Original Article

Morphological and Molecular Detection of Tick-Borne Haemoparasites in Ruminants from Al Gabal Al Akhdar, Libya and Ismailia Governorate, Egypt

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Abstract:

Tick-borne diseases (TBDs) are important factors that affect the development of animal husbandry worldwide, in both tropical and subtropical area. The aim of this study is to investigate the prevalence of the Piroplasma species in both Al Gabal al Akhder region in northeastern Libya and Ismailia, Egypt. Blood samples were collected randomly from 748 apparently healthy animals from various localities in Libya and Egypt during the period extending from January 2020 to January 2021. Giemsa-stained thin blood films were used for direct microscopy, conventional PCR was used for molecular identification of the samples. The results showed the prevalence of Piroplasma in Egypt was 23.9% in cattle and 19.5%in buffaloes, while in Libya the cattle was 27.6%, 31% in sheep, and 21% in goats. Real-time PCR in the diagnosis of blood parasites may give better results than conventional PCR.

Key words: Molecular, Microscopic, Haemoparasites, Microscopic, *Babesia spp.*, *Theileria spp.*

Introduction

Tick-borne diseases (TBDs) are important factors that affect the development of animal husbandry worldwide. They have an effect on wild and domesticated animals, and most are considered neglected and/or emerging zoonotic diseases (Homer et al., 2000). Bovine production and health are significantly impacted by hemoprotozoans, particularly *Theileria*, *Babesia*, and *Trypanosome* (Rajput et al., 2005). These protozoa have the ability to cause significant economic losses for the livestock industry worldwide (Ananda et al., 2009) such as mortalities, decreased production and lowered working efficiency (Uilenberg, 1995), in addition to increasing the costs of control measures (Makala et al., 2003). *Babesia* spp. resembles short and long loop formation in RBC, whereas *Theileria* spp. RBC is annular, round, dot, and rod-shaped (Song et al., 2018).

In Libya, because of the economic significance of sheep in their inherited traditions, they have priority among all farm animals. Despite that, there is a lack of information about the haemoparasites prevalence in domestic animals in Libya.

In many countries around the world, sheep are a key source of the meat, milk, and wool that are produced. Africa produces up to 10.9 and 8.4% of its total meat from sheep and goats, respectively (Arafa, 2002).

In general, *Babesia bovis* is the most pathogenic (Adham et al., 2009). Nevertheless, *Theileria* species serve as the cause of a group of tick-borne diseases known as Theilerioses, among which *T. parva* and *T. annulata* are the two most virulent and economically important (Schudel et al., 2004), besides, they are considered the most common causes of theileriosis in bovine (Gebrekidan et al., 2016)**.**

T. parva, which causes East Coast fever (ECF), is found in 14 countries (Tarimo, 2013) in sub-Saharan Africa, ECF is still the most common tick-borne disease affecting cattle in that region (Demessie and Derso, 2015) while *T. annulata* which causes Tropical Theileriosis occurs in North Africa as well as Asia and southern Europe (Schudel et al., 2004).

Theileria and *Babesia* spp. are widely distributed (Uilenberg, 1995). Babesiosis in the ovine can cause an acute disease characterized by hemoglobinuria, icterus, fever, and hemolytic anemia. Meanwhile, ovine theileriosis is responsible for lymphoproliferative diseases which are characterized by high morbidity and mortality (Alessandra and Santo, 2012).

Usually, recovered animals act as carriers, which creates a potential source of infection (Zhou et al., 2017), identifications of *Babesia*, and *Theileria* spp. usually depend on a traditional blood smear examination, beside Polymerase chain reaction (PCR) which is becoming a common tool for molecular identification, for instance, multiplex PCR saves time and money in blood protozoa diagnosis (Kocan et al., 2004).

Material and Methods:

Animals and study area

748 blood samples were collected from apparently healthy animals (goats, sheep, buffaloes and, cattle) between January 2020 and January 2021 from different regions throughout Egypt and Libya.

