

An alternative PCR assay with high sensitivity and specificity for the detection of swine toxoplasmosis based on the *GRA14* gene

Xi He

South China Agricultural University College of Veterinary Medicine

Derong Zhou

South China Agricultural University College of Veterinary Medicine

Yanwu Sun

South China Agricultural University College of Veterinary Medicine

Yuan Zhang

South China Agricultural University College of Veterinary Medicine

Xiaogang Zhang

Foshan Standard Bio-Tech Co., Ltd., Foshan, Guangdong Province

Yabiao Weng

South China Agricultural University College of Veterinary Medicine

Ruiqing Lin (✉ rqlin@scau.edu.cn)

South China Agricultural University College of Veterinary Medicine

Research

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Abstract

Background

Toxoplasma gondii, an intracellular apicomplexan protozoan parasite, can infect all warm-blooded animals. Infected swine are considered one of the most important sources of *T. gondii* infection in humans. Rapidly and effectively diagnosing *T. gondii* infection in swine is essential. PCR-based diagnostic tests have been fully developed, and very sensitive and specific PCR is crucial for the diagnosis of swine toxoplasmosis.

Methods

To establish a high specificity and sensitivity PCR detection method for swine toxoplasmosis, we used *T. gondii* *GRA14* gene as target to design specific primers and established a PCR detection method for swine toxoplasmosis. A total of 5462 blood specimens collected from pigs in 5 provinces and autonomous regions in southern China during 2016–2017 were assessed by the newly established *GRA14* gene PCR method.

Result

Altogether, we used *T. gondii* *GRA14* gene as target to design specific primers and established a high specificity and sensitivity PCR detection method for swine toxoplasmosis; in particular, this PCR method could detect *T. gondii* tachyzoite DNA in the acute infection phase. The *GRA14* gene PCR assay detected a minimum of 2.35 tachyzoites of *T. gondii*, and it could be used for *T. gondii* detection in blood, tissue, semen, urine and waste feed specimens. The overall *T. gondii* infection rate was 18.9% (1033/5462) by the newly established *GRA14* gene PCR method. According to statistical analysis among different regions, the positive rates of swine toxoplasmosis in the Shaanxi, Fujian and Guangdong areas in China from 2016 to 2017 were the highest, at 31.7% (44/139), 21.9% (86/391) and 18.8% (874/4645), respectively ($\chi^2 = 84.2$, $P < 0.0001$). Specimens collected in 2017 had a higher positive rate (19.1% or 886/4639) than those collected in 2016 (16.1% or 155/963) ($\chi^2 = 4.5$, $P < 0.05$). Specimens collected in autumn (39.4% or 187/474), spring (22.8% or 670/2940) and winter (18.2% or 129/709) also had higher positive rates than those collected in summer (3.8% or 57/1479) ($\chi^2 = 427.7$, $P < 0.0001$).

Conclusions

These results indicate that the new PCR method based on the *T. gondii* *GRA14* gene would be useful for the diagnosis of swine toxoplasmosis and that it would facilitate the diagnosis of toxoplasmosis in clinical laboratories.

Background

Toxoplasmosis is a global endemic parasitic disease caused by *Toxoplasma gondii*, which infects humans and various animals via ingestion of raw or undercooked meat from chronically infected animals or by ingestion of sporulated oocysts found in food, soil and water contaminated with cat feces [1]. In humans, the majority of infections are asymptomatic; however, on rare occasions, mild flu-like symptoms are observed [2, 3, 4], but when mothers are contaminated during pregnancy, congenital toxoplasmosis ranges from asymptomatic cases to fetal death [5]. Additionally, in immunocompromised individuals, it also ranges from pauci-symptomatic cases to fatal visceral involvement [6]. Although toxoplasmosis is often associated with cats and their shedding of oocysts [7], meat is the most common source of infection in developed countries. Among livestock, pigs are susceptible to infection with *T. gondii*, and infected pigs are considered one of the most important sources of *T. gondii* infection in humans [3]. Research has reported that 41% of the cases of foodborne human toxoplasmosis in the USA are caused by the consumption of pork, which can thus be considered a main source of infection for humans [8, 9].

Pig production is spreading worldwide, and there are two main production systems: specialized intensive systems and free-range organic production. In most experimental studies on the epidemiology of swine toxoplasmosis, endemic outbreaks of swine toxoplasmosis still occur frequently, especially in intensive breeding pig farms, and the morbidity and mortality of pigs are high [10, 11]. With the development of the pig industry, the smooth circulation of breeding pigs and hogs has accelerated the global spread and prevalence of toxoplasmosis in pigs, causing severe economic losses to the pig industry and threatening human health. Therefore, the accurate diagnosis of toxoplasmosis in pigs is a prerequisite for the effective prevention and control of human toxoplasmosis.

Pigs are one of the intermediate hosts of *T. gondii*. Pigs can become infected by food or water contaminated with oocysts. Then, *T. gondii* sporozoites released from sporulated oocysts invade enterocytes and convert to tachyzoites, and tachyzoites rapidly divide by endodyogeny and disseminate through the blood to other organs and tissues during the acute phase of infection [12]. The immune response of the host causes tachyzoites to switch back to bradyzoites that encyst in skeletal muscle and brain tissues to establish a chronic infection [13]. Moreover, the current diagnosis of *T. gondii* infection relies mainly on serological detection of specific IgG and IgM [14, 15]. However, serological detection has been limited. Among them, IgM is the first antibody detected during infection, indicating that the host has recently been infected, and these antibodies can persist for months or even years after acute infection. High levels of IgG antibodies are present in sera, and serological detection does not distinguish a recent infection or previous infection [16]. In addition, serological detection may fail during the active phase of *T. gondii* infection because the antibody titers are low. PCR methods are useful for the identification and diagnosis of the *T. gondii* acute infection phase [17, 18]. Some PCR methods have been used to detect toxoplasmosis, such as those targeting the B1 gene [19], internal transcript spacer (ITS) sequence [20] and 529 bp repetitive element [21], but these methods are mainly used in laboratory research, and the

applicability of these methods is ambiguous because the data are limited regarding their use in large-scale clinical detection.

