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Genotyping and characterization of *Toxoplasma gondii* strain isolated from pigs in Hubei province, central China

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Abstract. *Toxoplasma gondii*, a ubiquitous pathogen that infects nearly all warm-blooded animals and humans, can cause severe complications to the infected people and animals as well as serious economic losses and social problems. Here, one local strain (TgPIG-WH1) was isolated from an aborted pig fetus, and the genotype of this strain was identified as ToxoDB #3 by the PCR RFLP typing method using 10 molecular markers (SAG1, SAG2, alternative SAG2, SAG3, BTUB, GRA6, L358, PK1, C22-8, C29-2 and Apico). A comparison of the virulence of this isolate with other strains in both mice and piglets showed that TgPIG-WH1 was less virulent than type 1 strain RH and type 2 strain ME49 in mice, and caused similar symptoms to those of ME49 such as fever in piglets. Additionally, in piglet infection with both strains, the TgPIG-WH1 caused a higher IgG response and more severe pathological damages than ME49. Furthermore, TgPIG-WH1 caused one death in the 5 infected piglets, whereas ME49 did not, suggesting the higher virulence of TgPIG-WH1 than ME49 during piglet infection. Experimental infections indicate that the virulence of TgPIG-WH1 relative to ME49 is weaker in mice, but higher in pigs. This is probably the first report regarding a ToxoDB #3 strain from pigs in Hubei, China. These data will facilitate the understanding of genetic diversity of *Toxoplasma* strains in China as well as the prevention and control of porcine toxoplasmosis in the local region.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a widespread parasitic protozoan infecting humans and almost all warm-blooded animals, including poultry and livestock (Elmore *et al.*, 2010). Approximately one third of people around the world are infected by this parasite (Tenter *et al.*, 2000; Dubey and Jones 2008). *T. gondii* is a fairly successful pathogen that can invade and survive in all nucleated cell types of the hosts. As an opportunistic protozoan, it mainly causes miscarriage and stillbirth in pregnant women and animals, leading to serious social problems and economic losses worldwide (Montoya and Liesenfeld 2004; Dubey 2009a; Dubey 2009b; Dubey *et al.*, 2005).

The domestic pig is a kind of animal that is susceptible to *T. gondii*. In China, approximately 30% to 50% of domestic pigs are infected with *T. gondii*, with the infection rate as high as up to 70% in some regions (Li *et al.*, 2015; Yu *et al.*, 2011; Wu *et al.*, 2012; Xu *et al.*, 2014), leading to significant economic losses and also posing a potential threat to people. However, the genetic data of *T. gondii* strains isolated from pigs are limited in China. The ToxoDB #9 (Chinese 1) is considered as the main genotype in almost all examined hosts (Pan *et al.*, 2017), followed by ToxoDB #10 (Type I) (Pan *et al.*, 2017; Wang *et al.*, 2016). Additionally, the

ToxoDB #213 strain has been only reported in the Auhui province of China (Wang *et al.*, 2012) and ToxoDB #3 in Yunnan, Liaoning and Guangdong provinces of China (Wang *et al.*, 2016; Zhou *et al.*, 2011; Jiang *et al.*, 2013). Note that only a live ToxoDB #3 strain was isolated and preserved in Liaoning, China, and in the other two studies, only the DNA corresponding to the ToxoDB #3 strain was detected (Wang *et al.*, 2016; Zhou *et al.*, 2011; Jiang *et al.*, 2013).

In China, despite a high infection rate of *T. gondii* in pigs, genotyping data are still absent for some regions. In this report, we isolated some *T. gondii* strains from aborted piglets in Wuhan, Hubei, China, and one isolate (TgPIG-WH1) was identified as ToxoDB #3 by PCR-restriction fragment length polymorphism (PCR–RFLP) based on 10 molecular markers (Su *et al.*, 2006; Su *et al.*, 2010). The TgPIG-WH1 strain displayed a fairly low pathogenicity in mice, but the highest virulence in domestic pigs among all the strains tested. The results obtained from this study provide a new insight into *Toxoplasma* genotyping and may contribute to the prevention and treatment of toxoplasmosis.