Blood examination

Blood samples were randomly collected from a total of 209 (113 buffaloes and 96 cattle) from different regions in Ismailia, Egypt, and 539 (105 cattle, 372 sheep, and 62 goats) from different regions in Al Gabal al Akhdar province, Libya. These animals are apparently healthy and some cases show some clinical signs such as fever (41 °C), swelling lymph nodes, lacrimation, loss of appetite, diarrhea, emaciation, anemia, and hemoglobinuria, with degrees of jaundice appearing in vaginal and conjunctival mucous membranes, and a drop in milk production and abortions. The samples were collected using a sterile syringe,

approximately 2 ml from the jugular vein of the animals infested with hard ticks, before being dispersed into EDTA containing tubes, and transported in an icebox to the laboratory, each sample divided into two parts, the first part stored at -20 °C for molecular identification and the other part stored at 4 °C for morphological identification.

Thin blood smear preparation and microscopic examination

Blood samples were prepared for microscopic examination according to the standard protocol (Benzamin, 2010), and according to Soulsby (1968) The samples were morphologically identified.

Molecular identification

DNA Extraction

Genomic DNA was extracted and purified from 200µl of blood using the QIAamp® DNA blood Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted DNA was stored at -20 °C until use.

PCR amplification of the 18S rRNA gene of *Babesia* spp.

The following ingredients were added to a 25µl PCR reaction tube: 2mM MgCl2, 0.2mM dNTPs, Taq polymerase (0.05µl), 7µl of DNA, 4µl of nuclease-free water, a set of primers (2 pmol. oligonucleotide primers (Piro-18-F2 ACT GTC AGA GGT GAA ATT CTT AG and Piro-18-R all AAT AAT TCA CCG GAT CAC TCG) were used to amplify the 18S rRNA.

The PCR was carried out in a thermal cycler (C1000 Thermal Cycler, Bio Rad, USA) using 35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 45 seconds, and a final extension step of 72 °C for 5 minutes. The Power Pac (BioRad, USA) was used for (1.5%) agarose gel electrophoresis on the PCR products (5 µl) under predetermined guidelines (90 volts and 50 minutes).

Sequence analysis

Gel Bands were excised, purified, and sent for sequencing. The South Korean company Solgent Co. Ltd. carried out the Sanger sequencing. Sample Sequences were analyzed using BLAST®.

Phylogenetic analysis

Based on the 18S rRNA sequences of several closely related *Babesia* species, phylogenetic analysis was performed. MEGAX was used in the phylogenetic tree construction (Kumar et al., 2018).

Statistical analysis

Statistical analysis was carried out using Chi Square (X2) test for comparing infested and non-infested animals in each location of tick collection.

Results

The characteristic features of the piroplasma using a light microscope:

Morphology of *Babesia* spp. in cattle buffalo, sheep and goat

Babesia is classified into two groups based on morphology: small babesia (1.0–2.5µm long), which includes *Babesia bovis*, and large *Babesia* (2.5–5.0µm long), which includes *B.bigemina*. *Babesia bovis* is a minute parasite that often lives in the middle of the erythrocyte. It is typically found in pairs that are at an oblique angle to one another and is roughly 1-1.5µm long and 0.5-1.0µm wide (Fig. 2D). While *B. bigemina* is a considerably longer babesia and is typically seen in pairs with an acute angle to one another, it normally has a pear-shaped shape but can also be found in a variety of forms (Fig. 1). It is 3 to 3.5µm long and 1 to 1.5µm wide. Paired forms frequently exhibit two distinct red-staining spots in each parasite (*B. bovis* usually has only one) (Fig. 2A, 2B). *B. ovis* is small parasite which is 1–2.5µm in length (<2.5µm), round or rare pyriform, having obtuse angle present at the margin of the red cells. (Fig. 3).

