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Survey on the contamination of *Toxoplasma gondii* oocysts in the soil of public parks of Wuhan, China

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ABSTRACT

Toxoplasma gondii of warm-blooded animals and humans is an important pathogenic agent throughout the world. Soil is increasingly recognized as an important source in the transmission of *Toxoplasma*. To attain the contamination status of *T. gondii* in the soil of public parks, a total of 252 soil samples were collected from September 2009 to August 2010 at different sites located in 6 public parks of Wuhan, Hubei, China and detected by PCR and loop-mediated isothermal amplification (LAMP). The detection limit of PCR/B1, PCR/529 and LAMP was determined to be 50, 5, and 5 tachyzoites in soil, respectively. Forty-one samples were found positive for *Toxoplasma* DNA by PCR on both genes, whereas LAMP products were generated in 58 samples ($\chi^2 = 3.6328$, $P = 0.0567$). All parks were found contaminated and no significant difference was found among the parks (PCR: $\chi^2 = 0.0072$, $P = 0.9325$; LAMP: $\chi^2 = 0.6101$, $P = 0.4347$). However, contamination was found with significantly different among the four seasons (PCR: $\chi^2 = 11.6066$, $P = 0.0007$; LAMP: $\chi^2 = 12.4636$, $P = 0.0004$), with a gradual decrease in the prevalence from spring to winter on both analyses. This is the first investigation on soil contamination of public parks in China by *T. gondii* oocysts. The results indicate that the soil of public parks contaminated with *T. gondii* oocysts may play a role in the epidemiology of toxoplasmosis and effective preventive measures should be considered. Moreover, the conventional PCR and LAMP used in the present study are applicable to detect *T. gondii* oocysts in soil samples.

1. Introduction

Toxoplasma gondii, an apicomplexan protozoal parasite capable of infecting all warm-blooded animals worldwide, including humans, is the causative agent of toxoplasmosis. This is one of the most prevalent parasitic infections in animals and humans (Dubey and Beattie, 1988). However, infections with *Toxoplasma* are generally asymptomatic except after congenital transmission, when associated with abortions or clinical diseases, and in immunocompromised individuals such as patients suffered organ transplantation and AIDS (Dubey, 2008; Dubey and Jones, 2008; Dumètre and Dardé, 2003). *T. gondii* infection in pregnant women can result in fetal diseases, with severe problems, including abortion, encephalitis, malformations, mental retardation, loss of vision and sub-clinical symptoms (Afonso et al., 2008; Dubey and Jones, 2008; Yang et al., 2009). *T. gondii* infection in AIDS and other immunocompromised patients can cause life-threatening disease (Dubey, 2008; Lass et al., 2009). Moreover, severe infections may lead to neonatal

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deaths and abortion in animals (Dubey and Jones, 2008; Dumètre and Dardé, 2003).

T. gondii infections seem to be strongly associated with soil contact, as the suspected source of outbreaks was the oocysts from soil. So far, soil-borne transmission toxoplasmosis outbreaks have been documented in USA and Brazil (Coutinho et al., 1982; Stagno et al., 1980). The oocysts were isolated from the soil during an outbreak of toxoplasmosis in a rural area of Brazil in spite of no direct evidence of association of outbreak with soil (Stagno et al., 1980). Dabritz and Conrad (2010) summarized the association of prevalence of *T. gondii* with soil in humans. Thus, soil is increasingly considered as an important source in the transmission of *Toxoplasma*.

So far, a few studies have been conducted worldwide to determine the status of environmental contamination of this parasite. Contamination was maximal at cat defecating sites. Afonso et al. (2008) found soil samples contaminated by *T. gondii* oocysts only at the defecation sites of the cats, where the level of contamination was high. Similarly, contaminated soil samples were only found in areas used by cats (Lass et al., 2009). dos Santos et al. (2010) found that *T. gondii* oocysts were widely distributed in the soil of elementary public schools from the northwest area of the state of São Paulo using mouse bioassays. However, the report regarding seasonal variations on soil contamination of public parks by *T. gondii* oocysts is still unavailable. Thus, the objective of present study was to determine the frequency of occurrence of *T. gondii* oocysts in the soil of public parks in Wuhan city, China with respect to health risks to animals and human beings, and to determine the possible existence of seasonal variations.

2. Materials and methods

2.1. T. gondii strain

T. gondii of RH strain was obtained from National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China. Tachyzoites of *T. gondii* (RH) strain were harvested from the ascites of BALB/c mice infected 5–6 days earlier by injecting 1 ml of 0.1 M phosphate buffered saline (PBS) pH 7.2 as described previously (Fang et al., 2009). *T. gondii* of the Prugniaud strain was provided by Xinxiang Medical College, Henan, China.