Dense granule protein (GRA) is a type of secreted metabolic antigen released from dense granules into nasal cysts after *T. gondii* invades host cells. It plays an important role in parasite survival and intracellular replication [22] and is considered a potential target for the diagnosis and prevention of *T. gondii* infection [23, 24]. The *GRA14* gene has been found in multiple stages of the life cycle of *T. gondii*, such as tachyzoites, bradyzoites, schizonts, etc. [25], and it can be used as a new diagnostic target for toxoplasmosis. In this study, we used *T. gondii* *GRA14* genes as targets to design specific primers for *T. gondii* and established a high-specificity and high-sensitivity PCR detection method for swine toxoplasmosis. By the new PCR methods, we examined the prevalence of *T. gondii* in intensive breeding pig farms in Guangdong, Fujian, Guizhou, Shaanxi, and Anhui. The data generated improve our understanding of the transmission of *T. gondii* between pigs and humans.

Methods

Specimens

T. gondii, *Staphylococcus aureus*, *Clostridium welchii*, *Salmonella*, and *Escherichia coli* kept in the Laboratory of Veterinary Parasitology of South China Agricultural University; *Isospora suis* and *Eimeria suis* were donated by Foshan Standard Bio-Tech Co., Ltd., Foshan, Guangdong Province.

From January 2016 to December 2017, a total of 5602 blood, semen, wastewater, waste feed, feces, urine, and tissue specimens were collected from intensive breeding pig farms in 7 provinces, Guangdong, Fujian, Guizhou, Anhui, Shaanxi, Anhui and Hunan, China (Fig. 1). Among them, 4645 specimens were collected from 54 farms in Guangdong, 391 specimens were collected from 3 farms in Fujian, 280 specimens were collected from 3 farms in Guizhou, 139 specimens were collected from 3 farms in Shaanxi, 95 specimens were collected from 2 farms in Anhui, 32 specimens were collected from 1 farm in Hunan and 20 specimens were collected from 1 farm in Jiangxi. These farms were selected based on willingness to participate in the study. The main specimens were blood, including 5462 blood specimens. Blood specimens were obtained by venous blood collection and saved in blood protection solution (Foshan Standard Bio-Tech Co., Ltd., China), and the freshly collected semen, wastewater, waste feed, feces, urine, and tissue specimens were placed into clean plastic bags and transported immediately to the laboratory. The blood, semen, wastewater, waste feed, feces, and urine specimens were stored at 4 ~ 8°C, and the tissues were stored at -20°C.

Genomic DNA extraction

The extraction of *T. gondii* DNA used different protocols for different samples. For blood specimens, 300 µL of blood was collected and mixed with three times the volume of Red Blood Cell Lysis Buffer (Qcbio Science & Technologies Co., Ltd., China), gently shaken, stored at room temperature for 5 min, and centrifuged at 10,000 rpm for 1 min; then, the supernatant was removed, leaving leukocyte sediment. For

semen specimens, frozen semen was removed from liquid nitrogen and immediately placed in a 37°C water bath, shaken gently for 10 s, placed for 20 ~ 30 s and thawed. The DNA of blood and semen specimens was extracted by using a TIANamp Blood DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., China). For fecal, feed, urine and wastewater specimens, before the DNA was extracted, these specimens were allowed to rest undisturbed at room temperature overnight. The next day, the supernatant fluid was removed by vacuum aspiration, and the precipitation liquid was centrifuged at 2,000 ×g for 10 min. Two hundred milligrams of precipitation liquid was used for DNA extraction by using a QIAamp DNA Mini Kit (QIAGEN, Korea). For tissue specimens, the tissue was shredded with sterilized scissors, and 200 mg of tissue specimen was used for DNA extraction with the Wizard SV Genomic DNA Purification System (Promega (Beijing) Biotech Co., Ltd., China). The DNA was stored at -80°C before PCR analyses.

Primer design

A conserved region of the dense granule protein 14 (*GRA14*) gene (GenBank accession number: MH213492.1) was selected to design specific primers for detecting toxoplasmosis. Primers were synthesized and purified by Sangong Company (Shanghai, China). After experimental verification, one primer set designated GRA14-3 was screened for further development of the PCR assay. PCR amplification was performed using the primers GRA14F-3: (5'-ATGCAGGCGATAGCGCG-3') and GRA14R-3: (5'-CTATTCGCTTGGTCTCTGG-3') for reactions with expected PCR products of ~ 1000 bp based on the genome sequence of *T. gondii*.

PCR conditions

PCR was carried out in a total volume of 25 µL of PCR mixture containing 12.5 µL of PCR Master Mix (Premix ExTaq Version 2.0 Loading Dye Mix, cat. No.: TAKARA, China) with a MgCl₂ concentration adjusted to 3 mM, 1.25 µL of each primer at a concentration of 10 pmol/µL, 2 µL of DNA and 9.5 µL of sterile distilled water. Reaction conditions were initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 50°C to 66°C, which was chosen to optimize the PCR annealing temperature, and 72°C for 1.5 min; followed by a final extension step at 72°C for 7 min.

