DETECTION OF *TOXOPLASMA* OOCYSTS FROM SOIL BY MODIFIED SUCROSE FLOTATION AND PCR METHODS

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Abstract. A detection method of *Toxoplasma gondii* oocysts from soil was evaluated using the sucrose flotation technique with modification involving addition of 0.1% gelatin into washing and floating solutions. PCR was performed on untreated samples and after treatment with polyvinylpyrrolidone (PVP), heating and cooling, and NaCl. The addition of gelatin in the sucrose solution yielded a higher number of oocysts. A very thin band was observed when DNA extract was diluted to 1:1024, indicating the presence of PCR inhibitor in the soil. PCR performed on untreated DNA, on PVP-treated, and on PVP-treated with heating and cooling without added bovine serum albumin (BSA) showed a band only at higher dilutions (1:1024 and 1:512) but at a much lower dilution (1:8) with BSA. In contrast, DNA treated with all three agents showed a band at a much lower dilution (1:64), even without added BSA, and no dilution was required when BSA was added. The PCR inhibitors present in the soil were removed by employing various treatment procedures during DNA extraction, and BSA in PCR. Furthermore, the detection limit with the method was 1 oocyst/g of soil, indicating that this method is useful in epidemiological studies.

INTRODUCTION

Toxoplasma gondii is an anthropozoonotic coccidian parasite infecting a wide range of animals, including human. Infection in human occurs both as a result of being acquired and congenitally, but most remain asymptomatic. Symptomatic infections include lymphadenopathy, choriretinitis and reproductive disorders such as cerebral calcifications, hydrocephalus, microcephaly and abortion. Recently, it has been implicated in the causation of encephalitis in immunocompromised patients. Hence, in recent years, the *Toxoplasma* parasite has emerged as one of the re-emerging and opportunistic infections.

The *Toxoplasma* oocyst detection rate in cat feces in Japan ranges from 0.3 to 0.9% (Ito *et al*, 1974; Oikawa *et al*, 1990). Oocysts released in cat feces survive for a considerably long period of time, and are resistant to various chemical and physical treatments (Frenkel *et al*, 1975). *Toxoplasma* oocysts reportedly survive for 24 hours in 0.1% sodium dodecyl sulfate (SDS), 6 hours in 10% formalin, and 30 minutes in 1% ammonium hydroxide (Dubey *et al*, 1970). *Toxoplasma* oocysts were found to remain infective in mice after being kept at 35ºC for 32 days, at 50ºC for 1 hour, and at 4ºC for 54 months (Dubey, 1998), suggesting that oral infection with oocysts is an important route to *Toxoplasma* infection. Therefore, the study of recovery of *Toxoplasma* oocysts from the soil has great significance in terms of understanding the infection dynamics and control. However, very few studies of this type have been conducted in the past. These studies include antibody response in mice against oocysts recovered from soil (Fleck *et al*, 1972) and the identification of *T. gondii* by intraperitoneal inoculation of soil extract into mice (Ruiz *et al*, 1973). These methods discriminate *T. gondii* oocysts from those of *Hammondia hammondi* having identical morphology but are time consuming in that they take about one month.

During recent years, because of its high sen-

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sitivity and specificity, the polymerase chain reaction (PCR) technique has been applied in the diagnosis of toxoplasmosis (Grover *et al*, 1990; Lebech *et al*, 1992). PCR has also been applied to the detection and identification of organisms that are difficult to identify by morphological and immunological methods. In this study, we studied the applicability of the PCR technique in the detection of *Toxoplasma* oocysts in soil extract yielded by the modified sucrose flotation technique.

MATERIALS AND METHODS

Oocysts

Oocysts of *T. gondii* (S273 strain) prepared as described by Arrowood and Sterling (1987) were obtained from the Obihiro University of Agriculture and Veterinary Medicine, Japan. The oocysts, suspended in distilled water, were stored at 4ºC until use. The numbers of *Toxoplasma* oocysts were enumerated using a hemocytometer.

