Prevalence, risk factors and genotype distribution of *Toxoplasma gondii* DNA in soil in China

Wei Cong[a,*], Nian-Zhang Zhang[b], Rui-Si Hu[b], Feng-Cai Zou[c], Yang Zou[b], Wen-Yan Zhong[d], Jing-Jing Wu[d], Christopher J. Fallaize[e,f], Xing-Quan Zhu[b], Hany M. Elsheikha[f,**]

[a] *Marine College, Shandong University at Weihai, Weihai, Shandong Province, PR China*

[b] *State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province, PR China*

[c] *College of Veterinary Medicine, Yunnan Agricultural University, Kunming, Yunnan Province, 650201, PR China*

[d] *Department of Mathematics and Statistics, University of Calgary, Calgary, Alberta, Canada*

[e] *School of Mathematical Sciences, University of Nottingham, Nottingham, United Kingdom*

[f] *Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough, United Kingdom*

***Correspondence:**

Wei Cong

messicw@163.com

Hany M. Elsheikha

hany.elsheikha@nottingham.ac.uk

ABSTRACT

In the present study, we performed a cross-sectional survey to determine the occurrence and genotype distribution of *T. gondii* DNA in soil samples collected from different sources from six geographic regions in China. Between March 2015 and June 2017, 2100 soil samples were collected from schools, parks, farms and coastal beaches, and examined for *T. gondii* DNA using three PCR assays targeting 529-bp repeat element (RE) sequence, B1 gene and ITS-1 gene sequences. Also, we investigated whether geographic region, soil source and type, and sampling season can influence the prevalence of *T. gondii* DNA in the soil. Soil samples collected from farms and parks had the highest prevalence, whereas samples collected from school playgrounds and coastal beaches had the lowest prevalence. PCR assays targeting 529-bp RE and ITS-1 gene sequences were more sensitive than the B1 gene-based assay. Positive PCR products were genotyped using multi-locus PCR-RFLP, and ToxoDB #9 was the predominant genotype found in the contaminated soil samples. Multiple logistic regression identified factors correlated significantly with the presence of *T. gondii* DNA in the soil to be the source of the soil, including farms (odds ratio 3.10; 95% confidence interval [CI], 1.52 to 6.29; $p = 0.002$) and parks (2.59; 95% CI 1.28 to 5.27; $p = 0.009$). These results show that Chinese soil hosts *T. gondii* of the most prevalent genotype in China (ToxoDB#9) and that the soil type influences infection patterns.

Keywords: Soil contamination; *Toxoplasma gondii*; Prevalence; Genotypes; China

1. Introduction

Toxoplasmosis is a zoonotic disease caused by the protozoan *Toxoplasma gondii*, which is capable of infecting a wide range of avian and mammalian species (Montoya and Liesenfeld, 2004; Dubey and Beattie, 2010). Toxoplasmosis can cause serious illness and even death in congenitally infected infants and immunocompromised patients (Dubey and Beattie, 2010). This disease can also cause economic losses attributed to abortion and stillbirth in sheep and goats (Dubey and Beattie, 2010; Robert-Gangneux and Dardé, 2012). In addition to con- genital transmission, *T. gondii* is a foodborne and waterborne pathogen, which often leads to human infection through the consumption of un- dercooked meat containing parasite cysts, or by ingestion of food or water contaminated with sporulated oocysts (Frenkel et al., 1970).

Soil-borne toxoplasmosis has been reported in many countries, such as USA and Brazil (Stagno et al., 1980; Coutinho et al., 1982), high- lighting the importance of avoiding accidental ingestion of these en- vironmentally resistant oocysts (Alvarado-Esquivel et al., 2010; Dabritz and Conrad, 2010). Although *T. gondii* oocysts spread in the environ- ment is an important source of toxoplasmosis to humans and animals, little is known about the level of soil contamination with oocysts and how prevalence varies across soil types in China.

Understanding the ecology of *T. gondii* in the soil can inform our environmental and public health policy. In order to understand the factors contributing to oocyst adaptation and survival in soil, an asso- ciation between the variables that influence the distribution of *T. gondii* oocysts in soil, such as soil heterogeneity and climatic conditions, is required. Despite the availability of some tools that can be used for the detection of *T. gondii* oocysts in the soil (Dubey and

Beattie, 2010; Dubey, 2010; Jones and Dubey, 2010; Salant et al., 2010), the pre- valence of oocysts in the soil remains poorly defined (Afonso et al., 2008; Lass et al., 2009; Dos Santos et al., 2010).

The objectives of this study were to (i) identify *T. gondii* oocyst's DNA in soil samples and compare infection prevalence and genotype distribution in six different geographic regions in China and (ii) identify the possible risk factors associated with soil contamination with *T. gondii* oocyst's DNA. Comparative analysis of the soil samples was performed using three PCR-based assays, targeting 529-bp RE, B1 gene and ITS-1 region of rDNA gene, for the detection of *T. gondii* oocyst DNA in soil samples. The study shows that 10.9% of the tested soil samples from various regions in China contained *T. gondii* DNA, which may pose a risk to humans.

