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Investigation on the co-infections of *Toxoplasma gondii* with PRRSV, CSFV or PCV-2 in swine in part of China

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**Abstract**

The objective of the present investigation was to estimate the prevalence of *Toxoplasma gondii* infection and co-infection with porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV) and porcine circovirus type 2 (PCV-2) in pigs in China. A total of 372 tissues or serum samples collected from pigs distributed in 9 provinces/municipalities of China during the period from February 2011 to November 2012 were assayed for *T. gondii* antigens and antibodies using enzyme linked immunosorbent assay (ELISA) technique, while the PCR was designed for the detection of the PRRSV, CSFV and PCV-2, respectively. The total positive rate of *T. gondii*, PRRSV, CSFV and PCV-2 was 9.14% (34/372), 50.00% (186/372), 37.10% (138/372) and 3.23% (12/372), respectively. Among the 34 *T. gondii* positive samples, 26 samples were simultaneously infected with *T. gondii* and viruses, while the remaining eight samples were infected with *T. gondii* alone. In addition, the co-infection rate of *T. gondii* with PRRSV, *T. gondii* with PRSSV and CSFV, *T. gondii* with PRSSV and PCV-2, *T. gondii* with CSFV and PCV-2, *T. gondii* with PRSSV, CSFV and PCV-2 was 1.61% (6/372), 4.03% (15/372), 0.27% (1/372), 0.27% (1/372) and 0.81% (3/372), respectively. The results of the present survey revealed that PRRSV and CSFV were the common pathogens co-existing with porcine toxoplasmosis in China, and both of them could increase the chances of *T. gondii* infection in pig. This is the first report of *T. gondii* co-infections with viruses in pigs. It is very important to understand the interactions of parasite and virus, and can be used as reference data for the control and prevention of co-infections of *T. gondii* and viruses in pigs.

**Keywords:** *Toxoplasma gondii*, coinfection, PRRSV, CSFV, PCV-2, pig

1. Introduction

*Toxoplasma gondii* infections are widely prevalent in human beings and animals worldwide (Dubey 2010). In humans, infection is usually either asymptomatic or the cause of mild flu-like symptoms. However, toxoplasmosis can be life threatening in immunocompromised individuals. Moreover,
if acquired during pregnancy, toxoplasmosis can cause miscarriage or congenital malformations affecting the brain, eyes or other organs of the foetus. Consumption of raw or undercooked meat products containing *Toxoplasma gondii* tissue cysts or ingestion of food or drink contaminated with infectious oocysts from cat feces are risk factors associated with *T. gondii* infection (Dubey 2010).

Toxoplasmosis was an important cause of neonatal deaths and stillbirth in pigs (Dubey et al. 1991; Green and Morgan 1991; Kim et al. 2009). Pigs infected transplacentally with *T. gondii* may be born premature, dead, or weak, or die soon after birth (Jungersen et al. 2001), which has devastated the swine industry, causing significant economic losses. In addition, *T. gondii* infection in pigs poses risk for human infection with *T. gondii* through consumption of undercooked pork of the infected pigs (Huang et al. 2010). The serological prevalence of *T. gondii* in pigs has been reported in different provinces of China, ranging from 4.6 to 53.4%, and indicated a common exposure (Huang et al. 2010; Zhou et al. 2010; Tao et al. 2011; Yu et al. 2011; Li et al. 2012; Wang et al. 2012; Wu et al. 2012; Chang et al. 2013).

In addition to *T. gondii*, classical swine fever virus (CSFV), porcine circovirus type 2 (PCV-2), and porcine reproductive and respiratory syndrome virus (PRRSV) also cause reproductive and respiratory failure in pigs, resulting in significant economic losses to the pork industry. These three viruses were frequently reported in China (Tu et al. 2001; Li et al. 2011; Ge et al. 2012), and it was common for pigs to be simultaneously infected with two or three of them, especially under typical conditions of intensive pig production.

