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RESEARCH ARTICLE

Comparison of Recombinase Polymerase Amplification with Polymerase Chain Reaction for Species Identification of *Trypanozoon* Members Based on Minicircle and Maxicircle Genes

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ABSTRACT

Surra, caused by *Trypanosoma* (*T.*) *evansi*, causes significant economic losses in many parts of the world including Indonesia. Unfortunately, *T. evansi* is very difficult to distinguish morphologically from other members of the *Trypanozoon* subgenus (*T. equiperdum* and *T. brucei* sensu lato). Molecular identification using polymerase chain reaction (PCR) with minicircle primers followed by maxicircle primers is a possible approach to be applied in *Trypanozoon* endemic area such as Asia and Africa. However, PCR is not suitable for application in laboratories with limited resources. The recombinase polymerase amplification (RPA) technique is a promising alternative to PCR for resource-constrained laboratories or point-of-care (POC) settings. In this study, we compared the diagnostic capabilities of RPA and PCR. To achieve that, a total of 39 isolates, comprising 12 isolates of *T. evansi* and 27 isolates of *T. equiperdum*, were tested using PCR and RPA. RPA and PCR had an agreement coefficient of >0.9, categorized as a very good agreement according to Altman's criteria. The comparison between RPA and PCR showed an agreement of 97.4 and 98.7% for identification and detection, respectively, while the level of detection of RPA and PCR was 10^1 trypanosomes/mL. These results indicate that RPA is fundamentally promising technique for detection of *Trypanozoon* members in laboratories with limited facilities as an alternative to PCR.

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INTRODUCTION

Trypanosomiasis, especially surra, is reported to cause enormous economic loss due to mortality, decreased performance and productivity. These economic losses have been estimated to reach US$223 million in Somalia and IDR 25.7 billion (equivalent to US$1,7 million) in East Nusa Tenggara province of Indonesia (Subekti *et al*., 2024a). *Trypanosoma* (*T.*) *evansi*, is difficult to distinguish from other species of the *Trypanozoon* subgenus, such as *T. brucei* and *T. equiperdum*, due to morphological similarities (Wen *et al*., 2016; Gizaw *et al*., 2017; Subekti *et al*., 2024b). However, using the polymerase chain reaction (PCR), species of *Trypanozoon* can be distinguished with minicircle and maxicircle primers following a precise identification algorithm (Subekti *et al*., 2023; 2024b).

PCR is also considered sensitive because it only requires a trace amount of DNA to generate enough copies for detection. Unfortunately, its limitations are also related to its high sensitivity. If the sample is contaminated by even trace amounts of DNA, misleading

results can be produced (Smith and Osborn, 2009; Garibyan and Avashia, 2013). Another limitation of PCR is that the requirement for a thermal cycler to enable the cyclic heating and cooling process has largely restricted its application in laboratories with limited resources (Li *et al*., 2019).

Unfortunately, in several developing countries such as Indonesia, the application of PCR using a thermal cycler is still limited, especially at points of care (POC), which are usually located in remote villages with very limited resources. PCR has several limitations, especially dependence on a PCR machine, skilled operators, and an adequate laboratory environment, thereby preventing its application (Li *et al*., 2019), especially at POC and mobile laboratory units in the field.

An alternative that can overcome these limitations is recombinase polymerase amplification (RPA). RPA is an isothermal amplification that can run at single temperature ranging 37–42°C (Lobato and O'Sullivan, 2018; Lv *et al*., 2022). Compared with PCR, isothermal amplification, especially RPA, can achieve rapid detection in limited facilities (Zhang *et al*., 2024). RPA technology is an isothermal nucleic acid amplification technology developed by Peipenburg and co-workers from TwistDx, England in 2006 (Zhang *et al*., 2024). RPA is a nucleic acid amplification technology (NAAT) that uses three main proteins, namely recombinase, recombinase loading factor, and single-strand binding protein, to open the double strand of DNA to complete nucleic acid amplification, thus replacing the thermal cycle process in PCR technology (Li *et al*., 2019; Zhang *et al*., 2024). RPA is very suitable for amplifying genes with a length of around 100-400 bp (Zhang *et al*., 2024).