Specificity and sensitivity of the GRA14 gene PCR assay

PCR specificity was evaluated using extracted DNA from the blood and tissue of *T. gondii*-free pigs and other pathogens, such as *I. suis*, *E. suis*, *S. aureus*, *C. welchii*, *Salmonella* and *E. coli*. To determine the sensitivity of the PCR assay, tenfold serial dilutions of *T. gondii* DNA (RH strain) from tachyzoites at concentrations ranging from 2.35×10⁴ to 0.023 tachyzoites were prepared, DNA was extracted from each dilution, and PCR of these dilutions was carried out for each gene target.

Comparison of the GRA14 PCR assay with the RE sequence and B1 gene PCR assays

A comparison of the traditional detection primers (RE sequence, *B1* gene) and the primers screened in this study (GRA14) showed that 15 pig blood specimens from different farms were detected at the same time. The targets were the B1 gene (GenBank accession number AF179871) and the 529-bp repeat element sequence (RE) (GenBank accession number AF146527) [21]. The primers were 5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3' (TOX4) and 5'-CGCTGCAGACACAGTGCATCTGGATT-3' (TOX5) for the RE sequence and 5'-GAAAGCCATGAGGCACTC CA-3' and 5'-TTCACCCGGACCGTTTAGC-3' for the B1 gene [26]. The RE sequence reaction conditions were initial denaturation at 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, annealing at 58°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The *B1* gene reaction conditions were initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, annealing at 58°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. PCR was carried out in a total volume of 25 µL of PCR mixture containing 12.5 µL of PCR Master Mix (Premix ExTaq Version 2.0 Loading dye Mix, cat. No.: TAKARA, China) with a MgCl₂ concentration adjusted to 3 mM, 1.25 µL of each primer at a concentration of 10 pmol/µL, 2 µL of DNA and 9.5 µL of sterile distilled water.

Clinical specimen detection by the GRA14 PCR assay

A total of 5602 specimens collected from 67 intensive breeding pig farms in 7 provinces (specimen types included blood, semen, wastewater, waste feed, feces, urine, and tissues) were tested to date by PCR assay, and the primers were the GRA14 primers screened in this study. These specimens were collected from 7 provinces, Guangdong, Fujian, Guizhou, Anhui, Shaanxi, Anhui and Hunan, China.

PCR product sequencing and statistical analysis

Some positive PCR products of the expected size were sequenced on an ABI3730 autosequencer by Sangon Biotech (Shanghai, China) in both directions using the PCR primers. DNA sequence analysis was undertaken using the National Center for Biotechnology Information (NCBI) BLAST programs and database.

The χ^2 test implemented in SPSS Statistics 21.0 (IBM Corp., New York, NY, USA) was used to compare differences in infection rates among sampling date, season, and sample types. Differences were considered significant at $P < 0.05$.

Results

Optimization of GRA14- PCR assays

Conserved regions inside the *GRA14* gene were used to design candidate primer pairs; among them, one of the primer pairs named GRA14-3 had a high positive rate and no nonspecific bands (Fig. 2a). The optimal reaction annealing temperature was 65°C for the established PCR assay (Fig. 2b).

Specificity and sensitivity of the PCR assay

The PCR assay was found to be highly specific for the detection of *T. gondii* DNA, and there was no amplification from any of the heterologous control samples, including pig blood DNA, pig tissue DNA, *I. suis*, *E. suis*, *S. aureus*, *C. welchii*, *Salmonella* and *E. coli*. (Fig. 3a).

The sensitivity of the GRA14 PCR assay was determined by 10-fold serial dilution of *T. gondii* tachyzoite amount ($2.35 \times 10^4 \sim 0.0235$). The results showed that the method had a limit of detection of 2.35 *T. gondii* tachyzoites (Fig. 3b).

Specificity and sensitivity comparison of the B1, RE and GRA14 PCR assays

The specificity of the GRA14 PCR assay was compared with that of the protocols used to amplify B1 and RE. We performed PCR using the same 15 blood specimens from the different farms and showed that the GRA14 PCR assay had better specificity than the B1 and RE PCR assays. Amplification was observed in 15 DNA blood specimens by the RE sequence PCR assay, but there were nonspecific bands, and it was not easy to determine positivity (Fig. 4a). Only two specimens were positive, and 11 *T. gondii*-positive samples detected by the *GRA14* gene were negative by the *B1* gene PCR assay (Fig. 4b and 4c). Furthermore, the positive PCR products were confirmed by sequencing.

Occurrence of *T. gondii* in pigs

Of the 5602 specimens collected from pigs on different intensive breeding pig farms, 1044 (18.6%) were positive for *T. gondii*. By area, the infection rates of *T. gondii* in pigs were 18.8% (874/4645), 21.9% (86/391), 11.7% (33/280), 31.7 (44/139), 0% (0/95), 12.5% (4/32) and 0% (0/20) in Guangdong, Fujian, Guizhou, Shaanxi, Anhui, Hunan, and Jiangxi, respectively. Thus, Shaanxi had higher infection rates of *T. gondii* than other cities ($\chi^2 = 84.2$, $P < 0.0001$). Specimens collected in 2017 (19.1% or 886/4639, $P < 0.05$) had a higher infection rate than those collected in 2016 (16.1% or 155/963, $P < 0.05$). There were significant differences in detection rates for samples collected during the seasons (Table 1), and the infection rates of *T. gondii* in pigs were 22.8% (670/2940), 3.8% (57/1479), 39.4% (187/474) and 18.2% (129/709) in spring, summer, autumn and winter, respectively.

