

BIOCHEMICAL, PATHOLOGICAL AND MOLECULAR STUDIES ON BABESIOSIS IN CALVES

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Abstract

Bovine babesiosis is caused by the tick-borne blood parasite, *Babesia bovis* (*B.bovis*) and *Babesia bigemina* (*B.bigemina*). Many studies from 1980's to 2015's revealed that annual incidence of disease in summer season especially in adult cattle has associated with considerable economic losses. The present study were made on 115 Holstein calves at period from November 2014 till January 2015. Clinically affected calves showed signs of babesiosis as fever (41c°), anorexia, depression, weakness, pale mucous membrane, emaciation, weight loss, hematuria with accelerated heart and respiratory rates. Parasitological examination using Giemsa-stained thin blood films revealed presence of *B. bovis* in 54 out of 115 of examined calves while molecular examination by using regular Polymerase Chain Reaction (PCR) technique for same calves showed positive cases in 66 out of 115 and 14 out of 20 groups, *Boophilus annulatus* (*B. annulatus*) samples that affect these calves. There were significant decrease in erythrocytic count, hemoglobin content, packed cell volume, albumin, globulin and total protein but significant increase in serum bilirubine in clinically affected cases of *Babesia bovis*. Pathological examination of liver and spleen showed golden yellow to brown hemosiderine pigment with inflammatory cells infiltration. In conclusion, the present study indicates that the change of some epidemiological aspects of clinical disease as age and season could be occure. Infected cases can occur at any time of the year in young calves less than three months. It could be occure in winter season, in contrast to some previous studies.

Key words: *Babesia bovis* - biochemical analysis - histopathology - PCR

INTRODUCTION

Babesiosis is a tick-borne disease caused by the protozoan parasites of the genus *Babesia*, order Piroplasmida and hamper the growth of the bovine sector and impose serious constraints on the health and productivity of domesticated cattle in tropical and sub-tropical regions of the world. All *Babesia spp.* are transmitted by ticks

with a limited host range in tropical and subtropical countries (OIE Terrestrial Manual, 2012).

Bovine babesiosis is principally maintained by sub-clinically infected cattle that have recovered from disease. Morbidity and mortality vary greatly and are influenced by prevailing treatments employed in an area, whereas high morbidity and mortality values are observed when mature animals are infected for the first time (OIE Terrestrial Manual, 2012). Calves generally exhibit a milder form of infection and animals infected at a young age are resistant to severe disease when rechallenged as adults (Levy *et al.*, 1982).

In endemic areas, cattle become infected at a young age and develop a long-term immunity. However, outbreaks can occur in these endemic areas if exposed to ticks by young animals. The introduction of *Babesia* infected ticks into previously tick-free areas may also lead to outbreaks of disease. Babesiosis, like most haemoparasites, has generally been shown to cause destruction of red blood cells resulting in anemia, jaundice, anorexia, weight loss and infertility but vary according to agent and host factors (Radostits *et al.*, 2000).

In Egypt two species of cattle *Babesia* were reported; *B. bigemina* and *B. bovis*. However, only the Boophilid ticks; *Boophilus annulatus* (*B. annulatus*) are responsible for their transmission (Ilemobade, 1991). Microscopy using Giemsa stained blood smears has been considered the "gold standard" for detection of *Babesia* in the infected animals, particularly in acute cases, but not in carriers where the parasitemia is low (Nayel *et al.*, 2012).

Polymerase chain reaction (PCR) had been proved to be very sensitive and specific technique particularly in detecting babesiosis in carriers (Salem *et al.*, 1999 and Zulfiqar *et al.*, 2012).

The aim of this study was to detect *Babesia* infection in young aged calves in unusual prevalence season.

MATERIALS AND METHODS

Animal population and clinical presentation

The owner of the two farms introduced 40 newly born calves infested with ticks from the market and reared in yard beside old calves in the farms. After 17 days from introducing the market calves, there was an outbreak in old ones.

During the November of 2014 and January of 2015, 115 Holstein calves (from 2 adherent dairy farms) located between El-Manzala lake and El-Salam branch of River Nile, Egypt, were clinically and parasitologically examined for the presence of *Babesia spp.* infection. The ages of calves were ranged between one to three months. Calves from both dairy farms had recent clinical cases of *Babesia* infections and a

history of tick infestation as well as sporadic cases of sudden deaths (n=5) in the respective herds. Various degrees of tick infestation were present. Eighty out of 115 calves were apparently healthy while 5 calves suffered from sudden death.