Morphology of *Theileria* in cattle, buffalo, sheep, and goat

In the red blood cells, forms of the piroplasm typically take the shape of circular, ring-

shaped, or oval ring-shaped (0.5-1.5µm) (Figs. 4, 5).

Prevalence of piroplasmosis

In this study, 209 randomly collected blood samples from Ismailia province, Egypt (96 cattle, 113 buffaloes) and 539 randomly collected blood samples from Gabal al Akhdar province, Libya (110 cattle, 72 sheep, and 62 goats) were morphologically examined using Giemsa-stained thin blood films.

In Ismailia Province, Egypt, the results revealed infection by *Babesia* spp. (*Babesia bigemina* and *B. bovis*) and *Theileria annulata* infection. The infection rate of both *Babesia* and *Theileria* spp. were (6.25% and 17.70%) in cattle, respectively, while (3.5% and 15.9%) in buffaloes respectively (Table 1), the total prevalence of piroplasma infection was (23.9%) in cattle and (19.5%) in buffaloes, at different localities was (41.2, 12.5, 33.3, 25.0, and 15.8%) in the following districts; Kasassin, Elabtal, Kantara, Abu atwa, and Sarabiom respectively. The total prevalence of the infected buffaloes at different localities was (27.3, 15.0, 10.7, 29.4, and 19.2%) from Kasassin, Elabtal, Kantara, Abu atwa, and Sarabiom respectively (Table 1)

In Gabal al Akhdar Province, Libya, the results revealed the infection by *Babesia* spp. (*B. bigemina* and *B. bovis*) in cattle and (*B. ovis*) in sheep and goats and the infection with *Theileria annulata* in cattle and *T. ovis* in sheep and goats. The total infection by *Babesia* spp. was (9.5, 6.5, and 6.5%) in cattle, sheep, and goats respectively, whereas the total infection with *Theileria* spp. was (18, 24.7, and 14.5%) in cattle, (Table 1). The total prevalence of piroplasma infection in the examined cattle at different localities was 27.6% in cattle, 116 (31%) in sheep and 13 (21%) in goats, and (20, 17, 35, 40.9, and 27%) in the following districts; Al-Bayda, Shahat, Faydyah, Massah and Gandula respectively. The total prevalence of infected sheep was (41, 19, 27, 29, and 40%)

respectively, from Al-Bayda, Shahat, Faydyah, Massah, and Gandula. The total prevalence of the infected goats at different localities was (13, 33, 30, 6, and 43%) from Al-Bayda, Shahat, Faydyah, Massah, and Gandula respectively (Table 1).

Molecular identification of Piroplasma

A total of 100 blood samples from Egypt and Libya that tested positive for piroplasm morphologically were used in the molecular diagnosis. In spite of *Theileria* spp. samples being positive morphologically many of them failed to amplify utilizing PCR. The samples which positive were identified as *Babesia bigemina* and *B. bovis* based on the 18S rDNA gene; their GenBank accession numbers are (OM662297, OM662298, OM662299, OM662300, OM662301).

Phylogenetic analysis:

Based on the 18S ribosomal DNA gene, the phylogenetic analysis was performed using MEGA X10.1 software and the tree using neighbor – joining (NJ) methods was constructed (Fig. 6). Our study sequences of *Babesia* species were clustered with species from Egypt, Turkey, and Cuba, and away from species from the USA, China, and Colombia, as they do not share their ancestor.

Discussion

The prevalence of piroplasma infection in cattle and buffalo in Ismailia, Egypt, according to the current study was 23.9 and 19.5 %, respectively. The findings showed that various researches have noted the varied frequencies of piroplasmosis among cattle and buffalo (Mohsin et al., 2022). The majority of research found that the infection in cattle was higher than in buffalo. These results reveal that water buffaloes are suspected to be persistent carriers that are undetected by microscopy or they possess a natural tolerance to the parasite. They also imply that additional restrictions on tick infestation of buffaloes may be related to their propensity to spend a lot of time submerged in water or rolling

around in the mud, which acts as a natural means of ectoparasites controls (Hasson and Al-Zubaidi, 2014).

At Al Gabal al Akhdar, Libya, there were 27.6% of cattle with piroplasma infection. Piroplasma infection was present in 29.7% of sheep and goats overall, however, it was higher (31%) in sheep than in goats (13%). Goats may have a reduced rate of piroplasma infection because they pasture on steep, rocky terrain that limits their contact with ticks and other livestock species (Alessandra and Santo, 2012). The climatic conditions of the study area and the absence of hygiene measures may affect tick life cycles, which might increase tick infestations, therefore enhance the prevalence of blood parasites. Furthermore, alterations in blood parasite prevalence may be due to the immunological condition of the animals and the absence of veterinarian supervision's guidance on medication administration, which results in variations in the prevalence rates of infection (Adham et al., 2009; Hussein et al., 2017)

The total prevalence of *Babesia* and *Theileria* spp. was 4.8 and 16.7% in cattle and buffaloes, respectively, in Ismailia, Egypt. In contrast, it was (9.5 and 18%) in cattle, (6.5 and 23.3%) in sheep and goats, respectively, in Al Gabal al Akhdar, Libya. Fluctuations in the prevalence rates may be due to the changes in the environmental conditions that affect both vectors and parasites.

Theileria spp. predominated among the two piroplasma species found in the current investigation in sheep (24.7%) and goats (14.5%). These variations can be attributed to the difference in vector distribution according to temperature and humidity. Besides, animals that recover from babesiosis develop resistance to re-infection, (Adamu and Balarabe, 2012). Additionally, *Babesia* spp. rarely persist in sheep and goats (Jatau et al., 2011). The purpose of this study was to use the 18S rDNA gene to molecularly diagnose

different piroplasma species. 38 of the blood samples failed to amplify using PCR even though they were positive on morphological screening. These findings concurred with those of (Mohamaden et al., 2018) and may be explained by the majority of the cases being sub-clinically infected.

Babesia species sequences in the current investigation have a range of identities with those species in the GeneBank (99 to 100 %). These fragmented sequences from *B. bigemina* in the GeneBank came from various areas (MN053036.1 from Cuba, MH047816.1 from the USA, and MG604302.1 from Argentina) and have (100%) similarity with our species sequences. MN053042.1 from Cuba, EF458208.1 from Germany and MH045747.1 from the United States show 99.71% identities with our sequences of *B. bovis* species.

As a result, the small subunit rRNA gene region provides a reliable diagnostic marker for *Babesia* spp. and exhibits no change related to geographic distribution. These results are consistent with (El-Dakhly et al., 2020). In contrast, *Theileria* spp., the small subunit rRNA gene was unable to amplify our *Theileria* species, These may be attributed to the variation of the diagnostic marker of *Theileria* spp. even among isolates from the same geographical districts, which may lead to the failure of the amplification of the species, therefore the small subunit rRNA gene is not a good diagnostic marker in *Theileria* spp (El-Dakhly et al., 2020).

Conclusion

Molecular identification will support and enhance the diagnosis of diseases and selecting a successful genetic marker for diagnosis purposes will give good results, using Real-time PCR in the diagnosis of blood parasites may give better results than conventional PCR.

Ethics

This study was approved by the Ethics Committee of Faculty of Veterinary Medicine

Science, Suez Canal University, Egypt with approval number (2018123).

Conflict of interest

The authors declare that they have no conflict of interest.