2.2. Collection of soil samples

Two hundred fifty-two soil samples were collected from September 2009 to August 2010 at different sites located in six public parks of Wuhan, Hubei, China, namely Zhongshan Park, Ziyang Park, Lotus Lake Park, Hongshan Park, Shouyi Square and Guqintai Culture Square (Table 2). Wuhan (29°58′and 31°22′ N latitude, 113°41′ and 115°05′ E longitude) lies in the subtropical zone with sufficient sunshine and abundant rainfall especially from May to July, and the mean relative humidity is high in all seasons of year. According to the climate of Wuhan, the whole year was divided into following four seasons: (i) spring (March to May) (ii) summer (June to August) (iii) autumn (September to November) and (iv) winter (December to February). Because of the influence of monsoon, summer is hot and rainy, while winter is cold and dry in Wuhan. In summer, the highest temperatures range from 37 to 40 °C, but the lowest temperatures are also higher than 29–30 °C and the extreme temperature of the ground may exceed to 50 °C at some places.

All parks are located in the urban area with high visitor flow rate. The soil samples were taken from areas where cats often appear. Soil was removed from the surface layer of the ground (top 5 cm) with stainless steel shovel, air-dried and sieved using a 20 mesh to remove stones and organic detritus. Finally, 0.5 g of the soil prepared in this way were taken for further analysis.

2.3. DNA extraction

Soil DNA extraction was performed using the commercial E.Z.N.A.™ Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. One hundred microliters of DNA was eluted per sample, and DNA was stored at −20 °C until use for detection.

2.4. Conventional PCR

T. gondii B1 gene (AF179871) and 529 bp repetitive fragment (AF146527) were selected as the targets for PCR. The B1 gene consists of 35 copies, and it is highly conserved among strains of *T. gondii* (Burg et al., 1989). The 529 bp repetitive fragment consists of 200–300 copies in the genome of *T. gondii* (Homan et al., 2000). The B1 and 529 primer sequences are shown in Table 1. Both PCR were performed according to the conditions described by Burg et al. (1989) with the exceptions that the reaction mixture volume was 25 μl and 400 ng/μl of non-acetylated bovine serum albumin (Sigma) were added in the reaction mixture (Jiang et al., 2005). PCR products were analyzed by electrophoresis on a 1.0% agarose gel stained with ethidium bromide for visualization under a UV transilluminator.

2.5. LAMP

Amplification was performed according to the conditions described by Nie (2010). Six oligonucleotide primers were used for the LAMP assay targeting eight conserved regions within the sequence of MIC3 gene of *T. gondii* (Table 1). The LAMP products were visualized by 1.5% agarose gel electrophoresis. Visual inspection of LAMP amplicons in the reaction tube was performed by adding 1 μl of 1/10 dilution of SYBR Green I (Invitrogen, Australia) after the reaction, and the fluorescent signals of the solutions were observed under UV light.

Each sample was amplified in triplicate in order to determine the reproducibility of the PCR and LAMP. Moreover, to verify the effect of residual PCR inhibitors in all DNA samples, 2 μl of each DNA template mixed with 1 μl of genomic DNA from the *T. gondii* RH strain were co-amplified in PCR and LAMP.

2.6. Specificity of PCR and LAMP

DNA derived from *Schistosome* eggs, *Toxocara cati* eggs, *Streptococcus pneumoniae*, *Escherichia coli*, *Actinobacillus*

Table 1
Primer sets used in the present study for amplification of *Toxoplasma* DNA.

Molecular method	Target	Nucleotide sequence (5′–3′)	Amplicon	Reference or source
PCR	B1 gene (AF179871)	TOXO1, GGAACTGCATCCGTTCATGAG TOXO2, TCTTTAAAGCGTTCGTGGTC	194 bp	Originally by Burg et al. (1989) Also used by others (Homan et al. (2000); Lass et al. (2009); Yang et al. (2009))
PCR	529 bp repetitive fragment	TOX4, CGCTGCAGGGAGGAAGACGAAAGTTG	529 bp	Originally by Homan et al. (2000)
	(AF146527)	TOX5, CGCTGCAGACACAGTGCATCTGGATT		Also used by others (Lass et al. (2009); Schares et al. (2008))
LAMP	MIC3 (EU572718)	F3, TAGATGTATTGATGACGCCTCG B3, TATTCATTTTTTCACTCAAGCTCC BIP, AATTCGGCATCAGCGCGTCCCCCGATTCTC-CTTTCCCAT FIP, GACTTCGACTCCTTCCACACACGGAGAAT-GCTACACCTGCGAGT LF, CTCCGCCTTGAAGTCACTC BF, GATCTGCTTCCGCAACCTCC	263 bp[a]	Nie (2010)

[a] The length between F3 and B3.

pleuropneumoniae, *Staphylococcus aureus*, *Bacillus subtilis*, *Toxoplasma* RH strain and *Toxoplasma* Prugniaud strain was subjected to PCR and LAMP to determine the specificity of the assay.