Soil

Soil (pH: 7.8; water content: 3.7%) collected from a sandpit in a public park was used. The soil was thoroughly mixed and repeatedly examined for *Toxoplasma* oocysts by the sucrose flotation technique. The oocyst-free soil thus obtained was sealed in a plastic bag and stored at room temperature until use. Soil samples of 30 g were spiked with oocysts (at a rate of 500 in isolation, 10,000 in detection, and 0, 10, 25, 50, 100, 500 in detection limit tests).

Isolation of oocysts

Firstly, the oocyst spiked soil sample (30 g) was filtered through a 150 μm mesh, suspended in 50 ml of 0.1% Tween 80 solution, centrifuged at 1,100*g* for 10 minutes, and then the supernatant was discarded. The sediment was then suspended in 5 ml of sucrose solution (specific gravity 1.200), centrifuged as described above, and the supernatant was transferred to another tube. The supernatant, after dilution with distilled water (1:10), was centrifuged as described above, and the sediment thus obtained (whole) was subjected to DNA extraction.

The effect of gelatin on the recovery of oocysts was examined by washing and floating with or without 0.1% gelatin in sucrose solution. Also, the sediment was subjected microscopically to the detection of oocysts [sediment transferred to a 10 ml plastic tube, diluted with floating solution and filled to the brim, covered with a cover slip and then centrifuged at 45*g* for 5 minutes. The cover slip was removed and placed onto a glass slide, and examined (x 200) by counting the number of oocysts in the whole cover slip].

Detection of oocysts

The DNA for PCR was extracted by the conventional method. Briefly, the centrifuged debris was suspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA-2Na; pH 8.0) containing 1.5% SDS, and allowed to stand at room temperature for 30 minutes. DNA was extracted by an equal volume of phenol-chloroform solution, precipitated by isopropyl alcohol at -20ºC, washed with 80% ethanol and finally the pellet was dried. The dried pellet, dissolved in 8 μl of sterile distilled water, was used for the PCR. The removal of the PCR inhibitors present in the soil was examined by performing the extraction procedure with or without following: 1% polyvinylpyrrolidone (PVP) in TE buffer, heating and cooling (at 98ºC for 10 minutes followed by 4ºC for 10 minutes) of sample, and by treating the sample with 100 mM NaCl.

The *Toxoplasma* DNA was amplified in 20 μl of reaction mixture consisting of template DNA, PCR buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), dNTP (0.25 mM each), primers (0.5 μM each), and 0.4 U *Taq* DNA polymerase (Takara Shuzo, Japan). In some experiments, 10 μM of bovine serum albumin (BSA) was added to remove the PCR inhibitors. The primers used in this study were 5′-GGAA CTGCATCCGTTCATGAG-3′ and 5′-TCTTT AAAGCGTTCGTGGTC-3′ (Burg *et al*, 1989) located on the B1 gene (at the positions 694-714 and 887-868, respectively). The reaction mixture was overlaid with 2 drops of mineral oil to prevent evaporation and subjected to a 35 cycle PCR (PC-700, Astec, Japan). One cycle of PCR consisted of heating at 95ºC for 1 minute (denaturation), at 55ºC for 1 minute (annealing), and at 72ºC for 2 minutes (extension). At the end, the mixture was incubated at 72ºC for 10 minutes for the final extension, followed by electrophoresis on 1.5% agarose gel and staining with ethidium bromide. The analysis was done by visualizing on a UV illuminator. To investigate the reduction of the levels of PCR inhibitors in the samples, a serial two-fold dilution of template DNA was made and examined by PCR.

RESULTS

Isolation of oocysts

The effect of gelatin added in the washing and floating solutions on the recovery of oocysts is shown in Table 1. The addition of 0.1% gelatin in washing and floating solutions yielded a significantly higher number of oocysts (n=105; 21%) compared with those recovered without gelatin (n=37; 7%) ($p < 0.005$). The addition of gelatin only in the washing and floating solution recovered 57 (11%) and 69 oocysts (14%), respectively. A repeat recovery test of the same sample showed a recovery rate of 98% the first time, 2% the second time, and 0% the third time. Based on these results, we added gelatin to both the washing and floating solutions at a concentration of 0.1% and the isolation process was done only once.