Co-infections with the three viruses, PRRSV, CSFV and PCV-2, have been demonstrated in a significant proportion of field cases in China (Diao et al. 2005; Jiang et al. 2010). However, until now, data on co-infection of *T. gondii* with PRRSV, CSFV and PCV-2 were missing. In this study, the presences of *T. gondii* only and co-infections with other viruses in pigs were investigated. This is the first report of *T. gondii* co-infections with viruses in pigs in China. It is very important to understand the interactions of parasite and virus, and can be used as reference data for the control and prevention of co-infections of *T. gondii* and viruses in pigs.

### 2. Results

In the present study, total 372 pig serum samples collected from 9 provinces/municipalities of China were tested for PRRSV, CSFV and PCV-2, respectively. The total seropositive rate of *T. gondii* was found in 34 (9.14%) out of 372 samples (Table 1).

A total of 186 samples were positive for PRRSV and the positive rate was 50.00%. The positive rates of CSFV and PCV-2 were 37.10% (138/372) and 3.23% (12/372), respectively (Table 1).

Among the 34 *T. gondii* sero-positive samples, 8 samples were infected with *T. gondii* alone, while the remaining 26 samples were simultaneously infected with *T. gondii* and one of the PRRSV, CSFV and PCV-2, or the combination of 3 viruses (Table 2). The co-infection rate of *T. gondii* with PRRSV, *T. gondii* with PRRSV and CSFV, *T. gondii* with PRRSV and PCV-2, *T. gondii* with CSFV and PCV-2, *T. gondii* with PRRSV, CSFV and PCV-2 was 1.61% (6/372), 4.03% (15/372), 0.27% (1/372), 0.27% (1/372) and 0.80% (3/372), respectively. Detail results were shown in Table 2.

### 3. Discussion

In the present study, 186 samples (50.00%) were found positive for the presence of PRRSV. This result was consistent with previous reports. In China, out of the 2981 clinical samples collected from different provinces/municipalities between May 2006 and December 2010, 1 814 samples (60.85%) were positive for PRRSV (Heaslip et al. 2011). In the northwestern part of China, Shang et al. (2012) found

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### Table 1 Results of *Toxoplasma gondii* and three viruses detection of pigs in China

| Province/Municipality | Number of samples | Number of positive samples<sup>1)</sup> |
|-----------------------|------------------|----------------------------------------|
|                       |                  | *T. gondii* | PRRSV | CSFV | PCV-2 |
| Beijing               | 20               | 2           | 20    | 0    | 0     |
| Hebei                 | 30               | 0           | 27    | 0    | 0     |
| Hubei                 | 100              | 1           | 0     | 11   | 0     |
| Shanghai              | 12               | 3           | 1     | 4    | 2     |
| Jiangsu               | 40               | 1           | 4     | 1    | 2     |
| Tianjin               | 10               | 0           | 2     | 1    | 0     |
| Shandong              | 88               | 10          | 87    | 83   | 3     |
| Sichuan               | 61               | 15          | 43    | 36   | 5     |
| Chongqing             | 11               | 2           | 2     | 2    | 0     |
| Total                 | 372              | 34          | 186   | 138  | 12    |
| Positive (%)          | –                | 9.14        | 50.00 | 37.10| 3.23 |

<sup>1</sup>PRRSV, porcine reproductive and respiratory syndrome virus; CSFV, classical swine fever virus; PCV-2, porcine circovirus type 2.
that 35.9% of pigs were PRRSV-positive, and the average prevalence in 2007–2008 and 2009–2010 was 46.5 and 29.3%, respectively. In southern China, Xie et al. (2014) collected 126 tissue samples from 2010 to 2013 and detected the PRRSV. The results showed that the PRRSV positive rate was 32.54%, that there are two main subgenotypes in southern China, and that the dominant strain is HP-PRRSV (Xie et al. 2014). The PRRSV infection was sustained at a high level over the 8 years, indicating PRRS continues to be a serious threat to the swine industry of China. Comprehensive measures are needed to strengthen further prevention and control of the disease.