2. Materials and methods

2.1. Study sites

This study was performed in six provinces in China, including Shandong (Eastern), Henan (Centeral), Jilin (northeastern), Gansu (Northwestern), Yunnan (Southwestern), and Guangdong (Southern). Data about the climatic conditions of the studied regions were obtained from the local government website of each province. Shandong province (34°23'~38°24′N, 114°48'~122°42′E) has warm temperate monsoon climate, with an annual average temperature of 11 °C–14 °C and an average illumination of 2290–2890 h. Average annual rainfall ranges between 550 and 950 mm, with an annual precipitation of 60%–70%, concentrated in the summer. Henan province (31°23'~36°22′N, 110°21'~116°39′E) has warm temperate to sub-tropical, humid to semi-humid monsoon climate. The annual average temperature ranges between 12 °C and 16 °C. Average annual rainfall is between 500 and 900 mm, and the annual precipitation is 50%, concentrated in the summer. Jilin province (40°50'~46°19′N, 121°38'~131°19′E) has temperate monsoon climate. This province has an average temperature of −11 °C in the winter, whereas in the summer the average temperature is 23 °C. Average annual rainfall is between 400 and 600 mm, and the annual precipitation is 80%, concentrated in the summer. Gansu province (32°31'~42°57′N, 92°13'~108°46′E) has temperate continental monsoon climate. The annual average temperature is between 0 °C and 16 °C. Average annual rainfall is between 36.6 and 734.9 mm, and the annual precipitation is 50%–70% concentrated between June and August. Yunnan province (21°8'~29°15′N, 97°31'~106°11′E) has tropical monsoon to subtropical monsoon climate. In the hottest month (July), the temperature ranges from 19 °C to 22 °C, in the coldest month (January) temperature is about 6 °C–8 °C. The distribution of precipitation in this province varies by season and region. Guangdong province (20°13'~25°31′N, 109°39'~117°19′E) has subtropical monsoon climate. The annual average temperature is between 19 °C and 24 °C and the average annual rainfall is between 1300 and 2500 mm.

2.2. Soil sampling

From March 2015 to June 2017, a total of 420 soil sampling spots were examined. These included sampling spots from 20 schools, 20 parks and 20 farms from each of the six provinces (i.e. 360 sampling spots), in addition to sampling from 60 coastal beaches in Shandong

province. From each sampling spot (~10 m^2), five soil samples from five randomly selected locations within each sampling spot were collected (i.e, a total of 2100 samples). Each soil sample of 20 g was collected 5 cm below the soil's surface using stainless steel scoops, air-dried, and passed through 20-mesh sieve (Du et al., 2012). The types of soil sample surveyed included brown soil, yellow brown soil, dark brown forest soil, chernozem (black-coloured soil), fine sand, coarse sand, red soil, latosolic red soil, and yellow cinnamon soil.

2.3. DNA extraction and PCR assays

Oocysts present in the soil samples were purified as described previously (Lélu et al., 2012). Isolation of oocyst DNA from the soil samples was carried out using E. Z.N.A.^{TM} Soil DNA Kit (OMEGA, USA), following the manufacturer's recommendations. Extracted DNA was stored at−20 °C until use. The presence of *T. gondii* DNA was examined by conventional, semi-nested and nested PCR assays that targeted a 529bp-repeat element (RE), a 98bp fragment of B1 gene and a 227bp fragment of the multicopy internal transcribed spacer-1 (ITS-1) region of rDNA, as described previously (Homan et al., 2000; Jones et al., 2000; Burrells et al., 2013). The three assays were run in triplicate and included negative (without DNA) and positive (DNA extracted from tachyzoites of *T. gondii* RH strain genotype I) controls. The primers used in the three PCR assays are listed in the Supplementary Table S1.

2.4. Genotyping of T. gondii

Positive PCR products were subjected to further genetic characterization. Genotyping was carried out using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of 11 genes and sequences – SAG1, SAG2, alter. SAG2, SAG3, BTUB, GRA6, c22–8, L358, c29-2, PK1, and Apico – previously used as markers for *T. gondii* genotypic dis- crimination (Cong et al., 2015, 2017). Nine reference *T. gondii* strains, GT1, PTG, CTG, MAS, TgCgCa1, TgCatBr5, TgWtdSc40, TgCatBr64, and TgToucan, were included as positive controls. PCRs were performed in 25-µl volumes. Each reaction was set up using 1 × PCR buffer, 0.2 µM of each primer, 200 µM deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 0.2 U of HotStart*Taq* DNA polymerase (TAKARA, Japan). The PCR products were amplified using a thermal cycler (PTC 200, Bio- RAD) under the following conditions: 95 °C for 5 min to activate the DNA polymerase, followed by 30 cycles of 95 °C for 30s, 55 °C for 60s, and 72 °C for 90s, and a final extension at 72 °C for 10 min. Multiplex PCR-amplified products were diluted 1:1 in sterile, double-distilled water, and used for nested PCR amplification with internal primers for each marker, separately. A similar amplification program was used for the nested PCR. The nested PCR amplifications were carried out with an annealing temperature of 60 °C for 60s for all the markers except Apico, which was amplified at 55 °C. The nested PCR products were digested with restriction enzymes for 1 h, but the temperature for each enzyme was used as per the manufacturer's instructions. The restriction fragments were resolved on 2% agarose gels, visualized by GoldenViewTM and photographed using a gel documentation system (UVP GelDoc- ItTM Imaging System, Cambridge, U.K.). Details of the oligonucleotide primers used in the Mutiplex PCR and nested PCR are shown in Table S2 and Table S3, respectively.