However, a direct comparison between PCR and RPA, especially for the species identification of *Trypanozoon* using two pairs of primers, has not been reported thus far. This study aimed to evaluate species identification of members of the subgenus *Trypanozoon* with RPA using minicircle and maxicircle primers in stages compared to PCR using the same primers. Although this research focuses more on *T. evansi* and *T. equiperdum*, this technique may also be adopted and applied in other countries where *T. brucei* sensu lato infections naturally occur.

MATERIALS AND METHODS

Trypanosome Isolates and Identification: A total of 39 isolates were used in this study (Table 1). All trypanosomes were propagated in DDY mice according to the animal ethics committee's guidelines. All trypanosomes were identified morphologically and molecularly following the algorithm shown in Fig. 1. The morphological and molecular identification confirmed by PCR using ESAG6/7 primers to identify the *Trypanozoon* subgenus. Species identification was performed using the Mini primer (targeting the minicircle gene) followed by the Maxi primer (targeting the maxicircle gene). Based on the algorithm, the trypanosomes used in this study were identified as *T. evansi* (12 isolates) and *T. equiperdum* (27 isolates). All primers and PCR programs used (Fig. 1) followed previous research (Subekti *et al*., 2023).

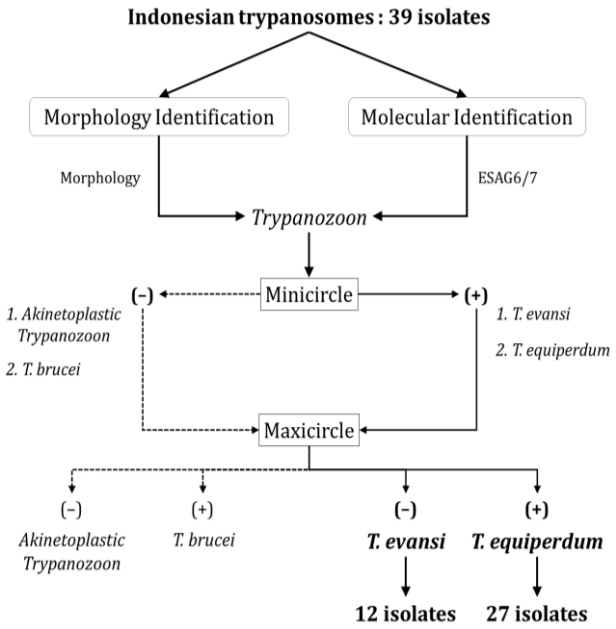

Fig. 1: Species identification algorithm of the subgenus *Trypanozoon.*

Trypanosome Propagation, Purification, and Dilution: Briefly, all trypanosomes were propagated in DDY mice and harvested when a parasite concentration of 10^8 trypanosomes/mL or more was reached (Subekti *et al*., 2023). Trypanosomes were purified by anion exchange chromatography using the Toyopearl 650M DEAE (Tosoh Bioscience, USA) as described previously (Subekti *et al*., 2023, 2024a). The experiments followed the guidelines of the Animal Ethics Commission of the Indonesian Agricultural Research and Development Agency (number Balitbangtan/BB Litvet/Rd/06/2021).

Before extraction, some of the harvested trypanosomes were serially diluted to obtain concentration of 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 trypanosomes/mL. The parasite concentration used for the level of detection assay was 10^1 to 10^6 trypanosomes/mL

DNA Extraction and PCR: DNA extraction was performed on 200µL of purified trypanosome suspension using DNAzol (Molecular Research Center Inc., USA) according to the manufacturer's guidelines. Molecular identification via PCR and RPA involved three primers, namely ESAG6/7, Mini, and Maxi (Table 2). ESAG6/7 primers: amplify the expression site-associated genes (ESAG) region 6 and 7 that encode transferin receptor of trypanosome (Subekti *et al*., 2023). The Mini primers amplified the *gRNA-kDNA minicircle* gene (Subekti *et al*., 2023), while the Maxi primers amplified the *nad5-kDNA maxicircle* gene (Li *et al*., 2006, 2007; Suganuma *et al*., 2016; Büscher *et al*., 2019).

