Indonesian Journal of Biotechnology, June, 2009 Vol. 14, No. 1, pp. 1124-1131

Analysis of *Toxoplasma gondii* Repeat Region 529 bp (NCBI Acc. No. AF146527) as a Probe Candidate for Molecular Diagnosis of Toxoplasmosis

Dyah Ayu Oktavianie A. Pratama[1,2]., Sumartono[3], and Wayan T. Artama[1,3] *

1. Research Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia
2. Program of Veterinary Medicine, Brawijaya University, Malang, Indonesia
3. Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia

Abstract

Toxoplasmosis is a disease caused by protozoan parasite *Toxoplasma gondii*. The infection is commonly asymptomatic. The availability of confirmative and accurate detection system is really needed. This research was aimed to develop a molecular diagnosis based on the conserved and high copy number repeat region of *Toxoplasma gondii* with hibridization method. Nucleic acid was isolated from tachyzoites. The repeat region of *T. gondii* was amplified using PuRe Taq Ready To Go-PCR Beads (Amersham Bioscience), *forward primer* 5'- GAC TCG GGC CCA GCT GCG -3' and *reverse primer* 5'- CCT CTC CTA CCC CTC CTC -3'. The amplicon was sequenced using ABI Prism 3100-Avant Genetic Analyzer (PT. Charoen Pokphand, Jakarta). Probe was labeled using *digoxigenin*-11-dUTP. Application of probe to detect it's complementary nucloeic acid was done by hibridization method. The research concluded that *probe toxo*-103 bp was highly homolog with several strain of *T. gondii* and it has no homology either with host's genome or other parasites which have close genetic relationship with *T. gondii*. Hybridization analysis showed that probe could detect the complementary nucleic acid up to 10 ng / ìl concentration.

Keywords: DNA probe, repeat region, *Toxoplasma gondii*, hybridization.

Introduction

Toxoplasmosis is an important parasitic infection of man and animals, caused by protozoan parasite *Toxoplasma gondii*. This disease is one of an extensively diseases spread worldwide in mammals (Dubey and Lindsay, 2004; Switaj *et al.*, 2005). Toxoplasmosis also causes significant veterinary losses. The disease prevalence is vary based on geographic and climate condition. It is well known-that the progression and severity of disease depend on the immunological status of the host, but recent studies suggest that the genetics of the parasite can also play a role (Switaj *et al.*, 2005).

The current methods for the diagnosis of toxoplasmosis have given dismayingly poor results in such patients and, therefore, there is a need for more sensitive procedures for the early diagnosis of *T. gondii* in body fluids or biopsy tissues (Savva and Holliman, 1990). The diagnosis of toxoplasmosis classically relies on serology and the demonstration of the pathogen in patient samples. Body fluids or tissues may also be inoculated intra peritoneal into mice or used to infect cell cultures *in vitro* (Homan *et al.*, 2000), but these methods are less effective. Serological diagnosis of active infection is unreliable because reactivation is not always

*corresponding author :Yuda Heru Fibrianto, Department of Physiology, Faculty of Veterinary Medicine, Gadjah Mada University, Jl. Olahraga Karang Malang, Yogyakarta, 55281, Indonesia, Tel. 62-274-649-62415; E-mail : yuda@ugm.ac.id

accompanied by changes in antibody levels, and the presence of immunoglobulin M (IgM) does not necessarily indicate recent infection. The ''gold standard'' for the detection of *T. gondii* organisms in clinical specimens is mouse inoculation and then the detection of *T. gondii*-specific antibodies. This method is sensitive and specific but time-consuming, taking up to 6 weeks to obtain a diagnosis. Currently, cell culture is the most practical method for the detection of *T. gondii* parasitemia, but this is also relatively slow and may lack sensitivity (Angel *et al*., 1997; Reischl *et al*., 2003; Switaj *et al*., 2005).

Over the past few years, the application of recombinant DNA procedures has led to the development of a number of DNA probes for potential use in the diagnosis of pathogen. Recent advances in recombinant DNA technology, and in particular the development of the polymerase chain reaction (PCR), allows certain pathogen. One of molecular diagnostic method for toxoplasmosis is the use of DNA probe to detect the complementary nucleic acid of infectious agent. This research was aimed to develop a DNA probe to detect toxoplasmosis based on a non-coding 529 bp DNA fragment that is repeated 200-300 times in the *T. gondii* genome. This fragment was highly conserved on several strains of *T. gondii* and it discriminates *T. gondii* DNA from that of other parasites (Homan *et al*., 2000), therefore it can be used as a sensitive and specific diagnostic tool for toxoplasmosis.

