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The Detection of *Toxoplasma gondii* in Wild Rats (*Rattus norvegicus*) on Mink Farms in Shandong Province, Eastern China

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Abstract

Toxoplasma gondii is a worldwide distributed zoonotic pathogen that threatens public health. However, there have been limited data for *T. gondii* infection in wild rats (*Rattus norvegicus*) in China. In the present study, a total of 227 wild rats were captured from three mink farms to investigate *T. gondii* infection in Shandong Province, eastern China. The DNA was extracted from 25 mg rats' brain tissues and subjected to a PCR amplification by targeting to the *T. gondii* B1. In 227 wild rat samples, 18 samples (7.93%) were positive for *T. gondii*. Then, the positive samples were further genotyped based on eight genetic markers, including eight nuclear loci (SAG1, 5′-SAG2, and 3′-SAG2, alternative SAG2, SAG3, GRA6, c29–2, and L358) and an apicoplast locus (Apico) by using the multilocus PCR-restriction fragment length polymorphism technology. Of these samples, eight were genotyped at nine nuclear loci, and two were genotyped at eight nuclear loci, forming three known genotypes (ToxoDB no. 43, ToxoDB no. 91, and ToxoDB no. 189) and two new genotypes. The closest ToxoDB genotypes were observed in wild rats, suggesting the differences in the population structure of the *T. gondii* between breed farm animals and wild rats. These data revealed the genetic variability of *T. gondii* in wild rats on mink farms in Shandong Province, with possible implication for public health.

Keywords: *Toxoplasma gondii*, RFLP, wild rats, closest ToxoDB genotypes, Shandong Province, China

Introduction

TOXOPLASMA GONDII IS one of the most important zoonotic foodborne pathogens that can infect almost all warm-blooded animals and humans (Dubey 2008, Mendez et al. 2017). The life cycle of *T. gondii* involves asexual stage (all warm-blooded animals, including rats) and sexual stage (felids). The infection of *T. gondii* in humans is through ingestion of the undercooked food and water containing tissue cysts and sporulated oocysts (Dubey 2008). An infection of *T. gondii* in hosts may cause severe symptoms, including abortion, encephalitis, and conjunctivitis, and even death in immunocompromised patients (Elsheikha et al. 2020).

To date, different genotypes of *T. gondii* exhibit different virulence. Some genotypes (*e.g.,* RH and GT1) can cause death of the inoculated mice regardless of the dose (Khan

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et al. 2009, Saraf et al. 2017). Therefore, an investigation of *T. gondii* genotypes in different animals is important for *T. gondii* research. A few lineages of *T. gondii*, that is, type II, type III, type 12, and Chinese I, have been identified recently (Dubey et al. 2021), such as synanthropic rats (*Rattus norvegicus*) in Jiangsu Province (ToxoDB no. 9, type II clonal lineage) (Yan et al. 2014), Edward's long-tailed rats (*Leopoldamys edwardsi*) in Chongqing municipality (ToxoDB no. 20, type II clonal lineage) (Zheng et al. 2019), Qinghai vole (*Lasiopodomys fuscus*) and Plateau pika (*Ochotona curzoniae*) in Qinghai Province (ToxoDB no. 10, type I clonal lineage, and a new genotype) (Zhang et al. 2013), voles (*Rattus flavipectus*) in Hubei Province (ToxoDB no. 9, type II clonal lineage) (Wang et al. 2013), Reed voles (*Microtus fortis*) in Jilin Province (ToxoDB no. 9, type II clonal lineage and ToxoDB no. 10, type I clonal lineage) (Zhang et al. 2014), Yunnan Red-backed voles (*Eothenomys miletus*) in Sichuan Province (a new genotype) (Wang et al. 2019), *Rattus rattus slade* and *Rattus flavipectus* in Yunan Province (ToxoDB no. 137, type II clonal lineage) (Wang et al. 2018) in China, rats (*Rattus norvegicus*) and mice (*Mus musculus*) in Belgrade, Serbia (unclear clonal lineage) (Vujanić et al. 2011), and *Rattus norvegicus, Rattus rattus*, and *Mus musculus* in Ahvaz district, southwestern Iran (unclear clonal lineage) (Saki and Khademvatan 2014).