Table 1
Occurrence of *Toxoplasma gondii* in pigs in China by the GRA14-PCR method

Variable	Category	Total no. of specimens	No. positive for <i>Toxoplasma gondii</i> (%)	P value
Region	Guangdong	4645	874 (18.8)	P < 0.0001
	Fujian	391	86 (21.9)	
	Guizhou	280	33 (11.7)	
	Shaanxi	139	44 (31.7)	
	Anhui	95	0	
	Hunan	32	4 (12.5)	
	Jiangxi	20	0	
	Collection year	2017	4639	
	2016	963	155 (16.1)	
Season	Spring	2940	670 (22.8)	P < 0.0001
	Summer	1479	57 (3.8)	
	Autumn	474	187 (39.4)	
	Winter	709	129 (18.2)	
	Total	5602	1041 (18.5)	

Of the 5602 specimens analyzed, the kind of specimens, including blood, wastewater, tissue, waste feed, semen and urine, varied, and the main specimens were blood; the infection rates of *T. gondii* in pigs were 18.9% (1033/5462), 3.8% (2/53), 3.0% (1/33), 33.3% (5/15), and 0% (0/95), and 12.5% (1/8) in blood, semen, wastewater, tissues, and urine (Table 2).

Table 2
Infection rates of *Toxoplasma gondii* in pigs by sample source

Sample source	Total no. of specimens	No. positive for <i>Toxoplasma gondii</i> (%)
Blood	5462	1033 (18.9)
Semen	53	2 (3.8)
Wastewater	33	1 (3.0)
Tissues	31	2 (6.5)
Waste feed	15	5 (33.3)
Urine	8	1 (12.5)
Total	5602	1044 (18.6)

Of the 963 blood specimens collected from pigs at different growth stages in 2016, including sows, boars, fattening pigs and piglets, 132 (16.3%) were positive for *T. gondii*. The detection rates among the different growth stages ranged from 0% (0/29) to 18.6% (122/655) (Fig. 5).

Discussion

The pig industry is important to the economy of many countries because pork is a main source of food for humans. Infected pigs are a source of *T. gondii* infection for humans and animals in many countries [27, 28]. Therefore, it is of great importance for public health to establish a rapid, highly sensitive and specific detection method for swine toxoplasmosis, especially during acute infection. In this study, based on the *GRA14* gene of *T. gondii*, a sensitive and specific PCR-based method for the rapid and direct detection of swine toxoplasmosis was developed.

A comparison of the detection of *GRA14* and previously reported genes (*B1* and the 529-bp RE) showed that PCR using *GRA14*-specific primers amplified *Toxoplasma*-specific DNA more efficiently. In the past few decades, different primer sets have been developed to detect *T. gondii*, including those targeting the *B1* gene and the 529-bp RE [21, 29]. *B1* amplification has been used more often for *T. gondii* detection, although it has shown lower specificity in some cases than other primer sets [30]. Although the 529-bp RE showed greater sensitivity, and some studies showed that it is 10 times more sensitive than *B1* [31, 32], some strains have partially or entirely lost repetitive units, which results in lack of detection of *T. gondii* infection. In this study, we developed a new PCR method to detect *T. gondii* in pigs based on the *GRA14* gene, which was more sensitive than PCR amplification of the *B1* gene and more specific than PCR amplification of the 529-bp RE. To ensure the validity of our investigation, all samples were randomly selected from clinical samples, including some that were positive samples. Evaluation of the detection sensitivities and specificity of PCRs using primers targeting the genes examined here demonstrated that *GRA14*-PCR is the most effective in amplifying *Toxoplasma* DNA.

GRA14-PCR could detect a minimum of 2.35 tachyzoites of *T. gondii*, and it can be used for detection during early *T. gondii* infection; the detection limit of diluted tachyzoites was four tachyzoites for 529-bp RE PCR and 40 for B1 PCR [21]. These results indicate that GRA14-PCR is an excellent method for the diagnosis of toxoplasmosis.

Furthermore, we found that GRA14-PCR was effective in amplifying *T. gondii* DNA in blood, tissue, semen, urine and waste feed. In this study, we used the GRA14-PCR method to detect mainly blood specimens, including 5462 specimens because, during the acute phase of *T. gondii* infection, the sporozoites released from sporulated oocysts invade enterocytes and convert to tachyzoites, and tachyzoites rapidly divide by endodyogeny and disseminate through lymphatic and blood circulation to other organs and tissues. Therefore, the detection of blood specimens can better identify the acute infection period of toxoplasmosis and result in timely treatment for pigs.

A total of 5462 blood specimens from 5 provinces in China were further applied in epidemiological studies with the GRA14-PCR method. The overall detection rate of *T. gondii* was 18.9% (1033/5462). This result is similar to detection rates in eastern China by PCR methods (18.03% or 75/416) [33] but higher than those reported in central China (2.06% or 34/1647 to 7.8% or 34/434) [34, 35] and southwestern China (13.6% or 56/412) [33] and lower than those reported in central China by ELISA (29.5% or 673/2277) [36] and those in three northeastern provinces by indirect hemagglutination (IHA) test (20.22% or 163/806) [37]. In relation to other countries, this rate is higher than that reported in Ireland (4.7% or 15/317) [38], Portugal (7.1% or 27/380) [39] and Brazil (12.5% or 38/296) [40] but lower than that reported in Mexico (50.8% or 218/429) [41] and Italy (57.1% or 12/21) [42]. The significant difference in the prevalence of *T. gondii* infection may come from the geographical distribution, animal species, or different detection methods.

In this study, statistical analysis showed that there was a significant difference in pigs among various sampling regions ($P < 0.0001$). According to our statistical analysis, the prevalence of *T. gondii* infection in pigs varied from 0 to 31.7%. Shaanxi had highest infection rates of *T. gondii* in this study, and Jiangxi and Anhui provinces had the lowest infection rates, where the specimen numbers were much different. We need to obtain additional specimens from Anhui, Jiangxi and Hunan in a later study.

