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Using PCR for detection of *Borrelia* sp. in Ticks and Ruminants in Egyptian Desert

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ABSTRACT

Objective: To investigate the prevalence of *Borrelia burgdorferi* infection in ticks and ruminant hosts.

Design: Cross sectional study.

Animals: 739 Ixodes ticks, 110 camels, 120 sheep and 40 cattle.

Procedures: All collected samples were tested by using nested PCR technique (nPCR) using *OspA* and *flaB* gene as the targets.

Results: Out of 739 adult Ixodes ticks, 111 (15%) were infected, the highest rate was in *A. variegatum* (43.24%) followed by *A. lepidum* (30.63%) and *B. annulatus* (26.13%). Shalatten was the most infected locality (18.52%) followed by Dakhla Oasis and Bir El-Abid in ratio 18.52% and 16.05%, respectively. Camel was the most infected animal (40.74%), then sheep (37.04%) and cattle (22.22%).

Conclusion and Clinical relevance: Medical and veterinary professionals in Egypt should consider *B. burgdorferi* as an emerging pathogen in the country. Future studies are needed to monitoring vectorial capacity of ticks in transmission *B. burgdorferi* to animals in parallel with discovering other reservoir host animals and the vector competence of tick species for *B. burgdorferi* and its public health and economic significance in Egypt.

Keywords: *Borrelia burgdorferi*, Borreliosis, nPCR, Ixodes ticks

1. INTRODUCTION

In the late twentieth century, the importance of infectious diseases has been increased. More than 25% of annual deaths worldwide are estimated to be directly attributed to the emergence and resurgence of infectious diseases [1]. Ticks are obligate hematophagous ectoparasite of vertebrates that have a limited mobility, but can be transported over large geographical distances by wild and domestic mammals and birds. Besides causing direct damage associated with blood feeding, they considered as the mainly vital vector in transmitting a great variety of pathogens, including bacteria, virus, protozoa and helminths that cause mild to lethal diseases in man and animals [2, 3]. From these pathogens, *Borrelia* spp. and *Coxiella* spp.

Borrelia spp. are best studied tick-borne pathogens. These spirochetes are motile Gram-negative, helical-shaped bacteria. Structurally, it is composed of inner and outer cell membranes, with 7-11 flagella located between them. The outer membrane comprises a variety of outer surface proteins (Osp) which are thought to be responsible for adaptation and survival of the spirochete in vector and host tissues. Flagellin is additional antigenic structures of *Borrelia burgdorferi*, which is considered the main component of flagella, in addition to, 60-kDa common antigen, which is a member of the heat-shock protein family [4].

Borrelia spp. are conserved in nature through complex interactions among ticks, particularly *Ixodes* spp., and a range of vertebrate hosts. They classified within the phylum spirochetes that embrace two major groups [5, 6]. The first is the Lyme borreliosis (*Borrelia burgdorferi sensu lato*), are transmitted by hard ticks (Ixodidae). The second group contains several *Borrelia* relapsing fever (RF) species, with the exception of *B. recurrentis* (louse-borne RF spirochetes), are transmitted via soft ticks (Argasidae).

Therefore, this study was designed to investigate the isolation, characterization, and incidence of *Borrelia* spp. in both *Ixodes* spp. ticks and ruminant livestock in different biocenoses of Egypt.

2. MATERIALS AND METHODS

2.1. Ethical considerations

The protocol was in accordance with the ARRIVE guidelines and the EU Directive 2010/63/ EU for animal testing and the NIH Manual for the Care and Use of Laboratory Animals (NIH Publications No. 8023 revised 1978). Moreover, it was agreed with the ethical guidelines approved by the Ministry of Higher Education and Scientific Research (50/4/10), National Research Center (10120507). The collection of ticks on ruminants did not involve national farms or other protected areas or endangered or protected species.

2.2. Study regions and animals

Between June 2014 and April 2016, Ixodid ticks were collected from 270 ruminants (120 sheep, 110 camels, and 40 cattle) in twelve localities from nine regions in Egypt, as shown in Figure 1. All the ticks were collected, either by hand or with pair of forceps[7], and taken to the laboratory for study.

2.3. Tick identification

All collected hard ticks were transferred to the laboratory where they were categorized and enumerated under magnification. Ticks were identified to the species level using previously established taxonomic identification keys for adults [8]. All the needed information about the collected ticks were organized to be on the tube as genera, species, host and animal number, collection site and date.