However, the information is limited for *T. gondii* genotypes in rats (*Rattus norvegicus*) in Shandong Province, China. Therefore, this study aimed at exploring the prevalence and population structure of *T. gondii* genotypes in rats (*Rattus norvegicus*) in Shandong Province.

Materials and Methods

Ethics statement

The study was approved by The Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (permit no. LVRIAEC-2019-007). All rats were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Tissue collection and DNA extraction

A total of 227 wild rats (*R. norvegicus*), including male rats ($n=212$) and female ($n=15$) rats, were tested. All the wild rats were randomly captured from three biggest mink farms in Shandong Province. The wild rats were divided into two age groups based on their carcass weight: sub-adult group (carcass weight: 120.0–180.0 g, $n=78$) and adult group (carcass weight: 180.1–390.0 g, $n=149$) (Yao et al. 2016). The brain tissues of ~25 mg were homogenized, and this was followed by a digestion with proteinase K. Then, the genomic DNA of each sample was extracted by using the TIANamp Genomic DNA kit (TianGen™, Beijing, China) according to the previous operation (Zhang et al. 2014). The DNA was maintained at −20°C until PCR amplification.

PCR detection and genotyping analysis

All DNA samples were screened by semi-nested PCR amplification of *T. gondii* B1 gene (Hill et al. 2006). Direct PCR was amplified by using the primer pair (forward: 5′-GGAACTGCATCCGTT-CATGAG-3′ and reverse: 5′-TCTTTAAAGCGTTCGTGGTC-3′). Semi-nested PCR was performed by using the forward primer 5′-TGCATAGG TTGCAGTCACTG-3′, and the reverse primer was used in the first round PCR (Hill et al. 2006). A total of 25 μL reaction system contained 2 μL of genomic DNA, 0.5 μL of dNTP mixture (2.5 mM each), 2 μL of $MgCl_2$ (25 mM), 2.5 μL of 10×PCR buffer, 0.2 μL of TaKaRa r-Taq® (5 U/μL) (Takara Bio Inc., Dalian, China), and 0.25 μL of primers (10 μmol/μL).

The PCR reaction conditions were set as follows: initial denaturation at 94°C for 5 min; 30 cycles including 94°C for 10 s, 57°C for 10 s, and 72°C for 30 s; and an additional extension at 72°C for 5 min. The second round of PCR reaction was performed by a small modification from the first round. The modifications included: (1) 1 μL of primary PCR production was used as a template; and (2) annealing temperature was increased to 62.5°C. Each reaction included positive (DNA from *RH* strain) and negative (reagent water) controls.

The positive samples were further used to identify *T. gondii* genotypes by the PCR-restriction fragment length polymorphism technology (RFLP) analyses of 8 nuclear loci (SAG1, 5′-SAG2, and 3′-SAG2, alternative SAG2, GRA3, GRA6, c22–8, and L358) and an apicoplast (Apico) as previously described (Su et al. 2010). Six reference *T. gondii* strains, namely GT1, PTG, CTG, MAS, TgCgCa1, and TgCatBr5, were used as controls. Then, the nested PCR products were digested with appropriate restriction endonucleases, and the reaction was conducted as previously described (Su et al. 2010). The enzyme-digested products were observed by 2–3% agarose gel electrophoresis. The genotypes of *T. gondii* were confirmed by a comparison of RFLP genotypes listed in the ToxoDB genotyping database (www.toxodb.org).

Statistical analysis

The data for the detection rate of *T. gondii* between different gender and age groups were statistically analyzed with the chi-square test (χ^2) by using SPSS version 25.0 (IBM

Table 1. Factors Associated with Prevalence of *Toxoplasma gondii* in *Rattus norvegicus*

Factor	*Category*	*No. tested*	*No. positive (%) [95% CI]*	*OR (95% CI)*	p
Age	Sub-adult (CW: 120.0–180.0 g)	78	6 (7.7) [1.78–13.61]	Reference	0.92
	Adult (CW: 180.1–390.0 g)	149	12 (8.1) [3.68–12.42]	1.05 (0.38–2.92)	
Gender	Female	15	1 (6.7) [0–19.29]	Reference	0.85
	Male	212	17 (8.0) [4.36–11.67]	1.22 (0.15–9.85)	
Total		227	18 (7.9) [4.41–11.44]		

95% CI, 95% confidence interval; CW, carcass weight; OR, odds ratio.