Our data showed that there were significant differences in detection rates for specimens collected during the different seasons ($P < 0.0001$). Autumn (39.5%, 187/474) had higher infection rates of *T. gondii* than the other seasons, followed by spring and winter infection rates of 22.8% (670/2940) and 18.2% (129/709), respectively. Lower rates were observed in the summer (3.9% or 57/1479). This result is different from that in a previous report in which higher infection rates of *T. gondii* were observed in autumn and summer, and lower rates were observed in winter [37]. We need to obtain additional specimens from different climates in future studies.

This study showed that samples collected in 2017 (19.1% or 886/4639, $P < 0.05$) had a higher rate of exhibiting *T. gondii* infection than those collected in 2016 (16.1% or 155/963, $P < 0.05$), possibly due to the rapid development of the pig industry in China and the weak public awareness of sanitation

husbandry practices in pig production; the agricultural sector's propaganda and guidance need to be strengthened.

Conclusions

In this study, we demonstrated that the *GRA14* gene would be an excellent target for primer design for swine *T. gondii* infection detection, particularly in the acute infection period. The GRA14-PCR assay targeting conserved regions in the *GRA14* gene showed more sensitivity and specificity than the 529-bp RE and B1 PCR assays. Based on this assay, the higher the *T. gondii* infection rates in the studied intensive breeding pigs are, the higher the risk of eating undercooked or raw pork meat in some southern and northern regions of China, raising a major public health concern. It is essential to establish efficient strategies to prevent and control *T. gondii* infection in intensive breeding pigs and humans in these investigated regions. These findings contribute to improving the diagnosis of toxoplasmosis, especially in the incubation period when the correct and precise diagnosis is fundamental to early treatment in areas.

Abbreviations

PCR: polymerase chain reaction; *GRA*: Dense granule protein.

Declarations

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Ethics approval and consent to participate

The research protocol was reviewed and approved by the Research Ethics Committee of South China Agricultural University. Blood, semen, urine and waste feed specimens from pigs were collected with the permission of the farm manager. During specimen collection, pigs were handled in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

YW and RL conceived and designed the experiments. DZ performed the experiments. YS, YZ and XZ provided technical assistance. DZ, YZ and XZ analyzed the data. DZ, YW and RL wrote the paper. All authors read and approved the final manuscript.

Author details

¹ College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province 510642, PR China. ² Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, Guangzhou 510642, PR China. ³ Foshan Standard Bio-Tech Co., Ltd., Foshan, Guangdong Province 528138, PR China

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Figures

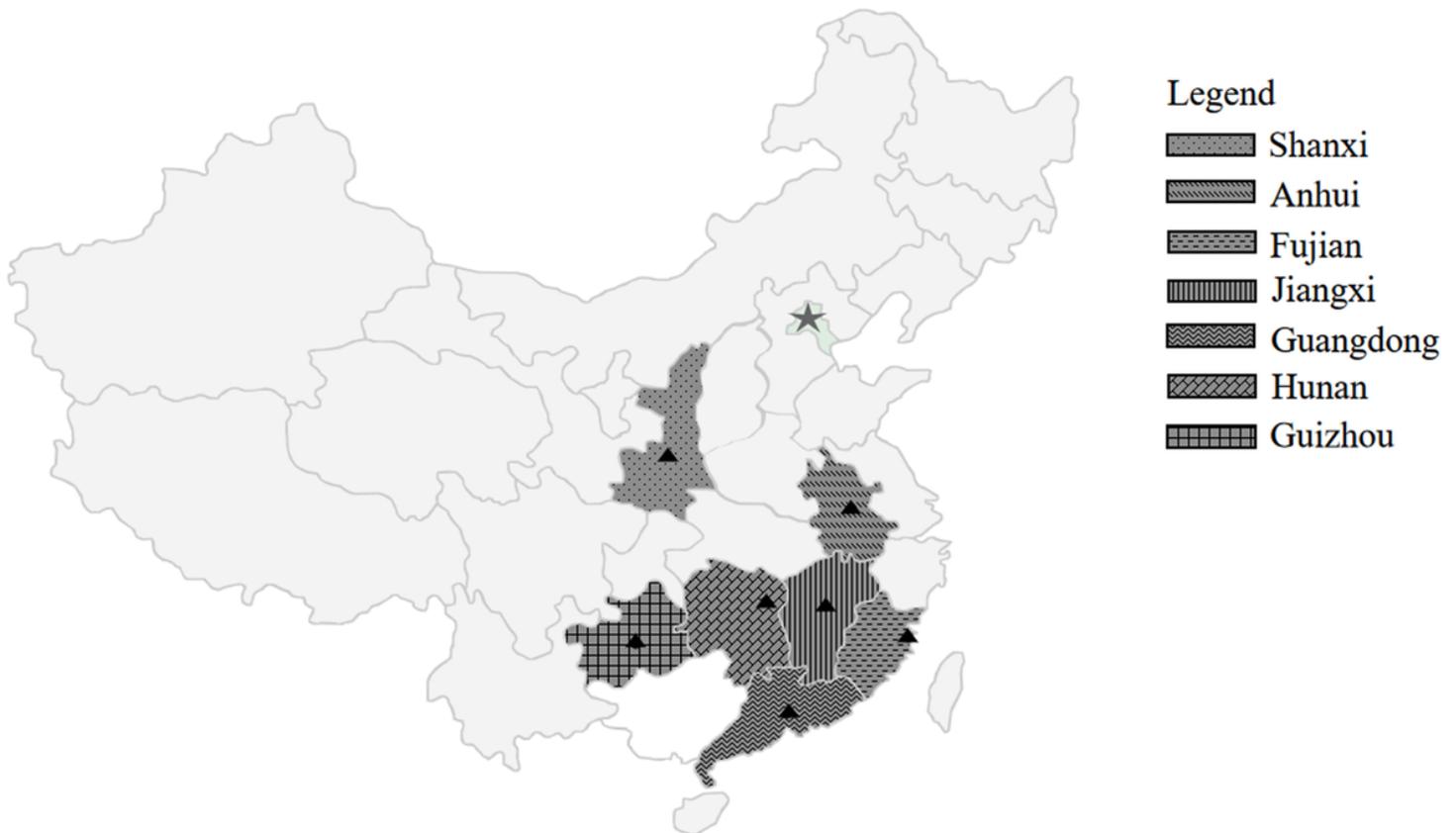


Figure 1

Map of districts in Shaanxi, Anhui, Fujian, Jiangxi, Guangdong, Hunan and Guizhou in China. The different colors show the specimen collection provinces, and the triangles represents the provincial capitals. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