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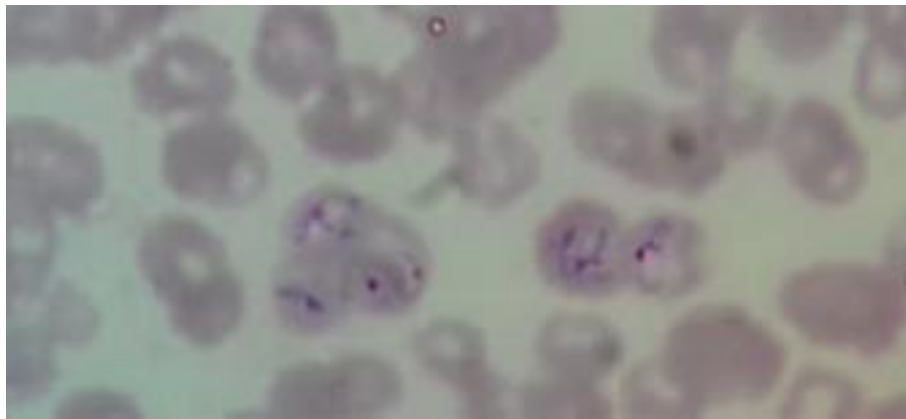
Fig. (1) Microphotograph of different shapes of *Babesia bigemina* with ovoid and amoeboid shapes inside the erythrocytes in giemsa stained blood smear.
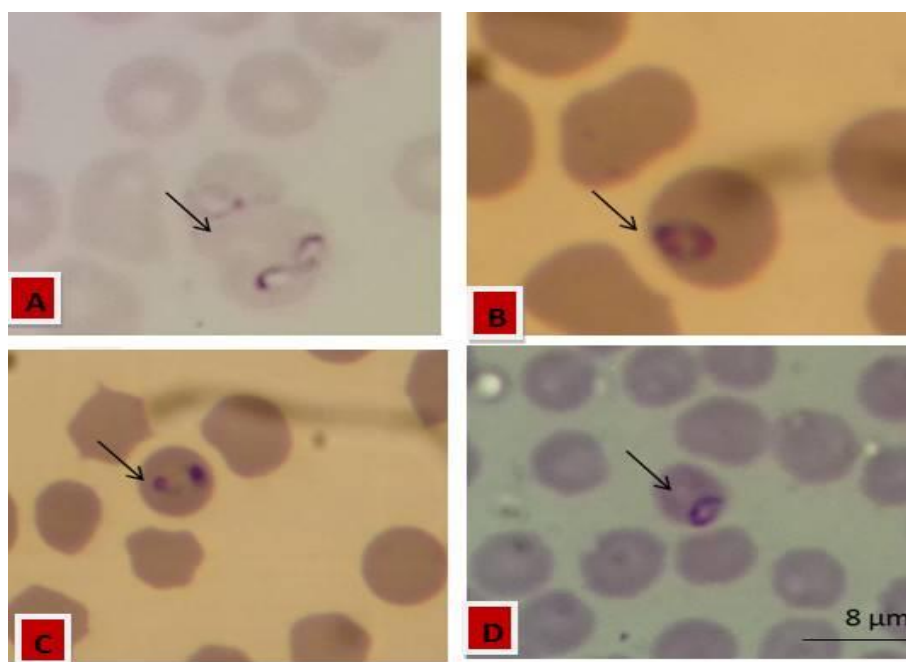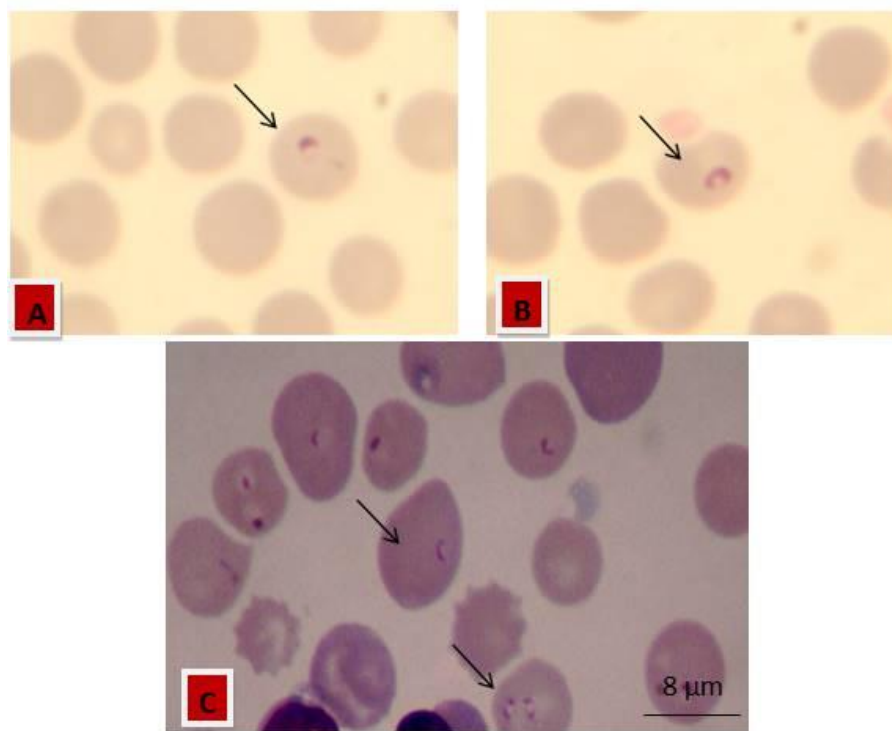

