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A highly sensitive molecular diagnosis method for detecting *Toxoplasma gondii* tachyzoite: a PCR/dot blot hybridization

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Abstract

This study aimed at finding a fast, sensitive, and efficient protocol for molecular identification of intracellular protozoa *Toxoplasma (T.) gondii*. For molecular detection of *T. gondii*, we developed a polymerase chain reaction coupled with dot blot hybridization assay (PCR/DBH). For DBH analysis, the amplified DNA of *T. gondii* tachyzoite was labeled by incorporation of digoxigenin. The DBH assay alone was capable of detecting down to 1×10^4 pg of *T. gondii* genomic DNA. The PCR alone was capable of detecting down to 1×10^3 pg of *T. gondii* genomic DNA, whereas the PCR/DBH assay was capable of detecting down to 1×10^2 pg of *T. gondii* genomic DNA, indicating that sensitivity of the PCR/DBH method was approximately 10 to 100 times higher than PCR or DBH alone. Our PCR/DBH assay will be useful for confirming the presence of *T. gondii* on the samples and differentiating *T. gondii* infection from other intracellular protozoa infections.

Key words : *Toxoplasma gondii*, Toxoplasmosis, Polymerase chain reaction, Dot blot hybridization, PCR/DBH

Toxoplasma (T.) gondii, an obligate intracellular apicomplexan parasite protozoan, is widely distributed and can infect many species of warm-blooded animals; thus, it is considered a significant zoonotic pathogen (Weiss and Dubey, 2009). Toxoplasmosis is a parasitic disease of great importance for veterinary medicine, husbandry, and public health because it causes productive and economic losses and, further, damages to human health due to consumption of contaminated meat and milk (Jittapalapong et al., 2005). This disease often induces huge economic losses in raising livestock because it is a frequent cause of early embryonic death and resorption, fetal death and mummification, abortion, stillbirth, and neonatal death in livestock (Buxton et al., 2007; Dubey 2009).

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T. gondii is one of the most prevalent zoonotic parasites worldwide. While only felidae can act as definitive hosts and thus shed oocysts in their faeces, almost all warm-blooded animals can serve as intermediate hosts. A tachyzoite stage of *T. gondii* appears on the primary infection, and, thereafter, the bradyzoite-containing tissue cysts occur primarily in brain or muscles (Paquet and Yudin, 2013; Tenter, 2009). Humans may acquire a *T. gondii* infection via oral uptake of sporulated oocysts from the environment, consumption of raw or undercooked meat containing tissue cysts, or transplacental transmission of the parasite from the non-immune mother to the foetus. Studies in Europe have shown that 35 ~58% of women at child-bearing age were seropositive for *T. gondii* (Tenter, 2009).

Experimental infections of food animals such as cattle, pigs, sheep and goats, have shown that these animals are susceptible to *T. gondii* contamination by in-

take of oocysts or tissue cysts, and that following experimental infection *T. gondii* can be isolated from their tissues, with the exception of beef (Zia-Ali et al., 2007). *T. gondii* cysts in pork can persist for a long time, and has been considered an important source of infection for humans (Bayarri et al., 2012). Raw or undercooked lamb meat is considered a delicacy in certain countries such as France and is therefore considered an important source of infection in that country (Bayarri et al., 2012). Birds can serve as a potential source of infection for humans. In chickens, *T. gondii* was found in skeletal muscles, heart, brain, ovary, oviduct, kidney, spleen, liver, lung, pancreas, gizzard, proventriculus, intestine and retina, and even in eggs (Kaneto et al., 1997).

Polymerase chain reaction (PCR) provides a powerful technique of identifying *T. gondii* and studying homology between their nucleic acids. However, PCR has a limitation of their susceptibility to contamination or to enzymatic inhibitors (Switaj et al., 2005). In order to avoid problems related to nucleic acid amplification, efforts have been made to obtain specific hybridization assays like as dot blot hybridization (DBH) and *in situ* hybridization (McNicol and Farquharson, 1997). DBH is a simple and specific method for detection of pathogens and has been reported as a method with higher specificity and lower sensitivity as compare as PCR assay (Duggan et al, 1994; Xia et al, 1995).

This study aimed to find a fast, sensitive and efficient protocol for molecular identification of intracellular protozoa *T. gondii*. For reliable and specific detection of *T. gondii*, we developed a PCR coupled with dot blot hybridization assay (PCR/DBH).

