



Evaluation of Loop-Mediated Isothermal Amplification (LAMP) for Diagnosis of *Theileria annulata* in both Cattle and Buffaloes in Upper Egypt

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ABSTRACT

Loop-Mediated Isothermal Amplification (LAMP) assay was used for detection of *Theileria annulata* infection in field samples from both cattle and buffaloes. These samples were collected from three governorates in Upper Egypt that include Assiut, EL-Fayoum and EL-wady EL-Gaded governorates. Reverse Line Blot (RLB) assay was used as a reference test for evaluation of LAMP assay efficacy in the diagnosis of bovine theileriosis. The obtained results revealed that according to the results of LAMP assay the infection rates were 65.24% and 43.24% in cattle and buffaloes, respectively. The evaluation study of LAMP test revealed high sensitivity in cattle (78.10%) if compared with buffaloes (47.37%). Specificity was higher in buffaloes (61.11%) if compared with cattle (57.53%). This study concluded that the LAMP assay was sensitive and specific assay in diagnosis of *Theileria annulata* infection. So, it is recommended to use LAMP assay, especially during molecular epidemiological surveys, which should be applied on a wide range because it will give a clear picture about the epidemiology of the disease, which helps in its prevention and control.

Introduction

Bovine theileriosis in Egypt is an important tick-borne disease occurs in Egypt since 1947 (Nagy, 1947). It is considered as destructive obstacles to livestock production because it causes severe losses, especially in indigenous breeds (Dolan, 1989; AL-Hosary, 2009). Early and accurate diagnosis plays an important role in the prevention and control of this disease. Conventional diagnosis of this disease usually based on the microscopic examination of Giemsa stained thin blood films

and/or lymph smears, but it usually limited to the acute phase of the disease when the parasitemia is high enough to be detected microscopically (AL-Hosary, 2009). During the chronic and carrier states of the disease molecular tools play an important role in the detection of infection. Reverse line blotting (RLB) is a test used to detect and differentiate all known *Theileria* and *Babesia* species as described by Gubbels *et al.* (1999). Recently, Loop-mediated isothermal amplification (LAMP) assay was developed and considered as a novel method for DNA amplification, where it is amplified with high specificity, efficiency and rapidity under isothermal conditions according to Notomi *et al.* (2000); Nagamine *et al.* (2002); Salih *et al.* (2008).

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The present study was designed to evaluate the LAMP assay for the diagnosis of *Theileria annulata* infection of cattle and buffaloes in Upper Egypt.

Materials and methods

Animals

A total number of 210 cattle and 37 buffalo belong to different localities in EL-Fayoum, Assuit, and EL-Wady EL-Gaded governorates were subjected to this study.

Sampling

Whole blood sample from suspected animals by jugular vein puncture on vacutainer tubes containing EDTA as anticoagulant (1mg/1ml) and marked with numbered labels in the field (Coles, 1986), and then stored at -20°C till use in DNA extraction.

DNA Extraction

DNA extraction from whole blood and lymph samples was carried out according to commercial kits (QIA amp blood kit, Qiagen, Ltd, UK).

Loop-Mediated Isothermal amplification assay

Six specific primers (F3, B3, FIP, BIP, LF and LB) for detection of *Theileria annulata* were derived from the sequence of the unique gene of *Theileria annulata* strain Ankara hypothetical protein (GeneDB TA04795) (Pain *et al.*, 2005). LAMP primers (F3, B3, FIP and BIP) were designed using

Primer Explorer V4 program (<http://primerexplorer.jp/e/>), while loop primers (LF and LB) were designed manually. The primers were synthesized by MWG Biotech AG (Munich, Germany). The sequence of each primer and location within the target sequence are shown in Table 1.