In order to confirm the compatibility of obtained product sequence with *T. gondii* B1 gene sequence (accession number AF179871), four randomly selected positive samples were sequenced. Sequencing was performed by an ABI automated sequencer, according to a standard procedure described by the manufacturer. Nucleotide sequences obtained were aligned with reference sequences using the ClustalX 1.81 package (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

2.7. Sensitivity of PCR and LAMP

The sensitivity of PCR and LAMP assay was determined in the following way: *in vivo* cultivated RH *Toxoplasma tachyzoites* were counted by hematocytometer, and then the tachyzoites were serially diluted from 5×10^3 to 5×10^{-1}. Half gram of soil was seeded with the known numbers of *T. gondii* tachyzoites. The soil DNA extraction and detection methods were preformed as described above.

2.8. Statistical analysis

Statistical analysis was performed by SAS version 8.0. Chi-squared test was used to compare percentages. A P value <0.05 was considered as a significant difference.

3. Results

3.1. Specificity of PCR and LAMP

For the specificity test, only *Toxoplasma* RH and Prugniaud strain DNA was amplified, whereas DNA of other pathogens including *Schistosome* eggs, *T. cati* eggs, *S. pneumoniae*, *E. coli*, *A. pleuropneumoniae*, *S. aureus*, *B. subtilis* that may be present in soil was negative by PCR or LAMP.

Four positive PCR products randomly chosen were subjected to DNA sequencing. Then the sequences were aligned with *T. gondii* B1 gene sequences available in GenBank (AF179871) using the ClustalX 1.81 package. These results confirmed that PCR products were *T. gondii* B1 gene fragments (data not shown).

The above results suggested that PCR and LAMP assays are specific for *T. gondii*.

3.2. Sensitivity of PCR and LAMP

As shown in Fig. 1A–C, the detection limit of PCR/B1, PCR/529 and LAMP is 50, 5 and 5 tachyzoites in soil,

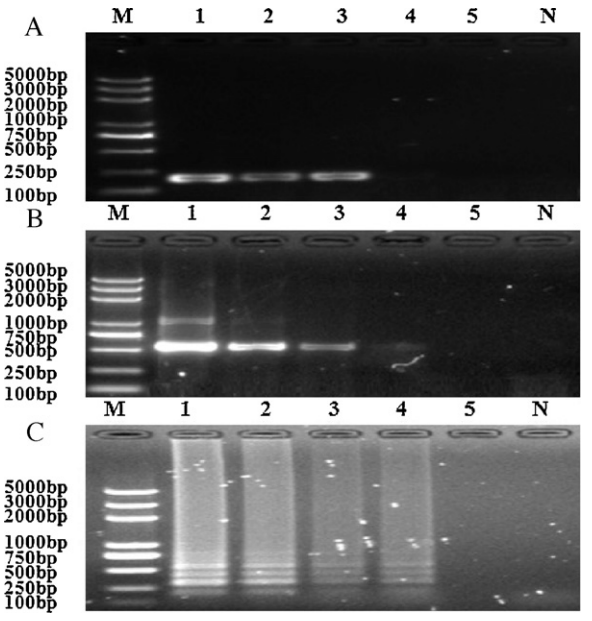

Fig. 1. Comparative sensitivities by PCR (A), PCR (B) and LAMP (C) for the specific detection of *Toxoplasma* tachyzoites DNA based on the B1 gene, 529 bp repetitive fragment and MIC3 gene amplification, respectively. Lanes 1 through 5, dilution set (5×10^3, 5×10^2, 5×10^1, 5×10^0 and 5×10^{-1} *Toxoplasma* tachyzoites, respectively); lane N, negative control; lane M, molecular size marker.

Table 2
Comparison between PCR and LAMP for detecting *T. gondii* oocysts in soil samples collected from different public parks.

