilts
2003	60	60	30
2004	60	60	360
2005	60	60	360
2006	60	60	360

3.3 Antimicrobial agent

Marbofloxacin (Marbocyl®, Vetoquinol) is a broad-spectrum bactericidal antibiotic in a third generation fluoroquinolone distinguished from others by its oxadiazine cycle. It gives the molecule a long elimination half-life. The basis structure of the molecule consists of a heterocyclic aromatic nucleus. Marbofloxacin acts directly on the bacterial enzyme DNA gyrase by penetrating the bacterium by simple diffusion. It affects bacteria during the multiplication phase, inhibits cellular replication, and blocks respiration, leading to bacterial death. The MIC 90% for *A. pleuropneumoniae* is low (0.027 µg/ml). Marbofloxacin's excellent diffusion in the tissues and can passes into the milk.

3.4 Vaccine

The APP sub-unit vaccine (Porcillis APP®, Intervet International Boxmeer, The Netherlands) contains Apx I, Apx II and Apx III toxoids, which are the major virulence factors in *A. pleuropneumoniae.* It also contains 42kDA outer membrane proteins, common to all serotypes and biovars of *A. pleuropneumoniae.* Alfa-tocoferal-acetate is used as adjuvant.

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3.5 Study design

The study period extended from March 2004 through December 2006 (Table 3.2). Before the beginning of the study, the serological profile within the herd was determined to assess the prevalence of PRRS and APP (year 2003).

Table 3.2: Study timeline from March 2004 to December 2006

Year	Month	Activity
2004	March	- First serological testing for PRRS and APP status within herd
		- Herd closed to incoming pigs
	April	- Routine use of aerial disinfectant spraying in breeding herd
		- Medication program
	May	- APP sub-unit vaccine program initiated
		- Unidirectional pig flow implemented in site-2 production
	October	- Seromonitoring for PRRS and APP antibodies
2005	October	- Seromonitoring for PRRS and APP antibodies
2006	October	- Seromonitoring for PRRS and APP antibodies
		- PCR testing for the persistence of PRRS and APP infection

Next, intervention strategies were conducted using the following management techniques.

3.5.1 Closed-herd system started on March 2004 to stop incoming replacement gilts for at least 2 years. Then, the herd raised their own replacement gilts which were PRRS seropositive but cooled down (S/P ratio lower than 1.2) at the acclimatization period. In essence, the herd attempted to augment stringent biosecurity practices by preventing disease entry into the herd. These included 5 main strategies as follow:

- **Transportation**: Clean and disinfect all transportation vehicles and equipment off site; segregate marketing transportation from internal pig movements; eliminate visitor vehicles; load the pigs at loading bay: use one-way gates.
- **Pig flow**: All-in, all-out (AIAO) flow maximizes health benefits; segregate/eliminate light pigs that remain on site post-marketing; eliminate rendering visits to sites.
- **People movement**: Control site visits/work flow; ban visitors; change boots and coveralls, wash hands between different age/phase/health status groups of pigs.
- **Equipment use and cleaning**: Segregate equipment among barns/rooms and wash and disinfect between groups.
- **Nutritional risks**: Stock only 1 month's feed; eliminate contaminated compounded feed; clean the water.

3.5.2 Disinfectant spraying was done to decrease the pathogens' load in the environment and to prevent direct transmission from droplets. All barns within site-1 production were washed, disinfected, and left empty for 1 week before subsequent production. Then, each unit of the farrowing and nursery rooms on site-1 production

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were sprayed with 800-fold dilution of Di-decyl dimethyl ammonium chloride 20% w/v at 4 times a day for 2 weeks before starting the medication program. After that, it was done routinely in the morning and evening throughout the study period. This is a special tool to prevent aerial spreading from sows to their offspring during the lactation period.

3.5.3 Medication elimination program was done only in site-1 production on April 2004 to decrease the amount of secondary bacteria, especially APP. The breeding herd was treated with medicated feed containing 200 ppm of Tiamulin together with 150 ppm of Amoxicillin for 14 days to decrease respiratory pathogens inside the pigs' bodies.

At the 3rd week of April 2004, the medication program was done in all site-1 pigs (except piglets less than 3 days old) to control the transmission of *A. pleuropneumoniae*. The pigs were injected intramuscularly twice with 5 mg/kg of Marbofloxacin within a 2-day interval.