LAMP reaction conditions

The reaction was performed in a final volume of 25 µl, which contained 12.5 µl 2xLAMP reaction buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄ and 0.2% Tween 20), 125 Im each deoxynucleoside triphosphate, 0.8 M betaine (Sigma-Aldrich Chemie, Munich, Germany), 8 U of the Bst DNA polymerase large fragment (New England Biolabs, Frankfurt am Main, Germany), 40 pmol each FIP and BIP primers, 20 pmol LF and LB primers, 5 pmol each F3 and B3 primers, and 2 ll of target DNA. The mixture was incubated at 63°C for 45 minutes using Biometra thermo cycler (TECHNE TC-312), and then heated at 80°C for 5 minutes to terminate the reaction.

Detection of the LAMP product

An aliquot of 8 µl of LAMP product was subjected to electrophoresis on a 1.5% agarose gel in a Tris-acetic acid-EDTA (TAE) buffer at 90 V for 1 hour, and then visualized under UV light after staining with ethidium bromide. In addition, LAMP amplicons were detected directly by the naked eye by the addition of 1.0 µl of 1:10 diluted SYBR Green I (Roche Diagnostics, Mannheim, Germany) to the mixture and observation of the solution color. The solution turned green in 1 min in the presence

Table 1. LAMP primers designed for *Theileria annulata* and used in this study

Primer name	Type	Length	Sequence (5'-3')
F3	Forward outer	24-mer	CGCATTGATCTTCTTATCGTTAT
B3	Backward outer	18-mer	CACTGGACATCGTGATGA
FIP	Forward inner primer (F1 - TTTT-F2)	45-mer; F1, 20mer F2, 25mer	GCGGGATTACCTTTGGTGICTTTT CTAGATGTCATTTTTGTATACTCAC
BIP	Backward inner primer (B-TTTT-B2)	40-mer; B1, 22mer, B2, 18mer	AGTGGCGGTGCTAGTTTTAATGTTTT TAACCTCACCCTGAGCG
LF	Loop Forward	23-mer	GTAGAGATCAGTGAAAAATTGAC
LB	Loop Backward	20-mer	GGCTGCCATCACAAATCAAAG

of a LAMP amplicon, while it remained orange when no amplification occurred (Figs. 1).

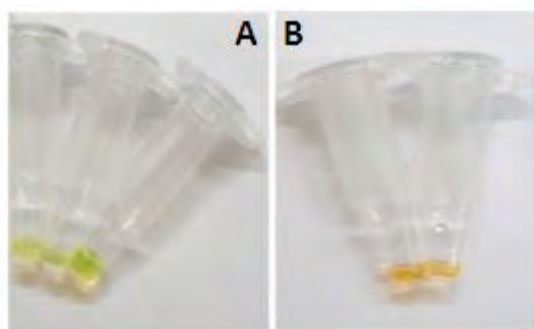


Fig. 1. Detection of LAMP amplicons by using SYBR Green. A) Showing positive result with SYBER Green (green color). B) Showing negative result with SYBER Green (orange color).

Evaluation of Loop-Mediated Isothermal amplification assay

Evaluation of LAMP as a method of diagnosis against RLB as reference test was carried out in the current study. Evaluation parameters included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and combined predictive value (CPV) according to Thrusfield (2005).

Results

The results of the current study confirmed the infection rates by using the LAMP assay as following 65.24 % (137/210) and 43.24 % (16/37) among cattle and buffaloes, respectively. RLB assay was

used for confirmation of *T. annulata* infection among examined cases. The infection rates were confirmed 65.24% (137/210) and 51.35% (19/37) among cattle and buffaloes, respectively (Table 2).

The results of the LAMP assay (Fig. 2) were evaluated by using the results of the RLB assay as a reference test. The finding of the evaluation study in cattle recorded true positive, true negative, false positive and false negative as 107, 42, 31 and 30, respectively. Accordingly the estimated sensitivity, specificity, PPV, NPP and CPV were 78.10%, 57.53%, 77.54%, 58.33% and 70.95 %, respectively (Table 4). The finding of the evaluation study in buffaloes recorded true positive, true negative, false positive and false negative results as following 9, 11, 7 and 10, respectively. Accordingly the estimated sensitivity, specificity, PPV, NPP and CPV of the conventional diagnostic method were 47.37%, 61.11%, 56.25%, 52.38% and 54.05%, respectively (Table 3).

