Journal of Advanced Veterinary Research Volume 7, Issue 3 (2017) 71-74

Journal of Advanced Veterinary Research

http://advetresearch.com/index.php/avr/index

Loop-Mediated Isothermal Amplification (LAMP) Assay for Diagnosis of Bovine Babesiosis (*Babesia bovis* infection) in Egypt

Amira A.T.A. AL-Hosary

Department of Animal Medicine (Infectious Diseases), Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

ARTICLE INFO

Original Research

Received:
19 April 2017

Accepted:
13 July 2017

Keywords:

Cattle
Babesia bovis
Tick
nPCR
LAMP

ABSTRACT

Bovine babesiosis is one of the destructive diseases affecting cattle worldwide especially in tropical and subtropical areas. In Egypt, small livestock holder represents the majority of livestock owners affected by the devastating impact of this disease including costs of diagnosis, treatment, control and prevention as well as limitations of production and reproduction of the affected animals. Early and accurate diagnosis of Babesia spp. infection plays an important role in treatment and control. The current study aimed to evaluate the efficacy of Loop-Mediated Isothermal amplification (LAMP) assay as a new molecular technique used for diagnosis of bovine babesiosis in naturally infected cattle. The confirmation of this infection was depended on blood smears, LAMP and Nested-Polymerase chain Reaction (nPCR) assays, which confirmed the infection in 19%, 47.62% and 52.38% of the examined animals, respectively. Tick samples were collected and identified as *Rhipicephalus (Boophilus) annulatus*, which is the vector of Babesia spp. Evaluation of blood smears and LAMP assay was carried out against nPCR as a reference test. The obtained results revealed that LAMP assay is a sensitive, specific and cost effective test and will be one of the near future applicable tests in epidemiological and diagnostic studies on babesiosis especially in developing countries endemic with this disease.

Introduction

Tick borne diseases (TBD) are responsible for worldwide great economic loss in terms of mortality and morbidity of livestock. This group of diseases has adverse effects on meat and milk production. Bovine babesiosis is one of these TBD found worldwide; caused by different species of genus Babesia (Adham *et al*., 2009). In Egypt, this disease first described in 1947 (Nagati, 1947). The clinical picture usually includes fever (≥40ºC), hemoglobinuria, anemia and/or nervous signs. Survived animals usually become low-level carriers with low level parasitemia, which continuously stimulates the immune system to produce specific antibodies and this protect the animals against the recurrence of infection (Homer *et al*., 2000; Radostitis *et al*., 2010; Mosqueda *et al*., 2012). Routine diagnosis of bovine babesiosis depends on clinical examination and microscopic detection of Babesia spp in Giemsa stained blood smears. This is useful only during the acute stage of the disease because the number of intraerythrocytic parasites usually higher enough to be detected microscopically (Bock *et al*., 2004; OIE, 2008). In subclinical and chronic infection more advanced techniques like molecular methods should be used (Perez *et al*., 2010; AL-Hosary, 2016). This study aimed to test the value of using LAMP assay in diagnosis of Bovine babesiosis in naturally infected cattle.

Materials and methods

Animals and sampling

A total number of forty two cattle *(Bos taurus)* belonging to different localities in Assiut governorate in the middle of Egypt were sampled, animals' ages were ranged from one year to more than seven years of both sexes. Two blood samples were collected; one from the ear vein for preparation of blood smears, another sample was collected from the jugular vein on ethylene diamine tetera acetic acid (EDTA) tubes and stored at -20°C till DNA extraction (Charles, 2007).

Clinical Examination

All animals were subjected to clinical examination accord-

*Corresponding author: Amira A.T.A. AL-Hosary
E-mail address: amiraelhosary@yahoo.com

ISSN: 2090-6277/2090-6269/

ing to Radostitis *et al*. (2010).

Conventional Detection

Blood smears were prepared directly after sampling, fixed in absolute methanol then stained with 10% Giemsa stain and examined by using x1000 magnification of the oil immersion lens (Charles, 2007; OIE, 2008).

Tick Identification

Tick samples were collected and identified using dissecting microscope according to the taxonomic key of Hoogstraal (1965) and Estrada-Pena *et al*. (2006).