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SPSS, Inc., Chicago, IL). All tests were two-sided, and p value <0.05 was considered statistically significant. Odds ratios and 95% confidence intervals (95% CIs) were estimated to explore the strength of the association between *T. gondii*-infection and the gender and age groups.

Results and Discussion

Toxoplasmosis has been a matter of concern since *T. gondii* was discovered in 1908 (Dubey 2008). *Toxoplasma gondii* has become one of the most important zoonotic pathogens nowadays. Rats, as an important potential source of *T. gondii* infection for cats (such as domestic cat), could be an indirect source for *T. gondii* infection in humans. Although many studies involving genotypes and prevalence of *T. gondii* in different animals have been performed, the information regarding *T. gondii* prevalence in wild rats (*R. norvegicus*) is still limited.

Rattus norvegicus is a dominant rodent species that is widely distributed in the residential areas and farmlands all over the world (Guo et al. 2016). *Rattus norvegicus* can destroy crops, and it is also an important reservoir host of many zoonotic pathogens, including *T. gondii*, *Trypanosoma cruzi*, *Cryptosporidium* spp., and *Enterocytozoon bieneusi* (Guo et al. 2016, Zhao et al. 2018, Garcia et al. 2019, Izquierdo-Rodríguez et al. 2019).

In the present study, the overall detection rate of *T. gondii* in wild rats was 7.9% (18/227, 95% CI 4.41–11.44), which is higher than that of 3.4% (4/116) in *R. norvegicus* in Guangdong Province (Yin et al. 2010), 5.4% (6/111) in Edward's long-tailed rats in Chongqing municipality in China (Zheng et al. 2019), and it is also higher than that of 0.8% (2/238) in *R. norvegicus* in Grenada, West Indies (Dubey et al. 2006), 2.9% (4/136) in suburban rodents in the Slovenian and Croatian parts of Istria (Ivovic et al. 2019), and 2.7% (1/37) in rats in Missouri and east central Kansas (Smith et al. 1995); however, it is lower than that of 23.9% (22/92) in *R. norvegicus* in the urban area of Xuzhou city (Yan et al. 2014), 15.5% (9/58) in rats in Yunnan Province in China (Song et al. 2020), 12.8% (12/94) in *R. norvegicus* in Corsica (France) (Izquierdo-Rodríguez et al. 2019), 13.5% (27/200) in *R. norvegicus* in Riyadh, Saudi Arabia (Elamin et al. 2014), 22.7% (35/154) in rats in the Island of Fernando de Noronha, Brazil (Costa Viegas de Lima et al. 2019), and 56.0% (56/100) in wild rats (*Rattus rattus*) in Northern Iran (Hosseini et al. 2021).

The different detection rates may be related to the regions, species, life conditions, and detection methods (Meerburg et al. 2009). In addition, in the two age groups, the detection rate of *T. gondii* in sub-adult rats (7.7%, 95% CI 1.78–13.61) was similar to that in adult rats (8.1%, 95% CI 3.68–12.42). This result is in line with the finding in Xuzhou city of China, showing 19.0% in the sub-adult group and 19.2% in the adult group, respectively (Yan et al. 2014). However, old and juvenile groups of rats were not collected in this study.

The statistically significant difference was not found in the detection rate of *T. gondii* in *R. norvegicus* of different ages ($p = 0.92$) (Table 1). We cannot assess the strength of the association between the detection rate of *T. gondii* and age conditions in the investigated wild rats. More age groups will be collected to explore the relationship between *T. gondii* infection and age factor in the future studies. Regarding the

