Journal of the Egyptian Society of Parasitology, Vol. 47, No. 2, August 2017
J. Egypt. Soc. Parasitol. (JESP), 47(2), 2017: 403 – 414

MOLECULAR DETECTION OF BORRELIA SP. IN ORNITHODOROS SAVIGNYI AND RHIPICEPHALUS ANNULATUS BY FlaB GENE AND BABESIA BIGEMINA IN R. ANNULATUS BY 18S rRNA GENE

By

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Abstract

This study determined the ability of the soft tick, *Ornithodoros savignyi* and the hard tick, *Rhipicephalus* (formerly *Boophilus*) *annulatus* to serve as carrier for new genotypes of *Borrelia* and *Babesia*. *Ornithodoros savignyi*, was collected in the summer, 2015 from camel market at Shalatein, Red Sea Governorate, while *R. annulatus* was collected from cows at Salhia, Sharkia Governorate and Banha, Qalyoubia Governorate. Hemolymph smears were prepared and stained with Giemsa stain and examined by light microscope (LM) for the presence of spiral form of *Borrelia* and vermicule stage of *Babesia*. The tick specimens that revealed positive infection with either *Borrelia* or *Babesia* by LM were screened by PCR using *flaB* gene for *Borrelia* and *18S rRNA* gene for *Babesia.* The obtained amplicons were sequenced, registered in GenBank and the phylogenetic trees for the obtained sequences were constructed. Results showed that spirochetes (*Borrelia*) were found in *O. savignyi* and *R. annulatus*, while the vermicule form of *Babesia* was found in *R. annulatus* only. The PCR amplified *Borrelia* and *Babesia* at 350 &50 bp, respectively. The obtained amplicons were recorded in GenBank with accession number MF084762, MF084761 & MF004418 for *Borrelia* sp. in *O. savignyi*, *Borrelia* sp. in *R. annula-tus* and *B. bigemina* in *R. annulatus*, respectively. The genotype of *Borrelia* sp. recorded in *O. savignyi* is very close to *B. burgdroferi* that causes Lyme disease in human. But, genotype of *Borrelia* sp. recorded in *R. annulatus* is close to *B. theileri* that causes a mild disease in animals. Genotype of *Babesia* in *R. annulatus* was 100% identical with *B. bigemina* that recorded before.

Keywords: FlaB, 18S rRNA, *Borrelia*, *Babesia*, *Ornithodoros savignyi*, *Rhipicephalus annulatus*.

Introduction

Persons and animals that become ill after a tick bite may be at increased risk because a tick bite may be considered as the source of the pathogen. Detailed knowledge of the causative agents, their distribution, and their relationship to potential vectors is also lacking (Williamson *et al,* 2010). In humans, Lyme Borreliosis occurs in all age groups, with equal prevalence in men and women which become infected from the bite of an infected tick (Adham *et al,* 2010).The clinical signs and symptoms of infection in cattle and other animals are mild and variable, but usually include fever and anemia (McCoy *et al,* 2014).The disease usually begins with a characteristic skin lesion, erythema migrans at the site of the tick bite. After several days or weeks, the spirochetes typically spread hematogenously, and patients may develop early-disseminated disease with dermatologic, cardiac, neurologic, and rheuma-tologic involvement. Late-stage disease can present chiefly as arthritis and/or neurological impairment (Steere *et al,* 1977; Elhelw *et al,* 2014). The tick-borne Relapsing fever endemic commonly transmitted by argasid soft ticks with the exception of *Borrelia* sp., transmitted by several species of *Ixodes* hard ticks (Parola *et al,* 2001; Krause *et al,* 2015). In Africa, relapsing fever borreliae cause mild to fatal septicemia and termination (El Bahnasawy *et al,* 2012). The genus *Borrelia* is composed of bacterial pathogens responsible for relapsing fever and Lyme borreliosis (Haitham *et al,* 2013). While the Lyme disease agent *Borrelia* sp. (Casjens *et al,* 2000) was transmitted by hard ticks, the re-

lapsing fever borreliae are transmitted by soft ticks (Vial *et al,* 2006; Trape *et al,* 2013). In Egypt, available data about the incidence and prevalence of Lyme disease are few (Haberberger *et al,* 1989). Only three recent studies have pointed out Lyme borreliosis (Elhelw *et al,* 2014), the molecular evidence of *B. burgdorferi* in ticks (Adham *et al,* 2010) and serological screening of Lyme disease in children (Hammoud *et al,* 1995). No studies documented the presence of Lyme borreliosis in Egyptian animals, but only in man (El Bahanasay *et al*, 2015). Borreliosis, is caused by the spirochete, *Borrelia theileri* transmitted by bite of an infected ticks of the subgenus *Rhipicephalus* (formerly *Boophilus*): *Rhipicephalus annulatus, R. hipicephalus microplus, R. hipicephalus decoloratus,* and *R. hipicephalus geigyi*. The mitochondrial 12S rRNA and 16S rRNA genes from selected ticks, and the *Borrelia* flaB, and 16S rRNA genes were PCR-amplified and sequenced (McCoy *et al,* 2014) A high infection rate (66%) of *B. burgdorferi* was observed in both nymph and adult soft ticks *O. savignyi* used OspC gene in PCR amplified (Adham *et al,* 2010). The identification of the *B. orrelia persica* with its vector *Ornithodoros tholozani* used sequences of different genes such as 16S rRNA, 12S rRNA, flaB, 18S rRNA and glpQ (Safdie *et al*, 2010).