MATERIALS AND METHODS

Sample collection and preparation of isolates from *T. gondii*

The lymph nodes (n=63) derived from aborted piglets or sick pigs were collected from 2015 to 2018 in Hubei province, China. Genomic DNA was extracted from these lymph nodes using the EasyPure Genomic DNA Kit (Transgen Biotech, Beijing, China) according to the manufacturer's recommendations, and the DNA samples were stored at -20°C until analysis. The PCR based on *T. gondii* 529bp repeat element was performed to detect infection as described previously (Meerburg *et al.*, 2012). The positive samples were used to feed 7-week-old female Kunming (KM) mice through oral administration. At 30 days post infection, mice were sacrificed to harvest the brains, followed by homogenization of the brains in 0.9% NaCl solution and digestion in pepsin. Then, the brain samples were detected by PCR based on *T. gondii* 529bp repeat element (Meerburg *et al.*, 2012). The positive mouse brain tissues were seeded on human foreskin fibroblast (HFF) culture flasks. Unfortunately, only one isolated strain was harvested from the medium and named TgPIG-WH1.

In vitro culture of tachyzoites

T. gondii type 1 strain RH and RH *Δhxgprt*, type a! strain ME49, type b! strain VEG, RFLP genotype ToxoDB #9 strain (Chinese 1 strain C7719) and TgPIG-WH1 strain were used in this study. They were propagated with human foreskin fibroblast (HFF) cells (purchased from ATCC, USA) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (LifeTechnologies, Inc., Rockville, MD, USA).

DNA extraction, PCR-RFLP assays

The genomic DNA was extracted from the tachyzoites using the EasyPure Genomic DNA Kit (Transgen Biotech, BeiJing, China). *T. gondii* isolates were genotyped by PCR-RFLP assays using 10 markers: SAG1, 5'+3' SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su *et al.*, 2006; Su *et al.*, 2010). The profile of the alleles was compared with the reference clonal strains RH (type I), ME49 (type II), VEG (type III) and RFLP genotype ToxoDB #9 strain (C7719). Amplification reactions for the genotyping with the external (Multiplex-PCR) and internal (nested-PCR) primers were performed as described by Cunha *et al.* (2016).

Virulence test of Tgpigwh1 tachyzoites in mice and pigs

All 7-week-old Kunming mice were purchased from the Hubei provincial center for disease control and prevention (Hubei, China), and all 35-45 day-old pigs were purchased from the pig breeding farm of Huazhong Agricultural University.

To check the virulence of the TgPIG-WH1, tachyzoites of indicated strains were used to infect KM mice (10^3 or 10^4 parasites/mouse, 10 mice for each strain) as previously described (Xia *et al.*, 2018) or pigs ($2*10^7$

parasites/pig, 5 pigs for each strain) by intraperitoneal injection. The survival of mice and pigs was monitored for 30 days.

After collecting the brain tissues of mice, the number of *Toxoplasma* cysts in each mouse brain was determined by DBA-FITC staining, as previously described (Buchholz *et al*., 2013).

The heart, liver, spleen and lymph node of pigs were collected for analysis of the pathologic changes using H&E staining. MIC3-based indirect ELISA was used to check the sera of mice and pigs to confirm the infection, and sera-negative mice and pigs were not included in the subsequent analysis. Cumulative mortality was graphed as Kaplan-Meier survival plots and analyzed in Prism 5 (GraphPad Software , Inc., La Jolla, CA, USA).

Measurement of *Toxoplasma* specific IgM and IgG levels

Serum samples were collected from pigs and stored at –20°C until analysis. The expression levels of *T. gondii* specific IgM and IgG were measured by ELISA as follows. Specifically, 96-well microtiter plates were coated with 100 µl of 0.34 µg/ml MIC3 or GRA1 diluted in PBS and incubated at 4°C overnight. Then, the plates were washed and blocked with 1% BSA. The serum samples were 200-fold diluted and added to each well, followed by incubation at 37°C for 45 min. After extensive washing, the bound IgM and IgG antibodies were detected by HRP conjugated goat anti-pig IgM and IgG secondary antibodies using tetramethylbenzidine (TMB) as substrate. Finally, the absorbance of each well was measured at 630 nm using a microplate reader (Bio-Rad Instruments, Hercules, CA, United States) and analyzed as previously described (Jiang *et al*., 2008). ELISA assay based GRA1 to detect *T. gondii* specic IgM antibodies in the sera of pigs was developed by our lab (unpublished data).