Molecular Detection

DNA Extraction

DNA extraction from whole blood samples was performed using QIAamp blood kit (Qiagen, Ltd, UK) according to manufacturer's instructions; the obtained DNA samples were stored at -20ºC till use.

Nested PCR (nPCR) assay

Nested PCR (nPCR) amplification was carried out by using primers specific to *B. bovis,* BBOV-IV005650 (BV5650). The nPCR products were subjected to electrophoresis at 75 volt for 90 minutes on 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich Chemie, Munich, Germany), then visualized under ultraviolet (UV) light; positive band was visualized at 561 bp according to Aboulaila *et al*. (2010a,b).

Loop-Mediated Isothermal amplification (LAMP) assay

Four specific primers (F3, B3, FIP and BIP) used for detection of *Babesia bovis* (Table 1).

The reaction 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 mM of each deoxynucleoside triphosphate, 0.8M 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 and 5 pmol each F3 and B3 primers, and 2 µl of target DNA. The mixture was incubated at 60ºC for 40 minutes using Biometera-thermo cycler (TECHNE TC–312, Germany) and then heated at 80ºC for 2 minutes to terminate the reaction. The obtained products were tested by adding 10 µl of SYBR Green I (Roche Diagnostics, Mannheim, Germany) to each tube contains LAMP product; positive samples gave green color and negative samples gave orange color for more confirmation an aliquot of 8 µl of LAMP product was subjected to electrophoresis on 2% agarose gel in a Tris-acetic acid-EDTA (TAE) buffer at 90 Volt for one hour and then visualized under UV light after staining with ethidium bromide, positive samples showing ladder like pattern (Liu *et al*., 2012).

Table1. LAMP primers for *Babesia bovis*

Primer name	Type	Sequence (5'–3')
F3	Forward outer	CACTAGCACCACACCAGTG
B3	Backward outer	CAAAAGGGGGTGCATCTCG
FIP	Forward inner primer	GCGTTGCTAGTAGTGGCACCGGAATTCCA GCTTCCACCCAACGAG
BIP	Backward inner primer	GCTACCCTAGTAGCCGGTTGGGGAATTCG AGCTTAACCCGGGTCGT

Evaluation of diagnostic assays

This evaluation was carried out against nPCR as reference test. The evaluation parameters include sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and combined predictive value (CPV) according to Thrusfield (2005).

Statistical analysis

Chi-squared test was used to evaluate statistical significance and $p<0.05$ was accepted as significant. The level of agreement between the methods was analyzed with a 95% confidence interval using SPSS 16.0 for windows (SPSS, 2016, Chicago, USA).

Results

Clinical examination revealed that some of the examined animals were suffered from acute stage of infection with specific clinical signs include fever and hemoglobinuria with different degrees of tick infestation. Tick samples were identified and all of them were related to *Rhipicephalus (Boophilus) annulatus* tick. Giemsa stained blood smears confirmed the infection in 19% of the examined animals by detection of the intraerythrocytic stage of *Babesis bovis* (Figs. 1, 2).

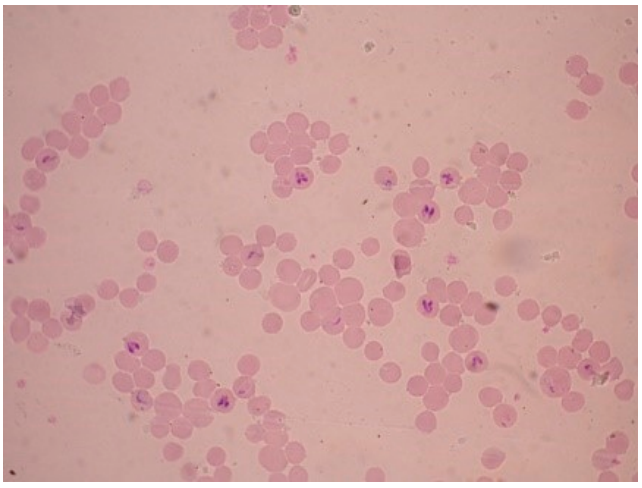

Fig. 1. Giemsa stained blood smear show *Babesia bovis*.