Detection of oocysts

Fig 1 shows the results of electrophoresis of the PCR product. *Toxoplasma* oocysts suspended in distilled water showed a prominent band (lane 2) at 194 bp, but none when the soil component was added to it (lane 3). A very thin band was observed when the DNA pellet was diluted 1,024 times (lane 4), indicating the presence of the PCR inhibitor in the soil. The PCR of the untreated (-/-/-), only PVP treated (+/-/-), and PVP and heating and cooling treated (+/+/-) DNA without added BSA showed a band only at the higher dilutions (1:1024 and 1:512), but at a much lower dilution (1:8) with BSA (Table 2). On the other hand, DNA treated with all three agents (+/+/+) showed a band at a much lower dilution (1:64), even without the added BSA, and no dilution was required when BSA was added (Fig 1; lane 5).

Fig 3 shows the detection limit of *Toxoplasma* oocysts from the soil. Positive bands could not be seen in the soil samples spiked with 0 and 10 oocysts (lane 2 and 3), but clear bands were seen in the samples spiked with 25 and more oocysts (lane 4 to 7), indicating that 25 oocysts in 30 g of soil, or 1 oocyst/g soil, were the detection limit of the present method.

DISCUSSION

The present study was carried out to investigate the usefulness of the PCR technique in the detection of *Toxoplasma* oocysts in soil. However, it is known that the soil itself or some factors such as humic acid (McGregor *et al*, 1996; Levesque *et al*, 1997; Khan *et al*, 1998) present

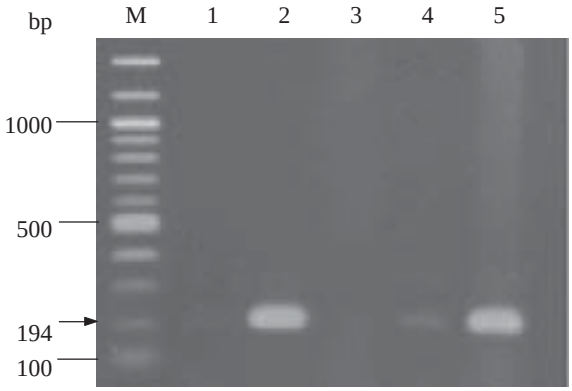

Fig 1–Inhibition of PCR by inhibitor present in soil. Lane M, size marker (100 bp DNA ladder); lane 1, negative control; lane 2, without soil components, lane 3: with soil components; lane 4, 1,024 times dilution of DNA pellet; lane 5, DNA extracted by various treatments.

Table 1
Effect of gelatin in the recovery of *Toxoplasma* oocysts from soil.

Gelatin (washing / floating)	No of oocysts recovered[a]			Recovery rate (%)
	min	max	mean	
-/-	29	48	37	7[b]
+/-	49	68	57	11
-/+	61	79	69	14
+/+	101	111	105	21[b]

[a]Figures are the number of repeated 3 experiments.
[b]Student's *t* test: $p < 0.005$.

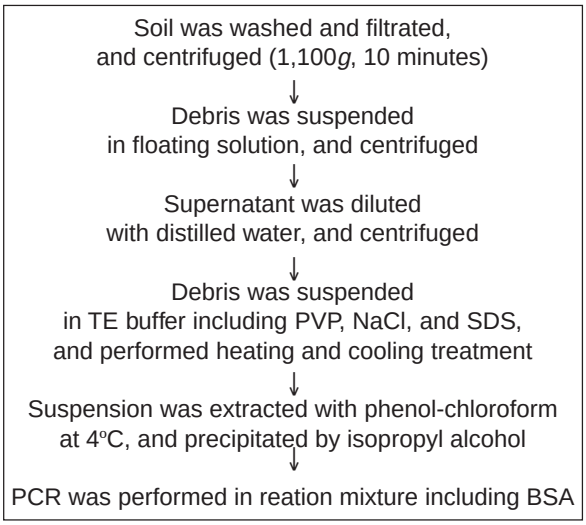

Fig 2–Flow chart for the detection of *Toxoplasma* oocysts from soil.