Babesia is an apicomplexan hemoparasite transmitted by ticks to a wide variety of mammalian hosts and cause significant mortality and morbidity to them. (El-Fayomy *et al,* 2013). In Egypt, zoonotic babesiosis was reported in man and dogs (Saleh *et al,* 2015). *Babesiosis* have long been recognized as economically important disease of cattle because they cause extensive erythrocytic lysis leading to anemia, icterus, fever, hemoglobinuria and death in not early treated cases (Vial and Gorenflot, 2006). *Babesia bigemina* was molecularly identified by using 18S rRNA gene in Uganda (Byaruhanga *et al,* 2016). It was detected by the same gene in Egypt (Elhaig *et al,* 2016). The first attempt to determine the prevalence of *B. bigemina* in *Boophilus annulatus* used 18 rRNA gene sequences in Egypt (Adham *et al,* 2009). The detection and specific discrimination of *Babesia* sp. and *Borrelia* sp. in ticks is of risky importance. Microscopic techniques for haemolymph examination remain the most appropriate for the diagnosis of acute disease (Almeria *et al,* 2001). Meanwhile, the main drawbacks for the microscopic detection of *Babesia* spp. and *Borrelia* spp. in haemolymph of ticks are the low sensitivity and the difficulty of differentiating between the species involved (Guglielmone *et al,* 1997). Polymerase chain reaction (PCR)-based detection methods were described that proved to be extremely sensitive and specific in the detection of *Babesia* and *Borrelia* organisms (Smeenk *et al,* 2000; Almeria *et al,* 2001).

This study was designed to determine ability of the commonest soft tick, *O. savignyi* that infest camels and the hard tick, *R. annulatus* that infest cows to serve as carriers for new genotypes of *Borrelia* in *O. savignyi* and *R. annulatus* as well as *Babesia* in *R. annulatus*.

Materials and methods

Tick collection: Soft tick *Ornithodoros savignyi* was collected from camel's body during summer 2015 from Shalatein, Red Sea Governorate (23°06'32.4"N 35°33'22.2"E).

However, the hard tick *Rhipicephalus* (formerly *Boophilus*) *annulatus* was collected from the body of cows and their pens, during the same season from Salhia, Sharkia Governorate (30°49'29.3"N 32°04'00.3"E) and Banha, Qalyoubia Governorate (30°25' 57.3"N 31°12'34.1"E). The tick specimens were brought alive to the laboratory of Animal Acarines Research Center, Faculty of Agriculture, Cairo University. The collected ticks were maintained alive at 28±1℃ and 75±5% relative humidity for further procedures. Locality (using Global Positioning System G.P.S.), host, date, climatic condition (temperature and relative humidity), and position of infestation of the ticks collected

were recorded. The identification of ticks was performed in the laboratory using the taxonomic keys (Hoogstraal, 1956; Walker *et al*, 2003).

Tick hemolymph smear preparation: Hemolymph was obtained from each tick by amputating the distal portion of one or more legs and smeared on microscopic slide (Burgdorfer, 1970). The hemolymph slides were dried in air and stained with Giemsa to investigate the presence of *Borrelia* in haemolymph slides prepared from *O. savignyi* and *R. annulatus* or *Babesia* from smears prepared from *R. annulatus* only. The prepared hemolymph slides were examined under oil emersion lens using ordinary microscope (Zeiss). The tick specimens that revealed positive infection with *Borrelia* or *Babesia* by light microscope were stored at -20℃ for molecular biology procedures.

DNA Extraction: The DNA of tick specimens that revealed positive infection with either *Borrelia* or *Babesia* was extracted. The frozen ticks were cut into small pieces using a disposable scalpel in 1.5µL Eppendorf tubes under a sterile laminar flow hood in Molecular Biology Laboratory, Zoology department, Faculty of Science, Menoufia University. The DNA was extracted from the ticks used the PureLink® Genomic DNA Kits (Invitrogen, USA). Briefly, each sample was covered in the tissue lysis buffer included in the kit (between 180µL & 540µL depending on the size of tick) and treated with proteinase K (20µL/180µL of tissue lysis buffer) incubated to 48 hat 56℃. Subsequent steps were carried out according to the manufacturer's instructions (Invitrogen, USA).