The 25µL PCR reaction of illustra™ PuReTaq Ready-To-Go contained 1µL (100ng/µL) template, 1µL (20µM) of each forward and reverse primers, and 22µL of nuclease-free water (Promega, USA). The Mini and Maxi primers were run in the same cycle conditions. The PCR cycle used in this study involved initial denaturation for 1 min at 94°C, followed by 35 cycles of denaturation (1 min at 94°C), annealing (2 min at 55°C), and extension (2 min at 72°C). The same PCR cycle was used for the LOD assay using ESAG6/7 primers.

Table 1: Indonesian trypanosomes identified and used in this study

No.	Isolate code	Species	Origin (City, Province)	Year isolated	Host
1	BTN01	*T. equiperdum*	Pandeglang, Banten	2013	Buffalo
2	BKN-EJ	*T. equiperdum*	Bangkalan, East Java	1988	Buffalo
3	BKLTZ	*T. equiperdum*	Bengkulu Selatan, Bengkulu	2015	Bali cattle
4	ACBT-YF1	*T. equiperdum*	Aceh Besar, Aceh	2015	Cattle
5	BOPKL	*T. equiperdum*	Pekalongan, Central Java	1982	Buffalo
6	SB-PR	*T. evansi*	Sumba Timur, East Nusa Tenggara	2014	Buffalo
7	TUBA-PR	*T. equiperdum*	Tulang Bawang, Lampung	2015	Buffalo
8	GKT-WB	*T. equiperdum*	Ketapang, West Kalimantan	2011	Buffalo
9	BRBS-9	*T. evansi*	Brebes, Central Java	2017	Buffalo
10	GWA-SC3	*T. equiperdum*	Gowa, South Sulawesi	1984	Buffalo
11	MNS-NC2	*T. equiperdum*	Minahasa, North Sulawesi	1984	PO cattle
12	PWJ	*T. equiperdum*	Purworejo, Central Java	1986	Horse
13	ERK-SC2	*T. evansi*	Enrekang, South Sulawesi	1986	Bx cattle
14	BTN05	*T. equiperdum*	Serang, Banten	2013	Buffalo
15	BRBS-7	*T. equiperdum*	Brebes, Central Java	2017	Buffalo
16	MNS-NC3	*T. equiperdum*	Minahasa, North Sulawesi	1984	Horse
17	SBWNT2	*T. evansi*	Sumbawa Besar, West Nusa Tenggara	1998	Cattle
18	BTN02	*T. equiperdum*	Pandeglang, Banten	2013	Buffalo
19	BTN03	*T. equiperdum*	Pandeglang, Banten	2014	Buffalo
20	BTN04	*T. equiperdum*	Pandeglang, Banten	2014	Buffalo
21	BTN11	*T. equiperdum*	Pandeglang, Banten	2014	Buffalo
22	BTN12	*T. equiperdum*	Pandeglang, Banten	2014	Buffalo
23	BRBS-11	*T. equiperdum*	Brebes, Central Java	1996	Buffalo
24	BRBS-12	*T. equiperdum*	Brebes, Central Java	1996	Buffalo
25	BRBS-14	*T. equiperdum*	Brebes, Central Java	1985	Buffalo
26	BOO	*T. equiperdum*	Bogor, West Java	1987	Cattle
27	DMK-CJ	*T. equiperdum*	Demak, Central Java	1994	Buffalo
28	BTN15	*T. evansi*	Pandeglang, Banten	2014	Buffalo
29	BTN16	*T. evansi*	Pandeglang, Banten	2014	Buffalo
30	BYW-EJ2	*T. evansi*	Banyuwangi, East Java	1992	PO cattle
31	BTN09	*T. evansi*	Pandeglang, Banten	2014	Buffalo
32	BTN18	*T. equiperdum*	Pandeglang, Banten	2023	Buffalo
33	PBN-CB2	*T. evansi*	Kotawaringin Barat, Central Kalimantan	2022	Horse
34	KDGN-SB4	*T. equiperdum*	Hulu Sungai Selatan, South Kalimantan	2023	Bali cattle
35	DPSU-EB4	*T. evansi*	Penajam Paser Utara, East Kalimantan	2016	Deer
36	DPSU-EB5	*T. evansi*	Penajam Paser Utara, East Kalimantan	2016	Deer
37	KDGN-SB5	*T. equiperdum*	Hulu Sungai Selatan, South Kalimantan	2023	Bali cattle
38	KDGN-SB6	*T. equiperdum*	Hulu Sungai Selatan, South Kalimantan	2023	Bali cattle
39	TBN-EJ	*T. evansi*	Tuban, East Java	1984	Buffalo