Histological examination

For histological studies, pigs infected with *T. gondii* were euthanized at 30 days post infection and the heart, liver, spleen and lymph node specimens were fixed in 4% buffered neutral formalin for 24 h, followed by embedding in paraffin blocks and slicing into 7–10 µm thick sections. After deparaffinage and rehydration, the sections were stained with hematoxylin and eosin following the manufacturer's instructions.

Statistical analysis

Statistical comparisons were performed in Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) using student's t tests and two-way analysis of variance (ANOVA) with bonferroni post-tests as indicated in figure legends. A p-value < 0.05 was considered as statistically significant, and the data were presented as the mean ± SEM of three independent experiments.

RESULTS

The *T. gondii* isolate was identified by PCR–RFLP typing

In this study, A total of 63 DNA samples were extracted from the lymph nodes of aborted piglets and sick pigs. 23.8% (15/63) of samples were positive for the *T. gondii* as detected by PCR based on the *T. gondii* 529 bp repeat element, which is repeated 200- to 300-fold in the genome of *T. gondii* (Homan *et al*., 2000). Although, the positive rate of *T. gondii* infection in the tissue samples was 23.8% by PCR detection, probably due to the low parasites load in the tissue samples, only one local strain was isolated from an aborted pig fetus. The *T. gondii* isolate genotyping was performed using 10 molecular markers and compared with the reference strains (see Table 1 for more information). The genotyping data are shown in Table 1. This isolate was identified as ToxoDB #3 and named TgPIG-WH1.

The *T. gondii* isolate derived from pig shows signicantly less virulence in mice

To evaluate the virulence of TgPIG-WH1 in mice, Kunming mice were intraperitoneally and separately infected with the tachyzoites of TgPIG-WH1, RH *Δhxgprt*, ME49 and VEG, followed by monitoring the survival of mice for 30 days. The results of virulence tests are shown in Fig. 1A-B. At the infection dose of

Table 1. PCR-RFLP genotyping of TgPIG-WH1 strain isolated from pigs in Hubei province, central China

Strains	Molecular markers											Gene type
	SAG1	(5'+3') SAG2	alt. SAG2	SAG3	BTUB	GRA6	C22-8	C29-2	L3358	PK1	Apico	
RH	I	I	I	I	I	I	I	I	I	I	I	ToxoDB #10
Me49	II or III	II	II	II	II	II	II	II	II	II	II	ToxoDB #1
VEG	II or III	III	III	III	III	III	III	III	III	III	III	ToxoDB #2
C7719	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgPIG-WH1	II or III	II	II	II	II	II	II	II	II	II	I	ToxoDB #3

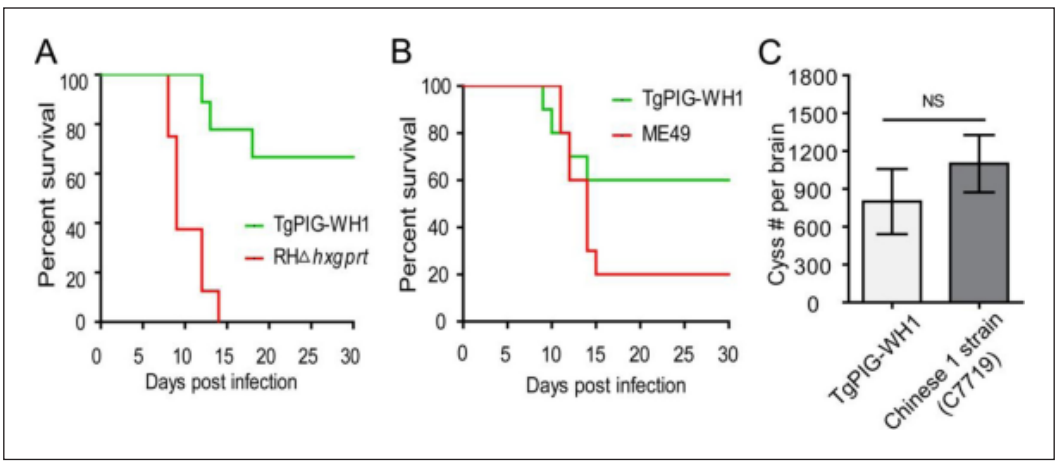

Figure 1. Virulence tests of TgPIG-WH1 in mice.
A-B. Survival curves of mice infected with tachyzoites of indicated strains. TgPIG-WH1 and RH strains (10^3 parasites/mouse) or TgPIG-WH1 and ME49 strains (10^4 parasites/mouse) were used to infect KM mice (10 mice for each strain) by intraperitoneal injection and the survival of mice was monitored for 30 days. **C**. Cyst loads in the brains of mice infected with TgPIG-WH1 or China 1 strain C7719. The mice survived at day 30 post infection were sacrificed, and the number of *Toxoplasma* cysts in the brain homogenate was determined. Student's t tests, not significant at $p > 0.05$.