Tachyzoites of *T. gondii* were obtained from peritoneal washings in mice inoculated with the QHO strain provided by Professor H. Park at Wonkwang University in Korea. DNAs were extracted from the tachyzoites using an AccuPrep Genomic DNA extraction kit (Bioneer Co., Korea) according to the manufacturer's instructions. The DNA was eluted in Tris-EDTA buffer (pH 8.0), and an aliquot was used for the PCR amplification. All DNA samples were stored at −20°C until the PCR assays were performed. The template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (*AccuPower* PCR PreMix; Bioneer Co., Korea) containing 2.5 U of Taq DNA polymerase, 250 μM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, and the gel loading dye. The volume was adjusted with distilled water to 20 μl. Detection of *T. gondii* DNA was based on amplification of the first internal transcribed spacer (ITS-1) of ribosomal DNA by one pair of primers; forward primer, 5′-AGTTTAGGAAGCAATCTGAAAGCACATC-3′, and reverse primer, 5′-GATTTGCATTCAAGAAGCGTGATAGTAT-3′ as described previously (Xie et al., 2005). The target size of PCR amplification was 529 base pairs. PCR using the ITS-1 primer pairs could be used to detect both bradyzoites and tachyzoites of *T. gondii* in the previous our study (data not shown). The reaction mixture was subjected to denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 7 min as described previously (Xie et al., 2005). Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer Co., Korea).

For DBH analysis, *T. gondii*-specific DNA probes were prepared by digoxigenin (DIG)-labeling after amplification of the genomic DNA by PCR as described previously (Kim, 2003). To prepare *T. gondii*-specific DNA probes, the PCR products amplified with ITS-1 primers were purified using Wizard PCR preps (Promega, Medison, WI, USA) and then labeled by random priming with DIG-dUTP (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Dot blotting was achieved by direct application on a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany).

The sensitivity of PCR assay with *T. gondii*-specific ITS-1 primers was evaluated. Purified *T. gondii* DNA samples ranging from 10^5 to 1 pg were used for the primary target amplification. For PCR/DBH analysis, the PCR products after primary amplification were dotted on the nylon membrane. The membrane was immersed in 0.4 M NaOH for 5 min and then in neutralizing buffer for 5 min. After rinsing in 2 x saline-sodium citrate buffer (SSC), cross-linking between the applied DNA and the membranes was done using UV cross-linker (Stratagene, La Jolla, CA, USA). Hybridization solutions contained 5 x SSC, 2% buffered blocking solution

(Roche Applied Science, Mannheim, Germany), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. DIG-labeled probe was denatured by boiling for 10 min and chilled in ice, and then added into hybridization solution at 0.1 g/mL. After pre-hybridization at 50°C for 1 h, the membrane was hybridized at 50°C for 3 h and then washed with 1 x SSC at 60°C for 10 min. For detection of hybridization, the membrane was incubated with anti-DIG conjugated with alkaline phosphatase (Roche Applied Science, Mannheim, Germany) and then colorized with nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl-phosphate (BCIP) (Roche Applied Science, Mannheim, Germany). The development of a dark purple positive reaction was allowed to proceed for 10～30 min in the dark. The specificity of PCR/DBH was evaluated by using the template DNA samples like as *Eimeria tenella* and *Eimeria maxima*, which DNAs were provided by Professor W. Min at Gyeongsang National University in Korea.

In our results, the DBH assay alone was capable of detecting down to 1×10^4 pg of *T. gondii* genomic DNA (Fig. 1). The PCR alone was capable of detecting down to 1×10^3 pg of *T. gondii* genomic DNA (Fig. 2). However, the PCR/DBH assay was capable of detecting down to 1×10^2 pg of *T. gondii* genomic DNA, indicating that sensitivity of the PCR/DBH method was approximately 100 times higher than the DBH method alone (Fig. 3). The specificity of PCR/DBH was confirmed by the study using other intracellular protozoa DNAs with high homology in their sequences. No positive signals were observed in the template DNA samples of *Eimeria(E.) tenella* and *E. maxima* in PCR/DBH assay. However, PCR/DBH using *T. gondii* template DNA resulted in strong positive signal (Fig. 4).