Polymerase chain reaction (PCR): PCR amplification was performed in a final reaction volume 2X (50 µL) containing 25µL 2X master mix solution (*i*-Taq™, *iNt*RON, Korea), 0.2 uM (2µL) of each primer, 4µL template DNA, 2.5µL Bovine serum albumin, and 14.5µL nuclease-free water. The designated primers were obtained from Macrogen, Korea. The oligonucleotide sequences of the used primers were listed (Tab.1). The PCR conditions of *Borrelia* were: an initial denaturation at 95°C for 7 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The PCR conditions of *Babesia* were: an initial denaturation at 94°C for 10 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The amplification reactions were carried out in a PCR thermal cycler Biometra T-personal/Germany S/N 1003507 and the corresponding amplicons were checked on 1% agarose gel using TAE buffer, stained with ethidium bromide, examined under UV transilluminator, and photographed using a digital camera.

Table 1: Nucleotide sequence of primer used for PCR of tick specimens

Region DNA	Primer's sequence (5′→ 3′)	Specificity	Annealing Tep./ PCR product	Reference
FlaB	5′- AACAGCTGAAGAGCTTGGAATG -3′	*Borrelia* sp.	55 °C/ 350bp	Williamson *et al*, 2010
FlaB	5′- CTTTGATCACTTATCATTCTAATAGC -3′			
18S rRNA	5′- GTTTCTGMCCCATCAGCTTGAC -3′	*Babesia* sp.	61 °C/ 450bp	Hilpertshauser *et al*, 2006
18S rRNA	5′- CAAGACAAAAGTCTGCTTGAAAC -3′			

Sequence analysis and construction of phylogenetic trees: Reaction products that contained an amplified fragment were purified using a GenElute PCR Clean-Up Kit (Sigma, Germany). The DNA sequencing was performed on one strand using the same primers employed for PCR. Sequences of *Borrelia FlaB* and *Babesia18S rRNA*, amplicons were aligned. The obtained sequences were assembled using Chromas Pro 1.5 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia). The resulting sequences were then subjected to Basic Local Alignment Search Tool (BLAST) analysis to determine similarities with those sequences available in the GenBank database hosted by the National Center for Biotechnology Information (NCBI). Phylogenetic relationships between new Egyptian isolates and other reference strains published in Gen-

Bank were inferred using the BioEdit sequence alignment editor (v. 7.2.6.). Two phylogenetic trees were constructed with the neighbor joining method (NJ) (Saitou and Nei, 1987). Phylogenetic analyses were conducted inMEGA5 (Tamura *et al*, 2007). Different approaches were evaluated in order to identify clades supported by the highest possible bootstrap values. Details are given in tables (1, 2, 3, 4 & 5).

Table 2: The accession number for sequences used for comparative analysis to *Borrelia*, obtained from GenBank.

Accession No.	Species	Country	Host	Reference
MF084762	*Borrelia* sp.	Egypt	*Ornithodoros savignyi*	This study
MF084761	*Borrelia* sp.	Egypt	*Rhipicephalus annulatus*	This study
EF141022.1	*Borrelia sp.*	Barazil	*Rhipicephalus microplus*	Yparraguirre,L.A.
KF569936.1	*Borrelia theileri*	Mali	*Rhipicephalus geigyi*	Mccoy,B.N.
KP191621.1	*Borrelia theileri*	Israel	*Ornithodoros sp.*	Kleinerman,G.
DQ100451.1	*Borrelia lonestari*	USA	*Carios capensis*	Reeves,W.K.
AY166716.1	*Borrelia lonestari*	USA	*Amblyomma americanum*	Bacon,R.M.
KR677091.1	*Borrelia sp.*	Portugal	Hard ticks	Nunes,M.
KU933526.1	*Borrelia sp.*	USA	*Amblyomma americanum*	Sayler,K.A.
KR677086.1	*Borrelia sp.*	Portugal	Hard ticks	Nunes,M.
KU749378.1	*Borrelia miyamotoi*	China	Human	Jiang, B. G.
DQ016626.1	*Borrelia sp.*	Poland	*Ixodes ricinus*	Wodecka,B.
AF529084.1	*Borrelia sp.*	France	*Ixodes ricinus*	Richter,D.
KR677089.1	*Borrelia sp.*	Portugal	Hard ticks	Nunes,M.
AY850064.1	*Borrelia lonestari*	USA	*Amblyomma americanum*	Pilgard,M.A.
KF894066.1	*Borrelia afzelii*	Poland	Mouse	Hildebrand,J.
JX257051.1	*Borrelia hispanica*	Morocco	*Ornithodoros erraticus*	Diatta,G.
D43777.1	*Borrelia miyamotoi*	Japan	Mammals	Fukunaga,M.
AY442142.1	*Borrelia lonestari*	USA	*Amblyomma americanum*	Varela,A.S.
KT592278.1	*Borreliellaburgdorferi*	USA	Lab mice	Esteve-Gassent,M.D.
AF007122.1	*Vibrio cholerae*	USA	Human	Klose,K.E.
EU979630.1	*Borrelia garinii*	Russia	*Ixodes persulcatus*	Beklemishev,A.B.
KX444534.1	*Borrelia theileri*	Congo	Lice	Amanzougaghene,N.
AY552536.1	*Borrelia lonestari*	USA	*Amblyomma americanum*	Varela,A.S.
KM875675.1	*Borrelia burgdorferi*	USA	*Ixodes scapularis*	Esteve-Gassent,M.D.
AB178780.1	*Borrelia afzelii*	Russia	Mouse	Masuzawa,T.
Tick species	Hosts	Stages*	Area	Total

Table 3: Accession number for sequences used for comparative analysis to *Babesia*,obtained from GenBank.