10^3 tachyzoites/mouse, RH *Δhxgprt* killed 100% of the mice within 30 days, whereas TgPIG-WH1 just killed 30% of the mice (Fig. 1A). At the infection dose of 10^4 tachyzoites/mouse, TgPIG-WH1 killed 40% of the mice within 30 days, whereas ME49 killed 80% (Fig. 1B). These results indicated that the virulence of TgPIG-WH1 tachyzoites was severely attenuated in mice.

In order to obtain the cyst formation of TgPIG-WH1 in mice, Kunming mice were intraperitoneally infected with 10^4 tachyzoites of TgPIG-WH1 and Chinese 1 strain C7719 (ToxoDB #9) separately. At 30 days post infection, the mice were sacrificed and the number of brain tissue cysts in the mice of positive infection with *T. gondii* was determined by DBA-FITC staining. As shown in Fig. 1C, the number of brain tissue cysts derived from these strains showed no significant difference (Fig. 1C).

TgPIG-WH1 has a strong pathogenicity to pigs

To check the virulence of TgPIG-WH1 in pigs, pigs were intraperitoneally infected with the tachyzoites of TgPIG-WH1 and ME49 separately, followed by monitoring their survival, body temperature and weight for 30 days. The results of virulence tests are

shown in Fig. 2A. At the infection dose of 2×10^7 tachyzoites/pig, TgPIG-WH1 killed 20% of the pigs within 30 days, whereas ME49 did not kill any pigs (Fig. 2A). As shown in Fig. 2B, the naïve pigs injected with PBS alone maintained a stable body temperature, while the pigs infected separately with TgPIG-WH1 and ME49 showed significantly higher body temperature during the acute stage (on days 4–6) than the naïve pigs.

To examine the impact of TgPIG-WH1 at the levels of IgM and IgG antibodies, ELISA assays based on GRA1 and MIC3 proteins were performed. The results showed that the levels of IgG antibodies from the stimulation of TgPIG-WH1 and ME49 were significantly higher than those of naïve pigs separately (at weeks 2–4, or at weeks 4, Fig. 3B), while the levels of IgM antibodies from the stimulation of TgPIG-WH1 were obviously higher than those of naïve pigs at 2 weeks post infection (Fig. 3A). Meanwhile, the levels of IgM antibodies from the stimulation of ME49 was not obviously higher, probably because of large variations between repeated assays (at weeks 1–3, Fig. 3A).

Clinical examination revealed that TgPIG-WH1-infected pigs displayed more severe pathological damages in the heart, liver, spleen and lung than naïve pigs and ME49-infected pigs (Fig. 4A). Additionally, PCR based on *T. gondii* 529bp repeat element showed that the positive rate was higher in the TgPIG-WH1-infected tissues (heart, spleen and lung) than in the ME49-infected tissues (3 samples for each group, see Table 2 for detailed information). To estimate the pathological damage of TgPIG-WH1 to the tissues *in vivo*, the survived pigs were sacrificed after the virulence test for the histopathologic analysis of the heart, liver, spleen, and lymph node by H&E staining. The micrographs are shown in Fig. 4B. In all tissue samples, there were no obvious pathological changes in naïve pigs in contrast to the pericarditis, bleeding and pericardial enlargement in the heart tissues from the TgPIG-WH1-infected pigs (Fig. 4B). Additionally, the liver morphology of TgPIG-WH1-infected pigs showed the disorder of a hepatic cord, disappearance of the lobule structure, irregularity of cell morphology and necrosis of liver tissue with a large number of inflammatory cells (Fig. 4B). In the spleen, the pigs infected with TgPIG-WH1 showed more severe lymphocyte depletion than the naïve pigs within the white pulp follicles of the spleen (Fig. 4B). Furthermore, the lymph node of pigs infected with TgPIG-WH1 also showed lymphocyte depletion (Fig. 4B). The

