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MOLECULAR DETECTION OF EHRLICHIA SPECIES IN IXODIDAE TICKS AND RUMINANTS IN SELECTED LOCALITIES IN EGYPTIAN DESERT

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ABSTRACT
Hard ticks are ectoparasites that infest animals, prompting severe transmittable infections. The aim of this study was identifying the presence of *Ehrlichia* spp. In ticks and their hosts from selected localities in Egypt. The 5223 adult ixodid ticks were collected from 270 ruminants; 110 camels, 120 sheep, and 40 cattle. Collected tick and animals' blood were examined. The statistical analysis of incidence of *Ehrlichia*spp. Within both ticks spp. And the examined animal soap. Was highly significant. The highest infested incidence in tick (24%) was in *Boophilusannulatus*, *Hylommadromedarii*, and *Rhipicephalus sanguineus* while the highest incidence of infection in animals was in camels (69.1%), followed by cattle (23.35%) and sheep (7.35%). The highest localities for ticks' Ehrlichiosis (14.7%) were Bir Al-Abid, Dakhla Oasis, and Shalateen whereas Dakhla Oasis showed the highest animal ehrlichiosis incidence (21.47%) followed by Tina Plain (12.88%) and Shalateen (12.27%). The positive *Ehrlichia* spp. Sequence were 100% identical to those in Africa and Iran. To conclude, ehrlichiosis seems to be endemic with Zoonotic potentials in the examined regions. Moreover, host specificity and transmission, including tick carriers, are requiring further researches and should not be neglected.

KEYWORDS: Ixodes, Ehrlichiosis, *Ehrlichia*spp., Tick-borne pathogen, Zoonosis.

1- INTRODUCTION

The relationship between Animals and pathogens is an old and intimate relationship, yet it has not taken its due attention. The risk of emerging infectious diseases remains a concern. Most emerging diseases are associated with tick-borne pathogens.[1] In many cases, their expansion is assumed to be due to changes in climate and habitat that alter the geographical distribution of vector and host. It is therefore necessary to identify the vector of pathogens that pose a major threat to human and animal health.

Over the past several decades, many researches have been done on both ticks and tick-borne diseases in many countries of the world,[2] which led to the need to answer questions about how the emergence and transmission of these diseases.

On a global basis, ticks come after mosquitoes as vectors of disease-causing agents to human and animals. Ehrlichiosis is a disease caused by members of the genus *Ehrlichia*that contains small, pleomorphic, Gram negative, obligate intracellular organisms, belong to the family Anaplasmataceae, order Rickettsiales, and established as an agricultural biothreat.[3,4]

Currently, the genus *Ehrlichia* contains five recognized species: *E. canis, E. chaffeensis, E. ewingii, E. muris and E. ruminantium. Ehrlichia* parasitise blood cells. In contrast with *Rickettsia*, *Ehrlichia* prevents the fusion of the phagocytic vacuole with lysosomes via interruption the expression of the appropriate receptors on the vacuole where it can live and multiply by binary fission.[5]

*Ehrlichia*are transmitted by ticks in the family Ixodidae. These bacteria are usually continued in closed cycles between ticks and wild or domesticated animal reservoir hosts, which can occasionally persist infected for long periods.

Since the *Ehrlichia*arean obligate intracellular bacterium, its detection requires specific techniques. Thus, the diagnosis of ehrlichiosis has been based on the

microscopic examination of Giemsa stained smears beside the presence of the tick vectors. On the other hand, many studies have depended on serological methods to detect antibodies against *Ehrlichia*proteins in ruminants[6] while other employed the detection of *Ehrlichia* in infected ticks using a DNA based assays.[7,8] Up to date, there is no confirmed evidence to what extent *Ehrlichia* infection present in farm animals in Egypt. This study pursued to fill this gap of knowledge and reinforce the existing reports on the presence of *Ehrilichia* in Egypt.

2- MATERIAL AND METHODS

2.1. Studied localities and animal population

A total number of 324 farm animals (168 sheep, 135 camels, and 71 cattle) were investigated for tick infestations at 6 selected localities in Egypt.

2.2. Sample collection

2.2.1. Ticks collection and identification: A total of 416 adult hard ticks were captured from host by forceps and orientated anticlockwise until the capitulum detaches from the host. Ticks were identified according to morphological characters by stereoscope using the available tick identification taxonomy keys[9,10] the hemolymph staining technique was done, and the remaining ticks were stored at -20 °C until DNA was extracted for molecular studies.

2.2.2. