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Optimization of deoxyribonucleic acid extraction and polymerase chain reaction methods for gene detection of *Toxoplasma gondii* in goat milk

MIRA FATMAWATI[1,2], LUCIA TRI SUWANTI[3,♥], MUFASIRIN[3,4], DIDIK TULUS SUBEKTI[5], FITRINE EKAWASTI[5], NUNUK DYAH RETNO LASTUTI[3], MIRNI LAMID[6], ENDANG SUPRIHATI[3], MUSTOFA HELMI EFFENDI[7], ANAM AL-ARIF[6]

[1]Student Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[2]Laboratory Public Health and Epidemiology, Faculty of Veterinary Medicine, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia. Tel./fax.: +62-856-908332, ♥email: mirafkh@ub.ac.id
[3]Laboratory of Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[4]Toxoplasma Study Group, Institut of Tropical Disease, Universitas Airlangga. Jl. Unair, Surabaya 60286, East Java
[5]Research Organization for Health, National Research and Innovation Agency. Jl. Raya Jakarta-Bogor Km. 47, Cibinong, Bogor 16912, West Java, Indonesia
[6]Division of Animal Husbandry, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia
[7]Division of Veterinary Pubic Heath, Faculty of Veterinary Medicine, Universitas Airlangga. Jl. Mulyorejo, Surabaya 60115, East Java, Indonesia

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Abstract. *Fatmawati M, Suwanti LT, Mufasirin, Subekti DT, Ekawasti F, Lastuti NDR, Lamid M, Suprihati E, Effendi MH, Al-Arif A. 2023. Optimization of deoxyribonucleic acid extraction and polymerase chain reaction methods for gene detection of* Toxoplasma gondii *in goat milk. Biodiversitas 24: 5905-5911.* Dairy goats (*Capra aegagrus hircus*) are intermediate hosts that transmit *Toxoplasma gondii* to humans through goat milk consumption. *T. gondii* can invade mammary glands and secrete into milk. The method of detecting gene of the *T. gondii* in milk is through a Polymerase Chain Reaction (PCR) test based on specific genes. The objective of this study was to compare the efficacy methods of the *T. gondii* molecular assay from the extraction, master mix for PCR assay, and primer that is possible to amplify the gene of *T. gondii* in goat milk for PCR assay. The research method for purification used flotation using concentrated sugar. This research compared the extraction kit from Kit Extraction 1 (K1), Kit Extraction 2 (K2), and Kit Extraction 3 (K3) with the extraction method listed in the brochure. Meanwhile, for the PCR assay, three types of master mix were used, including Master Mix 1 (M1), Master Mix 2 (M2), and Master Mix 3 (M3). The specific genes used included primers B1, ROP, GRA 1, GRA 7, BAG 1, and P30. Tris borate EDTA (TBE) or Phosphate Buffer Saline Solution (PBS) as buffer milk samples gave the same PCR test results. The results show that the principle of a flotation method test using PBS can be used to prepare the milk sample. The research showed that extraction kit 1 (K1) and extraction kit 2 (K2) gave positive results for the positive control. However, in terms of price, K2 is cheaper than K1. So, to optimize DNA extraction, K2 can be used. The master mix that can be used for PCR testing of milk samples is M1 because M1 gives positive results for positive controls and negative results for negative controls. Primers for optimal results used GRA 7 and B1 with denaturation temperature of 94°C, annealing at 56°C, and an extension of 73°C 33. This research concluded that the type of DNA extraction kit affects the PCR test results for detecting *T. gondii* in goat milk. It is necessary to optimize the type of master mix to be used in PCR testing and primers capable of amplifying the *T. gondii* gene.

Keywords: Dairy goats, gene, PCR assay, *Toxoplasma gondii*

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*. The definitive host of *T. gondii* is cats (*Felis catus*), while intermediate hosts include poultry, cattle (*Bos taurus*), goats (*Capra aegagrus hircus*), sheep (*Ovis aries*), pigs (*Sus domesticus*), and other wildlife (Delgado et al. 2022). Transmission of *T. gondii* to humans occurs by consumption of undercooked raw meat-contaminated tissue cysts, raw milk from dairy cattle, and ingestion of oocytes from environmental matrices such as soil, water, fruit, and vegetables (Fatmawati et al. 2022; Dumètre and Dardé 2003). Horizontally, there are three stages of *T. gondii* infection: oocytes, bradyzoites, and tachyzoites. Oocytes from cat feces can contaminate water and vegetables and reach shellfish or animals consumed from the sea. Bradyzoites Bradyzoite can be found in tissues, such as meat, and can infect humans if consumed in undercooked meat. Tachyzoites can be transmitted by consuming raw milk from goats (*Capra aegagrus hircus*), sheep (*Ovis aries*), camels (*Camelus*), rats (*Rattus*), and cats (*Felis catus*). The potential for transmission of goat milk toxoplasmosis to humans through the consumption of goat milk is due to the tachyzoite and bradyzoites stage (Gazzonis et al. 2019; Ross 2022).