2.4. Tick processing

The identified adult ticks were dissected to remove the distal portion of the legs for getting the hemolymph. The rest of the tick was longitudinally cut in two equal parts. One part for molecular biology, and the other was preserved frozen at -80°C as a backup sample for any additional analysis. Ticks were pooled together by species and separated into groups of up to five adult ticks per pool for testing.

2.5. Detection of the pathogen in blood and hemolymph samples

Hemolymph was bled directly on a glass slide and left to dry for 20-30 minutes. The glass adhered hemocytes were fixed in air for 15-20 min. Animal blood and tick hemolymph were stained with Fontana stain [9]. Stained smears were investigated microscopically for the presence of *Borrelia* spp.

2.6. DNA extraction, molecular detection and sequencing

The QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) was employed according to the manufacturer's instructions to extract the genomic DNA from collected animal blood samples and tick pools in each species. DNA concentration was estimated using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

DNA samples were analyzed for the presence of *B. burgdorferisensustricto* by nested PCR technique (nPCR) to increase the specificity. The first round of PCR to detect *Borrelia*-specific loci. Then, the product will be used as a new template to create internal amplified smaller fragment. Two *Borrelia* genes were subjected to amplification. *Flagellin B (FlaB)* which encodes flagellum major protein [10], and located on the linear chromosome, while the other gene was *Outer surface protein A (OspA)* that responsible for tick midgut colonization, and is plasmid-encoded [11, 12].

The nested PCR was subjected to determine the *Borrelia* spp. via amplification of 5S-23S rRNA Intergenic

Spacer Region and the flagellin gene (*flaB*). The sequence of the primers is shown in **Table 1**.

Reaction conditions for the tested genes were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 15 s, annealing temperature for 30 s, 72 °C for 45 s; 72 °C for 5 min; and hold at 4 °C. The annealing temperatures were different and shown in detail in table (1). The investigated samples were considered positive for *Borrelia burgdorferi* when the inner amplicons were detected on agarose gel electrophoresis from both *Borrelia* genes.

DNA sequencing and capillary electrophoresis were implemented for both strands (in both directions) of the PCR amplicons according the protocol previously published [13]. The same primers applied for the initial PCR amplification were used. Final sequence analysis and editing was performed by using Sequencer 4.7. Using BLASTN, version 2.2.10 (www.ncbi.nlm.nih.gov/blast/Blast.cgi), edited sequence data was compared with genetic sequences from characterized examples of *Borrelia* spp. published in GenBank.

2.7. Statistical Analysis

The number homogeneity of collected ticks was tested by the Chi-square depending on infected hosts with *Borrelia burgdorferi* in different localities and different infected *Ixodid* ticks that by using the FREQ Procedure Model of SAS [14]. The permitted method was applied consistent with Snedecor and Cochran [15]. Probability values (P-value) were judged statistically significant at < 0.05, 0.01 and < 0.001.

3. RESULTS

3.1. Microscopical detection of *Borrelia* spp. in stained smears

Spirochetes were not seen in any direct stained animal blood samples. However, the Fontana stain succeeded in detection of *Borrelia* spp. in hemolymph smears. So, prevalence of *Borrelia* spp. in animal hosts according to Fontana stain result was zero%, whereas their ticks species yield 3%. This result strengthens the value of the PCR-based technique in the detection of *Borrelia* spp. **Figure (1)** from arthropod and mammalian specimens.

3.2. Molecular detection of *Borrelia* spp.

As represented in **Figure 2**, the *BorreliaFlaB* and *OspA* genes loci were targeted for detection of the *Borrelia burgdorferi* bacteria through nPCR technique. The *FlaB* and *OspA* products from each tick were reported and compared against controls via gel electrophoresis. The *FlaB* primers (The amplicon appeared at 447 bp) capture *B. burgdorferi* s.s. and other closely related *Borrelia* geno species, while *OspA* primers (the amplicon appeared at 350 bp) detect only *B. burgdorferi* s.l. Amplification from both loci would indicate the presence of *B. burgdorferi* s.l.

Table1. Sequence of *Borrelia* Species-Specific Genes used during PCR Amplifications.