Accession No.	Species	Country	Host	Reference
MF004418	*Babesia sp.*	Egypt	*R. annulatus*	This study
EF458206.1	*Babesia bigemina*	Islands	Cattle	Vogl,S.J.
EF458205.1	*Babesia bigemina*	Puerto Rico	Cattle	Vogl,S.J.
DQ785311.1	*Babesia bigemina*	Spain	Cattle	Buling,A.
KU206296.1	*Babesia bigemina*	Uganda	Cattle	Byaruhanga,C.
LC125457.1	*Babesia ovata*	Japan	Cattle	Yokoyama,N.
FJ869902.1	*Babesia bigemina*	Mozambique	Cattle	Martins,T.M.
LC125456.1	*Babesia sp.*	Vietnam	Cattle	Yokoyama,N.
JQ993419.2	*Babesia bigemina*	China	*Ixodes persulcatus*	Zhang,Y.
HQ197740.1	*Babesia bigemina*	Turkey	Cattle	Inci,A.
KF429798.1	*Theileria annulata*	Iran	Cell line	Afshari,A.

Results

Total number of soft and hard ticks collected from different localities was 828 ticks (611 *O. savignyi*&215 *R. annulatus*).*O. savignyi* was collected from the camel market's ground at Shalatein, Red Sea Governorate (Tab. 4). But, cattle tick *R. annulatus* was collected from two localities; 172 ticks from Salhia, Sharkia Governorate &43 ticks from Banha, Qalyoubia Governorate.

Table 4: Tick species collected from host animals in all regions studied

Tick species	Hosts	Stages *	Shalatein	Birqash	Salhia	Banha	Total
O. savignyi	Off host	(M. & F.)	611	–	–	–	611
R. annulatus	Cows	(M. & F.)	–	–	172	43	215
Total			611	–	172	43	828

*M= Male & F= Female

Morphological detection of *Borrelia* and *Babesia* in tick hemolymph: The positive hemolymph smear with *Borrelia* was identified by the presence of spiral form of *Borre-*

lia (Fig. 1 A-C). Where, the positive haemolymph smear with *Babesia* was recognized by the presence of identical form of *Babesia* that called vermicule stage (Fig. 1 D). Percentages of infection with *Borrelia* and *Babesia* in the hemolymph smears prepared from *O. savignyi* and *R. annulatus*. A total of 828 ticks (611 *O. savignyi* and 215 *R. annulatus*) were investigated for the presence of *Borrelia* or *Babesia* in their hemolymph (Tab. 5). The overall infection with *Borrelia* and *Babesia* was 1.93% and 0.24% respectively. Borrelia was recorded in the two tested tick species with the infection rate 0.98% and 4.65% in *O. savignyi* and *R. annulatus*, respectively. *Ornithodoros savignyi* was free from *Babesia* infection, while only 2 *R. annulatus* ticks (0.93%) were positive with *Babesia*.

Table 5: Infection%of *Borrelia* and *Babesia* in smears and PCR prepared from *O. savignyi* and *R. annulatus*.

Tick species		No. of ticks	*Borrelia sp.* (%)		*Babesia sp.* (%)	
			Hemolymph.	PCR	Hemolymph.	PCR
O. savignyi	Shalatein	611	6 (1)	6 (1)	-	-
R. annulatus	Salhia	172	10(5.8)	10 (5.8)	2 (1.16)	2 (1.16)
	Banha	43	-	-	-	-
Total		828	16 (1.93)	16 (1.93)	2 (0.24)	2

Molecular detection of *Borrelia* and *Babesia* in tick tissues: PCR successfully amplified a single ~350 bp fragment of *Borrelia sp*. in *O. savignyi* and *R. annulatus*. PCR successfully amplified a single ~450 bp fragment of *Babesia sp.*in *R. annulatus* (Fig 2).