Materials and Methods

Toxoplasma gondii in vivo cultivation and isolation of tachyzoite DNA

A number of 1x10^{7} of *Toxoplasma gondii* tachyzoites was cultivated *in vivo* in Balb / C mice. Three days post infection, the intraperitoneal fluids was harvested and centrifugated. The tachyzoites was calculated, obtained 6x10^{6} tachyzoites / ml. Pellet was washed three times using PBS and resuspended with NTE solution. One hundred ìg / ml proteinase-K and 0,5% SDS were added, then incubated in waterbath at 37°C overnight. Phenol was added in equal volume, shaking 60 rpm for 20 minutes and centrifuged at 3000 rpm for 15 min at room temperature. The aqueous phase was pipetted out and equal volume of CIAA was added, centrifuged at 3000 rpm for 10 min and repeat until there was no interphase. *Deoxyribonucleic acid* was precipitated with 0,1 volume 3 M Na-acetat and 2x volume ice-cold ethanol absolute, incubated for 15 minutes in -20°C, then centrifuged at maximum speed for 5 min. The pellet was rinsed with 70% ethanol and dissolved in TE buffer.

Amplification of T. gondii repeat region

T.gondii repeat region was amplified using specific primers which was designed based on *T. gondii* repeat region 529 bp accessed from GeneBank (NCBI *Acc No.* AF 146527), as follows: forward primer 5'- GAC TCG GGC CCA GCT GCG -3' and *reverse primer* 5'- CCT CTC CTA CCC CTC CTC -3'. Primers were diluted at a concentration of 25 pmol / ìl. To amplify was used *puRe Taq Ready-To-Go PCR Beads* (Amersham Biosciences) and amplified using *thermocycler* (*Gene Cycler*, Biorad) with condition: (1) initial denaturation at 95°C for 5 min, (2) denaturation at 95°C for 45 sec, (3) annealing at 65°C for 30 sec, (4) extension at 72°C for 1 min, (5) repeat the cycle for 30 times, and (6) final extension at 72°C for 3 min. Amplification was done with sample (tachyzoite DNA) and negative control (without template).

Sequencing of PCR product

The PCR product was sequenced using ABI Prism 3100-Avant Genetic Analyzer

(PT. Charoen Pokphand, Jakarta) and sequencing results were analysed using BLAST program which available at http://www.ncbi.nlm.nih.gov.

Probe candidate analysis and probe synthesis

Probe candidate analysis was done by alignment the sequence with *T. gondii* genome from all strain and all isolate using BLAST program to show the homology. Probe candidate also analysed for it's specificity with hosts genome of *T. gondii* and the other parasites which have close genetic relationship with *T. gondii*. Probe candidate which chosen is the sequence which was highly homolog with *T. gondii* but has no homology with *T. gondii* genome and the other parasites. Probe candidate also determined based on the ideal qualifications of probe, include probe length, GC contents and sequence compositions. Probe was synthesized by Sigma-Aldrich.

Probe labeling using random primed labeling method

Probe labeling was done using *digoxigenin-11-dUTP* with random primed labeling method. In this method, as much as 1 ml template probe DNA with concentration 10 ng/3 ml was added to the reaction vial, then autoclaved double destilation water was added to a final volume of 16 ml . The DNA was denatured by heat the sample in a boiling water bath for 10 min and then chilled the sample in an ice/water bath quickly. Labeling was done by adding 4 ml mixed DIG-High Prime to the denatured sample, centrifuged brieftly, and incubated for overnight at 37°C. The reaction was stoped by adding 2 ml 0.2 M EDTA (pH 8.0) to the sample, and heated to 65°C for 10 min (Roche).