2.5. Statistical analysis

Associations between categorical variables were analyzed by Chi- square test. *P* values < 0.05 were considered to represent statistical significance. Univariate logistic regression was performed to determine associations between the presence of *T. gondii* DNA in the soil and various factors, such as soil source, soil type, geographic region, season, and climatic features. Multiple logistic regression (using generalized linear mixed models) was carried out to determine which of the variables, when all of them are considered in combination, explain the observed variation in prevalence of *T. gondii* DNA. To account for correlation between the samples collected at each of the 420 sites, the sampling site was included as a random effect. Although multiple sites were sampled in each region, there are only six regions and their potential effects are of intrinsic interest, so sampling region was considered as a fixed effect. However, this was found to be non-significant. Hence, the grouping structure in the random effects part of the model was by sampling site only. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) for the effects of the different levels of significant was performed using statistical software R (Version 3.4.4; http://www. R-project.org) (R Development Core Team, 2011). The generalized linear mixed models were fitted using penalized quasi-likelihood using routines from the R library MASS (Venables and Ripley, 2002).

3. Results

3.1. Contamination levels of T. gondii DNA in the soil

The prevalence of *T. gondii* DNA in 2100 soil samples from six provinces in China was determined. As shown in Table 1, 230 PCR positive *T. gondii* DNA samples were detected amounting to 10.9% overall prevalence of *T. gondii* DNA in all soil samples. The prevalence of *T. gondii* DNA detected in the individually tested samples ranged from 4.3% in high school playground to 16% in poultry farms (Table 1). Soil samples collected from farms (poultry [16%] and livestock [14.7%]) and parks (comprehensive [15%] and residential [14.7%]) had the highest prevalence with *T. gondii* DNA (Table 1). The lowest prevalence was detected in samples collected from school playgrounds (high school [4.3%] and elementary school [5.3%]) and coastal beach (6.7%).

Out of the 420 sampled sites, 136 were positive, meaning that 32.3% of the tested sites were contaminated by *T. gondii* DNA (Table 2). Considering all types of the 420 sampling sites, the proportion of soil samples tested positive for *T. gondii* DNA ranged from 21.7% in high school playground to 55% in residential park. Again, farms (livestock [41.6%%] and poultry [40%]) and parks (residential park [55%] and comprehensive [35%]) had the highest prevalence of contamination with *T. gondii* DNA (Table 2). Samples collected from school play- grounds (high school [21.7%] and elementary [23.3%]) and coastal beach (20%), had the lowest prevalence (Table 2).

3.2. Factors influencing T. gondii prevalence in soil

According to univariate logistic regression analysis, we identified the following risk factors for the presence of *T. gondii* oocysts in soil: costal beach (odds ratio, 14; 95% confidence interval [CI], 9.14 to 22.76; $p < 2e\text{-}16$), geographic region Gansu (8.37; 95% CI, 5.90 to 12.31; $p < 2e\text{-}16$), autumn season (5.81; 95% CI, 4.51 to 7.61; $p < 2e\text{-}16$), brown soil (7.82; 95% CI, 5.55 to 11.37; $p < 2e\text{-}16$); and subtropical monsoon climate (8.67; 95% CI, 6.08 to 12.83; $p <$

2e-16) (Table S4). Multiple logistic regression analysis identified soil source as the only significant factor influencing the presence of *T. gondii*. The reference level for soil source is "coastal beach" and the odds ratios relative to this reference level found to be statistically significant is: soil source farm (3.10; 95% CI 1.52 to 6.29; p =0.002) and soil source park (2.59; 95% CI 1.28 to 5.27; p = 0.009) (Table 3). Therefore, for ex- ample, we estimate that a soil source farm increases the odds ratio of presence of *T. gondii* DNA by a factor of 3.10 over that of coastal beach. Logistic regression analysis was also performed on the results obtained by individual PCR assays, and similar conclusions were obtained.

3.3. PCR-RFLP analysis

We compared the effectiveness of three different PCR assays targeting the sequences of 529-bp RE sequence, B1 gene and ITS-1 region of rDNA gene. Out of 2100 soil samples, amplification products were obtained from 179, 212 and 207 specimens using B1 gene, 529-bp RE and ITS-1 region of rDNA gene, respectively. Of these, 156 samples yielded amplification products using the three PCR assays, 10 samples were positive by 529-bp RE-based PCR assay, one sample was positive by B1 gene-(semi) nested PCR assay, and 9 samples were positive by ITS-1-nested PCR assay. The comparative performance of the three PCR assays in terms of detecting oocysts in soil samples is shown (Supplementary Fig. 1). Out of the 231 positive soil samples, 15 samples were successfully genotyped by PCR-RFLP and had restriction digest profiles consistent with that of the most prevalent Chinese *T. gondii* genotype ToxoDB#9 (Table 4).

4. Discussion

Better understanding of how environmental factors may influence the prevalence and genotype distribution of *T. gondii* DNA in the soil is essential for identification, assessment and management of the health and safety risks faced by members of the public. In the present study, we found that 29 (4.8%) out of 600 soil samples from public schools were positive for *T. gondii* DNA. This prevalence is less that reported in a previous report, where 15 (14.15%) out of 106 soil samples were found contaminated with *T. gondii* oocysts from schools in Lanzhou (capital city of Gansu province), northwest China using PCR method based on the 529-bp RE sequence (Wang et al., 2014). Also, 50% (60/ 120) of the soil samples examined from the parks in the present study were contaminated by *T. gondii* DNA. These findings support results obtained previously where *T. gondii* oocysts were found widely distributed in soil samples of public parks in Wuhan (Du et al., 2012) and Lanzhou (Wang et al., 2014) in China.

