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Loop-mediated isothermal amplification (LAMP) assays for detection of *Theileria parva* infections targeting the PIM and p150 genes

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ABSTRACT

We have developed two loop-mediated isothermal amplification (LAMP) assays for the detection of *Theileria parva*, the causative agent of East Coast fever (ECF), an economically important cattle disease in eastern, central and southern Africa. These assays target the polymorphic immunodominant molecule (PIM) and p150 LAMP genes. The primer set for each gene target consists of six primers, and each set recognises eight distinct regions on the target gene to give highly specific detection of *T. parva*. The detection limit of each primer set is 1 fg, which is equivalent to one copy of the PIM and p150 *T. parva* genes. These PIM and p150 LAMP primer sets amplify DNA of *T. parva* isolates from cattle and buffalo from different countries including Kenya, South Africa, Tanzania, Rwanda, Uganda and Burundi, indicating their ability to detect *T. parva* from different countries. With the advantages of simplicity, rapidity and cost effectiveness, these LAMP assays are good candidates for molecular epidemiology studies and for monitoring control programs in ECF-endemic, resource poor countries.

1. Introduction

Theileria parva is the causative agent of East Coast fever (ECF), a rapidly fatal lymphoproliferative disease of cattle in eastern, central and southern Africa. ECF is found only in areas where its tick vectors occur, particularly *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*. For susceptible cattle in an epidemic situation the rate of mortality can reach 95% (Medley et al., 1993). The main wildlife host of *T. parva* is the African Cape buffalo (*Syncerus caffer*) in which the parasite does not normally cause disease. A carrier state of *T. parva,* defined as the persistence of a tick transmissible infection, is common amongst naturally recovered host animals, both cattle and African buffalo (Dolan, 1999; Bishop et al., 2004).

Field diagnosis is normally achieved by observing clinical signs in infected animals: ECF is indicated by fever, enlarged lymph nodes and is associated with tick vector infestation. In addition, an acute disease with high mortality on farms without effective tick control may also indicate ECF. The disease can be confirmed by finding *Theileria* parasites in Giemsa-stained blood smears and lymph node needle biopsy smears (OIE, 2008). In addition there have been numerous diagnostic assays developed for the detection of *T. parva* infections, mainly for use in research, including vaccine and epidemiological studies. These include serological assays such as the indirect fluorescence antibody test (IFAT) and ELISA (Billiouw et al., 2005; Katende et al., 1998), which have been important tools for classifying the epidemiological states of ECF. The limitations of IFAT, an assay which uses whole schizont material as antigen, have included poor sensitivity and specificity. An ELISA that uses a defined recombinant *T. parva* antigen appears to perform better, with good sensitivity and specificity (Katende et al., 1998). However, serological assays are unable to differentiate between current and past infections due to the persistence of antibodies.

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Table 1
Theileria stock/isolate DNA used in this study.

Species	Stock/isolate	Host	Country	Year
Theileria parva	Muguga	Cattle	Kenya	ND
T. parva	Onderstepoort (UCTD)	Cattle	South Africa	ND
T. parva	Hluhluwe	Buffalo	South Africa	2005
T. parva	Pugu I Dar es Salaam	Cattle	Tanzania	1978
T. parva	Nyakizu	Cattle	Rwanda	1979
T. parva	Serengeti	Cattle	Tanzania	1978
T. parva	Entebbe	Cattle	Uganda	1980
T. parva	Katumba	Cattle	Burundi	1981
Theileria annulata	Tova	Cattle	Israel	1974
Theileria mutans	Intona	Cattle	Kenya	1990
Theileria taurotragi	R6	Cattle	Kenya	1992
Theileria orientalis	Chitose	Cattle	Japan	ND

ND, no data.

PCR diagnostic assays have been developed for the sensitive and specific detection of *T. parva,* including the detection of very low levels of the parasite in carrier animals (Bishop et al., 1992; Gubbels et al., 1999; Skilton et al., 2002). These assays are not in widespread use in the resource poor countries where ECF occurs due to the relatively complex nature of the assays, and the need for expensive equipment and well-trained personnel (Kuboki et al. 2003; Poon et al. 2006).