Probe labeling quantification

Quantification of probe labeling efficiency was done by direct detection procedure. Quantification was done by compared the labeled probe with *DIG-labeled control DNA*. At first, labeled probe and *DIG-labeled control DNA* was diluted serially until a certain concentration. Probe and control was spoted on nylon membrane, and then air dryied for at least 15 min. Membran was washed using washing buffer for 2 min and blocked for 30 min using 1x blocking solution. Let antibody bind DIG label on the membrane using 1:10 000 dilution of Anti-DIG alkaline phosphatase in 1x blocking solution for 30 min. The membrane washed to remove unbound antibody using washing buffer 2x15 min, equilibrated using detection buffer for 2 min, and incubated in NBT/BCIP substrate for at least 30 min. Quantification was done with comparing the colour intensity between labeled probe and *DIG-labeled control DNA*.

Hybridization of labeled probe and clinical sample

Hybridization was done on tachyzoite DNA as positive control, DNA from clinical sample and total DNA from healthy mice tissue as negative control. *Deoxyribonucleic acid* from clinical sample that have been measured for it's concentration was diluted to obtain concentration 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml and 1 pg/ml respectively. Sample DNA from clinical sample was denatured with boiled in 100°C for 10 min and then chilled in ice immediately. Two microliters of DNA sample that have been denatured was spoted on positively charge nylon membrane Hybond-N (Amersham Pharmacia), then airdryed for 1 h. The sample was immobilized on nylon membrane with UV *crosslinker* for 3 min.

Prehybridization was done on *DIG-Easy*

Hyb solution on shaker incubator at temperature 42°C for 3-4 h. Hybridization was done for overnight at temperature 42°C, with added 25 ng/ml labeled probe. The membrane was washed using 2x SSC which contain 0,1% SDS for 2 x 15 min at room temperature, and using 0,5x SSC which contain 0,1% SDS for 2 x 15 min at temperature 68°C (Roche®).

Hybridization of labeled probe and clinical DNA sample was detected with visualization using *antibody anti-dig-11-dUTP* conjugated with *alkaline phosphatase* and NBT/BCIP. At first, membrane was washed using *washing buffer* (0,1 M *maleic acid*; 0,15 M NaCl; pH 7,5; 0,3% Tween 20) for 3-5 min, then blocked using *blocking solution* (0,1 M maleic acid; 0,15 M NaCl; pH 7,5, 1% blocking reagent) for 1 h and washed again for 2-3 min. Membrane then incubated in *antibody anti-dig-11-dUTP* solution (stock antibody solution dissolved on blocking solution with ratio 1:2000) for 1 h. Membrane was washed for 2 x 15 min to remove the unbound antibody. Membrane was equilibrated on detection buffer (0,1 M Tris-HCl; 0,1 M NaCl; pH 9,5) for 15 min. Hybridization probe will be visualized after incubation on NBT/BCIP (*Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl phosphate*) substrate for at least 1 hour, protected from light (Roche®).

Toxoplasmosis detection of clinical DNA sample with PCR (for confirmation)

The clinical DNA sample was tested using PCR method as a confirmation for hybridization result. In this method, was used clinical DNA sample as template, and the same forward and reverse primers that were used to amplify the *Toxoplasma gondii* repeat region, under the same condition.

Results and Discussion

Toxoplasma gondii in vivo cultivation

Toxoplasma gondii tachyzoites was injected i.p to three Balb/C mices with each dose $1x10^7$ tachyzoites. After 72-96 hours mices were euthanized and cavum peritoneal was washed three times using physiologic NaCl to collect tachyzoites. The amount of tachyzoites was counted using *Neubauer* chamber, and resulted $6,8x10^8$ per ml tachyzoites.

Toxoplasma gondii tachyzoite DNA

Deoxyribonucleic acid isolation was done after a quite amount of tachyzoites were achieved. In this method were used non ionic detergent such as *sodium dodecyl sulfat* (SDS) to break the cytoplasmic membrane. The cell extract then centrifuged in order to get DNA in supernatant.

Amplification of T. gondii repetitive sequence

Toxoplasma gondii repetitive sequence was sequenced using primers which designed based on *T. gondii* repeat region 529 bp accessed from genebank (NCBI Acc. No. 146527), as seen in Figure 1.