TABLE 2. GENOTYPING OF *TOXOPLASMA GONDII* INFECTION IN *RATTUS NORVEGICUS* IN SHANDONG PROVINCE, EASTERN CHINA

Isolate ID	*Host*	*Location*	*SAG1*	*5′+3′ SAG2*	*Alternative SAG2*	*SAG3*	*GRA6*	*c29-2*	*L358*	*Apico*	*Closest ToxoDB genotype*
GT1	Goat	United States	I	I	I	I	I	I	I	I	Reference, ToxoDB no. 10
PTG	Sheep	United States	II/III	II	II	II	II	II	II	n	Reference, ToxoDB no. 1
CTG	Cat	United States	II/III	III	III	III	III	III	III	III	Reference, ToxoDB no. 2
MAS	Human	France	*u*-1*	I	II	III	III	I	I	I	Reference, ToxoDB no. 17
TgCgCa1	Cougar	Canada	I	II	II	III	II	*u*-1*	I	I	Reference, ToxoDB no. 66
TgCatBr5	Cat	Brazil	I	III	III	III	III	I	I	I	Reference, ToxoDB no. 19
WH-13	Rattus norvegicus	Shandong	I	II	I	III	II	III	II	I	New genotypes[1]
WH-14	Rattus norvegicus	Shandong	I	II	I	III	II	III	II	I	New genotypes[1]
WH-39	Rattus norvegicus	Shandong	*u*-1*	I	II	I	I	I	I	I	ToxoDB no. 189
WH-52	Rattus norvegicus	Shandong	I	I	II	I	I	I	I	I	ToxoDB no. 91
WH-76	Rattus norvegicus	Shandong	I	II	I	III	II	III	II	I	New genotypes[1]
WH-103	Rattus norvegicus	Shandong	*u*-1*	I	II	I	I	I	I	I	ToxoDB no. 189
WH-118	Rattus norvegicus	Shandong	I	II	I	III	II	III	II	I	New genotypes[1]
WH-119	Rattus norvegicus	Shandong	I	II	I	III	II	III	II	I	New genotypes[1]
WH-153	Rattus norvegicus	Shandong	I	I	II	n	I	I	I	I	ToxoDB no. 43
WH-190	Rattus norvegicus	Shandong	I	II	I	n	II	I	II	I	New genotypes[2]

[1,2]New genotypes identified in this study.
Apico, apicoplast; n, no amplification; *u*-1*, unique RFLP genotypes.