Sampling :

Two blood samples from each calf were drawn from the jugular vein by using sterile sharp needle. The samples that used for blood smear and PCR analysis were collected in clean and dry test tube containing EDTA as anticoagulant. On the other hand, blood samples were collected from dead animals which died recently less than 12 hours for preparation of blood smears. Also, small parts of spleen and liver were taken for histopathological examination. Thin blood films were prepared from blood samples, air-dried, fixed with absolute methyl alcohol for 15 minutes and then stained with Giemsa stain 10% for one hour. They examined microscopically by oil immersion lens (x1000) of light microscope according to Saleem *et al.*, (2014). The parasites were identified according to the characters described by Mohamed and Ebied (2014).

Ticks collection:

Ticks found on calves at the two farms and from floor were collected. The collected female ticks and nymphs were only incubated on saturated sodium chloride solution at 28 °C for 3 weeks then ticks were divided into 20 groups. Each tick group was macerated in 0.3 ml sterile phosphate buffer saline (PBS) using a sterile pestle and mortar. Tick suspensions stored at -20 °C until DNA was extracted for PCR analysis according to Halos *et al.*, (2004).

Hematological picture and biochemical analysis:

Hematological analysis was carried out within one hour of collection. The erythrocytic count (RBCs), hemoglobin (Hb), Packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were determined according to Schalm *et al.*, (1975). Serum was separated by centrifugation at 3000 rpm for 10 minutes then the clear supernatant serum aspirated carefully into dry sterile labeled vials and used for serum analysis in both clinically infected and contact apparently healthy, animals. The total protein, albumin, globulins and serum bilirubin were measured according to Lobetti *et al.*, (2000).

Pathological study:

Specimens from liver and spleen of the dead calves were collected after careful PM examination and fixed in 10% neutral buffered formalin, processed for light microscopic examination and then paraffin sections of 5 µ thick were cut and stained

with Hematoxylin and Eosin and Prussian blue stain for histopathological examination (Mulisch and Welsch, 2010).

Molecular analysis:

DNA extraction from blood and tick tissues:

DNA was extracted from each sample either blood or tick tissues by chloroform-isoamyl extraction method according to Sambrook *and Russell* (1989) and stored at -20°C.

Polymerase chain reaction (PCR):

Specific PCR has been used to detect the gene encoding the enzyme carbamoyl phosphate synthetase II for *B. bovis* within the DNA extracts of the suspected animals. Forward and reverse primers were designed according to Chansiri and Bagnara, (1995) to amplify 446 bp (F: TTTGGTATTTGTCTTGGTCAT, R: ACCACTGTAGTCAAACCTCACC). DNA amplification was done in 25 µl reaction volume containing PCR buffer (300 mM Tris, 75 mM ammonium sulfate, pH 9.0), 2.5 mM MgCl₂, 400 µM dNTPs, 20 pmol of each primer, 2 µl–1 taq DNA polymerase and 5 µl of each DNA sample. The thermal profile of PCR was started with an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 45 s. After amplification, 10 µl of each sample was electrophoresed in a 1.5% agarose gel and visualized under ultraviolet transillumination.

Statistical analysis:

Data were analyzed using T-test as described by Petrie and Waston (1999).

RESULT

Clinical examination:

One hundred and fifteen calves of one to three months age were examined. These calves were subjected for clinical examination. Thirty calves suffered from fever (41°C) anaroxia, depression, weakness, icteric mucous membrane, emaciation, weight loss and haematourea. Then the heart rate was increased, marked dyspnea was developed and visible mucous membranes were first congested but very soon became pale and in the terminal stages became icteric. Five calves infested with ticks were suffered from sudden death (figure 1). The rest of examined animals were apparently healthy.



Fig. 1. Dead calf infested with hard ticks.

Blood film:

In present study, parasitological examination of Giemsa-stained blood smears prepared from 115 calves, revealed intra-erythrocytic (pear shaped) *B. bovis* (figure 2) in 54 (46%) ones. All dead calves (n=5) had parasitemia more than 0.5% .

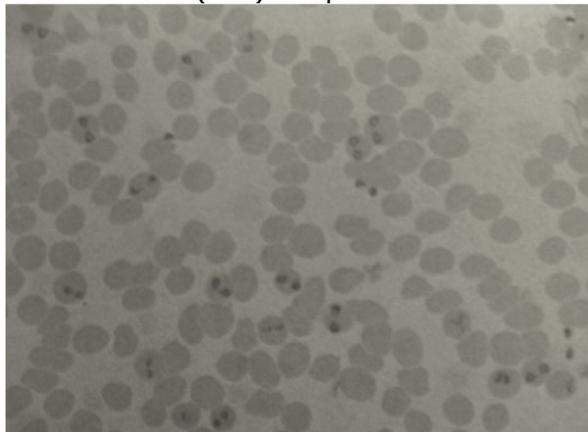


Fig. 2. *Babesia bovis* in Giemsa-stained blood smear of examined calve (x1000).

Identification of the collected ticks:

A total number of 500 ticks were collected from affected calves and farm floor. TICKS were identified according to hoogstraal (1956). 377 *females and nymphs* were found to be *Boophilus annulatus*. They were grouped into 20 groups before molecular examination, while the rest of the collected ticks (123) were males of *B. annulatus*.

Hematological analysis:

Blood parasites especially *B. bovis* were showed clear effects on the hematological picture of the infected calves as showed in table (1). The results in the table showed that, RBCs, Hb, MCV, MCH and PCV were significantly decreased in affected calves than that of apparently healthy ones.

Table 1. The effect of babesiosis on blood picture.

	Apparently healthy	Diseased animals
RBCS	7.9±0.3	4.4±0.2***
Hb (g/dl)	13.2±0.7	7±0.3***
MCV (FL)	46.8±2.2	54.5*±2.4
MCH (Pg)	16.7±0.7	15.9±0.8
PCV	37±1.4	24±1.1***

Significant variation at *P < 0.05 - ***P < 0.001

Biochemical analysis:

The biochemical changes in serum of both clinical and subclinical calves revealed significant decrease in globulin, albumin and total protein but significant increase in serum conjugated bilirubin in case of clinical cases compared with apparent healthy calves as showed in table (2).

Table 2. The effect of babesiosis on total protein, albumin, globulin and serum conjugated bilirubin.

Parameter	Control	Diseased
Total protein (g/dl)	6.79±2	5.01±0.11**
Albumin (g/dl)	4.81±0.12	3.05±0.09**
Globulins (g/dl)	2.2±0.07	1.040±0.06**
Serum bilirubin (m/dl)	0.325±0.03	0.951±0.04***

Significant variation at **P < 0.01 - ***P < 0.001.

Mean ±S.E conjugated bilirubin. Mean ±S.E. n=20.

Histopathological findings:

Liver showed degeneration of hepatic cells with severe congestion in blood vessels and sinusoids, hemorrhage and necrosis of hepatocytes with accumulation of inflammatory cells mainly lymphocytes and plasma cells around the portal area and central vein. There were proliferation of fibrous connective tissue in portal area. Golden yellow to brown hemosidrine pigments were scattered throughout the hepatic lobules which stained blue with Prussian blue stain (figures 3 and 4). Spleen showed marked golden yellow to brown stained hemosidrine pigments with extensive necrosis of lymphoid cells lead to depletion in lymphoid follicles with the presence of neutrophiles infiltration (figures 5, 6 and 7).

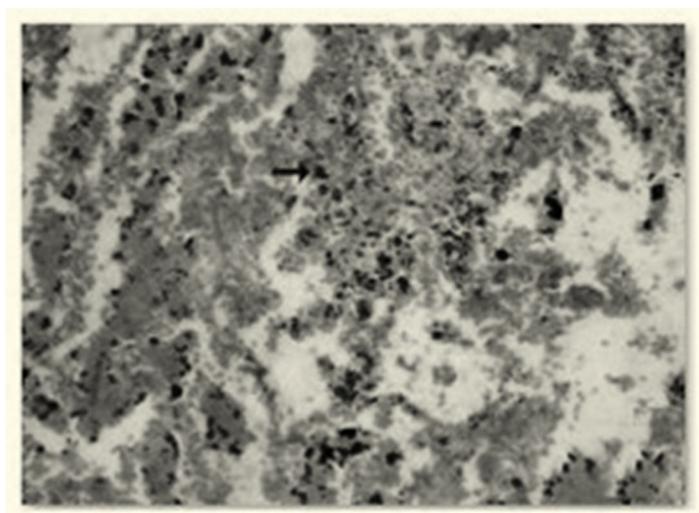


Fig. 3. Liver showed bluish coloration of hemosiderin pigments (Prussian blue, x400).

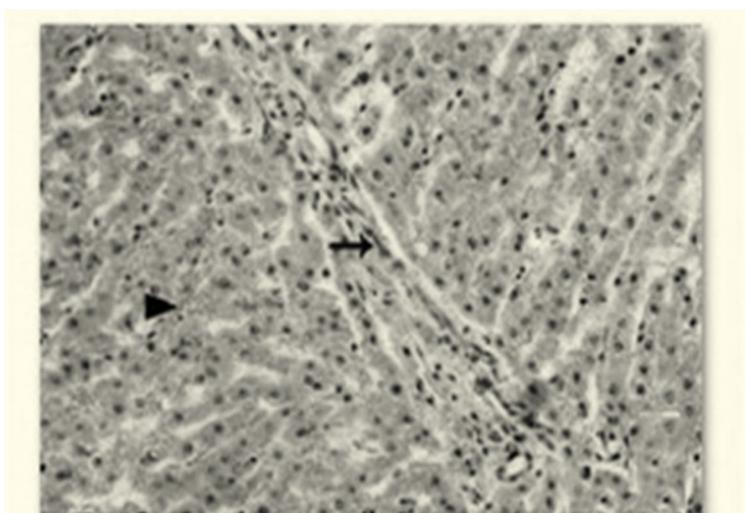


Fig. 4. Liver showed fibroblastic proliferation in portal area "arrow" (H&E, x200).

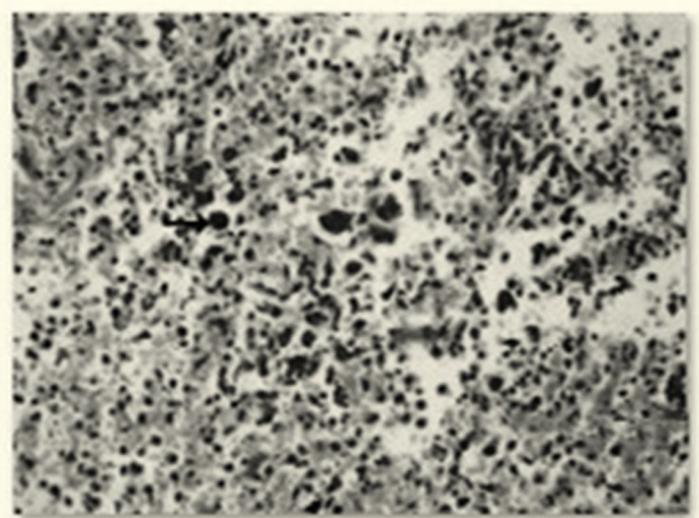


Fig. 5. Spleen showed marked golden yellow to brown stained siderocytes "arrow" with depletion in lymphoid follicles. (H&E, x400).

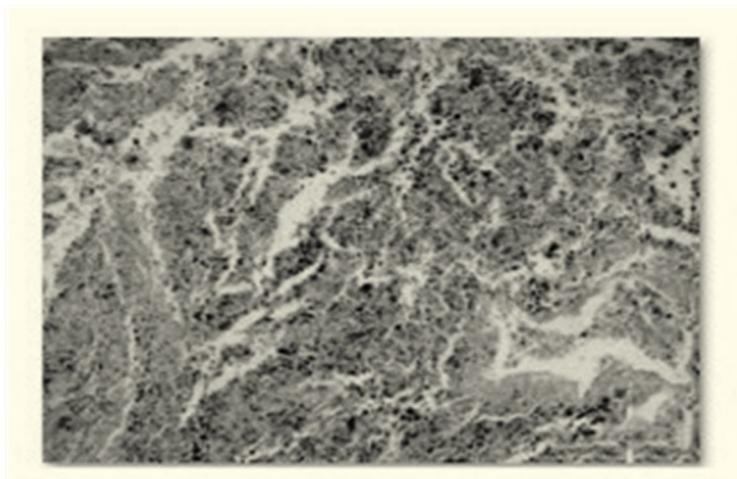


Fig. 6. Spleen showed intense blue colored hemosiderine pigments. (Prussian blue, x400)

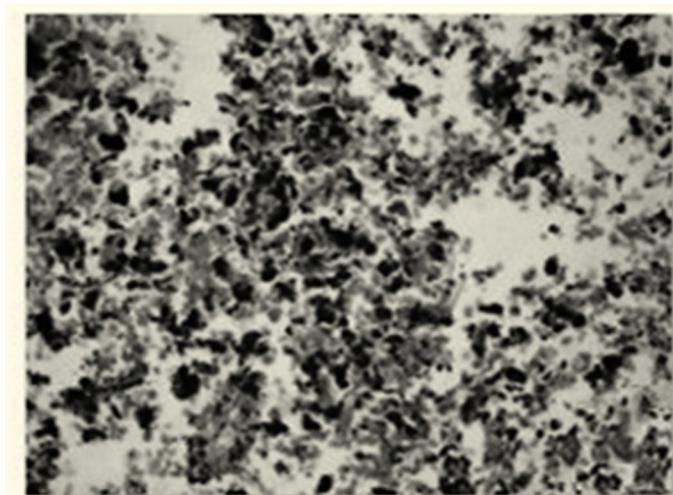


Fig. 7. Spleen showed siderocytes engulfed bluish stained hemosiderine pigments (Prussian blue, x400).

PCR- based molecular Diagnosis of *B. bovis*:

PCR detection of *B. bovis* DNAs in ticks:

Results showed that *B. annulatus* ticks, females and nymphs were positive for *B. bovis*, 14 (70%) out of 20 groups . Diagnostic bands were visualized at 446 bp that were specific for *B. bovis*.

PCR detection of *B. bovis* DNAs in blood:

PCR findings showed that 66 out of 100 blood samples (66%) were positive for *B. bovis* where specific bands were visualized at 446 bp .

Table 3. Comparison of the results of clinical signs, microscopy and PCR between clinically affected calves (group I n=35) and apparently healthy calves (group II n=80).

	Clinical signs	Blood film (+ve)	PCR(+ve)
Group I	35	35	35
Group II	80	19	31
Total	115	54	66

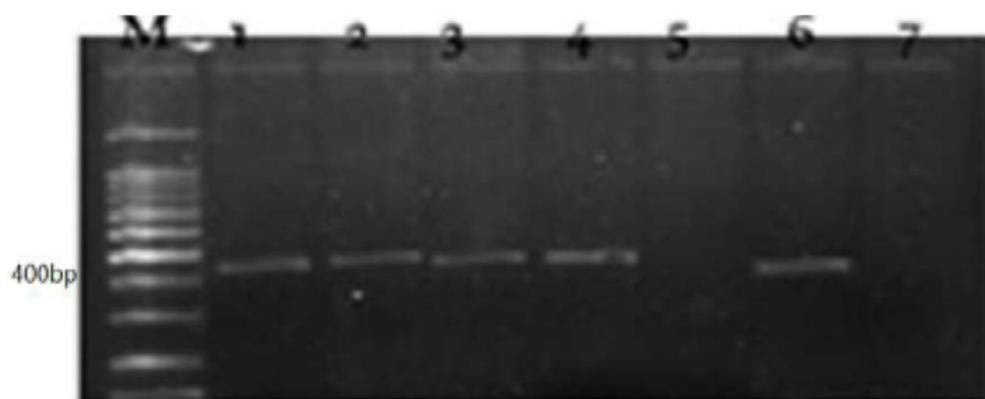


Fig. 8. PCR result showing positive samples on gel at 446pb for *Babesia*. Lane M: 100 bp DNA Ladder marker, Lane 1, 2, 3, 4, 6: Positive samples, Lane 5: Negative sample, lane 7: negative control.

DISCUSSION

Babesiosis is one of the most important diseases in our countries because it occurs sometimes in acute forms with serious recognized clinical manifestations and lowering the productive performance of the affected animals.

In contrast to most of authors, the present study documented the occurrence of *B. bovis* infection in calves less than 3 months in age suffering from babesiosis include high temperature (40-41°C), loss of appetite, cessation of rumination, anemia, labored breathing and haemoglobin urea. Such finding as in the present study could be due to destruction of large number of erythrocytes by blood parasite resulting in hemoglobinaemia and consequently hematuria. These findings are in agreement with Brown and kathLeen (1992). On other hand, Radostits *et al.*, (2000) attributed the sudden onset of high fever (40-41°C) as response to effect of unspecific toxic substances produced during the metabolism of *Babesia* on thermoregulatory center. Then, the heart rate was increased, marked dyspnea was then developed and visible mucous membranes were first congested but very soon became pale and in the terminal stages became icteric. This study showed that the occurrence of *B. bovis* infection in calves could happen in winter season while many studies carried in Egypt showed that seasonal prevalence of babesiosis in cattle was recorded in summer (Mohamed and Ebied, 2014). Also, El-Sawallhy, (1987) recorded the highest infection rate of babesiosis was in both summer and autumn while it was less in spring and low in winter. The occurrence of infection in winter may be attributed to the lowered immune status of calves in the farms and introduction of pathogenic strain of *B. bovis*.

This study showed that the occurrence of *B. bovis* infection happened in calves less than 3 months of age and this finding is in contrary with others who claimed that calves have a degree of immunity related to colostral derived antibodies and to age-specific factors that persists for 6 months (Radostits *et al.*, 2007).

The method of choice to detect *Babesia* in blood of infected animals in acute cases was blood film examination (Bose *et al.*, 1995). In the present work, examination of Giemsa-stained blood smear revealed intra-erythrocytic double pyriform (pear shaped) of *B. bovis* inside RBCs of infected animals. This is agreed with Zulfiqar *et al.*, (2012). In the present study, blood smear examination detected 46.9% and that was in agreement with Nayelet *et al.*, (2012) who demonstrated *Babesia* species in blood smears of 38.5% of tested cattle in Fayoum blood smears. Battsetseg *et al.*, (2002) in Brazil detected infection rates of 64 %. Fluctuation in the prevalence rates might be due to the variation of environmental conditions that affect parasites, vectors and host immunity.

The results of the hematological investigation in calves suffering from babesiosis revealed significant reduction in erythrocytic count, haemoglobin content and packed cell volume. All this might be due to destructive effect of parasites on erythrocyte in infected calves compared to that of apparently healthy ones and this result was agreed with Radostitis *et al.*, (2000) and Mahmued *et al.*, (2015).

The present study indicates that the serum albumin, globulin and total protein pattern were significantly decreased but bilirubin pattern was significantly increased. The changes in the protein picture in infected animals could be due to decrease protein production as a result of deprivation of diet protein resulting from anorexia and fever accompanied infection or due to disturbed hepatic functions or due to destructed RBCs and its excretion in urine as reported by AL-Aboud *et al.*, (2005) and Alam and Nasr (2010).

Initially, increased serum bilirubin level may be attributed to the massive haemolysis occurred during the early period of infection with *B. bovis* then hypoxia that lead to hepatic cell degeneration resulting in increased level of serum bilirubin as reported by Allen and Kuttler (1981).

Calves infected with blood parasites are characterized by parasitemia and an acute inflammatory response. In the present study, enlarged and swollen liver with enlarged dark spleen were reported which agreed with Demeter *et al.*, (2011), but didn't agreed with Yeruham *et al.*, (2003) who found neurological symptoms.

In the present study liver showed severe congestion, haemorrhage and necrosis with fibroblastic proliferation in hepatic tissue with neutrophilic infiltration and golden yellow to brown pigments. The spleen showed extensive haemorrhage with necrosis of lymphoid tissue, neutrophilic infiltration with golden yellow to brown hemosiderine pigments. These results were in agreement with Bock *et al.*, (2004), Schneider *et al.*, (2011) and Hamoda *et al.*, (2014). These results may be due to hemolysis of erythrocytes so lead to production of erythrocytes from bone marrow to compensate decrease in erythrocytes level. This lead to decrease serum level of iron

which is necessary for erythrocyte production. Pathogenesis is also related to rapid, massive, intravascular hemolysis and erythrocyte destruction with hemoglobinuria.

In the present study, PCR as a sensitive method was applied to detect *B. bovis* in ticks and in calves affected with those ticks. The amplification of DNA extracted from *Boophilus annulatus*, the main vector of *B. bovis* as mentioned by Divya *et al.*, (2014) revealed that 70% of examined ticks infected with *B. bovis*. The obtained results coincide with those recorded by Quintão-Silva *et al.*, (2007) who found that the infection rate in engorged *Boophilus microplus* tick females were 4.7% by *B. bovis* and with Adham *et al.*, (2009) who reported that the prevalence rate of *B. bovis* in *Boophilus annulatus* ticks was 55% in Egypt.