3.5.4 Mass vaccination was done in May 2004. All breeding stock was treated twice with APP sub-unit vaccine, with a 30-day interval between vaccinations. Nursery pigs aged more than 6 weeks received a second dose 4 weeks after first vaccination. Replacement gilts were vaccinated twice within 4 weeks of first mating. After that, the vaccination program was set up to routinely treat nursery pigs at 6 and 10 weeks of age, and replacement gilts were vaccinated twice within 4 weeks of first mating.

3.5.5 Good health management techniques were established for improving the herd's immune status and decreasing stress. These included low density stocking, proper ventilation and environmental temperature, unidirectional pig flow, and good manure and farm sanitation.

3.6 Laboratory test

Serum was harvested by centrifugation for 10 min at 3000 rpm and stored at -20°C until testing for the presence of antibodies. The detection of PRRSV-specific antibodies in serum samples was done by a commercial PRRS ELISA test kit (HerdChek-PRRS®IDEXX Laboratories Inc.; Westbrook, Maine). The CHEKIT-APP-Apx IV ELISA test kit was used to detect APP-Apx IV specific antibodies in serum samples.

The HerdChek PRRS virus antibody test kit is an ELISA kit for the detection of PRRS antibodies in swine serum which uses PRRS virus and normal host cell (NHC) antigen. It has 97.4% sensitivity and 99.6% specificity at 95% confidence interval level for the detection of PRRS virus antibodies in samples (Figure 3.1).

The results from the PRRS ELISA test were shown as a sample/positive ratio (s/p ratio) in which s/p ratio ≥ 0.4 was considered seropositive. It showed the presence of antibodies that responded to vaccinations or natural exposure. It can detect the most predominant European or American types of PRRS viruses that might be present at the farm.

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Figure 3.1: HerdChek test procedure

1. Dispense 100µL of undiluted negative control into PRRSV wells and NHC wells.
2. Dispense 100 µL of undiluted positive control into PRRSV wells and NHC wells.
3. Dispense 100 µL of diluted sample control into adjacent PRRSV and NHC wells. Run all samples in duplicate.
4. Incubate for 30 minutes at room temperature.
5. Aspirate liquid contents of all wells into an appropriate waste reservoir.
6. Wash each well with approximately 300 µL of phosphate-buffered wash solution three to five times. Aspirate liquid contents of all wells after each wash.
7. Dispense 100µL of anti-porcine HRPO conjugate into each well.
8. Incubate for 30 minutes at room temperature.
9. Repeat step 6-7.
10. Dispense 100µL of TMB substrate solution into each test plate well.
11. Incubate for 15 minutes at room temperature.
12. Dispense 100µL of stop solution into each well of the test plate to stop the reaction.

The CHEKIT-APP-Apx IV ELISA test kit is an enzyme immunoassay for the detection of antibodies against Apx IV toxin in serum and plasma of swine independently of the APP serotypes (99-100% specificity, 95% sensitivity). It can differentiate vaccinated (negative test result) from infected animals (positive test result), and is able to detect all serotypes with no cross reactivity because of the common and specific toxins. The fourth toxin (Apx IV) is produced only by infection (Figure 3.2).

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Figure 3.2: Chekit test procedure

1. Reagents preparation
   - Allow all reagents to equilibrate to the respective incubation temperature prior to testing.
   - Dilute the CHEKIT-10x-Concentrate 1;10 with water in order to prepare the ready-to-use CHEKIT-Washing & Dilution-Solution
2. Sample dilution
   - Dispense 90 µl CHEKIT-APP-Apx IV sample diluents in each well foreseen for samples and controls.
   - Add 10 µl samples or controls in the respective wells. Mix the contents within each well by shaking the microtiter plate.
3. Sample incubation
   - During 60 minutes (± 5 min.) at 37ºC (± 2ºC) or alternatively overnight (14-18 hrs.) between +2ºC and 8ºC in a humid chamber.
4. Washing the plate
   - 3x300 µl CHEKIT-Washing & Dilution solution
5. Conjugate distribution
   - Dispense 100 µl of the ready-to-use CHEKIT-APP-Apx IV-Anti-swine-Ig-PO-Conjugate in each well.
6. Conjugate incubation
   - During 60 minutes (± 5 min.) at 37ºC (± 2ºC) in a humid chamber.
7. Wash the plate
   - 3x300 µl CHEKIT-Washing & Dilution solution
8. Substrate distribution
   - Dispense 100 µl CHEKIT-TMB-Substrate into each well.
9. Substrate incubation
   - 15 minutes ± 5 minutes
10. Stop the reaction
    - Dispense 100 µl CHEKIT-stop-Solution-TMB-Substrate into each well.
11. Interpretation
    The optical density (OD) of the positive control (OD_{pos}) and that of the OD samples ($OD_{samples}$) are collected by subtracting the OD of the negative control (OD_{neg}). Analyze the samples in relation to the negative and the positive controls using the formula:

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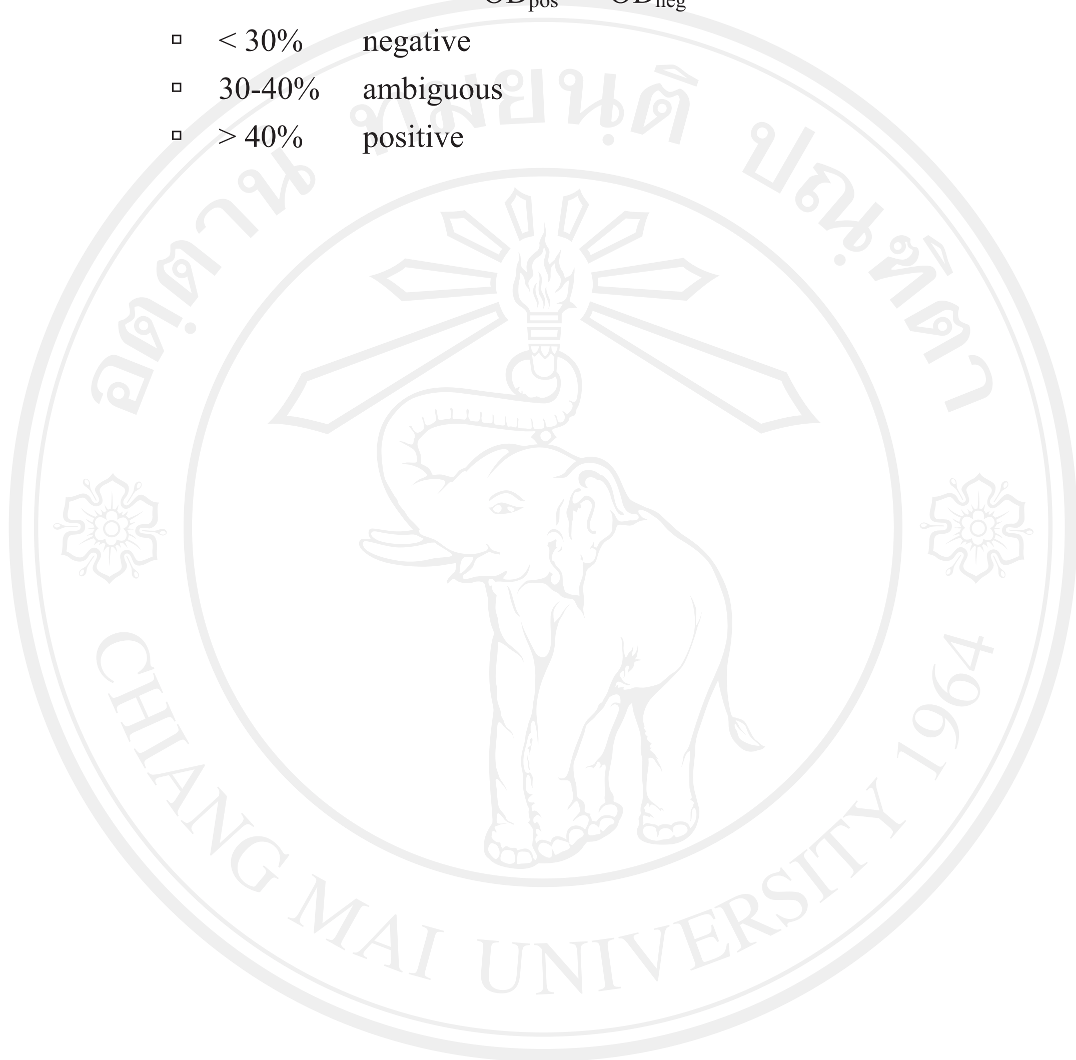
$$\text{Value (\%)} = \frac{OD_{samples} - OD_{neg}}{OD_{pos} - OD_{neg}} \times 100$$

- < 30% negative
- 30-40% ambiguous
- > 40% positive



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All collected tonsil tissue samples from 10 culled sows were investigated by nested RT-PCR using primers specific for American (US) and European (EU) PRRS virus strains. Total RNA was used as template in a single-tube reverse transcription nested PCR specific for ORF5 of EU-type and US-type PRRS virus.