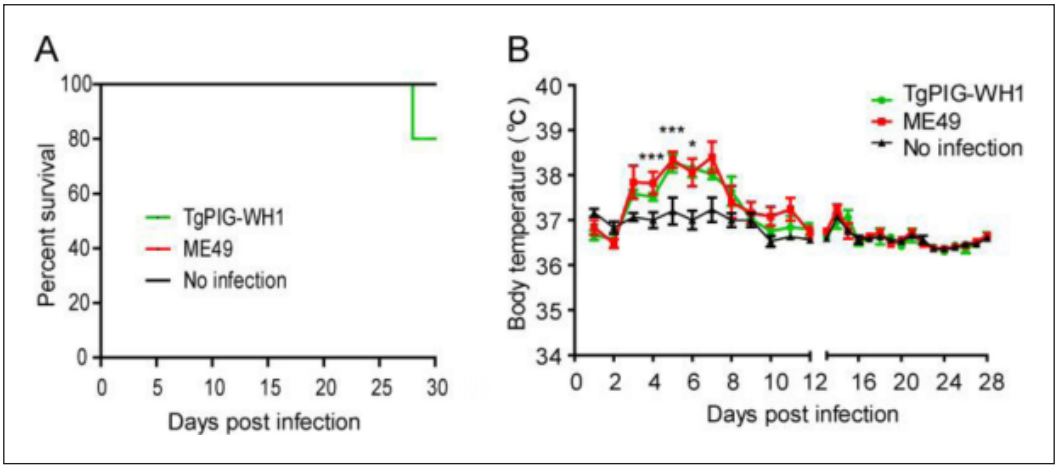

Figure 2. Virulence tests of TgPIG-WH1 in pigs.
A. The survival curves of pigs infected with tachyzoites of indicated strains. TgPIG-WH1 and ME49 strains were used to infect pigs (2×10^7 parasites/ pig, 5 pigs for each strain) by intraperitoneal injection and the survival of pigs was monitored for 30 days. No infection group (n=3 pigs) was included as a negative control. **B**. The body temperature of pigs was measured daily and compared with that of the No infection group using statistical analysis while all animals were alive. Two-way ANOVA, $^*p < 0.05$, $^{***}p < 0.001$.