In this study, the infection rate of CSF was 37.10% (138/372), which was significantly higher than that previously reported (Yao et al. 2011). Since 2007, the swine fever compulsory vaccination policy was implemented and the immune density reached 95%, which greatly improved the immune protection rate and reduced the large-scale outbreaks of CSF. From 2007 to 2009, the average infection rate remained at a lower level below 15% (Yao et al. 2011). The reason for this high infection rate from 2011 to 2012 remains unknown, but was assumed to be due either to an inadequate immunization protocol, or to genetic variation of the virus.

In the present study, only 12 samples (3.23%) were found positive for the presence of PCV-2, which was relatively lower than previous reports in China (Lang et al. 2000; Liao et al. 2011). In 2000, Lang et al. (2000) collected 559 serum samples from 22 pig herds in Beijing, Hebei, Shandong, Tianjin, Jiangxi, Shanxi, Jilin, Henan of China, and showed a positive rate of 42.9% for PCV-2 antibody in total samples. The latest report showed that PCV-2 infection was in 58.87% (249/423) of pig farms and 35.05% (687/1960) of clinical samples was positive for PCV-2 (Liao et al. 2011). All the above results suggested that PCV-2 was well controlled within the last years in China.

For T. gondii, the total seroprevalence in pigs from 9 provinces/municipalities was 9.14% (34/372), which kept at a relatively low level. Recently, sero-epidemiological surveys about the T. gondii infection in swine have been conducted in some parts of China, the infection rate of T. gondii was 4.6–53.4% (Huang et al. 2010; Zhou et al. 2010; Tao et al. 2011; Yu et al. 2011; Li et al. 2012; Wang et al. 2012; Wu et al. 2012; Chang et al. 2013). Among the 34 positive samples, 26 samples were simultaneously infected with T. gondii and viruses, while the remaining 8 samples were infected with T. gondii alone, which indicated that viral infections seem to increase the chances of T. gondii infection in pig. It is hard to precisely point out the relationship of the co-existence of T. gondii with PRRSV, PCV-2 or CSFV. However, among the T. gondii-seropositive subjects, the expected risk of co-infection by T. gondii/PRRSV and T. gondii/CSFV was high. PRRSV could cause immune function decline of pig, and the inhibition of immune function was very obvious especially in the early infection (Drew 2000; Renukaradhya et al. 2010). CSFV infection damaged the pig’s immune organ such as spleen and lymph nodes, and caused immune suppression, resulting in the concurrent infections with other diseases (Moennig et al. 2003; Lange et al. 2011). In a word, primary PRRSV and CSFV infection might reduce the body’s resistance to other opportunistic pathogens such as T. gondii, and predisposed to the development of secondary toxoplasmosis in pig.

In the practice, some farms might immunize the pigs with live virus vaccines. The vaccine strains might also result in the positive of the detections of PRRSV, CSFV or PCV-2 in this study by PCR method. However, up to now, there are no standard methods to distinguish wild strains and vaccine strains of these viruses. Thus, in this study, we did not distinguish the wild strains and vaccine strains.

As reported previously, T. gondii circulating antigen (TCA) was considered to be a direct evidence that an infection was present and could be easily detected during the acute phase (Zhao et al. 2012). However, in this phase, the antibodies may not produce. Therefore, if detection only relies on circulating antibodies, some positive samples might be neglected. So, in the present study, serological detection assays using both TCA and TCAb were performed by enzyme linked immunosorbent assay (ELISA) technique.

### 4. Conclusion

The results of the present survey indicated that T. gondii infection in pigs was as high as 9.14% in China, and the high infection of T. gondii in pigs might be a risk to the safety of animal origin food and public health. PRRSV and CSFV were the common pathogens co-existing with porcine toxoplasmosis in China, and both of them could increase the chances of T. gondii infection in pig. The research of co-infection of T. gondii with other pathogens still requests more works.