RPA was performed using the TwistAmp® Basic Kit (TwistDx Ltd., UK). The 50µL RPA reaction contained 2µL of each primer (20µM forward and reverse), 29.5µL of rehydration buffer, 2µL of 100ng/µL DNA template, and 12µL of nuclease-free water mixed together in the TwistAmp™ Basic reaction buffer. Before incubation, 2.5µL of 280mM magnesium acetate was added. The incubation temperature for each primer was optimized to 38°C, 40°C, and 42°C.

The PCR products (amplicons) were electrophoresed in a 1.5% agarose gel with the SYBR Safe DNA stain (Invitrogen, USA) using the RunVIEW visualization system (Cleaver Scientific Ltd., UK) and visualized using a Clear View UV Transilluminator (Cleaver Scientific Ltd., UK).

Table 2: PCR and RPA primers used in this study

Name	Sequence	Amplicon	Reference
ESAG6/7	F: 5'-ACATTCCAGCAGGAGTTGGAG-3' R: 5'-CACGTGAATCCTCAATTTTGT-3'	237 bp	Subekti et al., 2024b
Mini	F: 5'-CAACGACAAAGAGTCAGT-3' R: 5'-ACGTGTTTTGTGTATGGT-3'	357 bp	Artama et al., 1992
Maxi	F: 5'-TGGGTTTATATCAGGTTCATTTATG-3' R: 5'-CCCTAATAATCTCATCCGCAGTACG-3'	395 bp	Subekti et al., 2024b

F = forward; R = reverse

Data Analysis: The PCR and RPA results were compared, and their agreement was tested using AgreeStat360 (https://agreestat360.com/). The agreement coefficients obtained were interpreted using Altman benchmarking (Altman, 1991).

RESULTS

RPA Optimization: The temperature and incubation time of each primer for RPA have been successfully optimized. Based on this optimization (Table 3), DNA amplification using the RPA technique with ESAG6/7 and Mini primers was carried out at temperatures of 38°C and 40°C for 30 min, while RPA using Maxi primers was carried out at a temperature of 42°C for 60 min.

Analytical Sensitivity: In this study, PCR and RPA displayed the same detection of 10^1 trypanosomes/mL (Fig. 2). This detection level of RPA using the ESAG6/7 primer was adequate for detecting trypanosomes in blood samples. The ESAG6/7 primer was useful for detecting the *Trypanozoon* subgenus, while the Mini and Maxi primers were useful for distinguishing species.

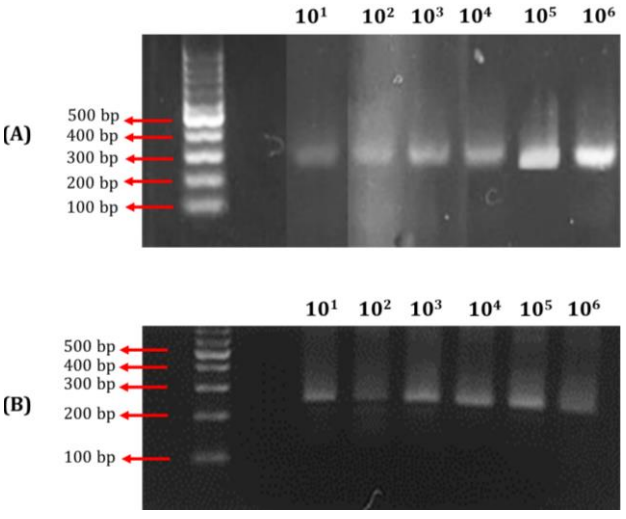

Fig. 2: PCR and RPA detection levels for increasing trypanosome concentration (10^n trypanosome/mL)

Table 3: RPA incubation temperature and time optimization of three primer pairs

	38 °C		40 °C		42 °C	
	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.
ESAG6/7	+	+	-	-	-	+
Mini	-	-	+	+	-	-
Maxi	-	-	-	-	+	++

Comparison of RPA and PCR: Amplification with RPA using Mini primers detected all isolates, like in case of PCR. Conversely, amplification with RPA using Maxi primers gave some negative results that were positive with PCR. This occurred for isolate BTN04, which was identified as *T. equiperdum* Isolate BTN04 came from Pandeglang, Banten province. Thus, the Maxi primers produced one error (2.6%) during amplification using RPA (Table 4).