Goat milk has a high nutritional content and has benefits such as anti-inflammatory, heart disease, antidiabetic and antihypertensive, strengthens bones, helps in metabolic efficiency, improves the immune system, and prevention for respiratory disorders such as asthma (Hammam et al. 2022). However, the consumption of raw goat milk has the potential to transmit toxoplasmosis, and this is an important focus in veterinary animal health. Research on the detection of *T. gondii* in milk has been carried out in various countries (Fatmawati et al. 2022; Stelzer et al. 2019). However, in Indonesia, there are no

data on detecting *T. gondii* in goat's milk. The Ministry of Agriculture of the Republic of Indonesia, through the Directorate of Veterinary Public Health in 2023, is planning to carry out surveillance for the detection of *T. gondii* in goat's milk. Therefore, the molecular test for *T. gondii* in goat's milk was optimized to support this program.

Toxoplasma gondii can be detected by serological testing, agent detection, and molecular analysis. The method of detecting the presence of the *T. gondii* gene in milk is through a Polymerase Chain Reaction (PCR) test based on specific genes. Literature studies that have been carried out show that there are differences in the methods of molecular testing for *T. gondii* in milk. Among others, starting from extraction kits, PCR techniques, and primers used in the test. The primers used for *T. gondii* goat milk molecular tests are diverse, such as SGA1, SGA2, TGR1, B1, TOX 4 and 5, ITS 1 and B1. Molecular detection in goat milk used various extraction kit methods, including kit (Nucleospin Macherey Nagel cloth, Germany), Nanodrop kit, QIAamp DNA mini kit (Qiagen, GmbH, Hilden, Germany), Cells and Tissues Genomic Prep TM (GE Healthcare), and iNtron Biotechnology Korea. These different extraction kits are used to extract genes from goat milk samples. The extraction kits were selected, considering that the kits can be easily obtained at an affordable cost. Research using PCR test to detect *T. gondii* in milk has been done by Amairia et al. 2016; Bezerra et al. 2015; Klauck et al. 2016; Luptakova et al. 2015; Martini et al. 2014; Ossani et al. 2017; Saad et al. 2018; Sroka et al. 2017; Tavassoli et al. 2013.

Indonesia Veterinary Science Research Center has a *T. gondii* isolate. Tachyzoites of *T. gondii* (RH virulent strain) were derived from cell culture. This isolate was performed with DNA extracted from tachyzoite cell cultures using 10 PCR-RFLP. Ekawasti et al. (2021) found that genetic markers that could be used for detecting the gene of *T. gondii* are B1#1, B1#2, B1#3, SAG1#1, SAG1#2, P30, BAG1, ROP1, GRA1, and GRA7. There has never been data on the detection of *T. gondii* in goat milk samples from Indonesia. Therefore, this study aimed to optimize the PCR assay to detect *T. gondii* in goat milk. This optimization includes DNA extraction, master mix solution, and primer preparation for best results.

MATERIALS AND METHODS

Sample preparation

Sample purification was carried out using the floating method by concentrated sugar. The principle of this method is based on the specific gravity (SG) of protozoa, which is lighter than the SG of the flotation solution. With this method, protozoa will float to the surface. First, 50 mL of milk was centrifuged at 4,000 rpm for 10 minutes. Then, take the pellet. Saturated sugar was added to the pellets as ½ of the total volume to be mixed and centrifuged at 4,000 rpm for 10 minutes. Then, the supernatant was transferred to another conical tube, added to three times the volume of H_2O, and centrifuged at 4,000 rpm for 10 minutes. The supernatant was removed from the centrifugation, and the pellet was given phosphate buffer saline solution (PBS) and centrifuged at 4,000 rpm for 10 minutes. The sample was then stored in the refrigerator at -18°C for further testing (Sroka et al. 2018; Mancianti et al. 2013).