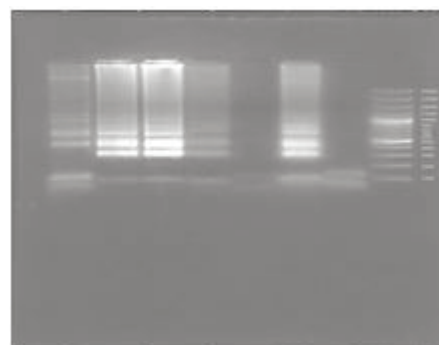


Fig. 2. Agarose gel showing the LAMP results (ladder like pattern) lines (1:4) were positive, line 5 was negative, line 6 was positive control, line 7 was negative control and line 8 was 100bp DNA marker.

Table 2. Rate of genetically confirmed infection among tested samples using RLB assay

Animal species and samples	LAMP assay		RLB assay	
	Positive	%	Positive	%
Cattle (210)	137	65.24	137	65.24
Buffaloes (37)	16	43.24	19	51.35

Table 3. Evaluation of LAMP test for diagnosis of *Theileria annulata* against RLB assay in cattle and buffaloes

Diagnostic methods	Test Results				Evaluation parameters (%)				
	TP ^a	TN ^b	FP ^c	FN ^d	Sensitivity	Specificity	PPV ^e	NPV ^f	CPV ^g
Cattle (210)	107	42	31	30	78.10	57.53	77.54	58.33	70.95
Buffaloes (37)	9	11	7	10	47.37	61.11	56.25	52.38	54.05

* RLB finding were considered as the reference test.

A: true positive, b: true negative, c: False positive, d - false negative, e: Positive predictive value, f: negative predictive value, g: Combined predictive value

Discussion

LAMP assay was used for the first time to detect *Theileria annulata* infection in field samples collected from cattle and water buffalo in Upper Egypt. The obtained results revealed that the infection rate in cattle and buffalo were (65.24% and 43.24%) respectively. These findings were evaluated against RLB assay as a reference test estimated sensitivity, specificity, PPV, NPP and CPV which were 78.10%, 57.53%, 77.54%, 58.33% and 70.95 %, respectively in examined cattle and 47.37%, 61.11%, 56.25%, 52.38% and 54.05%, respectively in examined buffaloes. According to the obtained results of this study this test is sensitive and specific especially during sub clinical and/or carrier cases which are most common in buffalo. This could be contributed to the ability of buffaloes to resist the infection through innate factors and cell mediated immune response in addition to low affinity of the protozoan parasite (*Theileria annulata*) to the buffaloes' cells as previously mentioned by Stagg *et al.* (1983); Baldwin *et al.* (1986). The low affinity of the parasite to the buffaloes cells makes the conventional and some molecular assay for example Tasm-1 target based PCR low sensitive to detect the infection in buffaloes except in acute stages as previously mentioned by AL-Hosary (2013). On the other hand, LAMP assay uses Bst polymerase rather than Taq DNA polymerase which used in standard PCR. This Bst polymerase enzyme has can't be inactivated by immunoglobulin G or any other blood components. So, it is better than other polymerase enzymes. LAMP assay can amplify DNA with high efficiency under isothermal conditions, it is highly specific for the target sequence and it is simple and easy to perform once the appropriate primers are prepared. LAMP method can be used to different biological substances with the determination of the suitable protocol to process the DNA template. This feature makes it easy to use in different laboratories conditions. This test provides a useful diagnostic tool in a clinical laboratory, particularly in resource-poor countries, in which bovine theileriosis is a serious endemic disease according to Notomi *et al.* (2000); Grab *et al.* (2005).

Conclusion

It could be concluded that the LAMP assay is

recommended for diagnosis as well as epidemiological surveys of *Theileria annulata* in order to prevent and control of this disease in Egypt especially in water buffaloes.

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