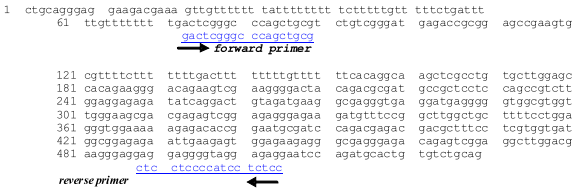

Figure 1. *Toxoplasma gondii* repeat region 529 bp and primers location

The total length of target repetitive sequence that should be amplified is 529 bp, but the primers was not designed from the beginning and the end of the sequence, so the fragment length that can be amplified is 434 bp.

The result of DNA amplification using specific primers designed based on *T. gondii* repeat region 529 bp (NCBI Acc. No. 146527) as seen in Figure 2 showed that in sample (tachyzoite DNA) showed one

dominant band at approximately 434 bp, but there was a second band at approximately 900 bp.

This electrophoregram was represented the characteristic of repetitive sequence, in which consist of two types which is tandem and disperse. There was no band was observed in negative control.

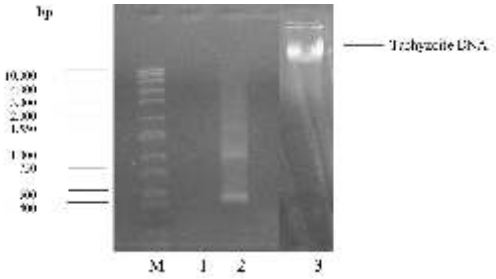

Figure 2. Amplification of *T. gondii* repetitive sequence using primers designed based on *T. gondii* repeat region 529 bp (NCBI Acc.No. 146527) on 1% agarose gel. M. Marker, 1. Negative control, 2. Sample (tachyzoite DNA), 3. Tachyzoite DNA.

The dominant band at approximately 434 bp, which is band of interest was isolated using electroelution technic in order to get the monomorphic band. The result is showed in Figure 3.

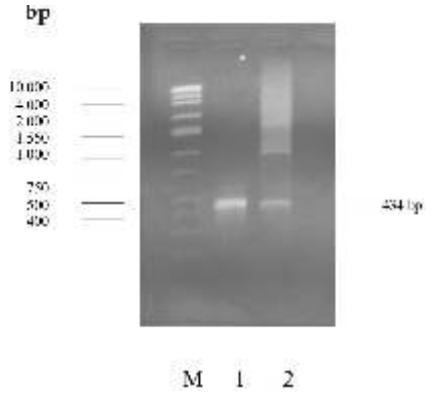

Figure 3. Isolation of band of interest (*T. gondii* repetitive sequence) M. Marker, 1. Monomorphic band, 2. DNA before electroelution.

Sequencing of monomorphic band

Sequencing of monomorphic band (*T. gondii* repetitive sequence) was done using the same forward and reverse primers which is used to amplify the *T. gondii* repeat region. The length of sequencing products was 424 and 470 nucleotides, respectively. But the requencing result from the forward primer can not be analyzed further, because of the abundant of non specific nucleotide. The sequencing result from the reverse primer can be seen in Figure 4.

```
1     CNNNATCNTN CANCNNCNTC TTNTNTCNNN CNANNCCTTT NCCTNNNNNT NNTNTNCTNA
61    NNNNNNNNNN NNNTTNTNTN TGGCCCNCAT GNGTCTNTCN GGATNAGACC CCGGAGCCNA
121   ANAGCGTTTT NTNNTTTTGA CTTTTTTTTG TTTTTTCACA GGCAAGCTCG CCTGTGCTTG
181   GAGCCACAGA AGGGACAGAA GTCGAAGGGG ACTACAGACG CGATGCCGCT CCTCCAGCCG
241   TCTTGGAGGA GAGATATCAG GACTGTAGAT GAAGGCGAGG GTGAGGATGA GGGGGTGGCG
301   TGGTTGGGAA GCGACGAGAG TCGGAGAGGG AGAAGATGTT TCCGGCTTGG CTGCTTTTCC
361   TGGAGGGTGG AAAAAGAGAC ACCGGAATGC GATCCAGACG AGACGACGCT TTCCTCGTGG
421   TGATGGCGGA GAGAATTGAA GAGTGGAGAA GAAGGGCGAG GGAGACAGAG T
```

Figure 4. Nucleotides sequence of *T. gondii* repetitive sequence (sequencing result)