The presence of such high levels of *T. gondii* DNA contamination in the soil samples obtained from the playground of schools and parks is worrying because these areas have become an increasingly important in the outdoor activities of the people in China; for the elderly to work out and for the children to play. Various factors can influence the level of soil contamination with *T. gondii* DNA, such as the density of the felid definitive host, hygienic standards implemented in the parks and schools, and climatic conditions (Dumètre and Dardé, 2003; Afonso et al., 2008; Meerburg and Kijlstra, 2009; Gotteland et al., 2014). Cats play a key role in the transmission of *T. gondii* by depositing oocysts into the soil with their feces. Cat-derived oocysts persist in the environment for long periods (Dumètre and Dardé, 2003; Dubey

and Beattie, 2010; Dubey, 2010) and can contaminate the estuarial water through surface runoff and soil washing. Given that cat-soil-intermediate host cycle presents a common mechanism by which *T. gondii* can reach new hosts, public health authorities should consider investigating the correlation between the presence of cats and the burden of *T. gondii* oocyst's contamination in the playground of schools and public parks.

The factors contributing to the flux of *T. gondii* oocysts into coastal waters have received some attention in the literature (Shapiro et al., 2010; Simon et al., 2013a,b). Anthropogenic changes (human-induced alterations in the coastal landscape and destruction of wetland habitats) and climate change (e.g. rising sea level, flooding and surface runoff) are among some of the mechanisms contributing to the contamination of nearshore waters with terrestrially derived zoonotic pathogens (Jones et al., 2008; Shapiro et al., 2010; Simon et al., 2013a,b; VanWormer et al., 2016). In the present study, we found evidence for high prevalence (20%) of *T. gondii* oocysts in soil samples from coastal/ bathing beaches (Table 2). *T. gondii* oocysts kept in seawater (15 ppt NaCl) at 4 °C for 24 months maintained their infectivity to mice (Lindsay and Dubey, 2009). Therefore, increased prevalence of *T. gondii* oocyst DNA contamination in coastal beaches highlights the potential contamination of nearshore waters with this terrestrially derived, water-borne zoonotic parasite and the subsequent increase in human exposure to *T. gondii* infection through recreation activities or consumption of seafood harvest (Jones et al., 2008, 2009; Simon et al., 2013a,b; VanWormer et al., 2016).

Animal farms have been considered as hotspot areas for the trans- mission risk of *T. gondii* oocyst infection in rural environments (Gotteland et al., 2014; Simon et al., 2017). In agreement with these studies, our data (Table 2) showed that soil samples from 58.3% of livestock farms and 46.7% of poultry farms are contaminated with *T. gondii* DNA. Other variables, such as the climatic feature subtropical monsoon and the autumn season also seem to influence the occurrence of *T. gondii* oocysts in soil in the present study (Table S4). The effect of spring conditions on the fate and dispersion of *T. gondii* oocysts from the melting snowpack to the Canadian arctic coast via the freshwater runoff has been appreciated (Simon et al., 2013a,b). More prevalence was detected in Gansu province and brown soil (Table S4), suggesting that geographic region and soil types are potential risk factors for soil contamination with *T. gondii* oocysts. Gansu province is characterized by a temperate continental monsoon climate with an annual average temperature between 0 °C and 16 °C. *T. gondii* oocysts seem to have a better survival rate at 4 °C compared to ambient temperature (Lindsay and Dubey, 2009; Gagelidze et al., 2018). The reason for the high as- sociation between brown soil and occurrence of oocysts in our study might be related to the possibility that soil type (brown soil) could also be linked to soil source (e.g. farm), however the exact reasons remain to be determined.

Currently, there is lack of knowledge on the nature or extent of any effect of soil type on *T. gondii* oocyst survival. A recent survey of 18 types of soils of Georgia has shown that the total number of bacteria and frequency of occurrence of certain bacterial genera vary by soil type (Li et al., 2015). The physical, chemical and biological parameters of the soil can vary with soil type and sampling season. Hence, knowledge of soil parameters that may influence the survival of *T. gondii* oocysts within soil is important for the development of risk assessment and management strategies aimed at reducing public health risks from activities such as land application of wastes containing human pathogens.

A few PCR-based methods have been developed for detection of *T. gondii* oocyst DNA (Salant et al., 2010; Mancianti et al., 2015; Chemoh et al., 2016; Liu et al., 2017). Of these, PCR assays targeting highly conserved repetitive DNA sequences, such as B1 gene, 529-bp repeat element (RE) and internal transcribed spacer-1 (ITS-1) region of ribo- somal DNA gene, seem to have good sensitivities (Salant et al., 2007, 2010; Chemoh et al., 2016). Other detection methods such as microscopy can not discern between oocysts of *T. gondii* and oocysts of other coccidian species due to the similarities between their morophological features. To our knowledge, this is the first study to analyze soil samples using, simultaneously, three PCR assays based on the amplification of 529-bp RE sequence, B1 gene and ITS-1 region of rDNA gene sequences.