Loop-mediated isothermal amplification (LAMP) is a simple technique which amplifies DNA with high sensitivity and rapidity under isothermal conditions (Notomi et al., 2000). LAMP products can easily be detected by the naked eye due to the formation of magnesium pyrophosphate, a white turbid by-product of DNA amplification which accumulates as the reaction progresses (Mori et al., 2001). Furthermore LAMP products can be detected by direct fluorescence by adding Loopamp® fluorescent detection reagent (FD) (Eiken Chemical Co. Ltd., Japan) at the start of the reaction (Tomita et al., 2008). Other fluorescent dyes such as ethidium bromide, SYBR green and Evagreen have also been used for visualisation of LAMP products under UV light (Qiao et al., 2007). LAMP can amplify different forms of templates including purified genomic DNA, heat-treated blood and blood dried on filter papers (Kuboki et al., 2003; Poon et al., 2006). In addition, we have recently reported that LAMP reagents are relatively stable even when stored at 25 and 37 °C, which supports the use of LAMP in field conditions and resource poor laboratories (Thekisoe et al., 2009).

Salih et al. (2008) have reported a specific LAMP assay for diagnosis of tropical theileriosis caused by *Theileria annulata*. We previously developed a LAMP assay based on heat shock protein 70 (HSP70) which amplifies major bovine *Theileria* species but is not specific for *T. parva* (Thekisoe et al., 2007). In this study we have developed two LAMP assays for specific detection of *T. parva* targeting the polymorphic immunodominant molecule (PIM) and p150 genes.

2. Materials and methods

2.1. Parasite DNA and field samples

Table 1 shows *Theileria* stocks or isolates used in this study. *Theileria parva* (Muguga) DNA was extracted from in vitro cultured schizont-infected lymphocytes. DNA of *T. annulata, Theileria mutans* and *Theileria taurotragi* was obtained from the International Livestock Research Institute (ILRI), Kenya. DNA of other *T. parva* isolates was obtained from Utrecht Centre for Tick-borne Diseases (UCTD), Utrecht University, Netherlands. The *Theileria orientalis* (Chitose strain) DNA was extracted from blood of an infected cattle in Chitose, Hokkaido, Japan. This study also used DNA from other protozoan parasites to check the specificity of the LAMP assays, namely *Babesia bovis* (Texas strain); *Babesia bigemina* (Argentina strain); *Trypanosoma brucei brucei* (GUTat 3.1) and DNA extracted from blood of an uninfected cattle from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. The 99 buffalo DNA samples from blood collected from various game parks in the South Africa, were supplied by the ARC-Onderstepoort Veterinary Institute. The buffalo samples were used as controls as data on their *T. parva* infection status was known prior to the commencement of the current study. A further 150 DNA samples were extracted from blood collected from cattle in Robanda village near Serengeti National Park in Tanzania.

2.2. DNA extraction

DNAs from *T. parva*-infected lymophocyte cell cultures and uninfected cattle blood as well as cattle samples from Tanzania were extracted by the phenol–chloroform method (Sambrook and Russell, 2001). Briefly, the extraction buffer (10 mM Tris–HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate) and 100 μg/ml proteinase K were added to the samples and incubated overnight at 55°C. DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with isopropanol. DNA was dissolved in 250 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0). The genomic DNA of field-derived buffalo blood samples was extracted using a MagNa Pure LC DNA isolation kit according to the manufacturer's procedures (Roche, Mannheim, Germany).

2.3. Preparation of plasmid DNA

The *T. parva* PIM and p150 genes of the Muguga stock were amplified by PCR using the F3 and B3 primers listed in Table 2. The PCR products were purified by QIAquick gel extraction kit according to the manufacturer's instructions (Qiagen, USA). Thereafter the PCR products were ligated into vector using the T-Vector

Table 2
The p150 and polymorphic immunodominant molecule (PIM) loop-mediated isothermal amplification (LAMP) primer sequences used in this study.