Sequence analysis

The sequence was analysed with BLAST program to show the homology of the *T. gondii* repetitive sequence (sequencing product) with *T. gondii* repeat region 529 bp from genebank. *Toxoplasma gondii* repetitive sequence from sequencing product alignment was showed 97% homology with *T. gondii* repeat region 529 bp of the 89th base to the 566th base (Figure 5).

```
gb|AF146527.1|AF146527 Toxoplasma gondii repeat region
 Length=529
 Score =  652 bits (353),  Expect = 0.0
 Identities = 368/379 (97%), Gaps = 1/379 (0%)
 Strand=Plus/Plus

Query  94   GTCTNTCNGGATNAGACCCCGGAGCCNAANAGCGTTTTNTNNTTTTGACttttttttgtt  153
            |||| || |||| ||||| ||||||| ||  ||||||| |  ||||||||||||||||||
Sbjct  89   GTCTGTCGGGATGAGACCGCGGAGCCGAAGTGCGTTTTCTTTTTTTGACTTTTTTTTGTT  148

Query  154  ttttCACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGAC  213
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  149  TTTTCACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGAC  208

Query  214  TACAGACGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGA  273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  TACAGACGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGA  268

Query  274  AGGCGAGGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGAG  333
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  AGGCGAGGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGAG  328

Query  334  AAGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGAAAAAGAGACACCGGAATGCGA  393
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329  AAGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGAAAAAGAGACACCGGAATGCGA  388

Query  394  TCCAGACGAGACGACGCTTTCCTCGTGGTGATGGCGGAGAGAATTGAAGAGTGGAGAAGA  453
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  389  TCCAGACGAGACGACGCTTTCCTCGTGGTGATGGCGGAGAGAATTGAAGAGTGGAGAAGA  448

Query  454  AGGGCGAGGGAGACAGAGT  472
            ||||||||||||||||||
Sbjct  449 -GGGCGAGGGAGACAGAGT 466
```

Figure 5. *Alignment* of *T. gondii* repetitive sequence (sequencing result with *reverse primer*) and *T. gondii* repeat region 529 bp.

The sequence was also aligned with repetitive sequence from the other strain of *T. gondii* to identify the homology. Alignment result showed that *T. gondii* repetitive sequence from sequencing product has 90% to 100% homology with

repetitive sequence several strain of *T. gondii*. The alignment result can be seen in Figure 6.

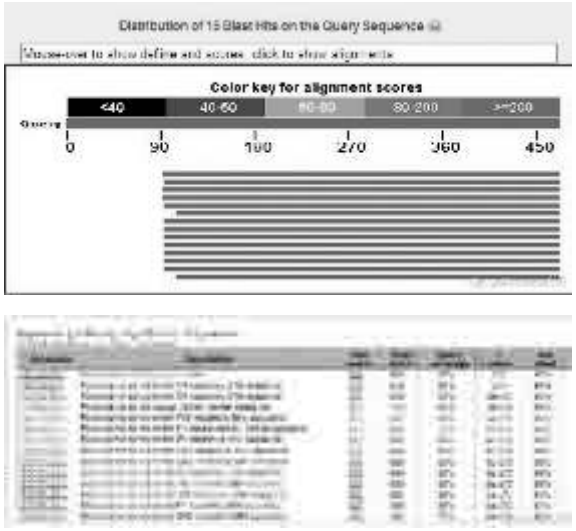

Figure 6. BLAST output with *query T. gondii* repetitive sequence (sequencing product).

The alignment result totaly showed 95,36% homology identity, therefore the repetitive sequence from sequencing result can be used as probe candidate that was highly specific for *Toxoplasma gondii*.