Fig. (2) Microphotograph (A) of *Babesia bigemina* with Pyriform body at acute angle inside the erythrocytes in giemsa stained blood smear.
Microphotograph (B and C) of *B. bigemina* (D) of *B. bovis* inside the erythrocytes in giemsa stained blood smear.
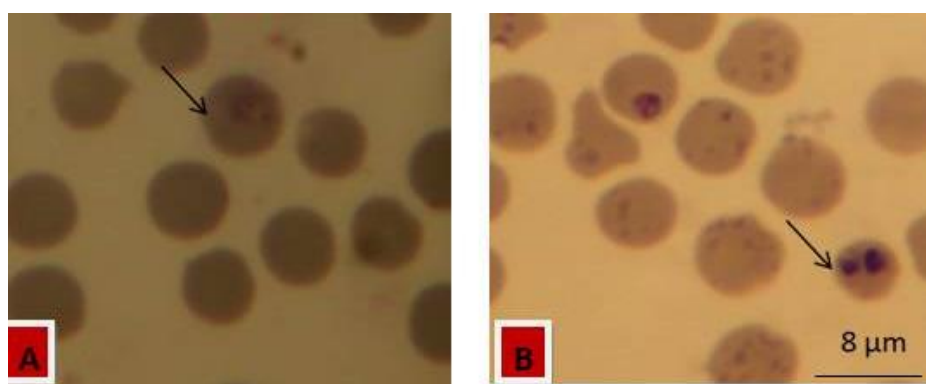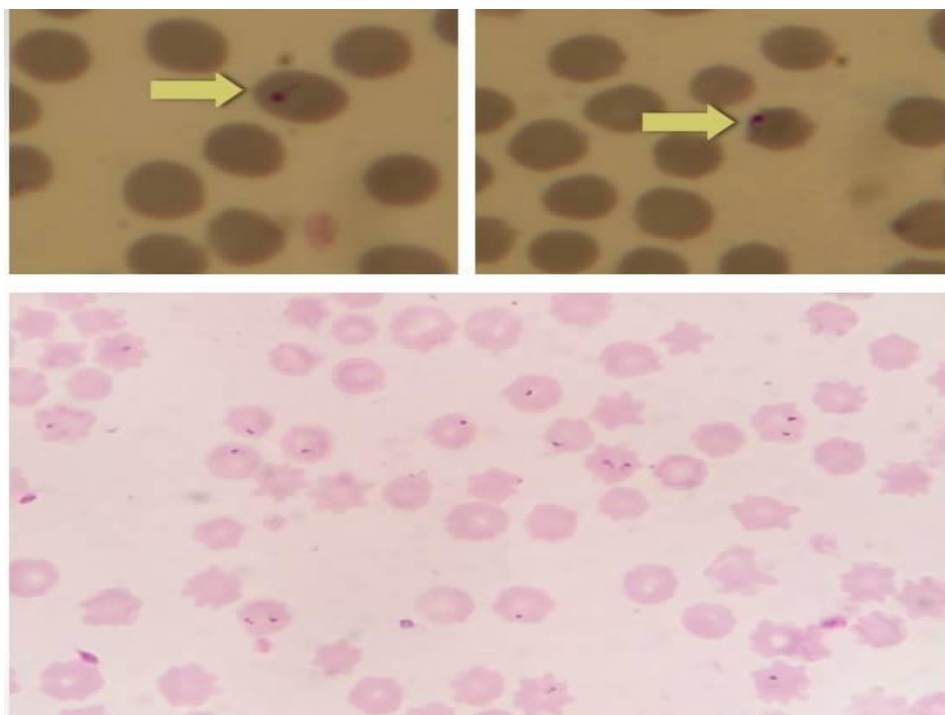