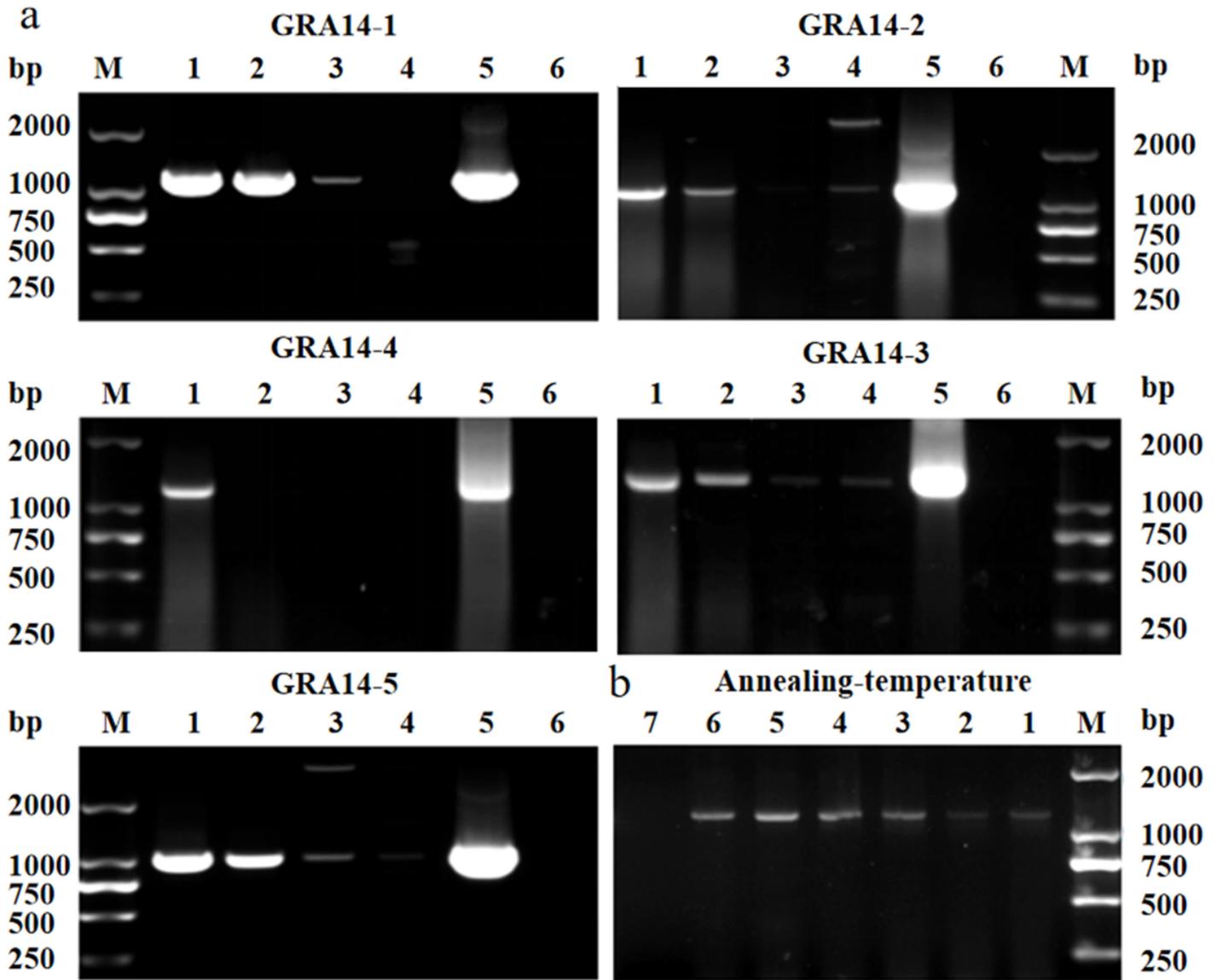


Figure 2

Optimization of the GRA14-PCR assay. a, agarose gel electrophoresis results for 5 pairs of primers designed based on the GRA14 gene, including the primers GRA14-1, GRA14-2, GRA14-3, GRA14-4, and GRA14-5. These primers were tested using total DNA extracted from *T. gondii* tachyzoites (lanes 1-5); lanes 6: negative control. b, agarose gel electrophoresis of the PCR products. 1: 50 °C; 2: 58 °C; 3: 61 °C; 4: 63 °C; 5: 65 °C, 6: 66 °C, 7: negative control, M: DL2000; the target segment was 1000 bp.