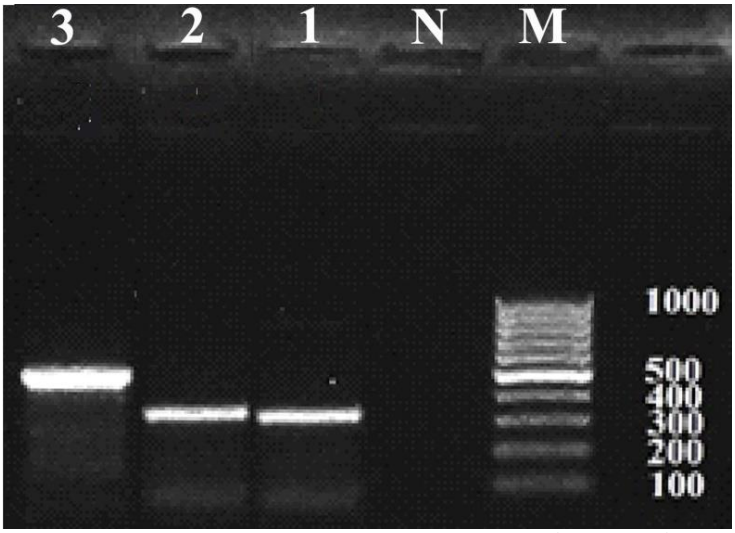

Fig. 2*:* Agarose gel electrophoresis (1 %agarose, stained with ethidium bromide): analysis of PCR amplification for detection of *Borrelia sp.* and *Babesia sp.* M: 100 bp DNA marker; N: negative control; 1) positive tick sample with *Borrelia sp*. from *O. savignyi* at 350 bp; 2) positive tick sample with *Borrelia sp*. from *R. annulatus* at 350 bp*;* and 3) positive tick sample with *Babesia sp*. from *R. annulatus* at 450 bp.

Nucleotide sequencing and phylogenetic analysis: The BLAST analysis confirmed the sequence was from *FlaB* gene of *Borrelia sp.* from *R. annulatus* and *O. savignyi*. Thus, the *FlaB* gene sequences generated from *R. annulatus* and *O. savignyi* isolates of *Borrelia sp.*, were submitted to GenBank data base under the accession numbersMF084761 and MF084762, respectively. Analyses revealed that these sequences are nearly identical with more than 99% similarity with the cognate gene sequences of Egyptian *Borrelia sp.* isolates from *R. annulatus* available in the database and share 99.2% nucleotide identity amongst them. The *FlaB* gene sequences of these Brazil *Borrelia sp.* isolates from *R. microplus* were found to have 99.2-97% nucleotide identity with *B. theileri* strain KAT from *R. geigyi* in Mali and Japan *Borrelia sp.* isolates from *Haemaphysalis flava* were found to have 95%. They have also a very close phylogenetic relation with isolates from USA and Portugal (Fig. 3). Analyses revealed that these sequences are

nearly identical with more than 99 % similarity with the cognate gene sequences of Egyptian *Borrelia sp.* isolates from *O. savignyi* available in the database and share 99.1 % nucleotide identity amongst them. The *FlaB* gene sequences of these Brazil *Borrelia sp.* isolates from *R. microplus* have 99.2, 97 % nucleotide identity with *Borrelia theileri* strain KAT from *R. geigyi* in Mali and USA *Borreliella burgdorferi* were found to have 95%. They have also a very close phylogenetic relation with isolates from USA and Portugal (Fig.3). Thus, close genetic relatedness was observed between *Borrelia sp.* isolates from this region of Egypt with Brazil and Mali rather than its neighbouring country Japan. In that respect, this study on phylogenetic relation of *Borrelia sp.* of cattle and camels isolate of Salhia & Shalatein regions with other isolates throughout the world may be considered first report from Egypt.