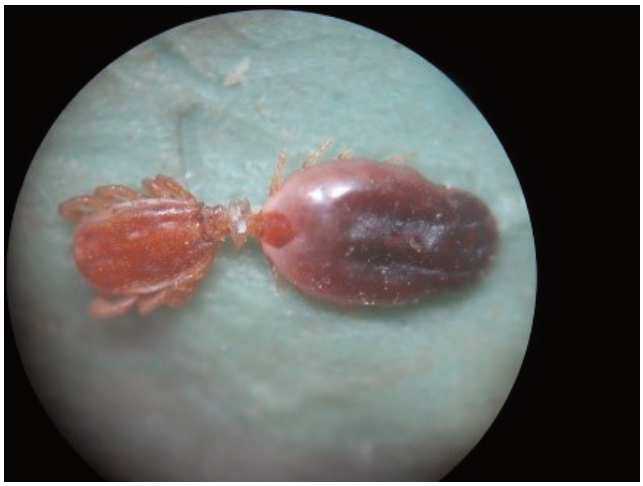

Fig. 2. Male and Female *Rhipicephalus (Boophilus) annulatus* tick

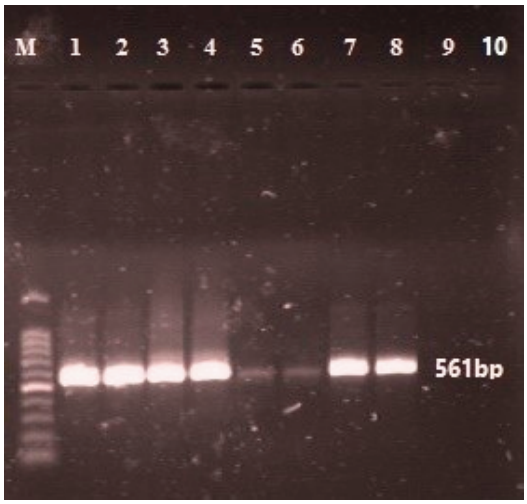

Fig. 3. *Babesia bovis* nPCR products. Lane M: DNA ladder 100 bp, Lanes 1: 8 positive PCR with bands of 561bp

LAMP and Nested PCR assays confirmed the infection in 47.62% and 52.38%, respectively (Table 2, Figs. 3, 4). Giemsa stained blood smears and LAMP assay were evaluated against nPCR as a reference test. The results concluded that blood smear recorded true positive, false positive, true negative and false negative as 8, 0, 20 and 14, respectively. Accordingly the estimated sensitivity, specificity, PPV, NPP and CPV were 36.36%, 100%, 100%, 58.82% and 66.67%, respectively. On the other hand, LAMP assay recorded true positive, false positive, true negative and false negative as 20, 0, 20 and 2 and accordingly the estimated sensitivity, specificity, PPV, NPP and CPV were 90.90%, 100%, 100%, 90.91% and 95.24%, respectively (Table 3).

Table 2. Infection rates according to Giemsa stained blood smears, nPCR and LAMP assays

Diagnostic assays	Positive	%
Blood smear	8	19
LAMP	20	47.62
nPCR*	22	52.38

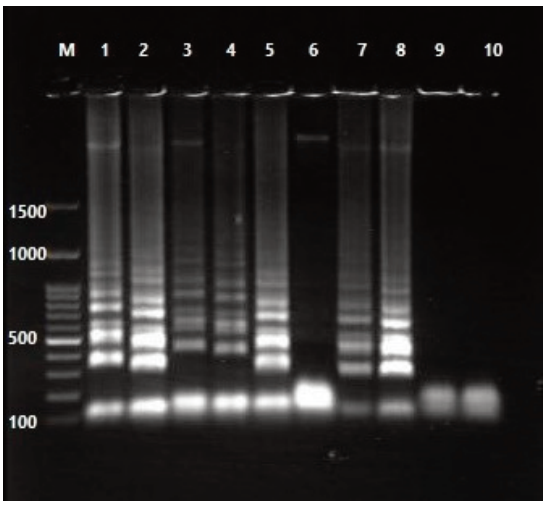

Fig. 4. *Babesia bovis* LAMP products. Lane M: DNA ladder 100 bp, Lanes 1: 5, 7 and 8 positive LAMP product with Ladder like pattern and lanes 6, 9 and 10 were negative