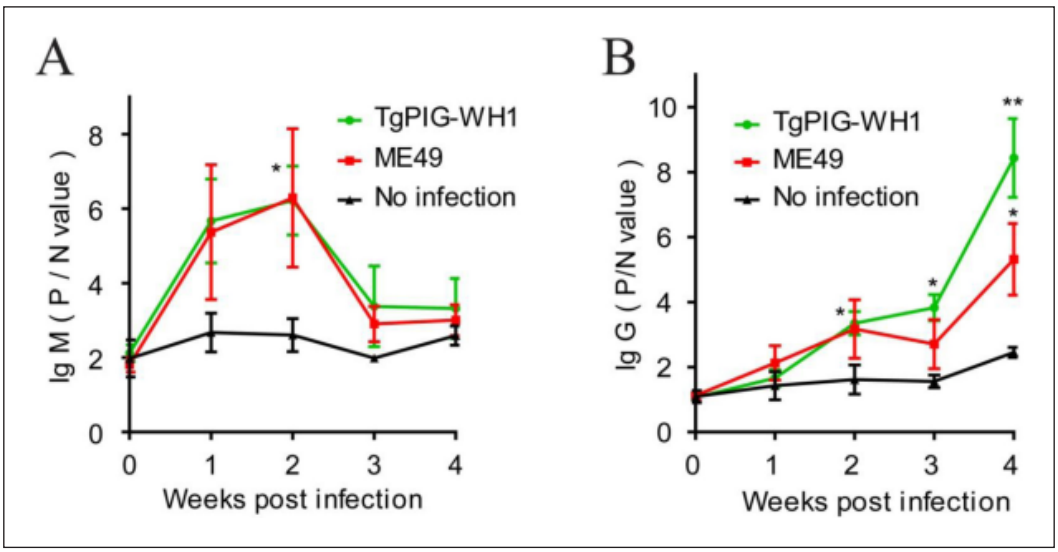

Figure 3. The expression levels of IgM and IgG antibodies in sera of pigs.
Relative expression levels of *Toxoplasma* specific IgM and IgG were determined by indirect ELISA. **A.** The expression levels of IgM in pigs from each group. **B.** The expression levels of IgG in pigs from each group. TgPIG-WH1 is the group of pigs infected with TgPIG-WH1 strain; ME49 is the group of pigs infected with ME49 strain; No infection group is the PBS treatment group. Sera from naïve pigs were used as controls and at least three pigs from each group were analyzed. Means ± SD from at least three independent experiments in triplicate. Student's t tests, $*p < 0.05$, $**p < 0.01$.

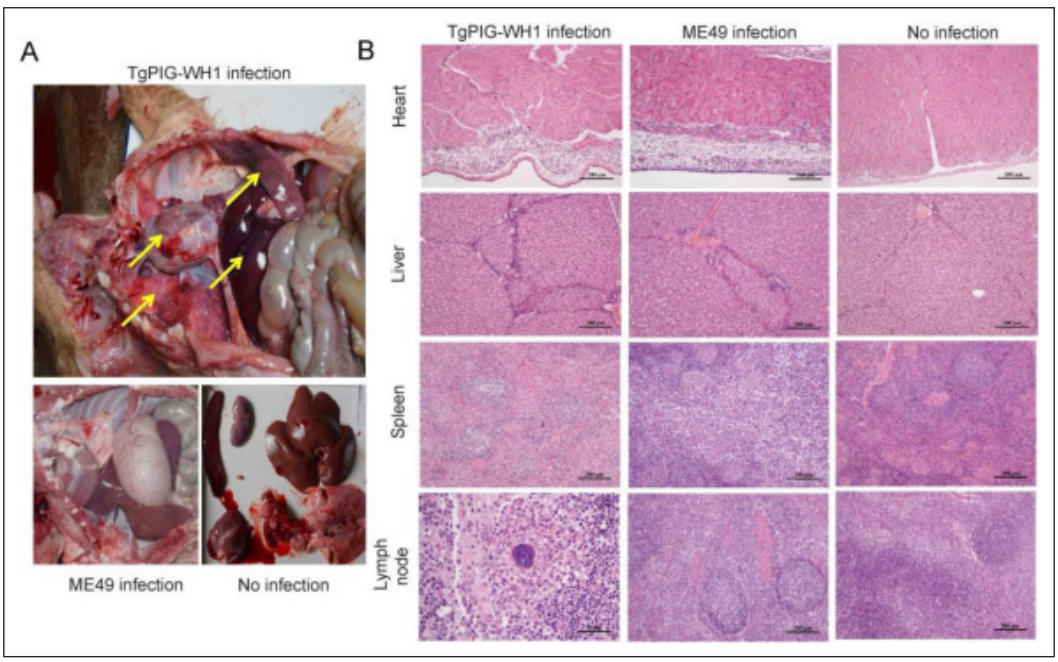

Figure 4. Pathological changes in pigs infected with TgPIG-WH1.
A. The pathological changes in the heart, liver, spleen and lung (the yellow arrows) in TgPIG-WH1-infected pigs. **B.** Tissue sections were stained with hematoxylin and eosin. TgPIG-WH1 infection is the group of pigs infected with TgPIG-WH1 strain; ME49 infection is the group of pigs infected with ME49 strain; No infection group is the PBS treatment group. Tissues from naïve pigs and ME49-infected pigs were used as negative and positive controls, respectively.

Table 2. The positive rate of *T. gondii* infection in the tissues of the pig infection model as detected by PCR based on the *T. gondii* 529bp repeat element

Tissue	Positive rate of TgPIG-WH1	Positive rate of ME49
Heart	3/3 (100.0%)	1/3 (33.3%)
Liver	2/3 (66.6%)	2/3 (66.6%)
Spleen	1/3 (33.3%)	0/3 (0.0%)
Lung	1/3 (33.3%)	0/3 (0.0%)
Brain	1/3 (33.3%)	1/3 (33.3%)

overall results from virulence tests, clinical examination and histopathologic analysis suggested that the TgPIG-WH1 strain displayed higher virulence than the ME49 strain in pigs.