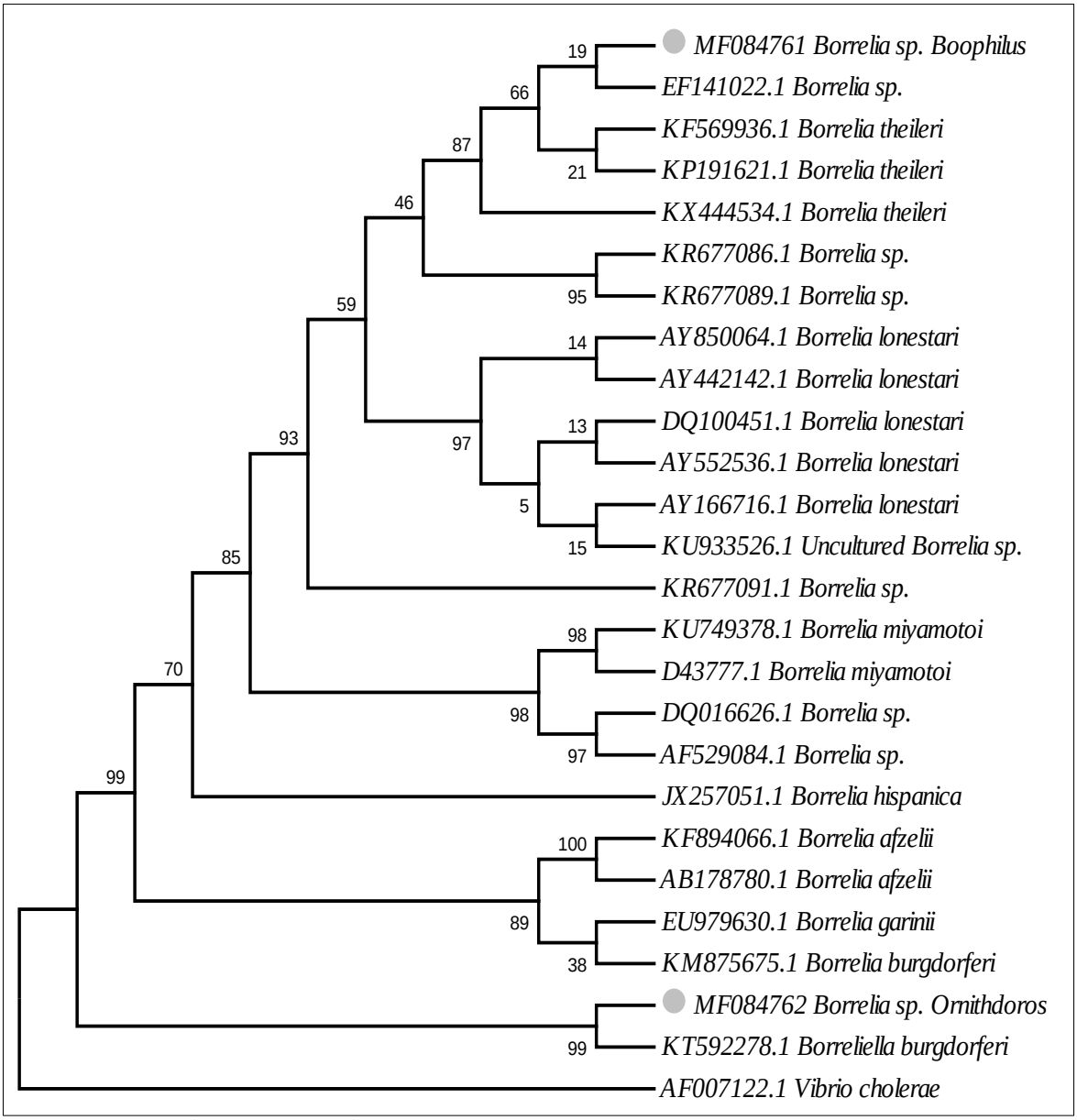

Fig. 3: Phylogenetic tree of *Borrelia* based on *flaB* gene. All sequences were aligned and Neighbor-joining tree was constructed. Upper black circle indicates to accession number of *Borrelia sp*. found in *R. annulatus*, lower black circle indicts to accession number of *Borrelia* sp. found in *O. savignyi*.

The BLAST analysis confirmed the sequence was from *18S rRNA* gene of *B. bigemina*. Thus, the *18S rRNA* gene sequences generated from *R. annulatus* isolates of *B.*

bigemina, were submitted to GenBank data base under accession number MF004418.

Analyses revealed that these RNA gene sequences of Egyptian *B. bigemina* isolates found to have 99.8 % nucleotide identities with *B. bigemina* from Puerto Rico and Spain, and they also have a very close phylogenetic relation 99 % with isolates from Uganda, and very close phylogenetic relation 99 % with isolates from China from *Ixodes persulcatus* ticks (Fig. 4). Thus, close genetic relatedness was observed between *B. bigemina* isolates from this region of Egypt with Puerto Rico and Spain rather than its neighbouring country China.