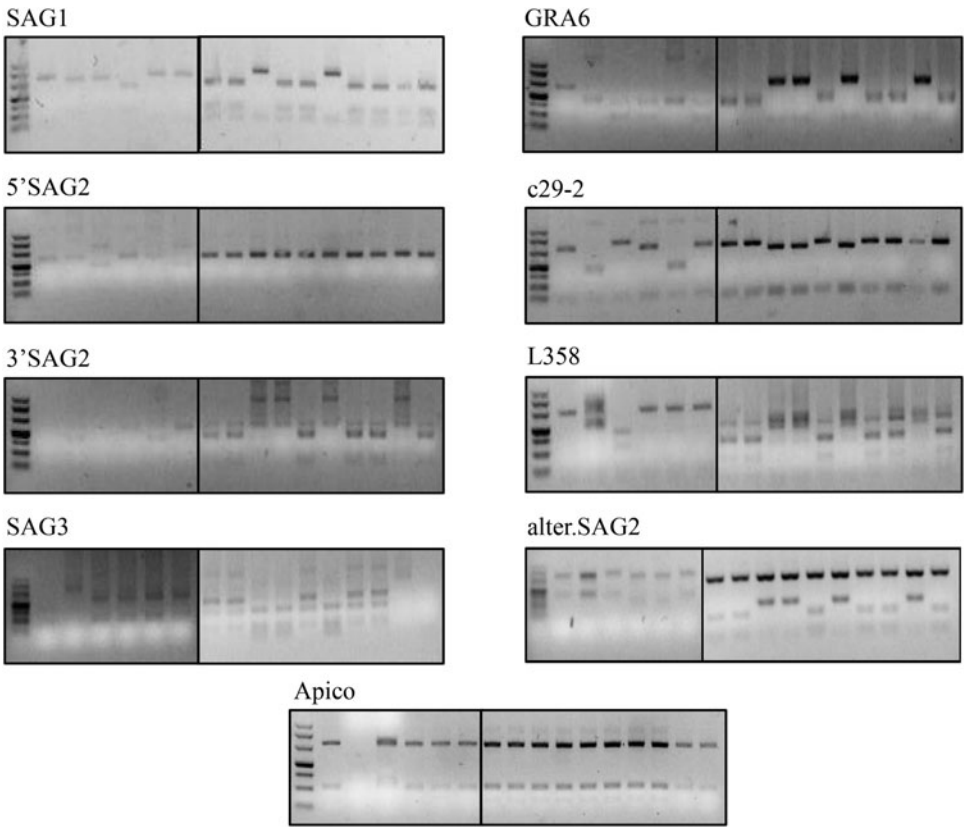

FIG. 1. PCR-RFLP polymorphism cleavage map of SAG1, 5′-SAG2, 3′-SAG2, alternative SAG2, SAG3, GRA6, c29-2, L358, and an Apico locus of *Toxoplasma gondii* isolates in rats (*Rattus norvegicus*) in Shandong province, eastern China. In the figure, Lane M represents DL500-bp DNA marker; Lane B1–B6 represents GT1, PTG, CTG, MAS, TgCgCa1, and TgCatBr5 controls; and Lanes a–j represent positive samples. Apico, apicoplast; PCR-RFLP, PCR-restriction fragment length polymorphism technology.

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gender of rats, the detection rates of *T. gondii* in male and female rats were 8.0% (95% CI 4.36–11.67) and 6.7% (95% CI 0–19.29), which were not statistically significant ($p = 0.85$) (Table 1). This result was consistent with the previous finding (Hosseini et al. 2021).

SAG2 locus was first described as being ideally suitable for the detection of *T. gondii* by Howe et al. (1997). Subsequently, this genetic marker has been exclusively used for the detection of *T. gondii* in many studies (Honoré et al. 2000, Fuentes et al. 2001, Aspinall et al. 2003, da Silva et al. 2005, Coelho et al. 2020, Paraboni et al. 2020). However, this method is outdated since it relies on only one marker. Thus, the RFLP method based on several markers (at least 5) has a good sensitivity level for direct typing (Su et al. 2010).

Consequently, RFLP has been widely employed in genotyping for the isolation of *T. gondii* from animals and humans (Dubey et al. 2002, 2003, Gallego et al. 2006). In the current work, we found that 10 samples were successfully genotyped for *T. gondii*, forming three known genotypes (ToxoDB no. 43, ToxoDB no. 91, and ToxoDB no. 189) and two new genotypes (Table 2 and Figure 1). In spite of this, the type III *T. gondii* has been identified in rats (*R. norvegicus*) in Riyadh, Saudi Arabia (Elamin et al. 2014). The present study identified closest ToxoDB genotypes in rats (*R. norvegicus*) in Shandong Province, China. This is probably because different animals could carry *T. gondii* of different genotypes, and the route and source of infection for animals or humans in each subject may be different in Shandong Province.

The type II *T. gondii* is the highly prevalent genotype in many food animals (Mondragon et al. 1998, Pappoe et al. 2017), which may underlie the human disease caused by the prevalence of *T. gondii* genotypes (Elamin et al. 2014). In addition, Dubey et al. (2021) found that type II, type III, type 12, and Chinese I *T. gondii* were common genotypes in wild rodents, which suggested that rats (*R. norvegicus*) could act as an intermediate host of *T. gondii*, thus causing a serious threat to public health.

However, some non-canonical genotypes were identified in wild rats (*R. norvegicus*) in previous studies (Vujanić et al. 2011, Saki and Khademvatan 2014, Wang et al. 2018), which was in agreement with that in rats (*R. norvegicus*). These findings further illustrate the genetic variability of *T. gondii* in wild rats.

Conclusions

The present study revealed the prevalence of *T. gondii* in wild rats (*Rattus norvegicus*) on mink farms in Shandong Province in China. Our data provided the preliminary information for a better understanding of the epidemiology and public health threats for *T. gondii* in wild rats in eastern China. It is noteworthy to explore the transmission route of *T. gondii* among wild rats, minks, and humans in Shandong Province.

Author Disclosure Statement

No conflicting financial interests exist.

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