# Development of Dot-blot Hybridization Based on 522 bp Repetitive Sequence (*R522*) for Detection of *Toxoplasma gondii*

**Asmarani Kusumawati[1,4]*, Harto Widodo[2], Tenri Ashari Wanahari[3], Nafratilova Septiana[4] and Sri Hartati[5]**

[1]Departement of Reproduction, Faculty of Veterinary Medicine, Gadjah Mada University, Jalan sekip unit II, Yogyakarta, 55281 Indonesia.
[2]Medicinal Plant and Traditional Medicine Research and Developtment Centre, National Institute of Health Research and Development, Ministry of Health Indonesia, Jalan percetakan negara no.29, Jakarta, 10560 Indonesia.
[3]Faculty of Medicine, Sebelas Maret University, Jalan Ir. Sutami 36 A, Surakarta, 57126 Indonesia.
[4]Centre of Biotechnology Study, Gadjah Mada University, Jalan teknika Utara, Yogyakarta, 55281 Indonesia.
[5]Department of Internal Medicine, Faculty of Veterinary Medicine, Gadjah Mada University, Jalan sekip unit II, Yogyakarta, 55281 Indonesia.

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**Toxoplasmosis, arising from infection by *Toxoplasma gondii*, is one of the most common parasitic diseases in humans and other warm-blooded animals. In humans, infections are usually asymptomatic but severe disease can occur in immunocompromized individuals and newborns. Due to the importance of the disease and in order to take suitable measures, an early diagnosis of the disease is essential, particularly for pregnant women and in industry of domestic animals. The genome of *T. gondii* contains repeat sequences *B1* and *R522* which constitute ideal targets for genome-based detection methods. The 522 base pairs repeat sequences *R522* are the most promising due to the high copy number, evaluated to be 200 to 300 units within the genome. We developed a simple dot-blot hybridization based on *R522* sequences. The method is simple and does not require sophisticated devices. The test of the method, using cloned *R522* as target, showed that the parasite detection method was sensitive and proved to be promising for use in routine health controls as well as for the survey of *Toxoplasma* infections.**

**Key words:** DIG-probe, dot blot hybridization, repeat sequences, *R522*, *Toxoplasma gondii.*

Infection with *Toxoplasma gondii* is one of the most common parasitic infections in humans and other warm-blooded animals, including birds. It has been found world-wide in almost one third of the human population (Dubey and Beattie, 1988; Tenter *et al.*, 2000). The infectious agent has been recognized as a category B priority pathogen by the American National Institute of Health (Bethesda, USA) (Weiss and Dubey, 2009). The disease burden of toxoplasmosis is considered as important as that of salmonellosis or campylobacteriosis (Kijlstra and Jongert, 2008).

*T. gondii* is an obligate protozoan intracellular parasite that belongs to the phylum Apicomplexa, subclass coccidia, and constitutes one of the most successful protozoan parasites on Earth, Due to its ability to manipulate the immune system and to establish a chronic infection (Kemp *et al.*, 2012). Transmission of the parsite occurs by ingestion of oocysts shed from feline feces, by ingestion of cysts from chronically infected tissues,

* To whom all correspondence should be addressed.
Tel.: +6281328867709; +6274560863;
Fax:+6274560863;
E-mail: kartapati_2008@yahoo.com

or by vertical transmission (Dubey, 2004; Wong and Remington, 1994). The parasite normally divides asexually to yield a haploid form that can infect virtually any vertebrate. However it also has a defined sexual cycle that occurs exclusively in cats (Sibley and Boothroyd, 1992). Felids, domestic and wild, are the only known definitive hosts.

In many species of animals, severe disease caused by *T. Gondii* infections includes embryonic death and resorption, fetal death and mummification, abortion, stillbirth and neonathal death. In healthy adult humans, toxoplasmosis is usually innocuous or asymptomatic. Yet, infection with *T. gondii* during pregnancy may lead to severe, if not fatal, infection of the fetus. Severe consequences include miscarriage, embryonic death and resorption, fetal death, retinochoroiditis, hydrocephalus, intracerebral calcification and mental retardation (Dubey and Beattie, 1988; Tenter *et al.*, 2000; Wong and Remington, 1994). In immunocompromized patients, *T. gondii* has emerged as an opportunistic infectious pathogen. Encephalitis is the most clinically important manifestation of toxoplasmosis in immunosuppressed patients (Zangerle *et al.*, 1991). This disease is the major cause of death among patients with AIDS. In goat and sheep industries, the disease may result in important economic losses (Ahmed *et al.*, 2008).