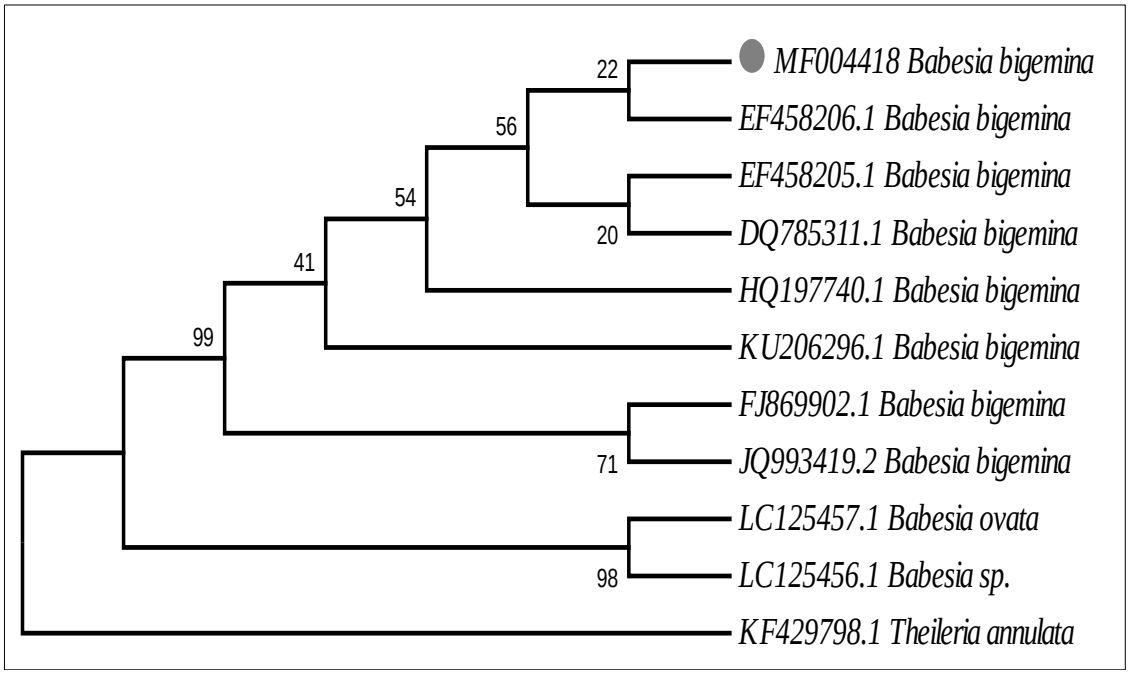

Fig. 4: Phylogenetic tree of *Babesia* based on *18S rRNA* gene. All sequences were aligned and Neighbor-joining tree was constructed. Black circle indicates to the accession number of *Babesia bigemina* that was found in *R. annulatus.*

Discussion

The most economic important of ticks is strongly due to their ability to acquire disease agents from infected hosts and transmit these agents to uninfected hosts that may be animals or humans. In this study, two tick species, one soft tick, *O. savignyi* and another hard tick, *R. annulatus* were chosen to determine their ability to serve as carriers for pathogens. It is well known that *O savignyi* has a zoonotic important and a previous study done (Helmy, 2000) confirmed the presence of this tick species in Shalatein that locate at the southern boundary of Egypt. The main camel market in this town includes thousands of camels imported from Sudan and other African countries. The persons accompanied with camels may be bitted by an infected *O. savignyi* with pathogens such as *Borrelia* (Shanbaky and Helmy, 2000). Thus, *O. savignyi* was investigated for the presence of *Borrelia* or other pathogens to achieve the goal of this study that concern the new genotype not isolated before from Egypt. On the other hand, the cattle tick, *R. annulatus* was the main tick on cattle in Egypt (El Kammah *et al,* 2001). Globally, the genus *Rhipicephalus* (formerly *Boophilus*) is the main vector of bovine borreliosis, such as *Borrelia theileria* (Yparraguirre *et al*, 2007, McCoy *et al*, 2014) and *Babesia* spp. to cattle (Adham *et al,* 2009). To fulfill this goal, *R. annulatus* was collected from Salhia and Banha and investigated for the presence of *Borrelia* and *Babesia*. Then the positive ticks were sequenced and the obtained sequences were analyzed to determine the new expected genotypes.

In Egypt, available data about the occurrence of Lyme disease are scarce and no

studies documented the presence of Lyme borreliosis in Egyptian animals and tick carriers verifying its zoonotic evidence. Also, no trial to isolate *B. burgdorferi* from the clinical samples was done (Elhelw *et al,* 2014).

Human infection with *B. persica* is transmitted by the soft tick *Ornithodoros tholozani* and was reported from Iran, Israel, Egypt, India, and Central Asia (Baneth *et al,* 2016). In Egypt, few articles reported the existence of Lyme disease in humans and animals. Samir *et al,* (2015) confirmed the occurrence of *B. burgdorferi* infection in Egyptian dogs in which the only identified tick species is *Rhipicephalus sanguineus*. *Borrelia sp.*, the causal organism of Lyme borreliosis.In the present study, 350bp fragment corresponding to *Borrelia sp.* was amplified from *R. annulatus* (5.8%). Similar data were by Adham *et al.* (2010) who found that 8% out of 882 *R. annulatus* ticks were infected with *B. burgdorferi*. There is a conflict between the identification of *Borrelia* in that finding and in this study because the present authors found that sequence of partial flab gene obtained from *Borrelia* sp. in *R. annulatus* (accession number: MF0847 61) is close to *B. theleria* (accession number: KF569936.1) and *Borrelia* sp (accession number: EF141022.1) without any correlation with *B. burgdroferi*. Dubinina *et al*, (2000) and Guiqing *et al*, (2003**)** found that of the 498 *I. scapularis* ticks collected from Rhode Island, Connecticut, New York, and New Jersey, 91 of 438 (20.7%) nymphal ticks and 15 of 60 (25.0%) adult ticks were positive by PCR assay, Daiva *et al*. (2004) using PCR found that (6.9%) *I. ricinus* ticks were infected with *B. burgdorferi*. In the present study, a low infection rate (1%) of *Borrelia sp.* was observed in both nymph and adult soft tick *O. savignyi*. The results agreed with Adham*et al,* (2010) who found *B. burgdorferi* infected 1541 *O. savignyi* collected from Dahshour, Giza Governorate. In the present study, *Borrelia* in *O. savignyi* (accession number: MF084762) is very close to *B. burgdorferi* (accession number*:* KT592278.1). Helmy (2000) recorded *Borrelia sp.* infection in 35.5% of *O. savignyi* collected from Dahshour, Giza Governorate. Shanbaky and Helmy (2000) found that Lyme borreliosis was a multiorgan infection caused by spirochetes of the *B. burgdorferi*, *Borrelia garinii,* and *Borrelia afzelii,* which are transmitted by ticks of the Ixodes species (Hengge *et al,* 2003). Disease burden within cattle is a concern around the world. The first observation of *B. theileri* in *R.geigyi*in Mali was related to and overlaps in distribution with *R. decoloratus* and *R. annulatus*, known vectors of *B. theileri*. However, minimal data exist on the infection rates in *B. theileri* tick vectors (Estrada-Peña *et al,* 2006).