DISCUSSION

Toxoplasma gondii has been recognized as an important food-borne pathogen in humans (Elmore *et al.*, 2010). Nearly one third of the human population worldwide is considered as chronically infected by consumption of undercooked meat with tissue cysts or ingestion of food or water contaminated with oocysts (Tenter *et al.*, 2000; Dubey and Jones 2008). In this study, a *T. gondii* prevalence of 23.8% was found in hilar lymph nodes of aborted and sick piglets from Hubei province, central China. Previously, the overall prevalence of 9.0% in hilar lymph nodes from slaughtered pigs in Jilin province, northeastern China, 65.8% in hilar lymph nodes from pigs in south China were reported using nested polymerase chain reaction (PCR) detection based on the *T. gondii* B1 gene (Jiang *et al.*, 2013; Jiang *et al.*, 2016). In Ireland, Portugal and Brazil, the prevalence *T. gondii* infection of slaughtered pigs was detected in 4.7%, 7.1% and 12.5%, respectively (Halova *et al.*, 2013; Esteves *et al.*, 2014; Samico Fernandes *et al.*, 2012). In Mexico and Italy, the prevalence *T. gondii* infection of fattening pigs and free-range, organic pigs was detected in 50.8% and 57.1%, respectively (Ortega-Pacheco *et al.*, 2013; Bacci *et al.*, 2015). The overall *T. gondii* prevalence of tissue samples is high in the world, suggesting the potential threat and risk for human infection.

The distribution of *T. gondii* genotypes varies in the world. Currently, three clonal strains (type I, II and III) are predominantly prevalent in north America and Europe (Lehmann *et al.*, 2000; Su *et al.*, 2003; Howe and Sibley 1995). In China, ToxoDB#9 is the major popular lineage (Pan *et al.*, 2017) and ToxoDB#3 has been found so far in a pig from Yunnan (Zhou *et al.*, 2011), Liaoning (Wang *et al.*, 2016) and Guangdong province (Jiang, *et al.*, 2013). This is the first report of ToxoDB#3 from pigs in Hubei, China. In China, ToxoDB#3 has also been identified in sparrows and pet birds in Gansu (Cong *et al.*, 2013; Cong *et al.*, 2014), wild birds in Xinjiang (Huang *et al.*, 2012), sheep in Qinghai (Zhou *et al.*, 2009) and cat in Yunnan (Tian *et al.*, 2014), suggesting that ToxoDB#3 is also a principal prevalent lineage in China.

Generally, the virulence of *T. gondii* isolates varies among their hosts and geographic regions. The three predominant types of lineages (I, II and III), such as RH, ME49 and VEG, have dramatically different virulence phenotypes in mice. The LD_{100} of type I strain RH was 1, in contrast to 10^3 for the LD_{100} of type II and III strains (Sibley and Boothroyd 1992). Currently, little information is available on the association of genotype and virulence of ToxoDB#3 from pigs in China. In this study, we have isolated and identified a ToxoDB#3 strain from pigs in Hubei province, China. At the infection dose of 10^3 or 10^4 tachyzoites/mouse, the TgPIG-WH1 tachyzoites just killed 30% or 40% of the mice, indicating that the TgPIG-WH1 tachyzoites display signicantly less virulence in mice. However, TgPIG-WH1 tachyzoites display a strong pathogenicity to pigs**.** In the pig infection model, PCR detected different tissues infected by TgPIG-WH1 or ME49. Our results showed that the positive rate of TgPIG-WH1-infected tissues (heart, spleen and lung) was obviously higher than that of ME49-infected tissues, suggesting strong infectivity of TgPIG-WH1 tachyzoites to different tissues of pigs and the potential risk of infection of the parasites to people who eat undercooked meat.

In this study, a TgPIG-WH1 strain was isolated from pigs in Hubei province, China and identified as ToxoDB #3. The virulence of TgPIG-WH1 is weaker in mice, but higher in pigs when compared with ME49. The data can enrich the genetic diversity of *T. gondii*. Further studies can focus on the differences of genetic expression using RNA sequencing.

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Conflict of Interest Statement
The authors declare that no potential conflict of interest exists.

Compliance with ethical standards
All animals were maintained under standard conditions according to the regulations specified by the Administration of Affairs Concerning Experimental Animals. Animal experiments were approved by the ethical committee of Huazhong Agricultural University (permit #: HZAUMO2016056).

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