The sample used was raw goat's milk. The results from the preparation of goat's milk with added TBE and PBS were used to test the type of extraction kit that will be used. For the positive control, tachyzoite isolate was added. Meanwhile, for testing the type of master mix and DNA primer that will be used, a goat's milk sample given the *T. gondii* isolate will be used as a positive control. Meanwhile, UPW and ultra-high temperature milk were used for the negative control because the *T. gondii* tachyzoites RH strain died in pasteurized milk at a temperature of 63°C for 30 minutes (Saridewi et al. 2013).

Toxoplasma gondii isolate

The isolate of the *T. gondii* RH strain was provided by The Indonesian Research Center for Veterinary Sciences, Ministry of Agriculture, Republic of Indonesia. Liquid nitrogen-containing isolates of *T. gondii* have been used for samples.

DNA extraction

Assessment of the efficiency of *T. gondii* DNA detection in goat milk using three variants of DNA extraction commercial kits such as Kit Extraction 1 (K1), Kit Extraction 2 (K2), and Kit Extraction 3 (K3). The manufacturer's instructions followed DNA extraction.

PCR assay

Three kinds of master mix were used, including Master Mix 1 (M1), Master Mix 2 (K2), and Master Mix 3 (K3). The primers used included B1, ROP, GRA 1, GRA 7, BAG 1, and P30 (Table 1).

RESULTS AND DISCUSSION

This research showed that PCR products from sample preparation given by Tris Borate EDTA (TBE) and PBS have the same result. This research also compares the three kit extractions for the same sample. Table 2 shows different PCR results from three different kit extractions.

PCR tests based on adding TBE and PBS showed the same results (Table 2). The difference in the extraction kit used distinguishes the above test results (Figure 1). Even when using the GRA1 primer, the test results were all positive. Meanwhile, using the ROP primer, the results showed no difference between the PCR test using K1 and K2 DNA Kit extraction. The PCR extraction kit using K1 DNA Kit Extraction showed that the GRA 1 primer showed positive results for all positive controls given tachyzoite isolates and samples given TBE and PBS.

PCR results using ROP primers showed positive results in positive samples when using the K1 and K2 extraction kits. However, the PCR results with the same primers but using the K3 extraction kit showed positive results for positive samples if added with PBS and negative results for

positive samples added with TBE. Moreover, a negative PCR result was shown if a positive sample was taken from the pellet and extracted with K3. The selection of extraction kits is the one that is cheap and could give a minimum false negative result. Therefore, K2 Kit Extraction was chosen for DNA extraction from goat milk.

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Primary optimization was performed using primers ROP, GRA 1, GRA 7, SAG 1 or P30 and GRA 1. This optimization of the master mix was also carried out using three master mixes: M1, M2, and M3.

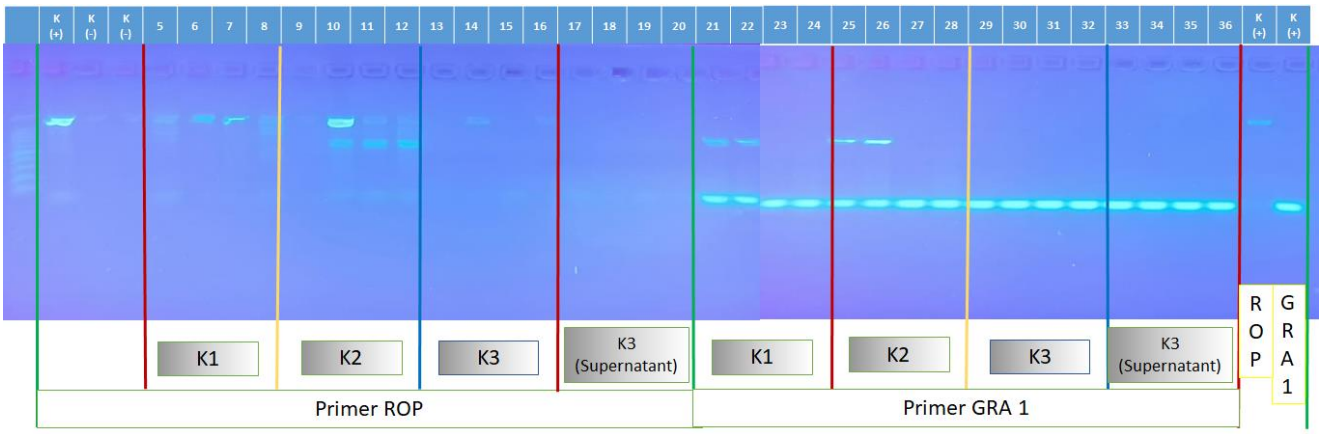

Figure 1. PCR result from different DNA kit extraction and 2 different primer (ROP and GRA1). Different kit extraction affects amplification of PCR result