In order to take suitable measures and to prevent disease outbreaks, an early diagnosis of toxoplasmosis is essential and the availability of efficient and sensitive diagnostic tools is of great importance, particularly for pregnant women and to prevent economic losses in farmings of domestic animals. Serological diagnosis is a widely used method for detecting *T. gondii* infections. It also allows to distinguish recent from chronic infections (Rilling *et al.*, 2003; Sensini, 2006). Among the currently used gene-targets in molecular diagnosis, repetitive non-coding sequences *B1* and *R522* (repetitive sequence composed of units of 522 bp) are undoubtedly the most promising, Due to their multiple copy number (Kong *et al.*, 2012; Rahumatullah *et al.*, 2012). *B1* contains 30 repeat units while *R522* was evaluated to contain 200 to 300 units within the genome (Homan *et al.*, 2000; Kong *et al.*, 2012; Reischl *et al.*, 2003). We developed a simple dot-blot hybridization method, based on *R522* sequences. The method is simple and does not require sophisticated devices, and several samples can be simultaneously processed on the same membrane. In test experiment, using cloned *R522* as template, the method proves to be sensitive. Due to its ease of use and for economical considerations, dot-blot hybridization can be easily used in routine health controls as well as for the survey or epidemiological investigations of *Toxoplasma* infections in humans and animals. The methodology is particulary suitable in conditions where sophisticated and expensive devices are not available.

## MATERIALS AND METHODS

### Extraction of Toxoplasma DNA and isolation and cloning of the repetitive sequence R522 in pCR2.1-TOPO

The extraction of *T. gondii* DNA and the isolation and cloning of *R522* sequence in pCR2.1-TOPO have been described earlier (Kusumawati *et al.*, 2010; Sri Hartati *et al.*, 2006). Briefly, tachyzoites of an Indonesian isolate IS-1 of *T. gondii* (Iskandar, 1988) were grown in the peritoneal cavity of Balb/c mice and collected from peritoneal exudate 6 days post-inoculation and purified according to Garberi *et al.* (1990). DNA was extracted using PureLink Genomic DNA Kit (Invitrogen).

The repetitive sequence *R522* was isolated and amplified by PCR and directly cloned in pCR®2.1-TOPO® plasmid (Invitrogen). Positive plasmids were purified from white bacterial colonies using High Pure Plasmid Isolation Kit (Roche®) and further analyzed by restriction enzymes and sequencing. Cloned *R522* in pCR2.1-TOPO was consequently named pCR2.1-R522.

### Synthesis of DIG-labeled probe

Probe, specific to the repetitive sequence *R522*, was designed using "Basic Local Alignment Search Tool" (BLAST) (http://www.ncbi. nlm. nih.gov) from the published *R522* nucleotide sequence of *T. gondii* IS-1 (Acc. no. EF195646.1; Kusumawati *et al.*, 2010; Sri Hartati *et al.*, 2006). The probe was isolated, amplified and labeled with digoxigenin by PCR using *R522* sequence cloned in pCR2.1-TOPO (pCR2.1-R522) as template and the primers : actacagacgcgatgccg (upstream); ctccactcttcaattctctcc (downstream) and PCR

Dig labeling mix (Roche®). Amplification was carried out in a final volume of 25 µl, as follows : denaturation at 94°C for 5 min ; 32 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and elongation at 74°C for 1 min then a final incubation at 72°C for 5 min. 1.0 to 2.0 µl of the PCR products was used in 1 ml hybridization solution.

**Dot-blot hybridization**

Dot-blot hybridization was performed on variable amounts of pCR2.1-R522 as target. Plasmids were first denatured by incubation at 100°C for 10 min then chilled on ice for 5 min. 1 µl of plasmid solutions was spotted onto Nylon membrane (Roche®). Hybridization was performed using DIG easy hyb. solution (Roche®) according to the manufacturer's recommendations. The membranes were first baked for 2 h at 80°C under vacuum then incubated for 30 min at 42°C in pre-heated DIG hybridization buffer (approximately 20 ml/100 $cm^2$ of membrane) with a gentle agitation. Hybridization was carried out by adding DIG-labeled probe, obtained by PCR, in 10 µl of buffer at 48°C, according to the formula of Keller and Manak (1989) for variable incubation times. The washing step was done at 68°C with 0.5x SSC 0.1%. Hybrids were detected by Fab fragments, anti-Digoxigenin-AP (Roche®) and revealed by NBT/BCIP (Roche®).

## RESULTS

**Synthesis of DIG-labeled probe**

Using a BLAST programme, as described in Materials and Methods, the best *R522* probe was determined. It is 237 nucleotides long and spans from nucleotide 201 to 438 on the nucleotide sequence of IS-1 *R522* unit (Acc. no. EF195646.1; Kusumawati *et al.*, 2010; Sri Hartati *et al.*, 2006) (Fig. 1). The sequence comparison did not show significant homologies with any of the nucleotide sequence data published in GenBank. Therefore the selected region of *R522* unit constituted an ideal probe for the detection of *T. gondii* by hybridization. Cross-reactions with other pathogens will also be avoided.