Sample preparation

The tonsils were excised from the carcass and gently rinsed under tap water. Tonsillar crypt debris was obtained from each pig by squeezing one of the two tonsils between two fingers until the debris rose to the surface. The crypt debris (0.1 g) was boiled 10 minutes in a lysis buffer and further processed for a PCR.

PRRS PCR test procedure:

1. 5 µl 22% trehalose was used to store and maintain the following mixture in the lids of 0.2 ml eppendorf tubes: 20 pmol of each inner PRRSV-EU primer ORF5 (5'-ATGAGA TATTCTCACAAATTGGGGCG-3') and ORF5R (5'-CTAGGCCTCCCATTGCTCAGCCGAAGT-3') or PRRSV-US inner primers US ORF5B (5'-GCTCCATTTCATGACACCTG-3') and US ORF5C (5'-AAAGGTGCAGAAGCCCTAGC-3') 1 µl of dNTPs (10mM), and 0.25 µl of Taq Polymerase. The tubes were left to dry for 2 h at room temperature prior to storage.
2. RT-PCR was performed in the bottom of the tubes containing the dried, trehalose-treated reagents within the lids. The amplification was carried out in 50 µl volume containing 5 µl of RNA and the following reagents: 5 µl of 10x PCR buffer, 5 µl of MgCl2, 2 µl of dNTPs, 5 pmol of each PRRSV-EU outer primers EUORF5B (5'-CAATGAGGTGGGCIACAACC-3'), and EUORF5C (5'-TATGTIATGCTAAAGGCTAGCA-3') or PRRSV-US primers 208F (5'-GTACGGCGATAGGGACACC-3') and 331R (5'-CCAGAATGTACTTGCGGCC-3'), 1 µl of 10% Triton X-100, 0.5 µl of Taq DNA polymerase, 0.25 µl of RNasin, and 0.5 µl of MMLV reverse transcriptase. Mineral oil was included to act as a vapor barrier between the RT-PCR reaction and the dried reagents within the lids. The tubes were then subjected to the following thermal cycling: 42ºC for 30 min, 95ºC for 5 min, and then 20 cycles at 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1.5 min.
3. The tubes were subsequently inverted several times to dissolve the dried reagents in the lids in order to initiate the nested PCR. The tubes were then centrifuged briefly before returning to the thermocycler for nested PCR, using 35 cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1.5 min. A single extension

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step of 72ºC for 10 min completed the amplification process. The RT-PCR resulted in a final amplicon of 606 bp for PRRSV-EU and 818 bp for PRRSV-US.

4. DNA sequencing: Electrophoresis of the PCR products was performed on 1.5% agarose gel. After the ethidium bromide staining and desalting in ultrafiltered water, bands of the expected size were excised from the gel, and the DNA was recovered using Nucleospin Extract II kit (Macherey-Nagel, Germany) following the manufacturer's recommendations. Gel purified PCR products were cycle sequenced using the BigDye™ Terminator Cycle Sequencing kit (v2.0, Applied Biosystems, USA).

The bacteria were detected from lymph node tissues by PCR assay [34].

APP PCR test procedure:

1. Cultivation of samples.

Tissue scrapings from seared cut surfaces of tonsils were suspended in phosphate-buffered saline and spread on four different agar media:

- two selective chocolate agar plates with added antibiotics and fungicide (300 µg of bacitracin/ml, 1 µg of lincomycin/ml, 1 µg of crystal violet/ml, 50 µg of nystatin/ml)
- one selective blood agar plate with added antibiotics and fungicide (100 µg of bacitracin/ml, 1 µg of lincomycin/ml, 1 µg of crystal violet/ml, 50 µg of nystatin/ml) and 0.07% NAD
- one nonselective blood agar plate with a nurse strain
- one nonselective chocolate agar plate

The plates were incubated at 37°C for 48 h. *A. pleuropneumoniae*-like colonies were subcultivated from all media and biochemically confirmed as *A. pleuropneumoniae*. The second selective chocolate agar plate was used for PCR.