A previous study comparing the prevalence of *T. gondii* DNA in cat feces using PCR assays targeting 529-bp RE sequence and ITS-1 gene showed that amplification based on ITS-1 gene was approximately seven times more sensitive than amplification based on 529-bp RE sequence (Chemoh et al., 2016). In another survey of *T. gondii* oocysts presence in cat feces using PCR assays targeting 529-bp RE sequence and B1 gene, the prevalence rate detected by PCR assay based on 529- bp RE sequence was 5.3%, which was lower than the detection level obtained by B1gene-based assay (17.95%). In our study, the concordance between the three PCR assays showed that PCR assay based on *T. gondii* 529-bp RE sequence or ITS-1 sequence can detect more positive samples than PCR assay based on B1 gene sequence. There are likely to be inhibitors within soil (e.g. humic acid), which can alter PCR performance, however in this study we did not use an internal control to monitor any inhibition.

We were also interested in defining the genetic diversity of the amplified *T. gondii* DNA from the soil samples because understanding the genetic population structure of *T. gondii* in soil samples can have epidemiological and public health relevance. Interestingly, PCR-RFLP analysis revealed only one ToxoDB#9 genotype in all analyzed PCR products. This result provides further support to previously reported finding where more than half of the examined *T. gondii* isolates in China were found to belong to genotype ToxoDB#9, which is the most predominant genotype detected in all examined hosts (Pan et al., 2017).

5. Conclusion

This work shows that *T. gondii* DNA is widely distributed in soil samples collected from schools, parks, farms and coastal beaches in six geographic regions in China. The performance of PCR assays based on 529-bp RE sequence and ITS-1 sequence for the detection of *T. gondii* DNA was better than that of the B1 gene-based PCR assay. ToxoDB#9 was the only genotype detected in the examined soil samples in China. The prevalence of soil contamination with *T. gondii* DNA varied by the level of sampling season, climatic feature, geographic region, and soil type. Implementation of measures to manage the risk of oocysts derived from cats may reduce soil contamination with *T. gondii* oocysts. More studies are warranted including more sensitive detection methods and sampling of cats for *T. gondii* with rigorous field work characterizing the ecology, movement, and behavior of potential wild felids. Our work provides baseline data to further risk assessment of *T. gondii* oocysts' contamination in Chinese soil. Future studies should explore the state of sporulation, viability and infectivity of the isolated oocysts.

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Declaration of competing interest

All authors have no conflict of interest to declare.

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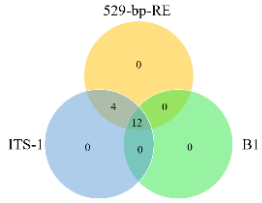
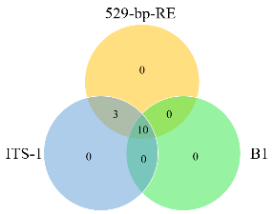
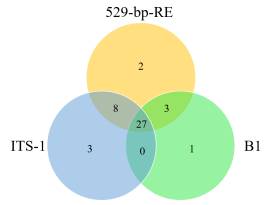
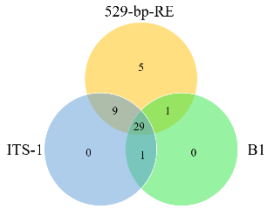
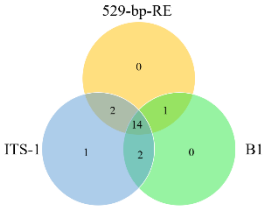
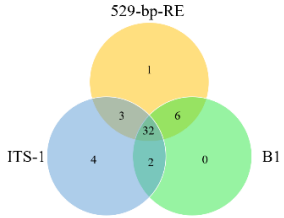
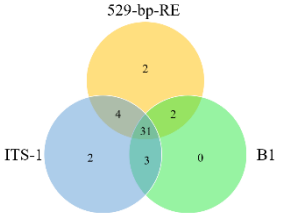
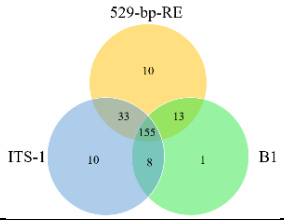
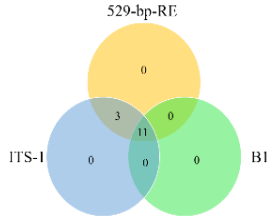
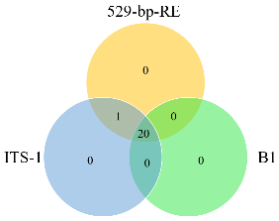
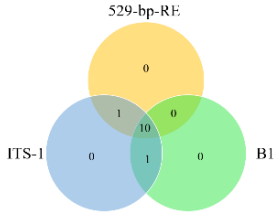
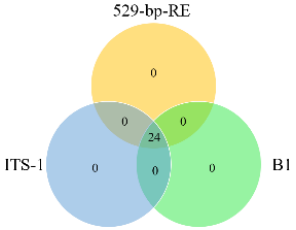
Table 1 Frequency of *Toxoplasma gondii* oocysts in 2,100 soil samples.

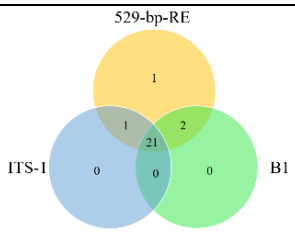
Source	Category (No. of samples)	No. of positive samples/PCR assay (529-bp-RE, B1, ITS-1)	Prevalence n (%) [a]	*p*-value Chi-square test
School playground				
	Elementary school (n = 300)		16 (5.3)	0.568
	High school (n = 300)		13 (4.3)	-
Park				
	Residential (n = 300)		44 (14.7)	-
	Comprehensive (n = 300)		45 (15)	0.909
Coastal beach				
	Bathing beach (n = 300)		20 (6.7)	-
Farm				
	Poultry (n = 300)		48 (16)	0.573
	Livestock (n = 300)		44 (14.7)	-

Total	n = 2,100		230 (10.9) -

[a] The prevalence values (presented inside parentheses) are the number of positive samples out of total number of samples analyzed per category.

