

PCR TECHNIQUE FOR DETECTING *TOXOPLASMA GONDII* IN ANIMAL AMNIOTIC FLUID

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Abstract. The goal of diagnosing congenital toxoplasmosis is early detection of maternofetal transmission, for early treatment to prevent unwanted sequelae. Polymerase chain reaction (PCR) is a method used recently for detecting toxoplasmosis during pregnancy. Amniotic fluid is a the clinical specimen used, since it provides a rapid, simple and safe method to obtain accurate results. The advantages of the PCR technique are high sensitivity, specificity and positive predictive value compared with other laboratory methods. To determine the sensitivity, specificity and lower detection limits in our laboratory, amplification of the B1 gene by nested PCR was performed on *Toxoplasma gondii* tachyzoites added to animal amniotic fluid samples. From 48 samples, our technique detected *T.gondii* in 30 out of 41 positive samples, and gave negative results for all the negative samples. The sensitivity for this nested PCR was 73%, the specificity was 100%, and the efficiency of the test was 77.1%. The nested PCR technique is recommended as a diagnostic method for detecting *T.gondii* in suspected congenital toxoplasmosis animals.

INTRODUCTION

Toxoplasma gondii cause illness in both animals and humans. In immunocompetent persons, the infection shows non-specific signs and symptoms. In immunodeficient persons and pregnant women serious disease occurs. Women infected during pregnancy may show a variety of clinical signs and symptoms depend on many factors, such as the number of the parasites, the virulence of strain, and the time period the mother acquires infection (Tenter *et al*, 2000). If the mother is infected in the first trimester, the result is abortion, stillbirth or severe disease of fetus (Lin *et al*, 2000). The severity of disease decreases if the infection occurs in the second or third trimester, but the risk for transmission from mother to fetus increases (Romand *et al*, 2001). Early diagnosis of infection is important in reducing the severity of the disease and the risk of transmission.

Similar to humans, infection in animals causes mild symptoms, but may produce an economic loss due to abortion or stillbirth if the infection occurs in pregnant livestock. The mechanism of vertical

transmission in the pregnancy is unclear. *T.gondii* tachyzoites from mother probably invade the placenta and cross into the fetal circulation (Tenter *et al*, 2000). Congenitally infected animals may be a source of transmission to humans and other animals. The ability to diagnose infection in farm or companion animals is thus useful.

Serologic tests are major diagnostic techniques in toxoplasmosis, but have limitations since serological dynamics in pregnant hosts may cause confusing or uncertain results. Diagnosis is delayed leading to undetected congenital toxoplasmosis or abortion. The PCR technique is now the method of choice for the detection *T.gondii* in pregnant patients (James *et al*, 1996; Montoya, 2002). The PCR technique can detect tachyzoites in cord blood, amniotic fluid, placenta or fetal tissue. The safest, fastest, simplest and most reliable simple is amniotic fluid (Hohlfeld *et al*, 1994). Previous studies indicate the high sensitivity and high specificity of this technique (Alanen, 1998; Jenum *et al*, 1998; Romand *et al*, 2001; Antsaklis *et al*, 2002). Information concerning congenital toxoplasmosis in livestock and companion animals is not available in Thailand due to the lack of a reliable diagnostic method.

We, therefore, used the PCR technique to detect *T.gondii* tachyzoites added to animal am-

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niotic fluid samples to evaluate the sensitivity and specificity of this method. Application of this method can be used for the diagnosis of *T.gondii* vertical transmission, to prevent further transmission in either animals or humans.

MATERIALS AND METHODS

Sample preparation

The amniotic samples were collected from the newborns of bitches coming for cesarotomy at Km8 Animal Hospital, Bangkok in April 2003. Each sample was kept at 4°C until used. Five Swiss mice were intraperitoneally inoculated with 3×10^4 RH strain *T.gondii* tachyzoites. The tachyzoites were harvested from the mice peritoneal fluid 3 days post-infection. Residual host cells were eliminated and the tachyzoites were washed with normal saline solution. A concentration of 1×10^4 tachyzoites per microliters was obtained using a hemocytometer.