Primer	Target gene	5'-Sequence-3'	Amplicon size (bp)	Annealing Temp.
FlaB1-Fw FlaB1-Rv	Flagellin gene (<i>flaB</i>)	GCATCACTTTTCAGGGTCTCA	503	55°C
		TGGGGAACCTTGATTAGCCTG		
FlaB2-Fw FlaB2-Rv		CTTTAAGAGTTCATGTTGGAG TCATTGCCATTGCAGATTGT		
OspA1-Fw OspA1-Rv	Outer surface protein A (<i>OspA</i>)	CTTGAAGTTTCAAAGAAGAT	487	55°C
OspA2- Fw OspA2- Rv		CAACTGCTGACCCCTCTAAT ACAAGAGCAGACGGAACCAG TTGGTGCCATTGAGTCGTA		

Table 3. Incidence of *Borrelia* spp. within species of *Ixodes* ticks in the investigated localities in Egypt.

Locality	Total collected hard tick species (n=416)						Total infected ticks/collected
	<i>A. variegatum</i> (n=39)	<i>B. annulatus</i> (n=84)	<i>H. a. excavatum</i> (n=76)	<i>H. dromedarii</i> (n=137)	<i>Rh. humeralis</i> (n=32)	<i>Rh. sanguineus</i> (n=48)	
	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)
Gharandal Valley	0 (0.00%)	0 (0.00%)	2 (2.63%)	3 (2.19%)	0 (0.00%)	0 (0.00%)	5 (1.20%)
Kharga Oases	1 (2.56%)	6 (7.14%)	3 (3.95%)	3 (2.19%)	0 (0.00%)	2 (4.17%)	15 (3.61%)
Marsa Matrouh	0 (0.00%)	5 (5.95%)	4 (5.26%)	4 (2.92%)	0 (0.00%)	0 (0.00%)	13 (3.13%)
Qantara Shark	0 (0.00%)	13 (15.48%)	2 (2.63%)	0 (0.00%)	0 (0.00%)	2 (4.17%)	17 (4.09%)
Shalateen	0 (0.00%)	0 (0.00%)	9 (11.84%)	11 (8.03%)	0 (0.00%)	0 (0.00%)	20 (4.81%)
Siwa Oasis	2 (5.13%)	3 (3.57%)	2 (2.63%)	6 (4.38%)	0 (0.00%)	1 (2.08%)	14 (3.37%)
Total infected ticks/species	3 (7.69%)	27 (32.14%)	22 (28.95%)	27 (19.71%)	0 (0.00%)	5 (10.42%)	84 (20.19%)
DF	20	**Highly Significant at P< 0.01 by using Chi-Square test according to each infected ticks' species per locality to total infected ticks' species in all localities.					
Chi-Square (X)	47.49						
Probability	**						

Table 3. Incidence of *Borrelia* spp. within ruminants in the investigated localities in Egypt.

Locality	Camel		Cattle		Sheep		Total infected animals/examined	
	Ex. No	infected No. (%)	Ex. No	infected No. (%)	Ex. No	infected No. (%)	Ex. No	infected No. (%)
Gharandal Valley	17	1 (5.88%)	0	0 (0.00%)	5	0 (0.00%)	22	1 (4.55%)
Kharga Oasis	11	2 (18.18%)	28	7 (25.00%)	7	0 (0.00%)	46	9 (19.57%)
Marsa Matrouh	20	3 (15.00%)	7	2 (28.57%)	61	5 (8.20%)	88	10 (11.36%)
Qantara Shark	11	1 (9.09%)	33	12 (36.36%)	11	0 (0.00%)	55	13 (23.64%)
Shalateen	58	36 (62%)	0	0 (0.00%)	63	8 (12.70%)	121	44 (36.4%)
Siwa Oasis	18	1 (5.56%)	3	0 (0.00%)	21	7 (33.33%)	42	8 (19.05%)
Total examined	135		71		168		374	
Total infected ticks/species	44 (32.6%)		21 (29.58%)		20 (11.90%)		85 (22.7%)	
DF	10	EX. No.= Examined number - **Highly Significant at P< 0.01 by using Chi-Square test according to each infected host per locality to total infected hosts in all localities.						
Chi-Square (X)	59.86							
Prob.	**							

3.3. Prevalence of *Borrelia burgdorferis* in *Ixodes* ticks in different localities

Hard ticks in the present study were collected from a total of 374 animals (168 sheep, 135 camels and 71 cattle) in six localities, as shown in Figure (1). A total of 416 *Ixodes* ticks, were collected and submitted to the laboratory for tick identification and *Borrelia* spp. detection. They are representing 6 tick species, *Hyalomma dromedarii* (n=137), *Hyalomma anatolicum excavatum* (n=76) *Amblyomma variegatum* (n=39), *Rhipicephalus sanguineus* (n=48), *Rhipicephalus humeralis* (n=

32), and *Boophilus annulatus* (n=84), as demonstrated in Table 2.