Table 3. Evaluation of different diagnostic assays against nPCR as a reference test

Diagnostic assays	Positive	%	Evaluation study								
			TP[a]	FP[b]	TN[c]	FN[d]	Sensitivity	Specificity	PPV[e]	NPV[f]	CPV[g]
Blood smear	8/42	19	8	0	20	14	36.36	100	100	58.82	66.67
LAMP	20/42	47.62	20	0	20	2	90.90	100	100	90.90	95.24

*nPCR was used as a 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

Bovine babesiosis caused by *Babesia bovis* is considered as pasture infection linked to tick vector known as *Rhipicephalus (Boophilus) annulatus*. This disease has adverse economic impact on milk yield and meat production in Egypt (Adham *et al*., 2009). Clinical and Giemsa stained blood smear examination are considered as conventional methods for diagnosis of this infection but they are sufficient tools only during the acute stage of the disease. Blood smear examination is useful in diagnosis if the blood sample collected directly from ear or tail veins because these small blood vessels contain higher percentage of infected erythrocytes (OIE, 2008). In this study, The conventional methods confirmed the infection in 19% of the naturally infected cattle infested with ticks if compared with previous literature, which confirmed the infection rate in 33.33% of the examined animals (Durrani and Kamal, 2008). This method is cheap and easily performed in short time by using simple instruments, but it usually requires some experience to differentiate the species of babesia. The findings of this study also, revealed that molecular assay includes nPCR and LAMP assays are sensitive and specific diagnostic tools. The highest sensitivity and specificity of nPCR

assay may be contributed to the presence of several copies of its target gene; moreover the sequence conservation among strains may be playing an important role in these findings (AbouLaila *et al*., 2010b). Nested PCR was used as a reference test in order to evaluate the efficacy of other diagnostic assays (Table 3). According to the previously mentioned results, sensitivity of LAMP assay (90.90%) was higher than sensitivity of the blood smear (36.36%). But the specificity of both assays was 100%. Predictive values also evaluated during this study because these parameters usually give an indication about the disease's probability in population during further investigation (Thrusfield, 2005). In the current study, the estimated PPVs were high for all diagnostic assays; these findings indicate that during future work, this tests will have the potentiality to detect infected animals correctly. On the other hand, NPV of blood smear was relatively low (58.82%), if compared with that of LAMP assay (90.90%), which proofed that LAMP assay able to detect the true negative samples and more sensitive and specific if compared with blood smear; the same finding was obtained by statistical analysis using Chi-squared test. This may be contributed to the ability of LAMP assay to detect the DNA even with low concentrations especially in case of chronic infection and carrier animals. Also, using of Bst polymerase with LAMP reaction is better than Taq DNA polymerase because this enzyme can't be inactivated by immunoglobulin G or any other blood components. LAMP assay can amplifies DNA with high efficiency under isothermal conditions; also, LAMP primers are highly specific for the target sequence. This feature makes it easy to use in different laboratories conditions and provides useful diagnostic aid particularly in resource poor countries. This means that using of this technology may have an important clinical applications (Notomi *et al*., 2000; Liu *et al*., 2012).

Conclusion

Tick eradication programs must be applied strictly with periodical examination of the animals. *Babesia bovis* specific LAMP assay is an inexpensive, rapid and sensitive technique. This assay has the potential to be one of the most applicable field test especially during epidemiological studies and day to day diagnosis in Egypt and other resource-poor countries endemic with this disease.

Acknowledgement

This work was partially supported by DFG project "Molecular epidemiology network for promotion and support of delivery of life vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa" (DFG project SE862/2-1).

References

Aboulaila, M., Yokoyama, N., Igarashi, I., 2010a. Development and evaluation of a nested PCR based on spherical body protein 2 genes for the diagnosis of *Babesia bovis* infection. Veterinary Parasitology 139, 45–50.