Table 4: Results of amplification with RPA and PCR using Mini and Maxi primers

No.	Isolate code	Species	Minicircle RPA	Minicircle PCR	Maxicircle RPA	Maxicircle PCR
1	BTN01	*T. equiperdum*	+	+	+	+
2	BKN-EJ	*T. equiperdum*	+	+	+	+
3	BKLTZ	*T. equiperdum*	+	+	+	+
4	ACBT-YF1	*T. equiperdum*	+	+	+	+
5	BOPKL	*T. equiperdum*	+	+	+	+
6	SB-PR	*T. evansi*	+	+	–	–
7	TUBA-PR	*T. equiperdum*	+	+	+	+
8	GKT-WB	*T. equiperdum*	+	+	+	+
9	BRBS-9	*T. evansi*	+	+	–	–
10	GWA-SC3	*T. equiperdum*	+	+	+	+
11	MNS-NC2	*T. equiperdum*	+	+	+	+
12	PWJ	*T. equiperdum*	+	+	+	+
13	ERK-SC2	*T. evansi*	+	+	–	–
14	BTN05	*T. equiperdum*	+	+	+	+
15	BRBS-7	*T. equiperdum*	+	+	+	+
16	MNS-NC3	*T. equiperdum*	+	+	+	+
17	SBWNT2	*T. evansi*	+	+	–	–
18	BTN02	*T. equiperdum*	+	+	+	+
19	BTN03	*T. equiperdum*	+	+	+	+
20	BTN04	*T. equiperdum*	+	+	–	+
21	BTN11	*T. equiperdum*	+	+	+	+
22	BTN12	*T. equiperdum*	+	+	+	+
23	BRBS-11	*T. equiperdum*	+	+	+	+
24	BRBS-12	*T. equiperdum*	+	+	+	+
25	BRBS-14	*T. equiperdum*	+	+	+	+
26	BOO	*T. equiperdum*	+	+	+	+
27	DMK-CJ	*T. equiperdum*	+	+	+	+
28	BTN15	*T. evansi*	+	+	–	–
29	BTN16	*T. evansi*	+	+	–	–
30	BYW-EJ2	*T. evansi*	+	+	–	–
31	BTN09	*T. evansi*	+	+	–	–
32	BTN18	*T. equiperdum*	+	+	+	+
33	PBN-CB2	*T. evansi*	+	+	–	–
34	KDGN-SB4	*T. equiperdum*	+	+	+	+
35	DPSU-EB4	*T. evansi*	+	+	–	–
36	DPSU-EB5	*T. evansi*	+	+	–	–
37	KDGN-SB5	*T. equiperdum*	+	+	+	+
38	KDGN-SB6	*T. equiperdum*	+	+	+	+
39	TBN-EJ	*T. evansi*	+	+	–	–

In general, the agreement between RPA and PCR using Mini primers showed a coefficient of 1. This means that RPA and PCR have an agreement categorized as very good based on Altman's criteria. The concordance between RPA and PCR using the Mini primers was 100% (Table 5). Likewise, the agreement between RPA and PCR using the Maxi primers showed an agreement coefficient of 0.941–0.956. Based on the coefficient values, RPA and PCR have an agreement categorized as very good based on Altman's criteria. The concordance between RPA and PCR using the Maxi primers was 97.4% (Table 5). Overall, the agreement between RPA and PCR (with both Mini and Maxi primers) showed an agreement coefficient of 0.952–0.983, with a percent agreement of about 98.7%, categorized as very good based on Altman's criteria.