Target gene	Accession No.	Target size	Primer name	Sequence (5′–3′)
p150	**L47230**	198 bp	FIP	GCGGACGAGATCATTAACTGTGATCCTTCTTCTTGATTGCGTA
			BIP	GACACTCGGCGATGGTCTTGGGGCAAGGTAATTAATCTTTCA
			F3	TTATCTGTTGGCCTTAGCA
			B3	CAATTTACCTCATTTGCACTG
			LF	CAAGGAGTCACCCTCGTC
			LB	GTTTCTAAATGCTGCGTTCCTAAAG
PIM	**L41833**	192 bp	FIP	GGCACAAAGTGCAATCAAAAAGAGAAGAAGTTTCATTTTCGACTGT
			BIP	GCATTGGACTTTTTGCTCATGCTCCTACGGAATAGCCCAAGA
			F3	GTTCTGAGAGCAAAGGCG
			B3	GGCTAACGAGGATAGTACGT
			LF	AGAGATGTGTCAAAAG
			LB	TTCAATGTTGGTGTATTCTTTGCCG

FIP, forward inner primer; BIP, backward inner primer; F3, forward outer primer; B3, backward outer primer; LF, loop forward primer; LB, loop backward primer.

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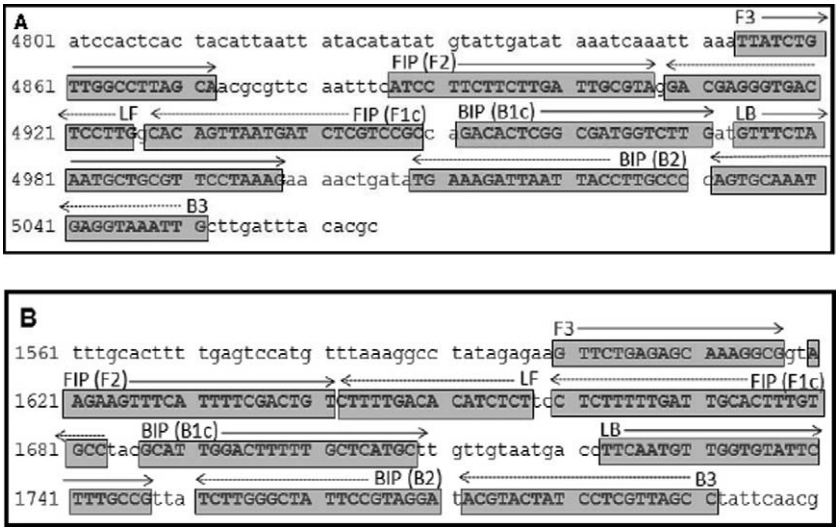

Fig. 1. Loop-mediated isothermal amplification (LAMP) primer positions on the target genes. (A) LAMP primers targeting the *Theileria parva* p150 gene. (B) LAMP primers targeting the *T. parva* PIM gene. F3, forward outer primer; FIP, forward inner primer; LF, loop forward primer; BIP, backward inner primer; LB, loop backward primer; and B3, backward outer primer. The solid arrows indicate the sense primers and the dotted arrows indicate the antisense primers.