Toxoplasmosis transmission by unpasteurized or inadequately processed milk or fresh cheese, important food sources in rural areas, can be a significant means of contamination by this agent (Hiramoto et al., 2001). In this study, we used a non-radioactive probe for DBH, which makes these techniques more attractive for diagnostic laboratories because the troublesome problems related to the short half-life of radioactive compounds, their disposal, and personnel safety can be avoided (Gauthier and Blais, 2003; Mansfield et al., 1995).

PCR with specific primers is considered sensitive assay for detecting *T. gondii* DNA from biological samples directly, especially if nested PCR is used (Su et al., 2002). However, PCR assays are subject to a high risk of contamination through DNA carry-over and may result frequently in false positive reactions (Borst et al., 2004; Maurer 2011; Szöllsi et al., 2008). To get around

Fig. 1. Dot blot hybridization with purified genomic DNA of *T. gondii*. A DIG-labeled probe derived from the genomic DNA of *T. gondii* was used for detection. 1×10^5 pg (lane 1), 1×10^4 pg (lane 2), 1×10^3 pg (lane 3), 1×10^2 pg (lane 4), 10 pg (lane 5), and 1 pg (lane 6) of sample DNA.

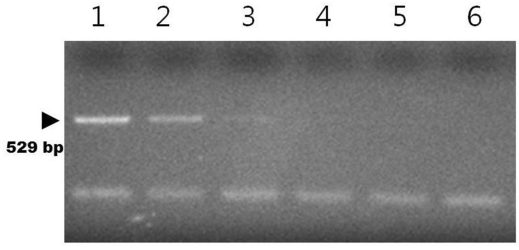

Fig. 2. PCR assay with *T. gondii*-specific ITS-1 primers. 1×10^5 pg (lane 1), 1×10^4 pg (lane 2), 1×10^3 pg (lane 3), 1×10^2 pg (lane 4), 10 pg (lane 5), and 1 pg (lane 6) of template DNA.

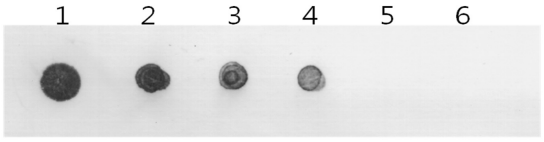

Fig. 3. PCR/dot blot hybridization with PCR-amplified DNA of *T. gondii*. A DIG-labeled probe derived from the genomic DNA of *T. gondii* was used for detection. 1×10^5 pg (lane 1), 1×10^4 pg (lane 2), 1×10^3 pg (lane 3), 1×10^2 pg (lane 4), 10 pg (lane 5), and 1 pg (lane 6) of PCR-amplified genomic DNA of *T. gondii*.

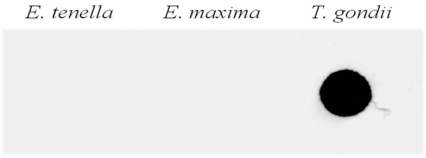

Fig. 4. Specificity of PCR/dot blot hybridization. The *T. gondii* probe was not reacted with other pathogens, *E. tenella* and *E. maxima*. However, PCR/DBH using *T. gondii* template DNA resulted in strong positive signal.

these problems, PCR/DBH assay may be an alternative choice for sensitive and specific detection of *T. gondii*, in which PCR sensitivity and specificity is increased by hybridization methods of the replicated DNA with specific labeled probe.

In this study, we used non-radioactive labels for DBH probe and it has made these techniques more attractive for diagnostic laboratories, because those avoid problems relative to the short life of radioactive compounds, their disposal, and personnel safety (Burns et al, 1987; Syrjanen et al, 1988). Described DNA probe labeling was used in this study and the method was shown to be rapid, sensitive and specific, making it suitable for the detection of primary amplified *T. gondii* species DNA products, which was allowed the increased sensitivity and specificity and *T. gondii* species DNA densitometry quantification. Complete time including PCR procedure and DBH detection is 8 hours. The PCR/DBH, which was established in this study, is much more sensitive and specific compared with one step PCR assay and DBH detection alone. Our PCR/DBH assay will be useful for confirming the presence of *T. gondii* on the samples like as meat.

In conclusion, the PCR/DBH assay is a more sensitive and specific method than PCR or DBH alone and will be diagnostically useful for detecting intracellular protozoa *T. gondii*.

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