2. Sequencing the *omlA* gene.

In total seven primers, designated LPF, LPF1, LPR, LPR1, LPR2, LPR3, and LPR4 (Table 3.3) were used for sequencing the *omlA* gene. For the production of amplification products from the *A. pleuropneumoniae* reference serotypes, PCR with primers LPF1 and LPR1 was performed under low-stringency conditions (denaturation at 94°C for 1 min, annealing at 40°C for 1 min, primer extension at 72°C for 2 min, 35 cycles). PCR with the rest of the primers was performed with annealing temperatures around 60°C, when used for production of amplification products for sequencing. The nucleotide sequences of the amplification products were determined by cycle sequencing with an Amplitaq FS dye terminator kit and a 373A

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automatic sequencer (Applied Biosystems Division, Perkin-Elmer, Foster City, Calif.). Analysis of the sequence similarities was performed with the HIBIO DNASIS program for Windows, Higgins and Sharp algorithm.

3. Preparation of samples for PCR.

Samples of pure cultures were prepared by suspending approximately 10 µl of bacterial culture in 200 µl of sterile water. Mixed bacterial cultures from selective chocolate agar plates were harvested for PCR by washing them in 2 ml of distilled sterile water. All samples were stored frozen at -80°C. Bacterial cells of both pure and mixed cultures were lysed. One microliter of supernatant was used in the PCR as described below.

Table 3.3: Position and sequences of primers used for PCR amplification of the *omlA* gene.

Primer	Sequence	Position (bp)
LPF	5'–AAGGTTGATATGTCCGCACC–3'	272-290
LPF1	5'–ATTGTAAACTTTAGAGCTTTATATT–3'	34-58
LPR	5'–CACCGATACGCCTTGCCA–3'	1223-1205
LPR1	5'–ATTAAAAAGTAAAAAAGCTATCCC– 3'	1312-1289
LPR2	5'–ATCTTTTACCGATGCACTATT– 3'	1340-1319
LPR3	5' –TAGATGATTACGATTAATCT ATCC– 3'	667-642
LPR4	5'–AAAAGTAAAAAAGCTATCCCG–3'	1308-1287

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4. PCR amplification

The LPF and LPR primers (Table 3.3) were used for amplification in the *A. pleuropneumoniae*-specific PCR. Before use the primers were purified by high-performance liquid chromatography (HPLC). The expected PCR amplification product was about 950 bp. The PCR was performed with an automated DNA thermal cycler. The PCR assay was performed with 0.5 U of *Taq* polymerase (Perkin-Elmer) in a total volume of 50 µl in a buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet P-40 detergent, 100 µM of each deoxynucleoside triphosphate, and 0.2 µM of each HPLC-purified primer. The PCR test tubes were subjected to initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 63°C for 20 s, and primer extension by DNA polymerase at 72°C for 2 min. To ensure complete strand extension, the reaction mixture was incubated for 10 min at 72°C after the last cycle. Twelve-microliter samples of the final reaction mixture were analyzed by electrophoresis in a 1.5% agarose gel in 1× Tris-borate-EDTA (TBE) buffer. The PCR products were stained with ethidium bromide (10 µg/ml) and visualized under UV light.

For determination of PCR detection limits, serial 10-fold dilutions of *A. pleuropneumoniae* (10^4 to 10^{-1} CFU/PCR test tube) in distilled sterile water were mixed with *Escherichia coli* in a concentration of 10^8 CFU/ml.

3.7 Outcome measurement

The health status of the pigs was monitored by the manager on daily basis and by a veterinarian at the time of visiting. The manager recorded health changes and therapeutic treatment. The farrowing rate of each group was calculated. Additional critical reproductive periods such as lactation length, weaning to estrus interval, days to return to estrus, and gestation length were estimated. Litter characteristics such as the number of piglets born alive, dead and mummified and the number of piglets weaned were recorded. The pigs' performances were recorded, such as average daily weight gain, feed conversion rate, average daily feed intake, and loss rate. The foundation of exposure was proven by the presence of antibody titers.

3.8 Statistical analysis

Bioassay results were also expressed as proportions (number positive/number tested). The level of significance was set at 0.05. The significance level of difference of seroprevalence between different groups of pigs was assessed by Chi-square test. The significance level of difference of growth parameters between different groups of pigs was determined using t-test.

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