Fig. (3): (A)&(B) Blood smear stained with giemsa showing *Babesia ovis* in the red blood cell
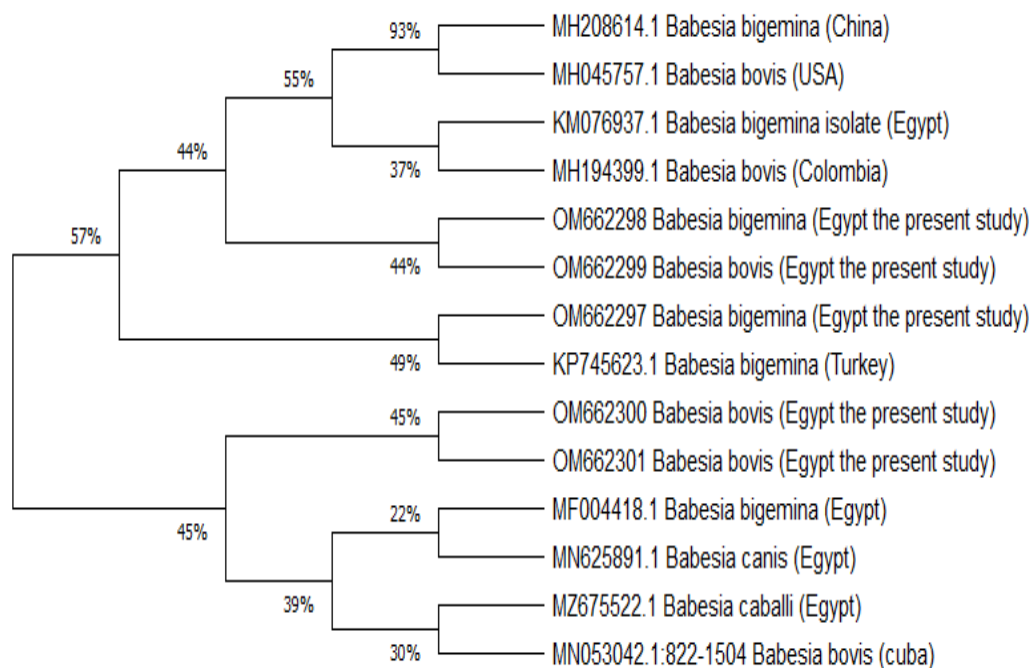
Fig. (4): Microphotograph (A and B) of *Theileria annulata* inside the erythrocytes in Giemsa stained blood smear, Microphotograph (C) of pleomorphic *Theileria annulata* with polychromatic (sever infection) in Giemsa stained blood smear

Fig.(5): Blood smear stained with giemsa showing *Thieleria ovis* inside the red blood cells

Fig. (6): The phylogenetic analysis was constructed using neighbor – joining (NJ) methods, to construct the phylogenetic tree of some of *Babesia* species sequences from the Genbank and our sequences samples are included based on 18S rDNA sequences.