Table 2. Prevalence of *Toxoplasma gondii* DNA in 420 sampling sites.

Source	Category (No. of samples)	No. of positive samples/PCR assay (529-bp-RE, B1, ITS-1)	Prevalence n (%) [a]	*p*-value Chi-square test
School playground	Elementary school (n = 60)		14 (23.3)	0.666
	High school (n = 60)		13 (21.7)	-
Park	Residential (n = 60)		27 (55)	0.273
	Comprehensive (n = 60)		21 (35)	-
Coastal beach	Bathing beach (n = 60)		12 (20)	-
Farm	Poultry (n = 60)		24 (40)	0.201

	Livestock (n = 60)		25 (41.6)	-
Total	n = 420		136 (32.3)	-

[a] The prevalence values (presented inside parentheses) are the number of positive samples out of total number of samples analyzed per category.

468 **Table 3** Parameter estimates and odds ratios obtained from the multiple logistic regression
469 model. The odds ratios presented for each remaining risk factor represent the odds ratio relative
470 to the reference level (source coastal beach) due to exposure to the factor.
471

Coefficient	Estimate	Standard error	Odds ratio (95% CI)	*p*-value
Intercept	-4.007	0.337	-	0.000
Source farm	1.130	0.362	3.10 (1.52 - 6.29)	0.002
Source park	0.953	0.362	2.59 (1.28 - 5.27)	0.009
Source school	0.066	0.384	1.07 (0.50 - 2.27)	0.864

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Table 4

Genotyping of *Toxoplasma gondii* DNA in various soil sources in China.

Isolate ID	Host/source	Location	SAG1	5'+3' SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype
GT1	Goat	United States	I	I	I	I	I	I	I	I	I	I	I	Reference, Type I, ToxoDB #10
PTG	Sheep	United States	II/III	II	II	II	II	II	II	II	II	II	II	Reference, Type II, ToxoDB #1
CTG	Cat	United States	II/III	III	III	III	III	III	III	III	III	III	III	Reference, Type III, ToxoDB #2
MAS	Human	France	u-1	I	II	III	III	III	u-1[a]	I	I	III	I	Reference, ToxoDB #17
TgCgCa1	Cougar	Canada	I	II	II	III	II	II	II	u-1[a]	I	u-2[a]	I	Reference, ToxoDB #66
TgCatBr5	Cat	Brazil	I	III	III	III	III	III	I	I	I	u-1[a]	I	Reference, ToxoDB #19
TgWtdSc40	White-tailed deer	USA	u-1	II	II	II	II	II	II	II	I	II	I	Reference, ToxoDB #5
TgCatBr64	Cat	Brazil	I	I	u-1	III	III	III	u-1[a]	I	III	III	I	Reference, ToxoDB #111
TgToucan	Toucan	Costa Rica	u-1	I	II	III	I	III	u-2[a]	I	I	III	I	Reference, ToxoDB #52
TgS1	Soil	Shandong	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS2	Soil	Shandong	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS3	Soil	Henan	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS4	Soil	Henan	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS5	Soil	Jilin	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS6	Soil	Yunnan	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS7	Soil	Guangdong	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgS8	Soil	Gansu	u-1	II	II	III	nd	II	II	III	II	II	I	ToxoDB #9
TgS9	Soil	Gansu	u-1	II	II	III	III	II	II	III	II	II	nd	ToxoDB #9
TgS10	Soil	Shandong	u-1	II	II	III	III	II	II	III	II	II	nd	ToxoDB #9
TgS11	Soil	Gansu	Nd	II	II	III	III	II	Nd	III	I	II	I	ToxoDB #9
TgS12	Soil	Guangdong	Nd	II	II	III	III	II	II	III	II	nd	I	ToxoDB #9
TgS13	Soil	Henan	u-1	II	II	III	III	nd	II	III	II	II	nd	ToxoDB #9
TgS14	Soil	Jilin	u-1	II	II	III	III	II	II	III	II	nd	nd	ToxoDB #9
TgS15	Soil	Yunnan	u-1	II	II	III	III	II	nd	nd	II	nd	nd	-

u-1 and u-2 denote novelRFLP genotypes.