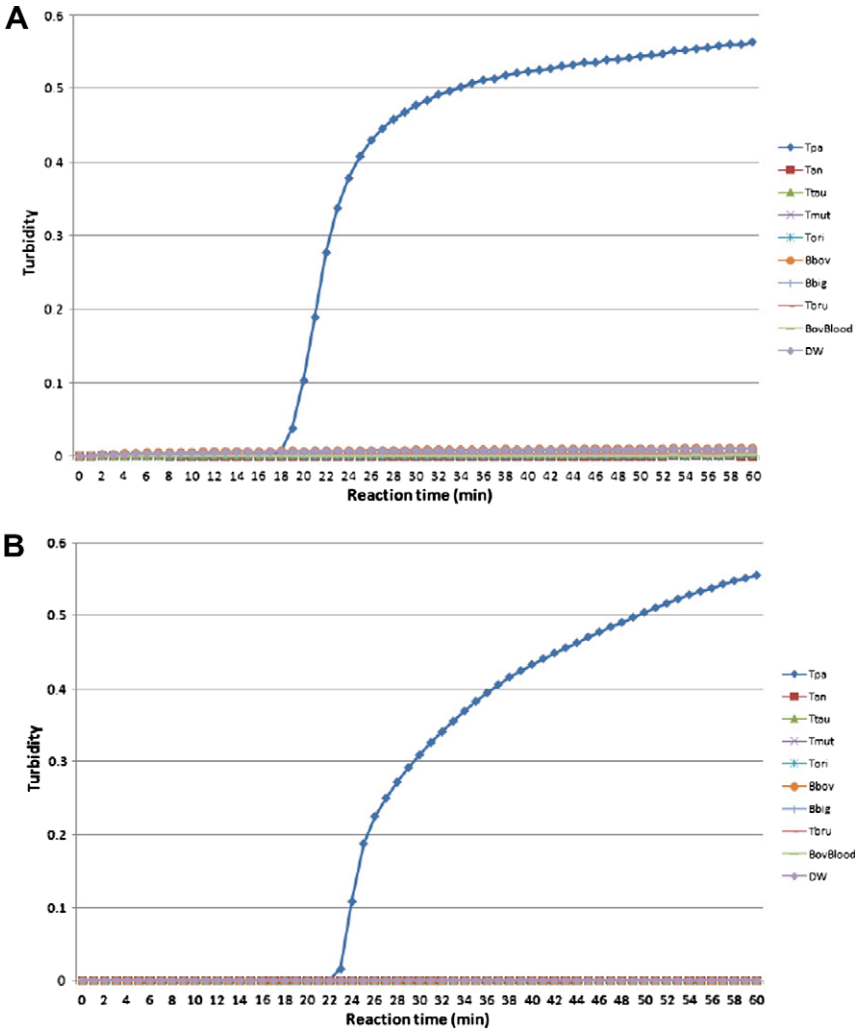

Fig. 2. Specificity tests of loop-mediated isothermal amplification (LAMP) primer sets for detection of *Theileria parva* genomic DNA. The standard positive reaction threshold is 0.1 value of the turbidity released (Mori et al., 2004). (A) Reaction with the p150 LAMP primer set. (B) Reaction with the polymorphic immunodominant molecule (PIM) LAMP primer set. Tpa, *Theileria parva*; Tan, *Theileria annulata*; Ttau, *Theileria taurotragi*; Tmut, *Theileria mutans*; Tori, *Theileria orientalis*; Bbov, *Babesia bovis*; Bbig, *Babesia bigemina*; Tbru, *Trypanosoma brucei brucei*; BovBlood, DNA extracted from uninfected cattle blood; DW, distilled water.

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Topo® Cloning 5 min PCR cloning kit (Invitrogen, USA). The recombinant plasmids were transformed into *Escherichia coli* DH5α and incubated for 12 h at 37 °C. The single colonies were cultivated in Luria–Bertani medium (LB) and plasmid DNA was purified using the Qiagen Midi kit (Qiagen, USA). Thereafter the concentration of plasmid DNA was quantified and used in sensitivity tests.

2.4. LAMP

The LAMP primer sets (Table 2) were designed from the PIM and p150 genes of *T. parva* (Muguga) using the Primer Explorer V4 Software (http://primerexplorer.jp/e/). Fig. 1A and B further show the primer positions on the target genes. LAMP reaction was conducted as described by Notomi et al. (2000) with minor modifications. For determination of optimal reaction temperatures of the newly designed LAMP primer sets, initial LAMP reactions were conducted at 60, 61, 62, 63 and 64 °C for the p150 primer set because the average melting temperature (T_m) of the six primers was 62.9 °C. For the PIM LAMP primer set reactions were conducted at 56, 58, 60, 61 and 62 °C because the average T_m of the six primers was 60.5 °C. The temperature in which the reaction would reach and cross the positive reaction threshold which is 0.1 of released turbidity (Mori et al., 2004) in a short period of time or faster than others was to be selected as the optimal reaction temperature (these reactions were done in five repetitions).

The LAMP reaction mixture with a total volume of 25 μl contained: 12.5 μl of 2× LAMP reaction buffer (40 mM Tris–HCl [pH 8.8], 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 μl (8 units) of *Bst* DNA polymerase, 2.6 μl primer mix (forward inner primer (FIP) and backward inner primer (BIP) at 40 pmol each, forward outer primer (F3) and forward outer primer (B3) at 10 pmol each, and loop forward primer (LoopF) and loop backward primer (loopB) at 20 pmol each), 3 μl of template DNA and 5.9 μl of double distilled water. For the reactions where 1 μl of FD was added, the volume of distilled water was reduced by 1 μl. All LAMP reactions were carried out for 60 min in a real-time turbidimeter (Loopamp LA200, Teramics, Japan) with a standard positive reaction threshold of 0.1 of the turbidity released (Mori et al., 2004). The LAMP products were also electrophoresed in 2% agarose gel, stained with ethidium bromide and visualised under UV light.

