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 teta acetic acid (EDTA) tubes and stored at -20ºC till DNA extraction (Charles, 2007).

Clinical Examination

All animals were subjected to clinical examination accord-
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 -20°C 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 using 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 F1P and B1P primers and 5 pmol each F3 and B3 primers, and 2 µl of target DNA. The mixture was incubated at 60°C for 40 minutes using Biometera-thermo cycler (TECHNE TC–312, Germany) and then heated at 80°C 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).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Type</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1P</td>
<td>Forward inner primer</td>
<td>ACCAGGCTAGTATGGCCGCAACAGCAGG</td>
</tr>
<tr>
<td>B1P</td>
<td>Backward inner primer</td>
<td>GTGCCGCTAGTTGGTGAAATTTCAG</td>
</tr>
</tbody>
</table>

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 *Babesia bovis* (Figs. 1, 2).

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

Table 1. LAMP primers for *Babesia bovis*
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**

<table>
<thead>
<tr>
<th>Diagnostic assays</th>
<th>Positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood smear</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>LAMP</td>
<td>20</td>
<td>47.62</td>
</tr>
<tr>
<td>nPCR</td>
<td>22</td>
<td>52.38</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Diagnostic assays</th>
<th>Positive</th>
<th>%</th>
<th>TP</th>
<th>FP</th>
<th>TN</th>
<th>FN</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPP</th>
<th>CPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood smear</td>
<td>8/42</td>
<td>19</td>
<td>8</td>
<td>0</td>
<td>20</td>
<td>14</td>
<td>36.36</td>
<td>100</td>
<td>100</td>
<td>58.82</td>
<td>66.67</td>
</tr>
<tr>
<td>LAMP</td>
<td>20/42</td>
<td>47.62</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>2</td>
<td>90.90</td>
<td>100</td>
<td>100</td>
<td>90.90</td>
<td>95.24</td>
</tr>
</tbody>
</table>

* nPCR was used as a reference test.

**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, these 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 proved 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

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References


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.