nd: no data

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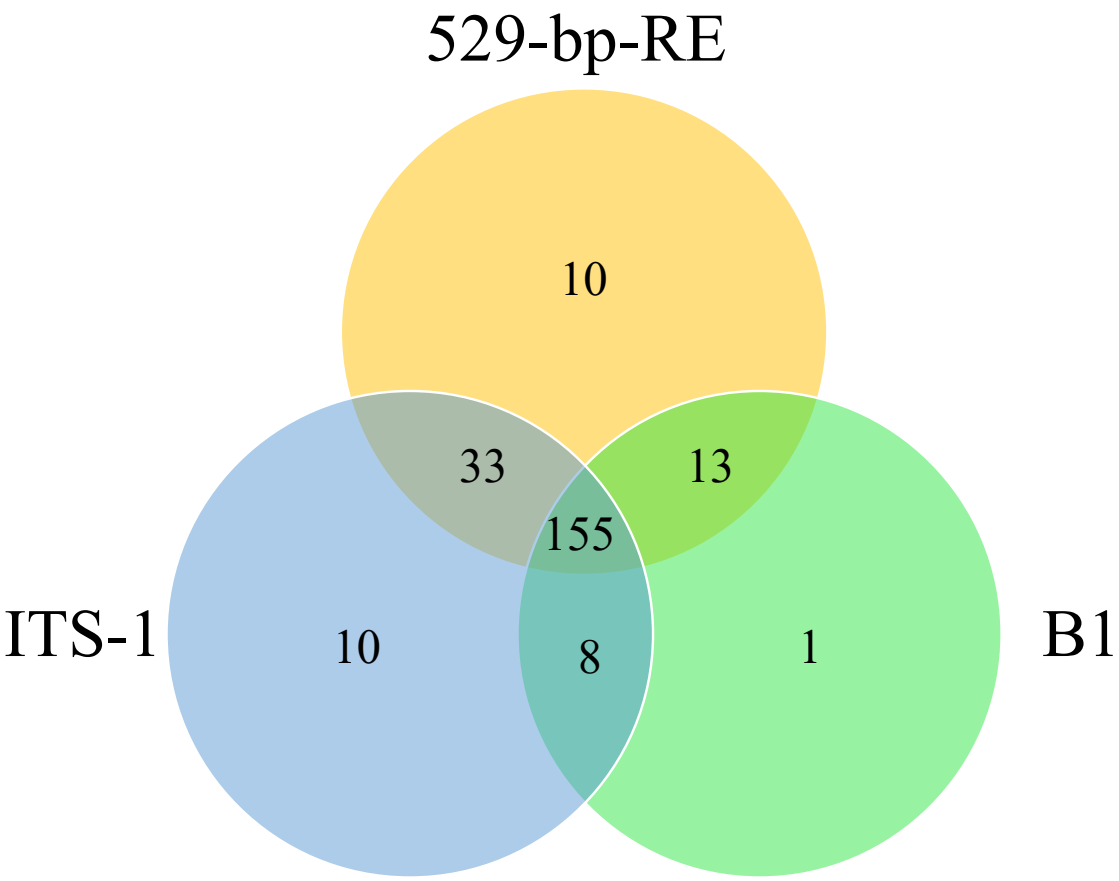
Additional files

Additional file 1: Fig. S1. Venn diagram showing the unique and shared positive results obtained by the three PCR assays based on 529-bp-RE, B1 and ITS-1 nucleotide sequences for detection of *T. gondii* DNA in soil samples.



Additional file 2: Table S1: Primers used in the PCR assays.

Method	Target	Nucleotide sequence (5'–3')	Amplicon	Reference
Conventional PCR	529 bp-RE	TOX4-CGCTGCAGGGAGGAAGACGAAAGTTG TOX5-CGCTGCAGACAGAGTGCATCTGGATT	529bp	(Lélu et al., 2012)
Semi-nested PCR	B1	TOXO1- GGAACTGCATCCGTTCATGAG TOXO2-TCTTTAAAGGGTTCGTGGTC TOXO4-TGCATAGGTTGCAGTCACTG	98bp	(Homan et al., 2000)
Nested PCR	ITS-1	Tg-NN1- TCAACCTTTGAATCCAAA Tg-NN2- CGAGCCAAGACATCCATT Tg-NP1- GTGATAGTATCGAAAGGTAT Tg-NP2- ACTCTCTCTCAAATGTTCCT	227bp	(Jones et al., 2000)

Additional file 3: Table S2: Primers used in the Mutiplex PCR-based genotyping of *T. gondii* strains.

Primer IDs	Primer sequence (5'–3')	Loci	Size (bp)
SAG1-Fext	GTTCTAACCACGCACCCTGAG	SAG1	503
SAG1-Rext2	AAGAGTGGGAGGCTCTGTGA		
5SAG2-Fext	GCTACCTCGAACAGGAACAC	5'-SAG2	334
5SAG2-Rext	GCATCAACAGTCTTCGTTGC		
3SAG2-Fext	TCTGTTCTCCGAAGTGACTCC	3'-SAG2	327
3SAG2-Rext	TCAAAGCGTGCATTATCGC		
P43S1	CAACTCTCACCATTCCACCC	SAG3	311
P43AS1	GCGCGTTGTTAGACAAGACA		
Btb (ext)F	TCCAAAATGAGAGAAATCGT	BTUB	529
Btb (ext)R	AAATTGAAATGACGGAAGAA		
GRA6-F1x	ATTTGTGTTTCCGAGCAGGT	GRA6	546
GRA6-R1	GCACCTTCGCTTGTGGTT		
c22-8Fext	TGATGCATCCATGCGTTTAT	c22-8	657
c22-8Rext	CCTCCACTTCTTCGGTCTCA		
c29-2Fext	ACCCACTGAGCGAAAAGAAA	c29-2	689
c29-2Rext	AGGGTCTCTTGCGCATACAT		
L358-Fext	TCTCTCGACTTCGCCTCTTC	L358	690
L358-Rext	GCAATTTCCTCGAAGACAGG		
PK1-Fext	GAAAGCTGTCCACCCTGAAA	PK1	1027
PK1-Rext	AGAAAGCTCCGTGCAGTGAT		
SAG2-Fext	GGAACGCGAACAATGAGTTT	SAG2	729
SAG2-Rext:	GCACTGTTGTCCAGGGTTTT		
Apico-Fext:	TGGTTTTAACCCTAGATTGTGG	Apico	846
Apico-Rext:	AAACGGAATTAATGAGATTTGAA		

Additional file 4: Table S3: Primers used in the nested PCR-based genotyping of *T. gondii* strains.