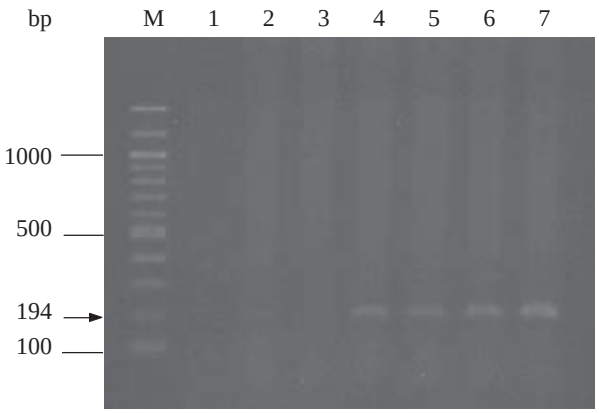

Fig 3–Detection limit of *Toxoplasma* oocysts in spiked soil. Lane M, size marker (100 bp DNA ladder); lane 1, negative control; lanes 2-7, soils containing 0, 10, 25, 50, 100, 500 of oocysts, respectively.

in the soil interfere with the flotation of oocysts and PCR results. Hence, gelatin was added to the washing and floating solutions to remove these factors. Also, the pre-treatment of DNA extract with various agents was performed. Furthermore, BSA was also added to the PCR mixture.

Previously, Kuczynska *et al* (1999) reported an increased recovery rate of *Cryptosporidium parvum* by using washing solution with surface-active agents such as Triton-X, Tween 80, and Tris-Tween 80 incorporated into it. Therefore, dispersants such as gelatin have been thought to detach the wanted organisms from the soil particles. On the other hand, in the field of bacteriology, methods using PVP and NaCl have reportedly been used for the detection of bacteria like *Pseudomonas putida* and *Desulfitobacterium frappieri* (Levesque *et al*, 1997; Khan *et al*, 1998). These reports recognized the effect of PVP, but the views about NaCl were different. Furthermore, the addition of BSA and the T4 gene-32 protein into the PCR mixture has also been reported to be useful (McGregor *et al*, 1996; Levesque *et al*, 1997).

This study revealed that the addition of gelatin in washing and floating solutions significantly increase the recovery of *Toxoplasma* oocysts from soil using the sucrose flotation technique, as expected. The number of oocysts recovered from the gelatin-containing solution was significantly higher than those recovered using gelatin-free solutions. As the random use of 0.1% gelatin yielded a good number of oocysts, the optimum concentration of gelatin may need to be investigated and determined.

None of the previous reports have mentioned the level of inhibitor removal by these various treatment procedures. Levesque *et al* (1997) simply

Table 2
Effect of pre-treatment of DNA on PCR results.

Treatments in DNA extraction (PVP / heating and cooling / NaCl)	PCR without BSA[a]	PCR with BSA[a]
-/-/-	1024	8
+/-/-	1024	8
+/+/-	512	8
+/+/+	64	1

[a]Dilution at which PCR was positive.

compared the removal level by observing the color of the DNA solution, but there was no correlation between the color of the DNA solution and the amount of PCR inhibitors. Hence, the method was less reproducible. In this study, an attempt was made to measure the removal level of PCR inhibitors semiquantitatively. BSA showed the highest level of inhibitor-removing effect followed by NaCl, and the method was reproducible. McGregor *et al* (1996) speculated that BSA blocks the PCR inhibitor to template DNA and DNA polymerase. Though the present study did not explore the inhibitor-removing mechanism, the results indicated the important role of protein (BSA) in removing PCR inhibitors present in soil.

None of the researchers such as Fleck *et al* (1972) and Ruiz *et al* (1973) have investigated the detection limit of *Toxoplasma* oocysts in soil. In this study, it was concluded that the detection limit of *Toxoplasma* oocysts in soil by this method was 25 oocysts in 30 g of soil or 1 oocyst/g of soil. To the best of our knowledge, this study is the first of its kind to report the detection limit of *Toxoplasma* oocysts in soil.

In the past, the surveillance of soil contamination with *Toxoplasma* oocysts was limited due to technical problems. The present study, however, revealed that the problems could be overcome by a combination of the sucrose flotation technique with gelatin and a PCR inhibitory substance removal procedure. Furthermore, it was possible to detect the oocysts with high sensitivity. It is, therefore, hoped that the present method will be useful in detecting *Toxoplasma* oocysts in soil and thereby useful in undertaking epidemiological studies in the future.

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