DIG-labeled probe was synthetized by PCR as described in Materials and Methods. Agarose gel analysis showed that the length of the PCR product corresponded to the calculated length (Fig. 2). As shown, due to DIG-labeling, the DIG-labeled probe exhibited a higher molecular weight compared to that of the non-labeled corresponding PCR product.

**Optimal conditions of dot-blot hybridization and detection threshold**

Optimal conditions of dot-blot hybridization were established by varying the amounts of the target, i.e. pCR2.1-R522, the DIG-labeled probe and the hybridization time.

An excellent result was already obtained when the hybidization was carried out for 3 hours with 1 ng of pCR2.1-R522. The same efficiency was achieved with either 2.5 µl, 2.0 µl, 1.5 µl or 1 µl of DIG-labeled probe in 1 ml of hybridization medium (Fig. 2.A). It appears so that 1 µl of probe was sufficient and increasing the amount of the probe did not seem to significantly influence the hybridization efficiency. A lower level of efficiency was observed with 0.1 ng of target. The less pronounced response with 1 ng of target and 1 µl of probe (Fig. 3.A.d) is certainly due to a technical error.

201

Acta cag acg cgat gccg ctcctcca gccgtcttgg agga gaga tatca ggac tgta gatg aaa ggcg agggtg agga tgag gggg gt

g gcgtg gttgg gaa gcga cgaga gtcg gaga gggag aaa gatgtttccg gcttgg ctg ctttcctgg agggtgg aaa aga gaca ccg

g aatg cgatcta gacga gaga cg ctttcctcgtggtgatg gcg gaga g aattg aaga gtg gaga agagg cga ggg aga cag agt

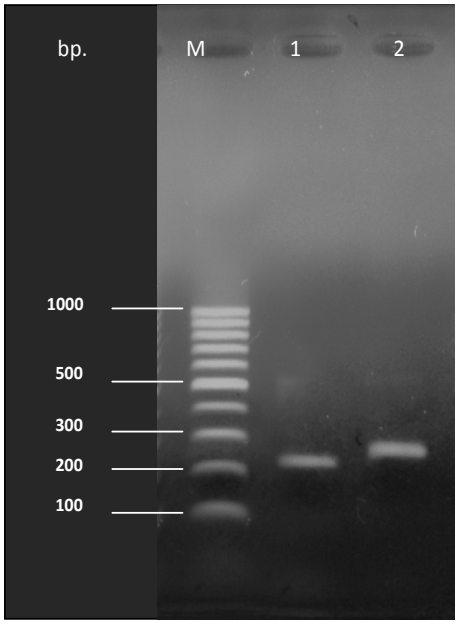
cgg a ggcttg gacg

438

**Fig. 1.** Nucleotide sequence of *R522* probe. The nucleotide sequence is from Kusumawati *et al.* (2010) and Sri Hartati *et al.* (2006); Acc. no. EF195646.1. The probe was obtained by PCR and DIG-labeled

By increasing the incubation times, it appeared that similar response was obtained with 5 hours or overnight (OVN) incubation (Fig. 3.C.D). No noticeable increase of response was obtained with an OVN incubation. The hybridization response was similarly saturated or quasi-saturated with 1 ng or 0.1 ng of target, contrarily to the result obtained with 3 hours incubation. An OVN incubation may be more convenient in practice. However as a 5 h incubation was sufficient, the whole process, including probe synthesis, denaturation-spotting of targets on membrane, and hybridization can be performed in less than ca. 7 hours. With high amounts of target, the process is still faster as a 3 h hybridization is sufficient. So, target detection can be easily achieved in one day work. Finally, it is worthwhile mentioning that with 1 ng of pCR2.1-R522, a saturated response was obtained whatever the time of incubation was. With 10-fold less pCR-*R522*, a saturated response was only observed with longer times (5 h and OVN).

Detection threshold of dot-blot hybridization was determined with 2 µl of probe, 10-fold serial dilutions of target, ranging from 1 ng to 1 pg of pCR2.1-R522, and 5 H or OVN incubation time. In both cases, the hybridization signal decreased with decreasing amounts of pCR2.1-R522 (Fig. 2.B.C). The dot-blot hybridization still allowed the detection of as few as 10 pg of pCR2.1-R522 in the hybridization conditions used. Considering the length of the plasmid, i.e. 4.4 x $10^3$ base pairs, and its molecular weight (ca. 2.6 x $10^6$g $mol^{-1}$) and the Avogadro number (6 x $10^{23}$ $mol^{-1}$), 10 pg of pCR2.1-R522 corresponded to ca. 2.3 x $10^6$ molecules of *R522* units. This number constituted so the detection threshold of the dot-blot hybridization based on the repeat sequence *R522* we developed. As *R522* repetitive sequence is composed of 200 to 300 repeat units in *Toxoplasma* genome, 2.3 x $10^6$ units correspond to ca. 9 000 parasites. This constitutes so the actual detection limit of the dot-blot hybridization we developed. It is in the order of sensitivity attained by hybridization methodology in general (Sri Widada *et al.*, 2003). It is certainly lower (by a factor of 1 000 or more) than that usually attained by gene amplification, as it is always the case but this is largely compensated by its ease of use. Its sensitivity is nevertheless largely sufficient for routine diagnosis of toxoplasmosis.