From this sequence analysis, the probe candidate was arranged, resulted in 103 bp length of probe candidate, as seen in Figure 7.

```
1   ctgcagggag gaagacgaaa gttgtttttt tatttttttt tctttttgtt tttctgattt
 61  ttgtttttttt tgactcgggc ccagctgcgt ctgtcgggat gagaccgcgg agccgaagtg
121 cgttttcttt ttttgacttt tttttgtttt ttcacaggca agctcgcctg tgcttggagc
181 cacagaaggg acagaagtcg aaggggacta cagacgcgat gccgctcctc cagccgtctt
241 ggaggagaga tatcaggact gtagatgaag gcgagggtga ggatgagggg gtggcgtggt
301 tgggaagcga cgagagtcgg agagggagaa gatgtttccg gcttggctgc ttttcctgga
361 gggtggaaaa agagacaccg gaatgcgatc cagacgagac gacgctttcc tcgtggtgat
421 ggcggagaga attgaagagt ggagaagagg gcgagggaga cagagtcgga ggctgggacg
481 aagggaggag gaggggtagg agaggaatcc agatgcactg tgtctgcag
```

Figure 6. BLAST output with *query T. gondii* repetitive sequence (sequencing product).

Probe candidate analysis

The probe candidate must have high specificity to the target nucleic acid. Moreover, the probe candidate also should be have no homology with the host's genome and the other parasites which have close genetic relationship with *T. gondii* to minimize cross hybridization. In order to fulfill this qualification, probe candidate was aligned with host's genome and with the other parasites.

The alignment result using BLAST program showed that the probe candidate was not homolog either with host's genome or other parasites which have close lineage with *T. gondii* (homology percentage 0%), thus this probe candidate can be used as diagnosis method for toxoplasmosis. Probe was synthesized in Sigma-Aldrich as the design above.

Probe labeling and labeling quantification

Probe was labeled using *digoxigenin-11-dUTP* with random primed labeling method. The labeling method resulted in labeled probe with total concentration approximately 51 ng/ml. From this concentration, furthermore was made a serial dilution for labeling quantification.

The result of labeling quantification can be seen in Figure 8.

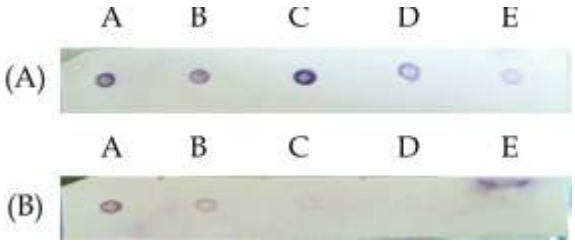

Figure 8. Result of probe labeling quantification
(A) DIG-labeled control DNA, with concentration:
A. 300 pg/ml, B. 100 pg/ml, C. 30 pg/ml, D. 10 pg/ml, E. 3 pg/ml;
(B) Labeled probe, with concentration: A. 15 ng/ml, B. 5,1 ng/ml, C. 1,5 ng/ml, D. 0,51 ng/ml, E. 0,15 ng/ml

This labeling quantification result showed that in DIG-labeled control DNA, the labeling reaction can be detected up to 3 pg/ml concentration, while in labeled probe the reaction labeling can be detected up to 0,51 ng/ml concentration. This quantification result was important to check the efficiency of each labeling reaction by

determining the amount of labeled product. From this result, can be determined the correct amount of probe that should be added to the hybridization solution.

Hybridization of labeled probe and clinical sample

Hybridization was done on tachyzoite DNA as positive control, DNA from clinical sample and total DNA from healthy mice tissue as negative control. The hybridization result is showed in Figure 9.

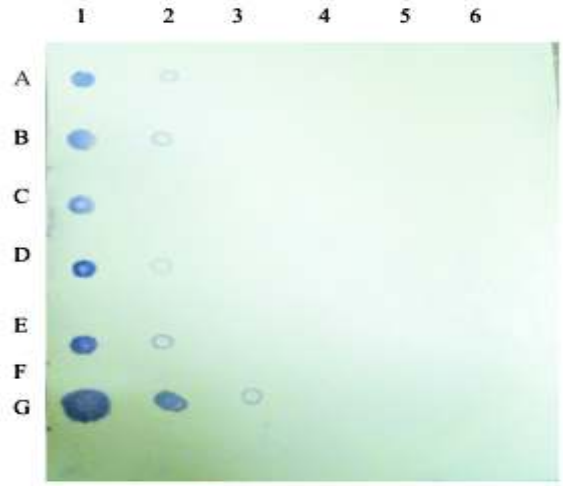

Figure 9. Hybridization of labeled probe and DNA clinical sample
A. DNA from chicken, B. DNA from pig, C. DNA from goat, D. DNA from cow, E. DNA from cat, F. Positive control (tachyzoite DNA), G. Negative control (DNA from healthy mice).
Concentration of sample DNA:
1. 100 ng / ml, 2. 10 ng / ml, 3. 1 ng / ml, 4.100 pg / ml, 5. 10 pg / ml, 6. 1 pg / ml.