### Table 2  Frequency of T. gondii alone or in combination with viruses in 372 pigs from February 2011 to November 2012

| Number of positive samples | Positive (%) |
|---------------------------|-------------|
| T. gondii+PRRSV           | 6           | 1.61        |
| T. gondii+CSFV            | 0           | 0           |
| T. gondii+PCV-2           | 0           | 0           |
| T. gondii+PRRSV+CSFV      | 15          | 4.03        |
| T. gondii+PRRSV+PCV-2     | 1           | 0.27        |
| T. gondii+CSFV+PCV-2      | 1           | 0.27        |
| T. gondii+PRRSV+CSFV+PCV-2| 3           | 0.81        |
| Total                     | 26          | 6.99        |
| T. gondii only            | 8           | 2.15        |
5. Materials and methods

5.1. Sample collection

From February 2011 to November 2012, a total of 372 samples were collected from different pig farms in 9 provinces/municipalities in China. The samples were mainly from lungs, kidneys, livers, blood, tonsil and lymph nodes of pigs showing clinical signs such as high fever, hemorrhages of skin, anorexia, lethargy, and abdominal breathing. The pigs showing no clinical signs from the same pig farms were also sampled. The 9 provinces/municipalities selected for the study covered eastern China (Shandong Province, Jiangsu Province and Shanghai Municipality), North China (Hebei Province, Beijing Municipality and Tianjin Municipality), central China (Hubei Province), and southwest of China (Chongqing Municipality, Sichuan Province). The location of the pig farms is shown in Fig. 1.

5.2. Sera preparation

Sera were obtained by centrifugation of clotted blood and kept at 4°C until being dispatched to the laboratory for evaluation with cold packs by air. After that, all the sera were stored at –20°C.

5.3. Serological examination of \( T. gondii \) antigens and antibodies

\( T. gondii \) antigens (Ag) and antibodies (Ab) were detected

![Fig. 1 The provinces/municipality in mainland China where pigs have been surveyed for Toxoplasma gondii, porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV) and porcine circovirus type 2 (PCV-2), respectively. Shadowed areas are the sampling locations for the present survey. BJ, Beijing Municipality; TJ, Tianjin Municipality; HeB, Hebei Province; SD, Shandong Province; JS, Jiangsu Province; SH, Shanghai Municipality; HuB, Hubei Province; SC, Sichuan Province; CQ, Chongqing Municipality.](image-url)
with the Pig Toxoplasma Antigen (Ag) ELISA Kit and Pig Toxoplasma Antibody (Ab) ELISA Kit (Aoqing Scientific, China), respectively, according to manufacturer’s instructions. Briefly, 50 μL of diluted pig sera (1:5) were added to each well (pre-coated with T. gondii Ab or Ag) and incubated at 37°C for 45 min. The wells were washed five times with washing solution (PBS containing 0.05% (v/v) Tween-20) and horseradish peroxidase-conjugated rat anti-T. gondii specific antibody or horseradish peroxidase-conjugated rabbit anti-pig antibody (IgG-HRP) was added to each well afterwards. The chromogen tetramethylbenzidine (TMB)-peroxidase substrate system was used for the colorimetric reaction. After stopped by 50 μL sulphuric acid (2 mol L⁻¹), plates were read at an optical density of 450 nm in the model 550 microplate ELISA reader (Bio-Rad, Hercules, CA, USA). Standard positive and negative controls were included on each plate. The serum with absorbance at least 2.1-fold higher than that of the negative control was considered as positive reaction.

5.4. Primer design

Genomic sequences of PCV-2, PRRSV and CSFV were obtained from genomic data bases. Oligonucleotide primers for PCV-2 were according to Zhang et al. (2009). Outer primer and inner primer for CSFV were designed on the base of previous reports (Lin et al. 2013). Sequences of PRRSV were aligned using the Megalign application (DNASTar) and Primer 6 were used for primer design. Oligonucleotide sequences of primer sets and their main characteristics are summarized in Table 3. Primers were commercially synthesized (Invitrogen, Shanghai, China).

5.5. RNA extraction of CSFV and PRRSV and cDNA synthesis

Total RNA was extracted from 200 μL of serum or 20 mg of organ tissue homogenates using the TRizol reagent (Life Technologies, MD, USA) according to the manufacturer’s instructions.