Aboulaila, M., Yokoyama, N., Igarashi, I., 2010b. Development and evaluation of two nested PCR assays for the detection of *Babesia bovis* from cattle blood. Veterinary Parasitology 172, 65–70

Adham, F.K., Abd-El-Samie, E.M., Gabre, R.M., El Hussein, H., 2009. Detection of tick blood parasites in Egypt using PCR assay *Babesia bovis* and *Babesia bigemina*. Parasitology Research 105, 721–730.

AL-Hosary, A.A.T., 2016. Comparison between Conventional and Molecular Methods for Detection of Bovine Babesiosis (*Babesia bovis* infection) in Tick Infested Cattle in Upper Egypt. Journal of Parasitic Diseases. doi:10.1007/s12639-016-0785-2, 1-4.

Bock, R., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129, S247-269.

Charles, M.H., 2007. Text Book for Laboratory Procedures for Veterinary Technicians fifth Ed. Mosby Elsevier science, St Louis Missouri. USA, pp. 187-252.

Durrani, A.Z., Kamal, N., 2008. Identification of ticks and detection of blood protozoa in Friesian cattle by polymerase chain reaction test and estimation of blood parameters in district kasur, Pakistan. Tropical Animal Health and Production 40, 441-447.

Estrada-Pena, A., Bouattour, A., Camicas, J.L., Guglielmone, A., Horak, I., Jongejan, F., Latif, A., Pegram, R., Walker, A.R., 2006. The known distribution and ecological preferences of the tick subgenus Boophilus (*Acari: Ixodidae*) in Africa and Latin America. Experimental Applied Acarology 38, 219-235.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clinical Microbiology 13, 451–469

Hoogstraal, H., 1965. African Ixodidae, Tick of Sudan, Cairo, Egypt. U.S Naval med Res Unit 3.110.

Liu, A., Guan, G., Du, P., Gou, H., Liu, Z., Liu, J., Ma, M., Yang, J., Li, Y., Niu, Q., Ren, Q., Bai, Q., Yin, H., Luo, J., 2012. Loop-mediated isothermal amplification (LAMP) method based on two species-specific primer sets for the rapid identification of Chinese *Babesia bovis* and *Babesia bigemina*. Parasitology International 61, 658–663.

Mosqueda, J., Olvera-Ramírez, A., Aguilar-Tipacamú, G., Cantó, G. L., 2012. Current Advances in Detection and Treatment of Babesiosis. Current Medicinal Chemistry 19, 1504-1518.

Nagati, H.E., 1947. Some new and rare records of piroplasmosis with a list of the species of Babesia and Theileria so far recorded from Egypt. Veterinary Record 59, 145–147.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acid Research 28, E63.

OIE, 2008. World Organization for Animals Health: Bovine babesiosis Chapter 2.4.2. pp. 1-3. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.02_BOVINE_BABESIOSIS.pdf

Perez de Leon, A.A., Strickman, D.A., Knowles, D.P., Fish, D., Thacker, E., de la Fuente, J., Krause, P.J., Wikel, S.K., Miller, R.S., Wagner, G.G., Almazan, C., Hillman, R., Messenger, M.T., Ugstad, P.O., Duhaime, R.A., Teel, P.D., Ortega-Santos, A., Hewitt, D.G., Bowers, E.J., Bent, S.J., Cochran, M.H., McElwain, T.F., Scoles, G.A., Suarez, C.E., Davey, R., Howell Freeman, J.M., Lohmeyer, K., Li, A.Y., Guerrero, F.D., Kammlah, D.M., Phillips, P., Pound, J.M., 2010. One Health approach to identify research needs in bovine and human babesioses. Workshop report. Parasites and Vectors 3, 36

Radostitis, O.M., Gay, C.C., Blood, D.C., Hinchcliff, K.W., 2010. Text book of Veterinary Medicine 9th Edn. Baieller Tindal and Co, pp. 1328-1329.

Thrusfield, M., 2005. Veterinary epidemiology. Third Edn. Blackwell science publishing. pp. 313-317.