Table 1. The sets of *Toxoplasma gondii* gene primers (Ekawasti et al. 2021)

Num	Primer	Nucleotides	Amplicon size (bp)
1	B1	F1 TGTTCTGTCCTATCGCAACG; R1 ACGGATGCAGTTCCTTTCTG	508
2	ROP	F CGTGACATATACTGCACTGAC; R CATCGTCAAACTCGATCAC	1220
3	GRA1	F CGGTTTGCTTGTGTTGTTTG; R CATGGGGTACGATCACAACA	802
4	GRA 7	F GCGGATCCGCCACCGCGTCAGATGAC; R CGGGATCCCTACTGGCGGGCATCCTC	616
5	BAG 1	F AGGAGAGAAGACCTCGAAAGAAG; R TGAACGCTAGGTTTCTGGATACG	460
6	P30	F CACACGGTTGTATGTCGGTTTCGCT; R TCAAGGAGCTCAATG TTACAGCCT	340

Table 2. PCR result from different DNA extraction kit with primer ROP and GRA 1

DNA extraction kit	Primer ROP				Primer GRA 1			
	Sample, TBE, Tachyzoites	Sample, PBS, Tachyzoites	Sample, TBE	Sample PBS	Sample, TBE, Tachyzoites	Sample, PBS, Tachyzoites	Sample, TBE	Sample PBS
K1	+ thick	+ thick	+ thin	+ thin	+	+	+	+
K2	+ thick	+ thick	+ thin	+ thin	+	+	+	+
K3	-	+ thin	+ thin	-	+	+	+	+
K3 (supernatant of sample)	-	-	-	-	+	+	+	+

Note: The "+" indicates PCR amplified product, and "-" indicates non. Thick and thin information showed bands resulting from DNA electrophoresis thick or thin as a PCR product

The results of the PCR test using master mix M1 showed that the positive control could amplify the *T. gondii* gene using various primers (Table 3). Whereas for the negative control, it showed that for the primary GRA 1 and ROP, the results were positive (false positive). As for the primers B1, GRA 7, and P30, the results were negative. However, for primary BAG 1, it shows that for UHT milk, the results are positive, while for UPW, the results are negative. These findings show the appropriate results' consistency if primers B1, GRA7, and P30 are used.

PCR test results using master mix M2 showed that the PCR test results for positive controls varied for primers B1, ROP, GRA1, GRA7, BAG1, and P30, likewise, with the results of the PCR test on the negative control. However, the PCR test showed consistent negative results using the GRA 7 primer (Table 4). But for other primer showed positive results (false positive).

PCR test results using master mix M3 showed that all results were negative (Table 5). The data showed that using master mix M3 gives a false negative for control positive. Optimization test results using a variety of primers showed that the master mix M1 gave consistently positive results for the positive control. Meanwhile, master mix M2 showed positive results in the positive control with GRA 7, BAG, and P30 primers. The results were negative for the other positive controls, unlike the PCR master mix M3, which showed negative results for both positive and negative controls. The primers that show positive, consistent results for positive samples and negative test results for negative controls are GRA7 and B1.

Discussion

The life cycle of *T. gondii*, which allows it to be excreted in milk, is tachyzoite or bradyzoite. The mechanism of entry of *T. gondii* into the mammary glands is the ability of *T. gondii* to secrete effector molecules from secretory organelles, microcinemas, rheptries, and solid granules. This protein allows *T. gondii* to affect signaling pathways with the host and allows entry into host cells. The ROP and GRA proteins form parasitophorous vacuole (PV), leading to an attack on the immune pathway of the host, in this case, the dairy goat. The difficulty in identifying *T. gondii* microscopic is due to its similarity with other protozoa, such as *H. hammond*, *Sryptosospora spp*., and *Sarcocystis spp*. Therefore, a molecular testing method was performed to confirm the diagnosis.