Table 5: Agreement coefficients of RPA and PCR based on Mini and Maxi primer use

Method	Coeff	SE	95% C.I.
RPA_{Mini} vs PCR_{Mini}			
Cohen's kappa	NaN	NaN	(NaN–NaN)
Gwet's AC1	1	1e-50	(1–1)
Brennan–Prediger	1	1e-50	(1–1)
Bangdiwala's B	1	1e-50	(1–1)
Percent agreement	1	1e-50	(1–1)
RPA_{Maxi} vs PCR_{Maxi}			
Cohen's kappa	0.941	0.058	(0.824–1)
Gwet's AC1	0.955	0.045	(0.863–1)
Brennan–Prediger	0.949	0.051	(0.846–1)
Bangdiwala's B	0.956	0.043	(0.868–1)
Percent agreement	0.974	0.025	(0.923–1)
$RPA_{Mini+Maxi}$ vs $PCR_{Mini+Maxi}$			
Cohen's kappa	0.952	0.047	(0.858–1)
Gwet's AC1	0.982	0.018	(0.947–1)
Brennan–Prediger	0.974	0.025	(0.924–1)
Bangdiwala's B	0.983	0.017	(0.948–1)
Percent agreement	0.987	0.013	(0.962–1)

Note: Coeff = coefficient agreement value

Species identification involved the interpretation of results using both primers in sequence, that is, Mini followed by Maxi, as shown in Fig. 1. Overall, one species identification error occurred using RPA compared to PCR, leading to a slight decrease in the agreement coefficient to 0.941–0.956. However, based on the coefficient value, RPA and PCR were still categorized as having very good agreement according to the Altman criteria. The concordance between RPA and PCR for species identification using two pairs of primers sequentially was 97.4% (Table 6).

Table 6: Agreement coefficient of species identification based on RPA and PCR using Mini and Maxi primers sequentially

Method	Coeff	SE	95% C.I.
Cohen's kappa	0.941	0.058	(0.824 - 1)
Gwet's AC1	0.955	0.045	(0.863 - 1)
Brennan–Prediger	0.949	0.051	(0.846 - 1)
Bangdiwala's B	0.956	0.043	(0.868 - 1)
Percent agreement	0.974	0.025	(0.923 - 1)

Note: Coeff = coefficient agreement value

DISCUSSION

PCR has been used for molecular detection or identification for more than 40 years since it was first established by Kary Mullis in 1983 (Li *et al*., 2019). An advantage of PCR is its capacity to raise and lower the temperature during the cycle, especially during annealing or hybridization, to ensure the specificity of primer binding to the target DNA (Garibyan and Avashia, 2013). In contrast, RPA, which uses low and single temperatures, causes non-specific amplification due to the mismatch tolerance property of RPA that poses a risk of amplifying non-targets and leading to false positivity (Munawar, 2022; Tan *et al*., 2022). In our hands/in this study, these concerns did not materialize as evidenced by the excellent agreement between PCR and RPA as will be discussed later.

RPA is known to tolerate a wide range of biological samples even containing the usual PCR inhibitors (Daher *et al*., 2016). The advantage of RPA over other isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP) that employs multiple primers, is that RPA primer design is similar to that of PCR, although a length of 30–35 bases is strongly

recommended for optimal recombinase or primer filament formation (James and Macdonald, 2015; Daher *et al*., 2016; Li *et al*., 2019). This is required by the UvsX recombinase for the incorporation of oligonucleotides into duplex DNA (James and Macdonald, 2015). Longer PCR primers (>45 bases) are not recommended (Daher *et al*., 2016), and shorter primers, typically 18–25 bases, can be used in the RPA but may decrease the sensitivity and reaction speed (Li *et al*., 2019). However, based on the evidence in this study, concerns about decreased sensitivity are unwarranted.

RPA technology has been reported to produce comparable results to PCR in detecting protozoa, such as *T. cruzi*, *T. evansi*, *Babesia gibsoni*, and *Leishmania* spp. causing cutaneous leishmaniasis (Zhang *et al*., 2024). RPA for detecting active *Trypanosoma evansi* infection has also been developed using RoTat 1.2 primers (Li *et al*., 2020). RoTat 1.2 primers detect the Rhode trypanosome antigen type (RoTat) 1.2 of variant surface glycoprotein (VSG) gene of the *Trypanozoon* subgenus, specifically *T. evansi* type A, as well as *T. brucei* and *T. equiperdum* (WOAH, 2021). Other researchers reported that RoTat 1.2 primers successfully amplified and detected 100% of *T. evansi* and 77.8% of *T. equiperdum*, while *T. brucei* reactions were all negative (Claes *et al*., 2004). Although opinions differ among researchers, the most accurate statement is that the RoTat 1.2 primer is not specific for *T. evansi* and, therefore, cannot be used for species identification of members of the subgenus *Trypanozoon*.