This study showed an overall frequency of ticks infection was 20.19% (84/416) of *Borrelia* spp. Of these infected ticks; 32.14% (27/84), 28.95% (22/84), 19.71% (27/84), 10.42% (5/84) and 7.69% (3/84) were in *Boophilus annulatus*, *Hyalomma anatolicum excavatum*, *Hyalomma dromedarii*, *Rhipicephalus sanguineus*, and *Amblyomma variegatum*, respectively. The species, *Rhipicephalus humeralis* showed no infection.

Concerning the distribution of the infection, the most infected localities were Shalateen, Qantara Shark, Kharga oases, Siwa oasis, and Marsa Matrouh recording 4.81%,

4.09%, 3.61%, 3.37%, and 3.13%, respectively. The lowest percentage of infection was recorded in Gharandal valley (1.2%). Moreover, the camels were the most susceptible animal type to borreliosis (32.6%) than other ruminants studied where cattle and sheep were recorded 29.58% and 11.9%, respectively. The highest location of infection was in Shalateen (36.4%) followed by QantaraSharq, Kharga oasis, Siwa oasis, MarsaMatrouh, and Gharandal valley with recorded percentage 23.64%, 19.57%, 19.05%, 11.36%, and 4.55%, respectively as shown in (Table 3).



Figure 1. *Borrelia* spp. appeared stained with Fontana stain (x=100)

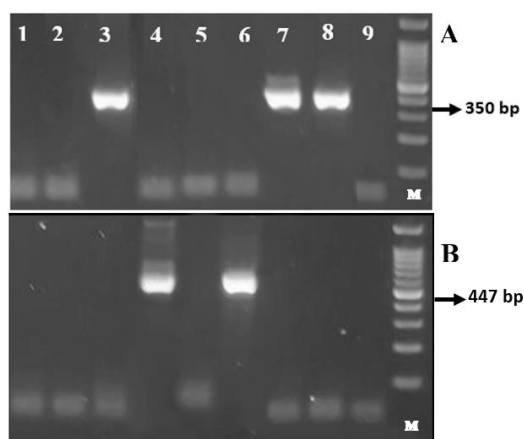


Figure 2. Molecular identification of *Borrelia* spp. by nPCR products of *flab* and *ospA* genes detected in 1.5% agarose gels stained with ethidium bromide. (A): Lane M: 100 bp DNA ladder. Lane 1: negative control and Lanes 3, 7, 8 positive samples at 350 bp amplicon of *ospA* gene (B): Lane M: 100 bp DNA ladder. Lane 1: negative control and Lanes 4, 6 positive samples at 447 bp amplicon of *flab* gene.

4. DISCUSSION

The distribution data of tick-borne pathogens and their possible association with emerging and distinct illnesses in Egypt are not extensively available. Animal who become diseased after a tick bite may be at increased risk of infection because the tick bite considered as the source of the pathogen. There is lacking in the detailed knowledge of the tick-borne pathogens, their distribution, and relationship to their vectors. Studies of pathogens carried in ticks in non-disease-known endemic regions might provide

epidemiological information about the pathogenic organisms, their vectors, and reservoirs. Moreover, these data might also deliver a chance to examine the causes of emerging zoonoses for which different factors for disease transmission may be present.

In this respect, despite the earlier detection of *Borrelia* spp. in Egypt since 2010 by using PCR reaction [16], little is known about their epidemiology and detrimental effect on animals and humans [17]. In the present study, our result revealed that camels were the most susceptible to borreliosis (32.6%) than other animals studied. Shalateen has the highest infection rate (62%) among examined camels where it comprises the main exported camel market in Egypt whereas thousands of camels imported from Sudan and other African countries. The importance of camels in that they are the main animals-structure in the Bedouin desert communities, therefore, camel grazing is the main activity practiced (shepherds) by the citizens at Eastern, Western and Southern Egyptian borders, and Sinai. So, they play an important and pivot role in the spread of ticks from one area to another as well as they serve as reservoir in transmitting the infection among the population of those areas and of course mainly to shepherds.