Many studies used the 18S rRNA gene in diagnosis *Babesia* species in animal hosts (Laha *et al,* 2015; Byaruhanga *et al,* 2016; Elhaig *et al,* 2016; Silveira *et al,* 2016; Liu *et al,* 2017) or tick vectors (De Vos *et al,* 1994; Bock *et al,* 2004; Adham *et al,* 2009; Wesonga *et al,* 2010; Rar *et al,* 2014). In the present study, the positive *R. annulatus* hemolymph with vermiculate stages of *Babesia* was screened by PCR and sequenced. The sequence (accession number: MF0044 18) was 100% identical with *Babesia bigemina* (accession number: EF458206.1). The absence of *B. bovis*, could be related to *B. bigemina* that had the higher rate of infection in the female ticks collected from cows and greater parasite density in their blood and the lack of active immunity. The prevalence of *B. bigemina* in the current study (1.16%) was lower than that reported previously by Ibrahim *et al,* (2013) and Mahmoud *et al,* (2015) they recorded 5.2% and 32.4%, respectively, in different Governorates of Egypt. These regional differences in prevalence may be due to differences in husbandry, tick distribution, detection methods, and time of sampling in Egypt. While in Banha, Qalyoubia Governorate *B. bigemina* positive samples were absent. Differences in animal management explain the absence of *B. bigemina* positive samples in Qalyoubia

Governorate as cattle in this Governorate are kept in small numbers, and well cared by their owners and being regularly treated with Diazinon compound that controls ticks (Ghosh and Nagar, 2014). In this study, the *18S rRNA* gene sequences of these Egyptian *B. bigemina* isolates found to have 100 % nucleotide identities with *B. bigemina* from Puerto Rico and Spain, and they also have a very close phylogenetic relation 99 % with isolates from Uganda, and very close phylogenetic relation 99% with isolates from China from *Ixodes persulcatus* ticks. So, close genetic relatedness was between *B. bigemina* isolates from this region of Egypt with Puerto Rico and Spain rather than its neighbouring country China. In that respect, this study on phylogenetic relation of *B. bigemina* of cattle isolate of Salhia region with other isolates throughout the world may be considered as the first report of its kind from Egypt.

The *Babesia* sp. detected by microscope was low but it might increase if PCR was used for all ticks. The infection rate, even if it is low in ticks it may be increased with the number of ticks in the field causing increase infection rate in animals where, 8.1% of cattle were infected with *B. bovis* in North Sinai Governorate (Mazyad, 2002), 11.5% of cow were infected with *Babesia bigemina* in Port Said Governorate (El-Fayomy *et al*, 2013), and 8.15% of cattle were infected with *Babesia* spp. in Menoufia Governorate (Nayel *et al*, 2012).

Conclusion

The present study investigated tick hemolymphs of the soft tick *O. savignyi* and the hard tick *R. annulatus* by Light microscope. Spirochetes were found in the two tick species while the vermicule form of *Babesia* was found in *R. annulatus* only. Tick positive samples were screened by PCR using *flab* gene for *Borrelia* and *18S rRNA* for *Babesia.* The PCR amplified *Borrelia* and *Babesia* at 350 and 450 bp, respectively. The obtained amplicons were sequenced and recorded in GenBank with accession number MF084762, MF084761 and MF004418 for *Borrelia* sp. in *O. savignyi*, *Borrelia* sp. in *R. annulatus* and *B. bigemina* in *R. annulatus*, respectively. In general, the genotype of *Borrelia* sp. recorded in *O. savignyi* is very close to *B. burgdroferi* that causes Lyme disease in human. However, the genotype of *Borrelia* sp. recorded in R. *annulatus* is close to *B. theileri* that causes a mild disease in animals. The genotype of *Babesia* found in *R. annulatus* was 100% identical with *B. bigemina* that recorded before.

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Explanation of figure

Fig. 1: Tick hemolymph smears stained by Giemsa: **A&B)**forms of *Borrelia* sp.in *R. annulatus*(black arrows), **C)**crowded forms of *Borrelia* sp.in *O. savignyi*, and **D)**vermicule stage of *Babesia bigemina* in *R. annulatus* (white arrow).