2.5. PCR

The PCR reactions were conducted using the F3 and B3 primers of the p150 and PIM LAMP primer sets as shown in Table 2. The PCR reaction mixture contained 10× PCR buffer (200 mM Tris–HCl [pH 8.0], 500 mM KCl, 50% (v/v) glycerol), 50 mM $MgCl_2$, 10 mM of dNTP mixture, 5 pmol of each primer, 5 U/μl of *Taq* DNA polymerase (Invitrogen, USA) and 3 μl of DNA in a final volume of 50 μl. The reaction mixture was heated at 94 °C for

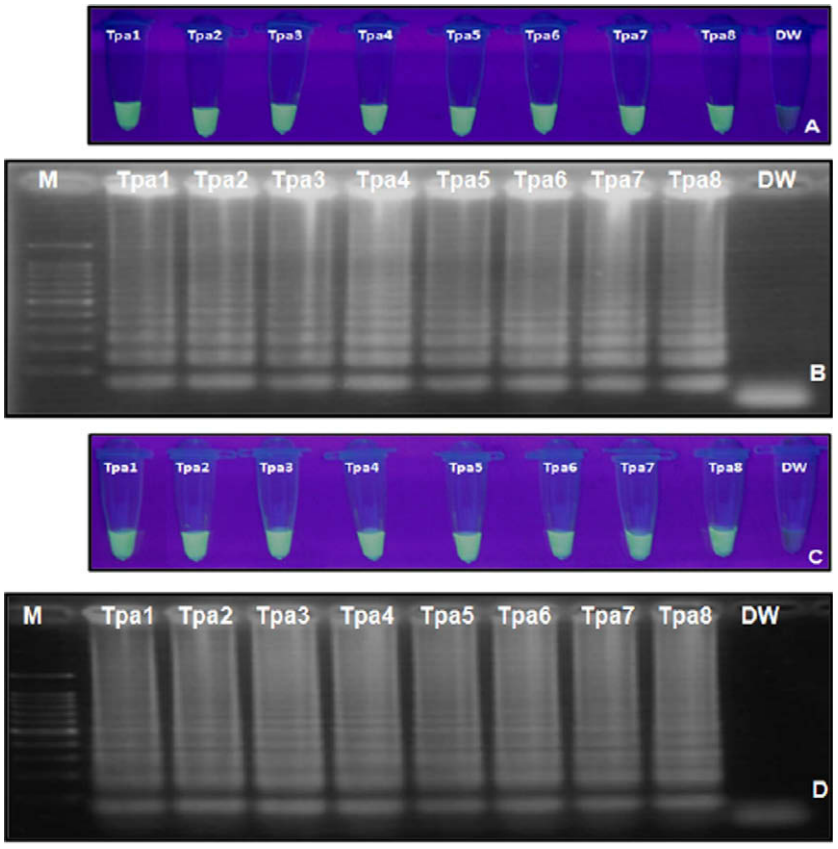

Fig. 3. Amplification of different *Theileria parva* isolates by p150 and polymorphic immunodominant molecule (PIM) loop-mediated isothermal amplification (LAMP) assays. Loopamp® fluorescent detection reagent (FD) (Eiken Chemical Co. Ltd.) was added in the reaction tubes prior to incubation and reaction results were detected by colour change of the FD after 1 h incubation. Observation was by manual, placing the reaction tube under UV light. The green colour/bright fluorescence indicates a positive reaction and the dark/less fluorescent colour indicates a negative reaction. (A and B) Reactions with p150 LAMP primers with detection under UV light using FD and agarose gel electrophoresis, respectively. (C and D) Reactions with PIM LAMP primers with detection under UV light using FD and agarose gel electrophoresis, respectively. Tpa1, *T. parva* Muguga; Tpa2, *T. parva* Onderstepoort isolate (UCTD); Tpa3, *T. parva* Hluhluwe (Buffalo); Tpa4, *T. parva* Pugu I Dar es Salaam; Tpa5, *T. parva* Nyakizu; Tpa6, *T. parva* Serengeti; Tpa7, *T. parva* Entebbe; Tpa8, *T. parva* Katumba; and DW, distilled water. (For interpretation of colour mentioned in this figure legend the reader is referred to the web version of the article.)

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10 min (denaturation step) and subjected to 40 cycles at 94 °C for 45 s, 1 min at 58 °C, and 1 min at 72 °C with a final extension at 72 °C for 7 min. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualised under UV light.