The confectionery method allows the microorganisms to be present in the milk. This test is used to identify worm eggs. Saturated sugar is used because this specific gravity can reach the egg's specific gravity range for the egg to float. Unlike saturated sugar, saturated NaCl cannot float eggs whose specific gravity exceeds the specific gravity of the solution. Therefore, the saturation line may be recommended for fecal examination by flotation. In general, the efficiency of fecal flotation is affected by the type of flotation solution, specific gravity, flotation time, and the homogeneity of the solution after centrifugation. The flotation solution plays an important role in making the eggs float, making it easier to observe. It works based on the difference in specific gravity of some chemical solutions (1,120-1,210 g/mL) and worm larvae eggs (1,050-1,150 g/mL) to make the eggs float to the surface of the water and separate the large particles in the feces out. Meanwhile, the density of *T. gondii* cyst is 1.056. The method of floating with sugar is common. Tissue cysts can also be separated on discontinuous gradients with 25-30% Percoll solutions (Watts et al. 2017).

The addition of TBE and PBS for sample preparation in this study showed the same PCR results. This research is aligned with Mancianti et al. (2013), which added EDTA and Tris HCl; Ahmed et al. (2014), adding PBS in the milk pellets before PCR testing. Tris-borate-EDTA (TBE) is a TE (Tris-borate-acetate) buffer consisting of 0.13 M Tris (pH 7.6), 45 mM boric acid, and 2.5 mM EDTA. In addition, TBE is a commonly used buffer for DNA agarose gel electrophoresis.

Table 3. PCR assay using master mix M1

Sample/primer	B1	ROP	GRA1	GRA7	BAG1	P30
Control positive						
- Tachyzoites	+	+	+	+	+	+
- The Isolate of *T. gondii* RH strain	+	+	+	+	+	+
Control negative						
- UPW	-	+	+	-	-	-
- Milk UHT	-	+	+	-	+	-

Note: The "+" indicates PCR amplified product, and "-" indicates non. UHT: Ultra High Temperature

Table 4. PCR assay using master mix M2

Sample/primer	B1	ROP	GRA1	GRA7	BAG1	P30
Control positive						
- Tachyzoites	-	-	-	+	+	+
- The Isolate of *T. gondii* RH strain	-	-	-	-	-	-
Control negative						
- UPW	-	-	-	-	-	-
- Milk UHT	+	+	+	-	+	+

Note: The "+" indicates PCR amplified product, and "-" indicates non. UHT: Ultra High Temperature

Table 5. PCR assay using master mix: PCR master mix M3

Sample/primer	B1	ROP	GRA1	GRA7	BAG1	P30
Control Positive						
- Tachyzoites	-	-	-	-	-	-
- The Isolate of *T. gondii* RH strain	-	-	-	-	-	-
Control Negative						
- UPW	-	-	-	-	-	-
- Milk UHT	-	-	-	-	-	-

Note: The "+" indicates PCR amplified product, and "-" indicates non. UHT: Ultra High Temperature

The research by Ossani et al. (2017) was conducted on direct DNA extraction from milk samples. However, research by Gazzonis et al. (2019) to reduce casein interference in milk, added 200 µL TE (1 mM EDTA, 10 mM Tris HCL - HCl (pH 7.6), and 300 µL EDTA 0.5 (pH=8) before DNA extraction. The research by Sroka et al. (2017) showed that after adding EDTA, the samples were resuspended and centrifuged. The same method was later adopted by Amairia et al. (2016), Vismarra et al. (2017), and Sadek et al. (2015). The basic concept of milk sample purification is to optimize subsequent tests so that when extracted, the cells match the target *T. gondii* gene. Goat's milk is a perishable food and an excellent growth medium for microorganisms. Based on the results of the microscopy, it can be observed that there are many bacteria in the milk. Raw goat milk contains contaminated bacteria (TPC), *S. aureus*, *Enterobacteriaceae,* and *coliform* bacteria, which can harm consumers (Wanniatie et al. 2019). In addition, goat milk can transmit many pathogenic bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter spp*., and *Coliform* (Suguna et al. 2012). However, microscopic examinations revealed no specific tachyzoite formation. The next project calls for conducting in silico research to find genetic primers for *T. gondii*, which has an affinity for bacteria found in milk.

The extractor choice depends on the target organism type and how to lyse the target DNA. Types of bacterial agents with thick walls will differ from those with thin walls, like protozoa. The main principles in DNA extraction are the lysis, binding, washing, and eluting stages. The lysis process includes physical, chemical, and enzymatic methods. For the enzymatic method, enzymes to lyse agents used are lysozyme, zymolase and lyticase, proteinase K, collagenase, and lipase. The use of proteinase k and RNAase can affect the final results of the DNA extract. Therefore, in this research, optimization was performed by extraction using 3 commercial kits, as shown in Tables 2, 3, and 4.