Public parks	No. of samples examined	PCR	LAMP
		Positive (%)	Positive (%)
Zhongshan Park	42	6 (14.29)	10 (23.81)
Ziyang Park	41	5 (12.20)	9 (21.95)
Lotus Lake Park	39	6 (15.38)	7 (17.95)
Hongshan Park	43	6 (13.95)	8 (18.60)
Shouyi Square	44	11 (25.00)	15 (34.09)
Guqintai Culture Square	43	7 (16.28)	9 (20.93)
Total	252	41 (16.27)	58 (23.02)

respectively. These results indicated that the sensitivity of conventional PCR/529 and LAMP is slightly higher than that of PCR/B1 for detection of *T. gondii*.

3.3. Contamination status of *T. gondii* oocysts in soil samples

The samples were considered as PCR positive when the results of both PCR/B1 and PCR/529 were positive. As shown in Table 2, *T. gondii* DNA was found in 41 samples (17.8%) by PCR, whereas LAMP products were generated in 58 samples (23.02%). However, no statistically significant difference was observed between the detection methods ($\chi^2 = 3.6328$, $P = 0.0567$). Co-amplification of all DNA samples and *T. gondii* DNA in PCR and LAMP reactions was positive. The residual PCR inhibitors could be neutralized by high concentration of BSA in the reaction mixture.

Overall, all of 6 public parks were contaminated (Table 2). The prevalence of *T. gondii* was highest in Shouyi Square and lowest in Lotus Lake Park, however, the difference was not statistically significant (PCR: $\chi^2 = 0.0072$, $P = 0.9325$; LAMP: $\chi^2 = 0.6101$, $P = 0.4347$).

A one year survey was conducted to determine the influence of seasonal variation on the contamination of *T. gondii* oocysts in soil (Table 3). Statistically, contamination of *T. gondii* in soil was significantly different among the four seasons (PCR: $\chi^2 = 11.6066$, $P = 0.0007$; LAMP: $\chi^2 = 12.4636$, $P = 0.0004$). The prevalence of *T. gondii* oocysts in soil gradually declined from spring to winter with both methods (Fig. 2).

4. Discussion

Soil is a potential environmental source of *T. gondii* infection for animals and humans (Alvarado-Esquivel et al., 2010; Dabritz and Conrad, 2010; Dumètre and Dardé, 2003). In natural environment, oocysts can remain infective for months to years (Dubey and Beattie, 1988; Dumètre and Dardé, 2003). Unfortunately, evaluation of *T. gondii* oocysts contamination in environment has been limited by lack of reliable detection methods (Dumètre and Dardé, 2003; Jones and Dubey, 2010). So far, little information has been gathered on the presence of oocysts in natural environment, especially in soil (Afonso et al., 2008; dos Santos et al., 2010; Lass et al., 2009). The present study is the first investigation on the soil contamination of public parks in China by *T. gondii* using rapid molecular detection methods.

Table 3
Seasonal contamination of *T. gondii* oocysts in the soil of public parks (September 2009 to August 2010).

Seasons	No. of samples examined	PCR	LAMP
		Positive (%)	Positive (%)
Spring	66	18 (27.27)	23 (34.85)
Summer	63	12 (19.05)	18 (28.57)
Autumn	60	7 (11.67)	10 (16.67)
Winter	63	4 (6.35)	7 (11.11)
Total	252	41 (16.27)	58 (23.02)

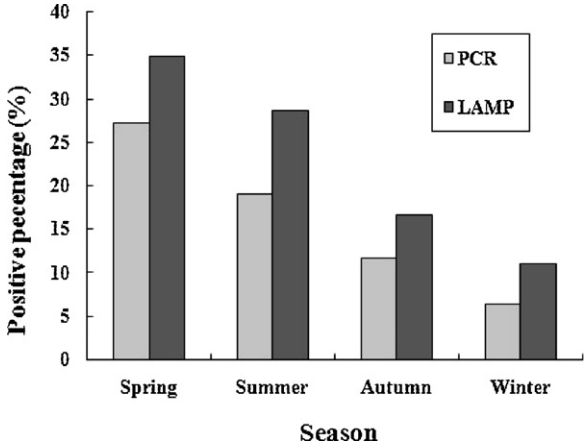

Fig. 2. Seasonal variation in the contamination of soil by *T. gondii* oocysts from different parks of Wuhan, China.

Although *T. gondii* oocysts persist for several months in the environment, some reasons may hamper their detection, including low concentration of oocysts in soil, lack of techniques with sufficient sensitivity to detect and identify oocysts (Dabritz and Conrad, 2010; Dumètre and Dardé, 2003). In the present study, we did not assess the infectivity of oocysts. Mouse bioassay is often unsuited to sensitive and simple detection, as a large amount of samples is needed. According to oocyst and sample features, molecular methods are necessary to recover low numbers of oocysts and to discriminate *T. gondii* from closely related coccidian (Dumètre and Dardé, 2003). On the other hand, molecular method reduce the detection time from weeks to 1–2 days and save the cost (Afonso et al., 2008; Lass et al., 2009; Sotiriadou and Karanis, 2008; Villena et al., 2004).