Primer IDs	Primer sequences (5'–3')	Loci.	Size（bp）
SAG1-S2	CAATGTGCACCTGTAGGAAGC	SAG1	390
SAG1-Rext	GTGGTTCTCCGTCGGTGTGAG		
5-SAG2F	GAAATGTTTCAGGTTGCTGC	5'-SAG2	242
5-SAG2R	GCAAGAGCGAACTTGAACAC		
3-SAG2F	ATTCTCATGCCTCCGCTTC	3'-SAG2	222
3-SAG2R	AACGTTTCACGAAGGCACAC		
P43S2	TCTTGTCGGGTGTTCACTCA	SAG3	225
P43AS2	CACAAGGAGACCGAGAAGGA		
Btb-F	GAGGTCATCTCGGACGAACA	Btub	411
Btb-R	TTGTAGGAACACCCGGACGC		
GRA6-F1	TTTCCGAGCAGGTGACCT	GRA6	344
GRA6-R1x	TCGCCGAAGAGTTGACATAG		
SAG2-Fa	ACCCATCTGCGAAGAAAACG	SAG2	546
SAG2-Ra	ATTTCGACCAGCGGGAGCAC		
L358-F2	AGGAGGCGTAGCGCAAGT	L358	418
L358-R2	CCCTCTGGCTGCAGTGCT		
PK1-F	CGCAAAGGGAGACAATCAGT	PK1	903
PK1-R	TCATCGCTGAATCTCATTGC		
c22-8F	TCTCTCTACGTGGACGCC	c22-8	521
c22-8R	AGGTGCTTGGATATTCGC		
c29-2F	AGTTCTGCAGAGTGTCGC	c29-2	446
c29-2R	TGTCTAGGAAAGAGGCGC		
Apico-F	TGCAAATTCTTGAATTCTCAGTT	Apico	640
Apico-R	GGGATTCGAACCCTTGATA		

Additional file 5: Table S4: Univariate logistic regression analysis of risk factors for the presence of *T. gondii* oocysts in soil samples in China based on the three PCR assays used in the present study.

Risk factors	Prevalence Positive/total (%)	Odds ratio (95% CI) *	*p*-value
Soil source			
Coastal beach	20/300 (6.7)	14.00 (9.14 – 22.76)	<2e-16†
Farm	93/600 (15.5)	0.38 (0.22 – 0.63)	0.000
Park	89/600 (14.8)	0.41 (0.24 – 0.66)	0.000
School	29/600 (4.8)	1.40 (0.77 – 2.51)	0.255
Region			
Gansu	32/300 (10.7)	8.37 (5.90 – 12.31)	<2e-16†
Shandong	54/600 (9)	1.20 (0.75 – 1.90)	0.423
Henan	44/300 (14.7)	0.69 (0.42 – 1.12)	0.142
Jilin	32/300 (10.7)	1.00 (0.59 – 1.68)	1.000
Guangdong	31/300 (10.3)	1.03 (0.61 – 1.75)	0.894
Yunnan	38/300 (12.7)	0.82 (0.49 – 1.35)	0.446
Season			
Autumn	71/450 (15.8)	5.81 (4.51 – 7.61)	<2e-16†
Winter	24/255 (8.7)	1.33 (0.84 – 2.16)	0.219
Spring	44/485 (9.1)	1.72 (1.15 – 2.59)	0.008
Summer	102/910 (11.2)	1.52 (1.08 – 2.14)	0.014
Soil type			
Brown soil	34/300 (11.3)	7.82 (5.55 – 11.37)	<2e-16†
Yellow brown soil	44/300 (14.7)	0.74 (0.45 – 1.19)	0.225
Dark brown forest soil	17/160 (10.6)	1.07 (0.58 – 2.03)	0.817
Chernozem	15/140 (10.7)	1.06 (0.56 – 2.07)	0.847
Fine sand	8/150 (5.3)	2.26 (1.07 – 5.38)	0.043
Coarse sand	12/150 (8)	1.46 (0.75 – 3.04)	0.273
Red soil	38/300 (12.7)	0.88 (0.53 – 1.44)	0.615
Latosolic red soil	31/300 (10.3)	1.10 (0.66 – 1.86)	0.693
Yellow cinnamon soil	32/300 (10.7)	1.07 (0.64 – 1.79)	0.794
Climatic feature			
Subtropical monsoon	69/600 (11.5)	8.67 (6.08 – 12.83)	<2e-16†
Continental Monsoon Climate	32/300 (10.7)	0.96 (0.57 – 1.62)	0.894
Temperate monsoon	32/300 (10.7)	0.96 (0.57 – 1.62)	0.894
Tropical monsoon	38/300 (12.7)	0.79 (0.47 – 1.31)	0.371
Warm temperate monsoon	54/600 (9)	1.16 (0.72 – 1.84)	0.519

Warm to subtropical, humid to semi-humid monsoon	44/300 (14.7)	0.67 (0.40 – 1.09)	0.110

* OR: Odds ratio; 95% CI: 95% confidence interval
† Reference against which other categories are compared