Hybridization was done on temperature 42°C, based on calculation of optimum hybridization temperature below:

Tm = 49.82 + 0.41 (% G + C) – 600 / l, T*hyb* = Tm – (20°-25°C), in which:

Tm: *melting temperature*, T*hyb*: optimum hybridization temperature, l: probe length.

This hybridization showed positive result for all sample, in which probe *toxo* could detect the DNA sample up to concentration 10 ng / ml.

Toxoplasmosis detection of clinical DNA sample with PCR (for confirmation)

The clinical DNA sample also tested using PCR method as confirmation for hybridization result. In this method were used the same primers that were used to amplify the *Toxoplasma gondii* repeat region, under the same condition. The result can be seen in Figure 10.

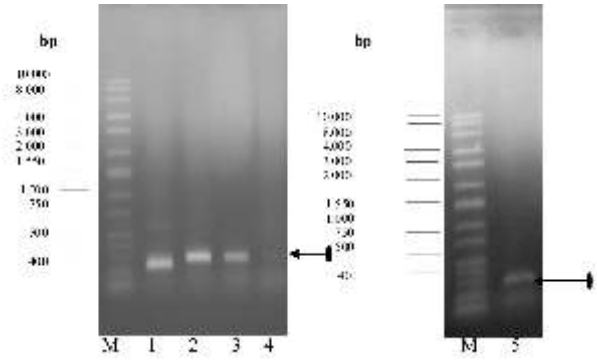

Figure 10. Electrophoresis of PCR result
M. Marker, 1. DNA from pig, 2. DNA from cow, 3. DNA from goat, 4. DNA from chicken, 5. DNA from cat.

The PCR results were agree with the hybridization result, in which all samples showed positive result and indicate that all samples were infected toxoplasmosis.

The research concluded that *probe toxo*-103 bp was highly homolog with several strain of *T. gondii* and it has no homology either with host's genome or other parasites which have close genetic relationship with *T. gondii*. Hybridization analysis showed that probe could detect the complementary nucleic acid up to 10 ng / ìl concentration.

Acknowledgement

We are very much indebted to Prof. Dr. Christian Bauer, DVM for his comment and

correction of this manuscript and also Mrs Arsiyah and Mr. Tukijo for their valuable contributions on this work and maintaining the *Toxoplasma* isolate.

References

Angel, S.O., M. Matrajt, . Margarit, M. Nigro, E. Illescas, V. Pszenny, M.R. Amendoeira, R. Guarnera and J.C. Garber. 1997. Screening for Active Toxoplasmosis in Patients by DNA Hybridization with ABGTg7 Probe in Blood Samples. *J. Clin. Microbiol.* 35 (3), 591-595.

Dubey, J.P. and Lindsay, D.S. 2004. Biology of *Toxoplasma gondii* in Cats and Other Animals in *World Class Parasite: Volume 9: Opportunistic Infections: Toxoplasma, Sarcocystis, and Microsporidia*. Kluwer Academic Publisher.

Homan, W.L., Vercammen, M., De Braekeleer, J., Verschueren, H. 2000. Identification of a 200- to 300- fold Repetitive 529bp DNA Fragment in *Toxoplasma gondii*, and Its Use for Diagnostic and Quantitative PCR. *Int. J. Parasitol.* 30(1), 69-75.

Reischl, U., S. Bretagne, D. Kruger, P. Ernault, J.M. Costa. 2003. Comparison of Two DNA Targets for the Diagnosis of Toxoplasmosis by Real-time PCR using Fluorescence Resonance Energy Transfer Hybridization Probes. BMC. *Infect. Dis*, 3(1), 1-9.

Savva, D and Holliman, R.E. 1990. Diagnosis of Toxoplasmosis Using DNA Probes. *J.Clin.Pathol.* 43, 260-262.

Switaj, K., Master, A., Skrzypczak, M., Zaborowski, P. 2005. Recent trends in molecular diagnostics for Toxoplasma gondii infections. *Clin Microbiol Infect,* 11, 170–176.