Extraction kits 1 (K1) and 2 (K2) have been provided with buffer fluid, washing water, elution buffer, and Proteinase. However, RNAase is added to Extraction Kit 1 (K1). For extraction kit 3 (K3), conventional extraction methods use lysis and homogenize systems using reagents; however, DNA precipitation uses lysate and ethanol. Using ethanol, which evaporates very quickly, requires expertise. So, it needs to be careful when precision is formed, and then the ethanol drying and washing process is carried out twice. Therefore, based on the components contained in the two extraction kits, this research shows that the one that provides the most optimal results is using extraction kit 3.

This research obtained different final PCR results for each master mix used. The basic concept of this test is a mixture of dNTP (needed as a substrate for the formation of new DNA strands), MgCl 2, Taq polymerase (an enzyme needed to build new DNA strands), pH buffer, and mixed in nuclease-free water. The master mix usually includes DNA polymerase, dNTPs, MgCl2, and buffer. Moreover, several brands of the master mix have different ingredients. The Use of DNA Polymerase in master mix 3 (K3) is a 94 kDa thermostable DNA polymerase, which was expressed in *E.coli* by cloning the Thermus aquaticus polymerase gene. DNA Polymerase is a protein derived from E. coli; this protein is purified to high purity by removing DNA. This master mix differs from master mixes 1 and 2, which do not provide information regarding Taq DNA Polymerase. The research showed that the master mix that gave optimal results for the PCR test on milk was Master Mix 1 (M1) (Table 2). This research found that the PCR test using Master Mix M1 showed the positive control could amplify the *T. gondii* gene using various primers. The DNA polymerase in M1 has been developed to provide stronger amplification than other commonly used polymerases, allowing it to work well with challenging templates in the presence of PCR inhibitors. Additionally, its highly efficient nature provides excellent results under fast PCR conditions.

Master mixes were produced to give more efficiency to the PCR method. Many ready-to-use PCR master mixes are available to help save time and avoid errors in estimation and proportions. Most commercial PCR master mixes are intended for a specific type of PCR with a specific temperature in certain thermocyclers. Some master mixes can be used for continuous PCR (or quantitative PCR or qPCR), which relies on a thermocycler that can recognize movement responses by comparing the increase in fluorescence of a memorized color to that of an expert mixture. Before using the master mix, preparation is required. Additionally, It may need to be liquefied, diluted with PCR-quality water, and mixed via a pipette or rotator. (Souza et al. 2023).

PCR assay optimization must be performed to obtain accurate results. There are several types of sample purification studies, and each step identifies weaknesses. This research shows that the primer that can be used in PCR examination with goat's milk samples is primer B1. It can be seen in Table 3, where primer B1 gives positive PCR results in positive controls and negative results in negative samples. PCR method for *T. gondii* targeting the B1 gene was established in 1989. This method has been widely used in the prenatal diagnosis of congenital toxoplasmosis and *T. gondii* infection in immunocompromised patients. Multicopy ITS-1 and 18S rDNA have also been used as targets in a few studies, showing similar sensitivity to the B1 gene [127-129]. Ekawasti et al. (2023a) stated that the assay using the B1 target gene is more sensitive than the P30 gene.

Primer B1 can be used for external and internal PCR tests (nPCR). The B1 gene can be amplified in all currently tested *T. gondii* strains. *Toxoplasma gondii* DNA was identified by amplification of the B1 gene in the PCR molecular assay. PCR method with target gene B1 has been used since 1989, and this method is widely used to diagnose congenital *T. gondii* in immunocompromised patients. According to other research, Multicopy ITS-1 and 18S rDNA have been used as targets in several studies showing similar sensitivity generation B1 (Liu et al. 2015).

Granule antigen (GRA) develops in the vacuolar space after the agent invades the host cell, interfacing the plasma membrane parasites with PV membranes. Likewise, the GRA protein released by bradyzoites was found to be associated with the ground substance of the cyst wall or

detected in its membrane barrier. All forms of *T. gondii* can express GRA7, and the anti-GRA7 antibodies are generated during infection, mainly in humans and rodents. GRA7 appears capable of inducing artificial liposome tubulation and has been implicated in the acquisition of nutrients by the parasite through a mechanism involving the uptake of host endolysosomes. Therefore, GRA7 is widely used for serological and molecular diagnosis of toxoplasmosis (Ekawasti et al. 2023b).

In conclusion, the type of DNA extraction kit affects the PCR test results to detect *T. gondii* in goat milk. It is necessary to optimize the type of master mix to be used in PCR testing and primers capable of amplifying the *T. gondii* gene.

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