In this study, RPA for species identification involved two pairs of primers, Mini and Maxi. Mini primers were designed to distinguish *T. evansi* and *T. equiperdum* from *T. brucei* (Artama *et al*., 1992: Subekti *et al*., 2023; 2024b). Likewise, Maxi primers were designed to distinguish *T. equiperdum* and *T. brucei* from *T. evansi* (Li *et al*., 2007; Subekti *et al*., 2023). This is because *T. evansi* has completely lost its maxicircle, only possessing a homogeneous minicircle (Lun *et al*., 2010; Gizaw *et al*., 2017). Identification using these two primers hierarchically, as in Fig. 1, allows for distinguishing *Trypanozoon* species.

RPA was found to cause the misidentification of the BTN04 isolate. Based on the identification algorithm using Mini followed by Maxi primers, RPA identified BTN04 as *T. evansi*, while it was identified as *T. equiperdum* using PCR followed by sequencing. This RPA identification error using Mini and Maxi primers on BTN04 may be due to the overly high concentration of the DNA template, which inhibits the RPA reaction, as indicated by Lobato and O'Sullivan (2018). Another possibility is related to the Mini and Maxi primers, which have a length of 18–25 bases and can potentially reduce sensitivity as indicated by Li *et al* (2019). However, both these explanations remain unlikely due to several considerations. First, researchers have successfully amplified templates with RPA using primers of 17–20 bases (Fuller *et al*., 2017; Li *et al*., 2019). Second, in our study, using RPA with ESAG6/7 primers (21 bases long) successfully detected trypanosomes at a concentration of 10^1 trypanosomes/ml (Fig. 2). This limit of detection is similar to that reported for the RPA-based detection of *Schistosoma japonicum* and *Babesia orientalis* (Sun *et al*., 2016; An *et al*., 2021). Third, the use of Mini primers that have a shorter length (18 bases) than Maxi primers (25 bases) still leads to successful amplification using RPA. Thus, the reason for the failure of DNA amplification of the BTN04 isolate using RPA is not yet known.

Overall, the performance of RPA for species identification of *Trypanozoon* members was very good, as revealed by the percent agreement for detection and identification between RPA and PCR using Mini and Maxi primers, which reached 98.7% (Table 5) and 97.4% (Table 6), respectively. These values are superior to that of RPA for *Angiostrongylus cantonensis* detection derived from larvae isolated from naturally infected slugs which reach only 65% (Jarvi *et al*., 2021). RPA and PCR using both primers for detection were categorized as having very good agreement, with agreement coefficients of 0.952, 0.982, 0.974, and 0.983 for kappa, AC1, Brennan–Prediger (BP) and B, respectively (Table 5).

In general, the comparison results of RPA with PCR were either similar or better than the comparison results of LAMP with PCR. The agreement between LAMP and PCR for the detection of *T. brucei* in the laboratory showed a kappa of 0.95 (0.64–1.00), but when used for the detection of *T. brucei* in the whole blood of horses in the field, the kappa value was 0.57 (0.42–0.72), categorized as moderate agreement (Gummery *et al*., 2020). Other studies showed that the agreement between LAMP and PCR for the detection of *Enterococcus hirae* from pure single colony only had an AC1 of 0.844 (0.68–1), while the detection of *T. brucei* in humans had a kappa and AC1 of 0.81 (0.73–0.89) and 0.82 (0.75–0.89), respectively (Mitashi *et al*., 2013; Dolka *et al*., 2019). Meanwhile, RPA and PCR for species identification of the *Trypanozoon* subgenus were also categorized as having very good agreement based on the agreement coefficients of kappa, AC1, BP, and B, which were 0.941, 0.955, 0.949, and 0.956 respectively (Table 6).

Conclusions: The molecular identification algorithm to distinguish species of *Trypanozoon* requires Mini and Maxi primers to be used sequentially. RPA provided excellent agreement with PCR for species identification of *Trypanozoon* members. Both primers performed very well with the RPA technique. Limitation that need attention are the stages of parasite purification. Providing a purification kit along with a genomic extraction kit is a challenge that needs to be studied further. Likewise, its application if using whole blood samples without parasite purification also needs to be evaluated further.

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