This study was documented that PCR is confirmatory to light microscopy, so results of PCR revealed that 57% of animals were positive for *B. bovis*. These findings were slightly high in contrast to many other authors such as Quintão-Silva *et al.*, (2007), M'ghirbi *et al.*, (2008), Chaudhry *et al.*, (2010) and El-Sify *et al.*, (2015) who recorded infection rates ranged between 10 to 15 % for bovine babesiosis using PCR, while Costa-Junior *et al.*, (2006) in Brazil found that 26.7 % of cattle were positive for *Babesia* infection using PCR. In Egypt, Aziz *et al.*, (2014) mentioned that the infection rate of *Babesia spp.* was 25.33%. The high infection rate in the present result compared to others could attribute to introduction of animals infected with ticks to clean free farms.

CONCLUSION AND RECOMENDATIONS:

It could be concluded that babesiosis is a life threatened diseases accompanied by disturbance in serum protein fractions and hepatic dysfunction. This study showed that the higher susceptibility of young calves less than 3 months to *B. bovis* infection were documented in winter season and this represent a high risk because the owners not expect that. The precise reasons for the change in some epidemiological aspects of the disease (age and season) are unknown, but changes in agricultural practice and rearing animal systems are likely to be of importance. A reversal of the trend could be devastating, as vigilance. It is advisable a lot of studies must be done on large scale in different areas and seasons in Egypt farms. It is advisable to investigate all newly introduced animals in any farm using PCR to detect low parasitemia as occurred in carrier animals or subclinical infection to avoid transmission of infection to primary inhabitant animals in the farm.

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دراسات بيوكيميائية وباثولوجية وجزيئية على مرض البابيزيا في العجول

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1. قسم البكتريولوجي (معمل المنصورة الفرعي)

2. قسم الباثولوجيا (معمل المنصورة الفرعي)

3. قسم الكيمياء و النقص الغذائي والسموم (معمل المنصورة الفرعي)

4. قسم الطفيليات (معمل المنصورة الفرعي)

تعتبر الإصابة بالببازيا في مصر واحدة من اهم طفيليات الدم المنقولة بواسطة القراد. لقد أثبتت العديد من الدراسات منذ 1980 حتى 2015 أن بابيزيا بوفيز و بابيزيا بيجيمنا من الأمراض الموسمية وبخاصة فصل الصيف في الماشية في الأعمار فوق 6 شهور مما يسبب خسارة إقتصادية فائقة. تم فحص 115 عينة دم من عجول الهولستين من مزرعتين متجاورتين وجد بهما قراد إلى جانب ظهور علامات المرض على العجول وهي الحمى (41 درجة مئوية) وفقدان الشهية والحمول والضعف و الشحوب في الأغشية المخاطية و الهزال و فقدان الوزن وظهور دم في البول. تم التأكيد بالفحص الطفيلي و الهستوباثولوجي والبيوكيميائي وكذلك استخدام الطرق الجزيئية للتعرف على الإصابة بالببازيا من خلال الدم والقراد. أظهر الفحص الطفيلي لشرائح الدم المصبوغة بصبغة الهميسا عن ظهور 54 حالة مصابه من 115 بينما الدراسة البيوكيميائية أظهرت انخفاض واضح في عدد كرات الدم ، الهيموجلوبين ، الألبومين والبروتين الكلى وزيادة واضحة في البيليروبين في العجول المصابة بالببازيا بوفيز. أما الفحص الجزيئي باستخدام طريقة أنزيم البلمرة المتسلسل أظهر وجود 66 حالة إيجابية من 115 حالة وكذلك 14 حالة إيجابية من 20 مجموعة قراد (بوفيلس أنيولاتس) المأخوذ من العجول المصابة. أوضح الفحص الهستوباثولوجي لعينات من الكبد والطحال وجود صبغة الهيموسيدرين داخل الخلايا البلعمية مع وجود خلايا التهابية. نستنتج من هذه الدراسة إمكانية إصابة العجول في سن صغير وإمكانية ظهور المرض في فصل الشتاء.