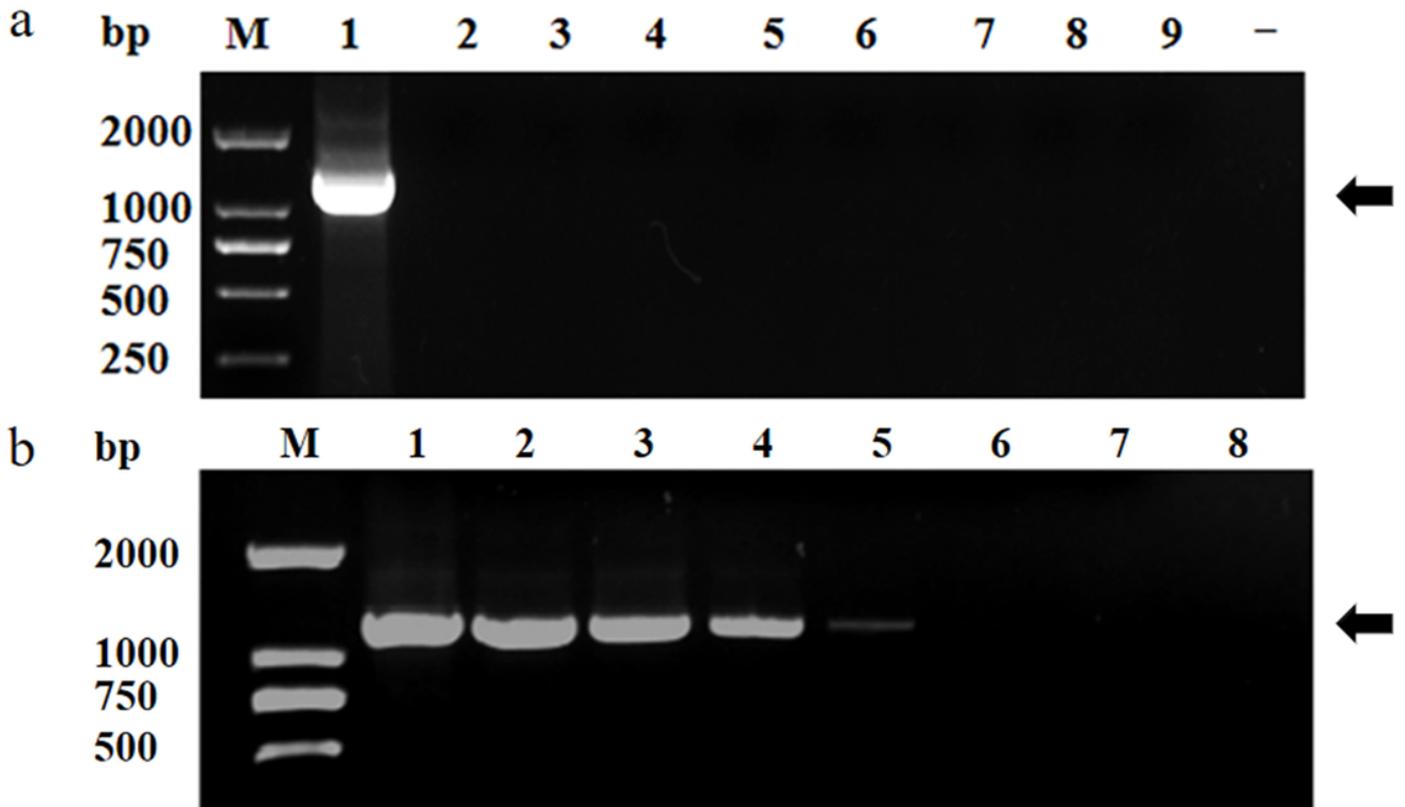


Figure 3

Specificity and sensitivity of the GRA14-PCR assay. a, detection specificity of the PCR using the GRA14-3 primers; 1, *Toxoplasma gondii*; 2, blood sample; 3, pork tissue; 4, *Eimeria suis*; 5, *Isospora suis*; 6, *Staphylococcus aureus*; 7, *Clostridium welchii*; 8, *Salmonella*; 9: *Escherichia coli*; "-": negative control. b, detection sensitivity of the PCR using the GRA14-3 primers. DNA was extracted from dilutions of tachyzoites, with tenfold serial dilutions (0.0235 - 2.35×10^4); lanes 1-7: dilutions of tachyzoites (2.35×10^4 , 2.35×10^3 , 235 , 23.5 , 2.35 , 0.235 , 0.0235), 8: negative control. M: DL2000; the target segment was 1000 bp.