3. Results

3.1. Optimal temperature and specificity of LAMP primers

The optimal temperature of the p150 LAMP primer set is 63 °C and that of the PIM LAMP primer set is 60 °C. Despite these optimal reaction temperatures of the respective LAMP primer sets, amplification reactions were also achieved with temperatures below and above the optimal temperatures, although with a slightly slower threshold time for a positive reaction (data not shown). The PIM and p150 LAMP assays amplified only *T. parva* DNA whilst negative control DNA of other bovine *Theileria* species, bovine *Babesia* species, *Trypanosoma*, uninfected cattle blood and distilled water (non-DNA control) were not amplified (Fig. 2A and B). Furthermore, both PIM and p150 LAMP assays amplified DNA of *T. parva* isolates of cattle from Katumba (Burundi), Onderstepoort (South Africa), Pugu I Dar es Salaam (Tanzania), Nyakizu (Rwanda), Serengeti (Tanzania), Entebbe (Uganda) and a buffalo isolate from Hluhluwe (South Africa) (Fig. 3).

3.2. Detection limit of LAMP

The detection limit of both the PIM and p150 LAMP assays is 100 fg as determined from serially diluted total DNA extracted from in vitro cultured *T. parva* (Muguga) schizont-infected lymphocytes (Fig. 4A and B). Furthermore, in serially diluted target gene fragments referred to as plasmid DNA (pDNA) in this study, the detection limit of both assays was as low as 1 fg (Table 3). In