It is established that the cattle tick, *B. annulatus* is the main tick species infest cattle in Egypt [18] and it is considered the main vector of bovine borreliosis [19]. Our findings are in line with the previous published work that the highest infection rate of *B. annulatus* (15.48%) was in Qantara Sharq where there are considerable count of cows and people depend on them in the field of agriculture and family income.

Unfortunately, In Egypt, data on Lyme disease are scarce, and there is no any study of Lyme disease in animals and ticks has been documented to verify its zoonotic evidence. In addition, no attempts were performed to isolate *B. burgdorferi* from clinical specimens [20]. While any level of prevalence may be worrisome, the current risk of Lyme disease in the regions we investigated appears to be in concordance if compared with the previous published results in different areas in Egypt; Dahshore, Abu Rawash, Kerdasa, Kafr Tohormos, Saft el Laban and Nahia, Giza Governorate where the infection rate was between 0.0-50.0% (*B. annulatus* 8%; *H. dormedarii* 25%; *H. excavatum* 50%; *R. sanguineus* 8%; *R. pulchellus* 0%; and *A. lepidum* 28%). Previous studies have reported positive correlations between *B. burgdorferi* prevalence and tick abundance [21]. However, the difference that we observed among our investigated areas was significant.

In our present work, we relied on the nested polymerase chain reaction (nPCR) in detection of *Borrelia* spp. where it is established that this technique is an excellent tool in increasing the specificity and sensitivity of pathogen detection, specifically when investigate samples from contaminated environments [22].

Our detection of *Borrelia* spp. was depending on *OspA* where it encodes an outer membrane protein which is a major antigen of the Lyme disease agent [23] to the extent that it is considering a promising candidate for an effective vaccine for Europe [23]. In addition to the Flagellin B (*FlaB*) gene. For ecological investigation and identification of the *B. burgdorferi* s.l. species, the nPCR technique, and the *FlaB* and *OspA* gene targets, have been extensively used since the early 1990s, [24-27] and still used up till now [28, 29].

Studies employing PCR in Lyme disease diagnosis have yield variable results [30, 31]. In our study, 350bp and 478 bp fragments corresponding to *Borrelia* sp. was amplified from investigated ticks (20.19%). Our obtained data are consist with those of previous work and inconsistent with others [16, 20, 32, 33]. These discrepancies in the results may be attributed to different environmental factors as tick habitat, geographic distribution of reservoir hosts, developmental and/or feeding state of the investigated ticks or by different use of methods, particularly the sensitivity of the primers used and the PCR technique conditions. All these factors have strong impact on the obtained data [34]. In addition to, site-specific ecological dissimilarity might elucidate the observed differences in collection rates in different studied areas. Continued collection will make clear the effects of local site variation, and on the degree to which ticks may be increasing in abundance, distribution, and transmit the infection.

Assessment of ticks for a range of bacterial agents has provided several additional associated factors. These findings deliver the highlights on the distributions and endemicity of emerging and potentially pathogenic tick-borne organisms. Some of these tick-borne agents may cause an unknown health risk. Given the wide distribution of these ticks, accurate assessments of the vectorial capacity, the frequency of the bacteria in these tick populations, their potential to

cause human and animal diseases, and their ability to act as competent vectors are warranted. Sustained study and monitoring will play a vital and crucial role in public health assessment for related disease threats.

Since the present knowledge on the ecology and epidemiology of different *B. burgdorferi* s.l. species is still inadequate. So, further information on the distribution of different *B. burgdorferi* s.l. species in their natural reservoir hosts and vectors is essentially needed [35]. This is a fundamental prerequisite for a better understanding of *borrelia* spp. endemic circulation and also, for the establishment of effective preventive strategies against Lyme borreliosis.

In conclusion, the findings of this study provide additional information on the geographic distribution of *B. burgdorferi* in different ticks in Egypt. The overall frequency of *B. burgdorferi* in ticks (20.19%) and animals (22.7%). Shalateen are recorded the highest incidence among the investigated localities.

Medical and veterinary professionals in Egypt should consider *B. burgdorferi* as an emerging pathogen in the country. Future studies are needed to monitor vectorial capacity of ticks in transmission *B. burgdorferi* to animals and humans in parallel with discovering other reservoir host animals and the vector competence of tick species for *B. burgdorferi* and its public health and economic significance in Egypt.

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