Serial dilutions of the tachyzoites to 1, 1:10, 1:100, 1:1,000 and 1:10,000 were performed. Each 100 µl amniotic fluid sample was added to 100 µl of tachyzoites using a blinded technique. Seven samples of 200 µl amniotic fluid each were prepared as negative samples. The forty-one added samples and seven negative samples were kept at -20°C until the PCR protocol was performed.

PCR technique

DNA extraction and purification of both positive and negative samples were done using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The PCR protocol was a nested PCR with the target being the B1 gene, previously performed by the Toxoplasma Reference Unit, Public Health Laboratory, Singleton Hospital, Swansea, United Kingdom.

Primary PCR

The sequences of primers used for the extracted DNA in the primary PCR reaction were: 5'-TCA AGC AGC GTA TTG TCG AG-3' and 5'-CCG CAG CGA CTT CTA TCT CT-3'. Each sample was mixed with 5 µl of 10x reaction buffer, 8 µl dNTP, 2.5 µl of each primer, 0.25 µl Taq polymerase and 6.75 µl deionized water, of a total volume of 50 µl. For the PCR amplification, initial denaturation was performed at 94°C for 30 seconds, followed by 50 cycles of denaturation at 94°C for 15 seconds, annealing at 45°C for 30 seconds, extension at 72°C for 45 seconds and

final extension at 72°C for 10 minutes.

Secondary PCR

The sequences of the primer of the secondary PCR, used were: 5'-GGA ACT GCA TCC GTT CAT GAG-3' and 5'-TCT TTA AAG CGT TCG TGG TC-3'. Each primary PCR product was mixed with 5 µl of 10x reaction buffer, 8 µl dNTP, 0.5 µl of each primer, 0.25 µl Taq polymerase and 34.75 µl deionized water, for a total volume of 50 µl. The PCR reaction was performed at similar temperature to the primary PCR, but the amplification cycles was carried out for 35 cycles. The Thermo Hybrid Px2 PCR machine was used throughout the experiment.

The PCR products were electrophoresed on 2.5% agarose gel in 1x TAE buffer and stained with ethidium bromide. Under a transilluminator with a 100 bp DNA ladder, the product length of the positive result was 197 bp.

RESULTS

Forty-one of forty-eight samples were prepared as positive sample at the different concentration as described in Table 1. The seven remaining samples were negative controls. The results obtained are shown as in Table 2 and Fig 1.

The test results showed a sensitivity of 73%, specificity of 100%, positive predictive value of 100%, and an efficiency test of 77.1%. The lower detection limit of our laboratory was 10^1 tachyzoites/µl.

DISCUSSION

The PCR technique for detecting *T.gondii* tachyzoites in amniotic fluid is a valuable diagnostic method for the prenatal diagnosis of congenital toxoplasmosis (Hohlfeld *et al*, 1994; Antsaklis *et al*, 2002) because it is safer, reliable and the results are more rapidly obtained than the conventional diagnostic method (Pelloux *et al*, 1996). It is more convenient for sample transportation than mouse inoculation since the PCR technique can detect even partially destroyed tachyzoites. However, this technique is recommended for use in combination with other diagnostic methods, particularly mouse inoculation (Antsaklis *et al*, 2002). Currently, there are many PCR protocols used at different laboratories, and the best protocol is still ambiguous (Pelloux *et al*, 1998). The sensitivity and specificity of the PCR

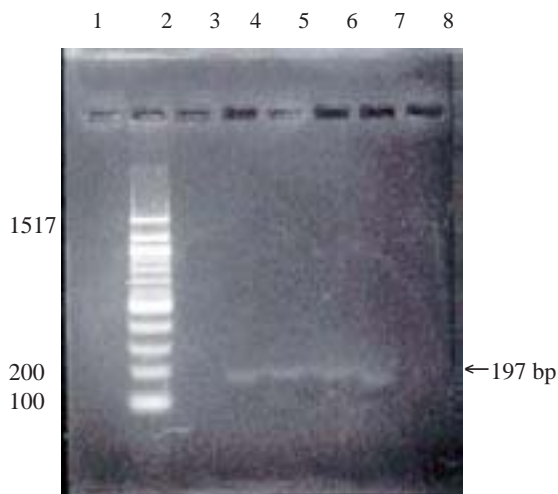


Fig 1—shows 100 bp MW ladder used as a marker. The 197 bp fragment was the product of the B1 gene amplification. Lane 2 shows the standard marker. Lane 3 was the PCR negative control. Lanes 4-7 contained the tachyzoite added samples.