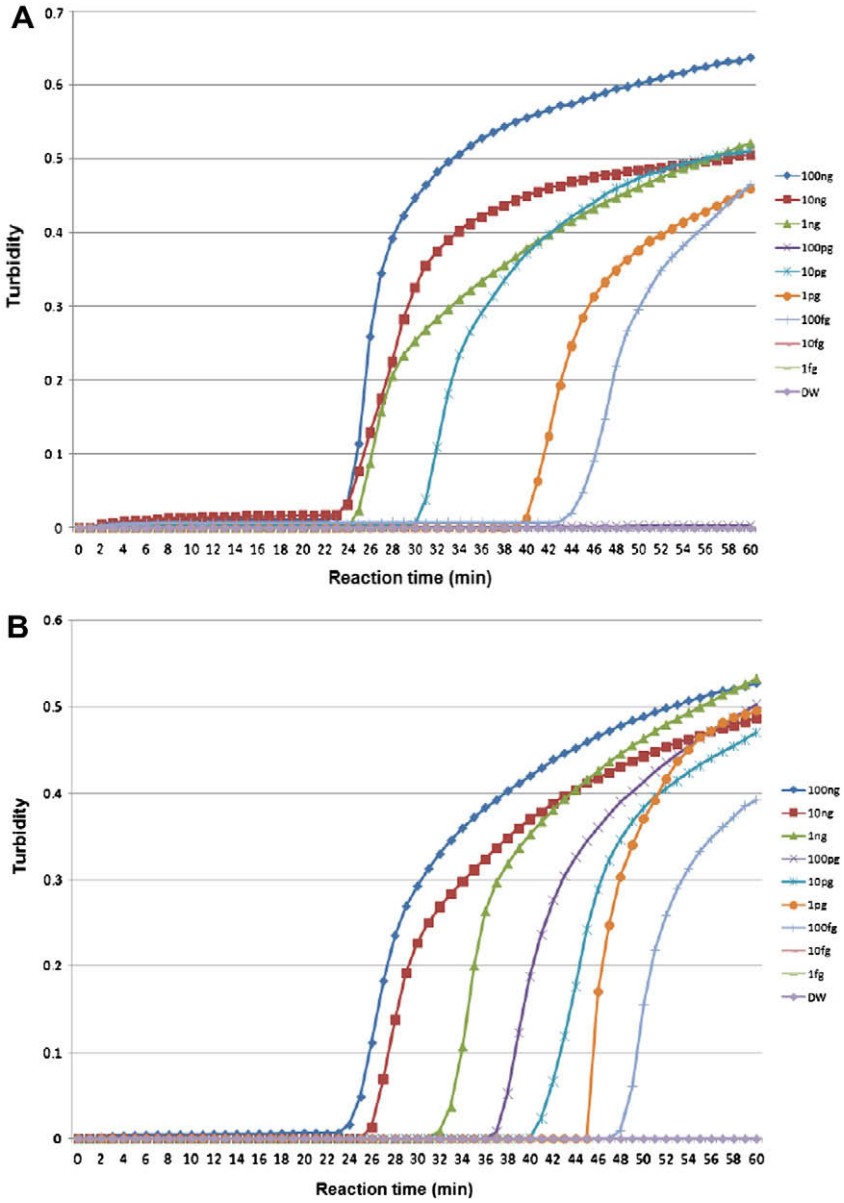

Fig. 4. Sensitivity reactions for both p150 and polymorphic immunodominant molecule (PIM) loop-mediated isothermal amplification (LAMP) primers on serially diluted *Theileria parva* genomic DNA extracted from in vitro cultured infected lymphocytes. The standard positive reaction threshold is 0.1 value of the turbidity released (Mori et al., 2004). (A) Reaction with the p150 LAMP primer set. (B) Reaction with the PIM LAMP primer set.

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addition to a real-time turbidimetry device which records the turbidity value versus time, we used the fluorescent detection reagent which enables detection of results by the naked eye (i.e. manually) or under UV illumination, and its detection results were consistent with those obtained from real-time LAMP turbidimetry (data not shown).

3.3. Detection of T. parva infections from field-derived buffalo and cattle samples

Out of 99 buffalo DNA samples from South Africa with previously confirmed *Theileria* infection status, 90 were positively amplified by both the PIM and p150 *T. parva* LAMP assays (Table 4). PCR with the F3 and B3 primers of both PIM and p150 genes gave identical results for these buffalo samples (Table 4). Out of 150 DNA samples collected from clinically healthy cattle in Robanda village of Tanzania, one sample (0.6%) was positively detected for *T. parva* infection by both the PIM and p150 LAMP assays as well as the PIM and p150-PCR assays (Table 4). The PCR products from this single Tanzanian positive sample were directly sequenced and shown to be derived from *T. parva* PIM and p150 genes, confirming that the products were specific (data not shown).

4. Discussion

In this study we report on development of *T. parva*-specific LAMP assays based on the PIM and p150 genes. Both the PIM and p150 LAMP primer sets specifically amplified the *T. parva* DNA, whilst DNA of other protozoan parasites known to infect cattle and buffalo were not amplified. Serological tests with p150 and PIM recombinant antigens have shown high specificity when tested against other protozoa (Katende et al., 1998). Both the p150 and PIM genes are polymorphic within their repeat sequence regions between isolates (Toye et al., 1995; Skilton et al., 1998). However, it is known that the PIM gene also contains conserved regions present amongst many isolates from different countries (Toye et al., 1996). In agreement with that notion, this study has demonstrated that the LAMP primer sets designed from the gene sequences of *T. parva* (Muguga) which was originally isolated in Kenya, could also amplify DNA of *T. parva* isolates from South Africa, Tanzania, Rwanda, Uganda and Burundi, indicating geographical conservation of the sequences. Njiru et al. (2008) reported that purified trypanosome DNA >200 ng had an inhibitory effect on LAMP reaction as monitored in real-time, and showed very weak bands on the agarose gel. Similarly, in the current study one of the *T. parva* genomic DNA isolates was initially not amplified due to highly concentrated DNA, however after dilution to ~100 ng LAMP amplification was achieved. It is therefore advisable to ensure DNA is appropriately diluted after DNA extraction and to use at least a 100 ng DNA template concentration as the maximum per reaction or two to three dilutions of extracted DNA.

The p150 and PIM LAMP primer sets amplified serially diluted *T. parva* DNA with high sensitivity whereby their detection limit was 100 fg for serially diluted *T. parva* genomic DNA extracted from schizont-infected lymphocytes. Although we used a real-time turbidimeter device in the current study, we have further demonstrated that LAMP reaction products can be detected by use of Loopamp fluorescent detection reagent which is added in the reaction tube before incubation. This mode of detection gave similar results to real-time turbidimetry whereby it also indicated positive reactions for serially diluted genomic and plasmid DNAs down to 100 fg and 1 fg, respectively.