Table (1): Prevalence of *Babesia* spp., *Theileria* spp. and total infection of piroplasma at different localities of Ismailia province, Egypt and Al Gabal al Akhdar province, Libya

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Ismailia province, Egypt.				Al Gabal al Akhdar province, Libya.					
Prevalence of *Babesia* spp., and *Theileria* spp. infections									
Parasite	Cattle		Buffaloes		Cattle		Sheep		Goat	
	No. infected	(%)	No. infected	(%)	No. infected	(%)	No. infected	(%)	No. infected	(%)
***Babesia* spp.**	6/96	6.3	4/113	3.5	10/105	9.5	24/372	6.5	4/62	6.5
***Theileria* spp.**	17/96	17.7	18/113	15.9	19/105	18	92/372	24.7	9/62	14.5

	Cattle		Buffaloes			Cattle		Sheep		Goat	
Locality	No. Examined	Infected (%)	No. Examined	Infected (%)	Locality	No. Examined	Infected (%)	No. Examined	Infected (%)	No. Examined	Infected (%)
Kasassin	17	(41.2)	22	(27.3)	Al-Bayda	30	(20)	80	(41)	15	(13)
Elabtal	25	(12.5)	20	(15.0)							
Kantara	15	(33.3)	28	(10.7)	Shahat	18	(17)	70	(19)	12	(33)
Abu atwa	20	(25.0)	17	(29.4)	Faydyah	20	(35)	85	(27)	10	(30)
Sarabiom	19	(15.8)	26	(19.2)	Massah	22	(40.9)	72	(29)	18	(6)
Total	96	(23.9)	113	(19.5)	Gandula	15	(27)	65	(40)	7	(43)
					Total	105	(27.6)	372	(31)	62	(21)

Percentage of infected animals in relation to total no. of animals examined in each location.
Total : Chi-Square (x^2) = 216.094[a], DF= 6, P. Value= 0.000 (highly Significant).

ملخص عربي

الكشف المورفولوجي والجزيئي للطفيليات الدموية المنقولة بالقراد في المجترات من الجبل الأخضر، ليبيا ومحافظة الإسماعيلية، مصر

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تعتبر الأمراض التي تنتقل عن طريق القراد من العوامل الهامة التي تؤثر على صحه الحيوانات في جميع أنحاء العالم، في كل من المناطق الاستوائية وشبه الاستوائية. الهدف من هذه الدراسة هو دراسة مدى انتشار أنواع البيروبلازما في كل من منطقة الجبل الأخضر في شمال شرق ليبيا والإسماعيلية بمصر. تم جمع عينات الدم بشكل عشوائي من 748 حيوانًا سليمًا ظاهريًا من مناطق مختلفة في ليبيا ومصر خلال الفترة الممتدة من يناير 2020 إلى يناير 2021. واستخدمت أفلام الدم المصبوغة للفحص المجهري المباشر وتم استخدام تفاعل البوليميراز المتسلسل التقليدي للتعرف الجزيئي للعينات. وأظهرت النتائج أن نسبة انتشار البيروبلازما في مصر بلغت 23.9% في الأبقار، و19.5% في الجاموس، بينما بلغت في الأبقار 27.6%، و31% في الأغنام، و21% في الماعز في ليبيا. إن اختيار دليل جيني وراثي جيد لأغراض التشخيص سيعطي نتائج جيدة، واستخدام تفاعل البوليميراز المتسلسل (الريف تيم) في تشخيص طفيليات الدم قد يعطي نتائج أفضل من تفاعل البوليميراز المتسلسل التقليدي.

الكلمات المفتاحية: جزيئية، مجهرية، طفيليات دموية، مجهرية، بابيزيا ، ثيليريا.