Table 1

The number of samples of each concentration.

Sample concentration	Numbers
10^4 tachyzoites/ μ l	4
10^3 tachyzoites/ μ l	9
10^2 tachyzoites/ μ l	9
10^1 tachyzoites/ μ l	11
1 tachyzoites/ μ l	8
0 tachyzoite (negative sample)	7
Total	48

Table 2

The number of positive and negative results with the tachyzoite added samples.

Test results	Added samples		
	Positive	Negative	Total
Positive	30	0	30
Negative	11	7	18
Total	41	7	48

technique using amniotic fluid varies widely depending on the PCR protocol used. The sensitivity was estimated to be around 70-80% and the specificity was never less than 94% (Bastien, 2002).

The wide range of sensitivities and specificities of the PCR technique has resulted in high false positive results and high false negative results. In our study, there were no false positive results, but there were relatively high false negative results particularly in the lower concentration samples. A possible reason for this may be the inadequate sensitivity of the primers used in our laboratory or a low affinity for the DNA target, since in the false negative lanes, there were obvious primer dimers each time.

The target used by our laboratory was the B1 gene, which is the most sensitive and widely used target for detecting both tachyzoites and cysts in all 21 strains of *T.gondii* (Bastien, 2002). Although the B1 gene had a 35-fold repetitive sequence, this may be insufficient when compared with the targets used for other microorganisms. To solve this problem, more repeated copy numbers are required. The 110-fold 18s rDNA is an interesting alternative. However, there is a previous study indicating a higher sensitivity for the B1 gene than the 18s rDNA (Jones *et al*, 2000). This may have resulted from the insufficient sensitivity of the primers.

Besides the false negative results obtained with the lower concentrations, the amplification products were absent many times (data not shown). The most likely reason is the PCR inhibitors were not eliminated from the amniotic fluid samples. To clarify this, positive internal controls should have been included in the reaction, as recommended by Bretagnes (2003). The occurrence of internal control amplified products, but absence of expected products indicates the presence of a PCR inhibitor.

In our results, expected PCR products were present, but in some lanes the unexpected non-specific products were also seen. Previous studies showed a very high sensitivity with this technique, detecting even a single tachyzoite (Pelloux *et al*, 1996; Robert-Gangneux *et al*, 1999; Hafid *et al*, 2001). Our laboratory showed a lower sensitivity. A possible reason is that the samples had been kept for quite a long time before the extraction and amplification process. This can reduce the sensitivity of PCR technique because of the degradation of frozen DNA (James *et al*, 1996). The washing with normal saline and preparation of the tachyzoites from mice peritoneal fluid may also have reduced the sensitivity of the PCR tech-

nique (Warnekulasuriya *et al*, 1998). This problem can be eliminated by replacing the washing agent with phosphate buffer saline (PBS).

We also monitored the amount of DNA with a spectrophotometer. The concentrations of DNA in some samples as measured by the spectrophotometer did not correspond with the concentrations of the tested samples. This might indicate a heterogeneous distribution of the tachyzoites added to each of the amniotic fluid samples. This can cause inconsistency in the results. To avoid this problem, DNA should be extracted before being added to each sample. A homogeneous distribution of DNA is easier than a homogeneous distribution of tachyzoites (Guy *et al*, 1996).

In our study, the animal amniotic fluid samples were collected at birth, so this technique should be used for neonatal diagnosis rather than prenatal diagnosis. However, in case of livestock, the amniotic fluid collection at birth is too late to prevent abortion or stillbirth. To avoid this economic loss, amniocentesis, as in humans, should be performed using ultrasonography as a directional guide.

A previous study found that in pigs congenital toxoplasmosis is less common than post-natal infection (Dubey, 1986). The PCR technique is useful for helping the veterinarian to differential diagnose the cause of abortion, stillbirth and neonatal abnormalities, which are often found in both companion animals and livestock.

ACKNOWLEDGEMENTS

We are grateful to Dr Anuwat Sritamanote for collecting the animal amniotic fluid samples and to Mr Amorn and Mr Saiyooth for helping in the laboratory and in caring for the animals used in our work. This study was financially supported by the Thanat-Molee Khoman Foundation.

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