African buffalo usually harbour *T. parva* parasites and act as reservoirs of infections for ticks and ultimately cattle (Dolan, 1999; Bishop et al., 2004) in domestic and wild animal interface areas (Grootenhuis and Olubayo, 1993). Archived total blood DNA samples from African Cape buffaloes in three provinces of the Republic of South Africa as well as DNA extracted from cattle in Robanda village of Tanzania were screened for *T. parva* infections with the p150 and PIM LAMP and PCR assays. Archived total blood DNA samples from buffaloes had previously confirmed positivity for *T. parva* (90 samples) or *Theileria* sp. (nine samples) by standard real-time PCR at the ARC-Onderstepoort Veterinary Institute. As a result 90% of the buffalo samples were *T. parva* positive when screened by both p150 and PIM LAMP primer sets. Identical results were obtained by PCR using the F3 and B3 primer pairs of the respective LAMP primer sets.

Primers designed from the p104 and p67 genes have been successfully used for PCR amplification and detection of *T. parva* infections (Skilton et al., 2002; Musoke et al., 2005). Prior to this study several LAMP primer sets were designed from the p67 and p104 genes of *T. parva*. Specific amplification of *T. parva* DNA with these p67 and p104 primers was also achieved, however LAMP reactions with the respective primers were slow or delayed for unknown reasons. As a result high sensitivity could not be achieved within 60 min of reaction time (data not shown). This observation brings to light the fact that performance of LAMP assays is also affected by the selected target sequence and its physical characteristics. Another important issue is that, since LAMP uses four to six primers, one has to take care that there is no significant difference in melting temperatures between the primers. In the case of the p104 and p67 primer sets (more than 10 LAMP primer sets designed in this

Table 3
Detection limit of the polymorphic immunodominant molecule (PIM) and p150 loop-mediated isothermal amplification (LAMP) assays.

LAMP assay	Type of DNA	Detection limit (fg)
PIM	gDNA[a]	100
PIM	pDNA[b]	1
P150	gDNA	100
P150	pDNA	1

pDNA, plasmid DNA.
[a] Total genomic DNA (gDNA) extracted from schizont-infected lymphocytes.
[b] Target sequence cloned into plasmid culture vector.

Table 4
Detection of *Theileria parva* infections by loop-mediated isothermal amplification (LAMP) from buffalo and cattle samples.

Country	Province	Host	Total no of samples	P150 LAMP +ve[a] (%)	PIM LAMP +ve (%)	P150 PCR +ve (%)	PIM PCR +ve (%)
South Africa	KwaZulu-Natal	Buffalo	85	85 (100)	85 (100)	85 (100)	85 (100)
	Limpopo	Buffalo	5	5(100)	5(100)	5(100)	5(100)
	Mpumalanga	Buffalo	9[b]	0 (0)	0 (0)	0 (0)	0 (0)
Tanzania	Robanda	Cattle	150	1 (0.6)	1 (0.6)	1 (0.6)	1 (0.6)

PIM, polymorphic immunodominant molecule.
[a] Positive detection.
[b] The nine samples were positive for *Theileria* sp. buffalo by real-time PCR targeting the 18S rRNA (unpublished data).

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study), most of the primers had high T_m differences of more than 10 °C between each of them. We therefore suspect that some of the primers would not melt at a single temperature used in the LAMP reaction. This could also be the reason why the reactions were slow or delayed.

The final amplified DNA products in a LAMP reaction are cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand which appear in a ladder-like pattern in agarose gel in contrast to a single band in PCR (Notomi et al., 2000). Furthermore, a LAMP by-product called magnesium pyrophosphate released in a positive DNA amplification reaction (Mori et al., 2001) can be detected using FD (Eiken Chemical Co. Ltd., Japan) which is added prior to incubation of the reaction mixture. The FD contains calcein bound with manganese ion so as to remain quenched. When the reaction proceeds the manganese ion is deprived of calcein by the generated pyrophosphate which results in emission of fluorescence. The free calcein then binds with magnesium ion in the reaction mixture so that it strengthens the fluorescence emission (Tomita et al., 2008).

In conclusion, the current study has developed specific, sensitive and rapid LAMP assays for detection of *T. parva* infections targeting the p150 and PIM genes. These LAMP assays are rapid as shown by low concentrations of *T. parva* DNA being amplified within 60 min. An additional advantage is that results can be observed in real-time when using a real-time turbidimetry device or manually immediately after the reaction using fluorescent detection dyes. There is still a need for further evaluation of these LAMP assays with more field samples representative of areas where *T. parva* is prevalent in Eastern, Central and Southern Africa.

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