Lane 1 : unlabeled PCR products; lane 2 : DIG-labeled PCR products; M : DNA markers and their corresponding lengths.

**Fig. 2.** Analysis of DIG-labeled probe by electrophoresis on a 1.8% agarose gel

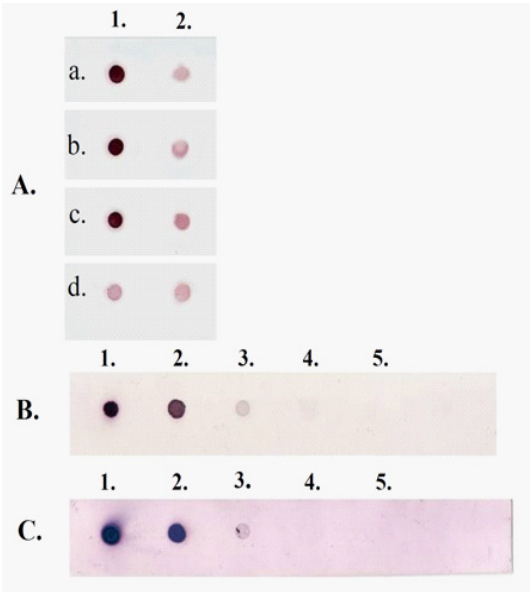



**Fig. 3.** Optimal conditions of dot-blot hybridization and detection threshold. Hybridization times : 3 h (A); 5 h (B); OVN (C). In (A) : 2.5 µl (a); 2.0 µl (b); 1.5 µl (c); 1.0 µl (d) of probe and 1.0 ng (1), 0.1 ng of pCR2.1-R522. In (B) and (C) : 2.0 µl of probe and 1 ng (1), 100 pg (2), 10 pg (3), 1 pg (4) dan 0 pg (5) of pCR2.1-R522

## DISCUSSION

Toxoplasmosis is a common parasite-born disease of humans and warm-blooded animals, birds included. The pathogenic agent, *T. gondii,* is an obligate protozoan intracellular parasite that appears to be one of the most successful protozoan parasites on Earth, Due to its ability to establish a chronic infection (Kemp *et al.*, 2012). Other genera of the phylum Apicomplexa include important pathogens such as *Plasmodium* or *Eimeria*. *T. gondii* is nevertheless the most tractable model for the study of intracellular Apicomplexan parasitism as it can be cultured, easily transfected and genetically studied *in vitro* (Kim and Weiss, 2004).

It is essential that *T. gondii* infections can be diagnosed as early as possible both in humans and domestic animals, by an easy and reliable parasite detection method, to prevent the disease spread and to avoid disastrous effects, particularly on immunocompromized individuals, foetus or neonates. All the more, *T. gondii* infections are in most cases inocuous and have no effect on the health. Numerous diagnostic tools of toxoplasmosis have been developed. Due to financial problems, sophisticated and expensive equipements are not always available and in some circumstances a simple detection method is preferable.

We developed a simple dot-blot hybridization technique which can be applied to field investigations. The method is fast and the whole process, including the synthesis of DIG-labeled probe, may last for less than 7 hours. The process may still take less time as the probe can beforehand be synthetized in lage amounts and stored till use and easily transported everywhere. Among the current gene-based detection methods, dot-blot hybridization is undoubtedly the simplest and the easiest to perform. As several samples can be processed on the same piece of membrane, the method also proves to be economic.

The repetitive sequence *R522* is an ideal target for hybridization methodology as it is composed of 200 to 300 units per tachyzoite cell. The sensitivity of the method has therefore to be increased by a factor of 200 to 300. The dot-blot hybridization we developed exhibits a detection threshold of ca. 9 000 parasites. It is in the same order of sensitivity as those published for other pathogens (Sri Widada *et al.*, 2003). The *R522*-based dot-blot hybridization proves so to be efficient enough. Besides, due to its ease of use, not requiring sophisticated equipments, *R522*-based dot-blot hybridization is highly recommended for use in field surveys of *Toxoplasma* infections as well as epidemiology investigations.

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