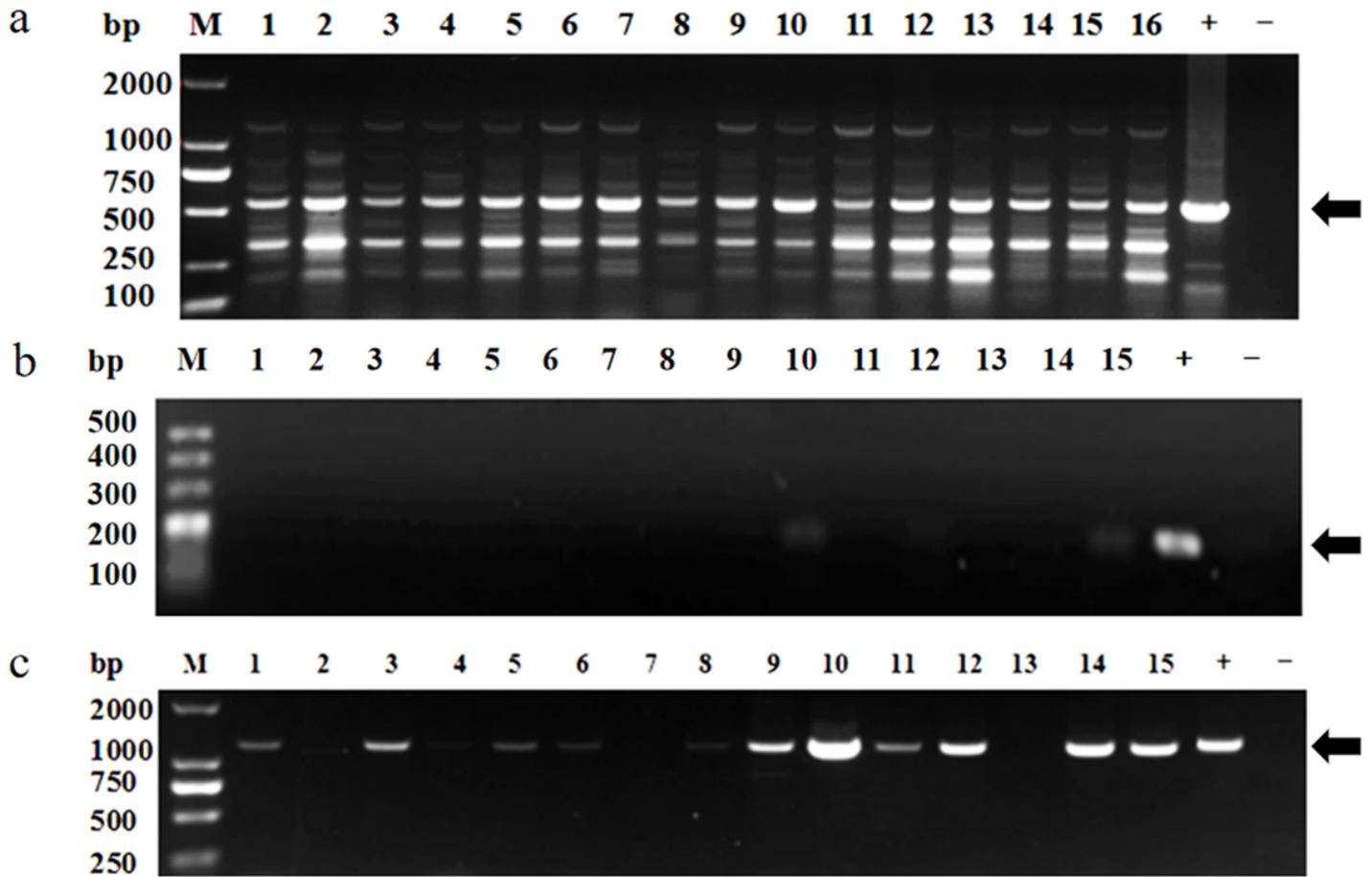


Figure 4

PCR detection of clinical samples targeting the B1 gene (a), RE sequence (b) and GRA14 gene (c). Lanes 1-15 were the clinical blood samples from 15 pigs. "+": positive control, "-": negative control. M: DL2000. The RE sequence target segment was 529 bp, the B1 gene target segment was 200 bp, and the GRA14 target segment was 1000 bp.

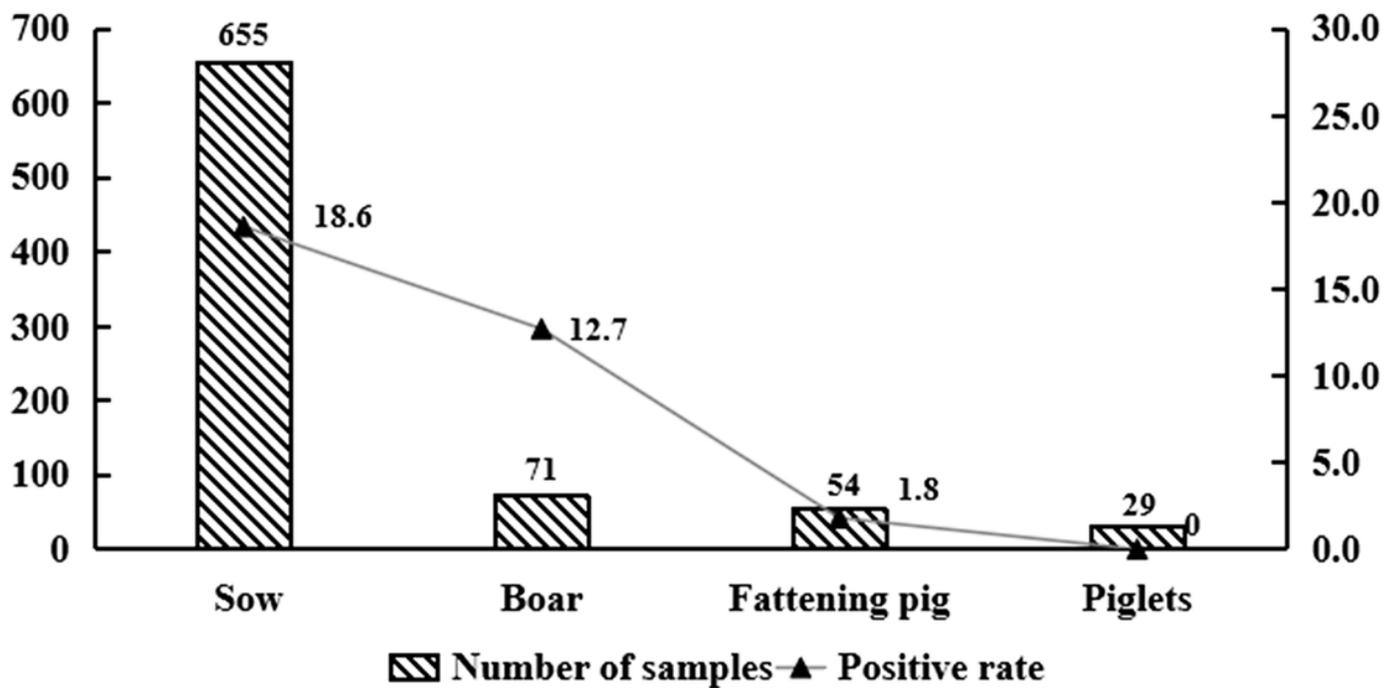


Figure 5

The positive rate of *Toxoplasma gondii* in pigs by different growth stages.

Supplementary Files

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