For the first-strand complementary DNA synthesis, 10 μL of the viral RNA were included in a total reaction volume of 20 μL containing 4 μL of 5× reverse transcriptase (RT) buffer (50 mmol L⁻¹ Tris-HCl, 8 mmol L⁻¹ MgCl₂, 30 mmol L⁻¹ KCl, 1 mmol L⁻¹ dithiothreitol pH 8.3), 0.5 mmol L⁻¹ each deoxynucleotide triphosphate (dNTP), 1 μmol L⁻¹ antisense primers of CSFV (SR2898) and PRRSV (QST), respectively, 20 U of RNase inhibitor and 10 U of avian myeloblastosis virus reverse transcriptase (TaKaRa, Dalian, China). After incubation for 60 min at 42°C, the mixture was then cooled in ice water for 2 min.

5.6. DNA extraction of PCV-2

The DNA was extracted from 200 μL of serum or 20 mg of organ tissue homogenates using a commercial kit (QIaamp DNA Mini Kit, Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations.

5.7. PCR system

For PCV-2, PCR amplification system contained 3 μL of virus DNA, 0.5 μmol L⁻¹ of each primer, 12.5 μL 2× PCR Taqmix (Tiangen, Beijing, China), and ddH₂O were added in a final volume of 25 μL. The cycling conditions were an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min (Zhang et al. 2009).

For PRRSV, PCR amplification system contained 2 μL of virus cDNA, 0.5 μmol L⁻¹ of each primer, 12.5 μL 2× PCR Taqmix (Tiangen, Beijing, China), and ddH₂O were added in a final volume of 25 μL. The cycling conditions were an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

For CSFV, the amplification procedure for the inner primers was the same as the outer primers. PCR amplification system contained 2 μL of virus cDNA, 0.5 μmol L⁻¹

Table 3 Oligonucleotide primer sequence and characteristics

| Virus | Primer¹ | Sequence (5’→3’) | Target products |
|-------|---------|------------------|----------------|
| PCV-2 | PCF1096 (F) | GGATATTGTAGTCCTGGTCG | ORF2 (493 bp) |
|       | PC1588 (R) | TCCCGGCACCTTCCGGATATAC |
| PRRSV | SFGP5 (F) | GCATTGTAGTCCTGGTCG | ORF4, ORF5 and ORF6 (633 bp) |
|       | SGP5 (R) | TCCCGGCACCTTCCGGATATAC |
|       | QST (RT) | TAAAGGTCCTGGATATAC |
| CSFV  | SF2228 (F) | AGGAGTGGACCTGGCTGGTGG | E2 (671 nt) |
|       | SR2898 (R) | TCGCTGAGTCCTGGCTGGTCGT |
|       | SF2477 (F) | TAAAGGTCCTGGATATAC |
|       | SR2848 (R) | TAAAGGTCCTGGATATAC |

¹F, forward primer; R, reverse primer; RT, reverse transcription. SF2228 (F) and SR2898 (R), outer primers; SF2477 (F) and SR2848 (R), inner primers.
of each primer, 12.5 μL 2× PCR Taqmix (Tiangen, Beijing, China), and ddH₂O were added in a final volume of 25 μL. The cycling conditions were an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min (Lin et al. 2013).

All the amplifications were performed in an Eppendorf Mastercycler 1 gradient thermal cycler (Eppendorf, Westbury, NY, USA). The final amplified DNA was analysed by electrophoresis in 1% agarose gels and stained with golden view followed by visualisation under ultraviolet light.

5.8. Determination for the positive ratio

The positive rate from a given locality was calculated using the following formula: Positive rate of T. gondii (%) = ([Numbers of positive by Ag + Numbers of positive by Ab] – [Numbers of positive by both Ag & Ab]) / Numbers of total samples
Positive rate of PRSSV/PCV/CSFV (%) = Numbers of positive samples/Numbers of total samples
Co-infection rate (%) = (Numbers of co-infection of T. gondii with PRSSV or PCV or CSFV) / Numbers of total samples

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