Two PCR assays based on the B1 gene and 529 bp repetitive fragment and LAMP based on the MIC3 gene were used to detect *T. gondii* oocysts in soil samples. The B1 gene and 529 bp repetitive fragment were selected for PCR assays because of their high copy number of the targets and the frequent use for *T. gondii* detection (Afonso et al., 2008; Burg et al., 1989; Homan et al., 2000; Lass et al., 2009; Sotiriadou and Karanis, 2008; Villena et al., 2004; Yang et al., 2009). The use of B1 gene in PCR detection of *T. gondii* oocysts of environmental samples is probably limited because of the non-specificity of some primers and lower sensitivity (Yang et al., 2009). Results of the study suggest that PCR with the 529 bp repetitive fragment was more sensitive than with

B1 gene (Fig. 1). Therefore, the samples were considered as PCR positive when the results of both PCR/B1 and PCR/529 were positive.

LAMP is the most recently developed method with highly sensitivity, specificity, efficiency, and rapidity under isothermal conditions for detection of organisms (Notomi et al., 2000; Sotiriadou and Karanis, 2008). The sensitivity is not significantly influenced by the presence of non-target DNA. It has been already evaluated for detection of *T. gondii* in water (Sotiriadou and Karanis, 2008). In the present study, as shown in Table 2, the result suggested higher detection ability by the LAMP assay (23.02%) when compared to conventional PCR (16.27%). These results could be explained by the ability of LAMP even the same sensitivity of LAMP and PCR/529 (Fig. 1).

In the present study, the overall prevalence of *T. gondii* oocysts in soil were 16.27% (41/252) and 23.02% (58/252) by PCR and LAMP, respectively (Tables 2 and 3). Similar prevalence of *T. gondii* oocysts in soil samples was documented previously, for example, 17.8% in Poland using PCR (Lass et al., 2009), and the prevalence of 22.58% in school playgrounds in Brazil using mouse bioassay (dos Santos et al., 2010). The presence of *T. gondii* DNA present in public parks indicates that the soil contaminated with oocysts may be a source of infection for animals and humans in Wuhan.

Many factors may impact on the contamination status of *T. gondii* oocysts in soil, including the density of cats, the hygiene management in the parks and the prevailing climatic conditions (Afonso et al., 2008; Dumètre and Dardé, 2003; Meerburg and Kijlstra, 2009). Cats are important in contamination status of *T. gondii* oocysts in soil because they are the only hosts that can excrete the resistant oocysts. Seroprevalence of cats provide a good estimate of environmental contamination with oocysts (Jones and Dubey, 2010). However, there is little information about the prevalence of cats infected with *T. gondii* in the parks. In spite of this, it is most likely that seropositive cats have already shed oocysts (Dumètre and Dardé, 2003). As there are numbers of stray cats in parks, the chance is high for *T. gondii* oocysts to enter the environment and be transmitted to animals and humans (Zhang et al., 2009). Therefore, a pilot survey should be launched to investigate the prevalence of *T. gondii* in stray cats and the contamination status of public parks at national level.

As shown in Table 3 and Fig. 2, the prevalence of *T. gondii* oocysts in soil changed throughout the year, but was always positive and gradually declined from spring to winter with both methods. For *T. gondii*, spring and summer might be the seasons with higher sanitary risk. On the other hand, the results may indicate that the risk for animals and humans to acquire *T. gondii* infection via soil could vary throughout a year, being higher during spring and summer. Cats are more frequently active in spring and summer than that in autumn and winter, increasing the chance of infection with *T. gondii* and the excretion of oocysts. It has been suggested that the presence of oocysts in the environment is likely to decline during warmer, drier seasons, especially in summer and early autumn, but the seasonal variations of contamination status in the present study is remarkably different (Dumètre and Dardé, 2003; Jones and Dubey, 2010). Environmental matrices may be heavily contaminated in summer by soil washing after peaks in rainfall in rainy season that includes May, June and July in Wuhan (Dumètre and Dardé, 2003; Miller et al., 2002).

In conclusion, this study showed that *T. gondii* DNA was widely distributed in environmental soil samples of public parks in Wuhan, China. The results of our findings provide evidence that contaminated soil may create a real risk to animals and human beings of being infected with *T. gondii*. In addition, it is possible to conclude that conventional PCR and LAMP used in this study are applicable to detect *T. gondii* oocysts in soil samples.

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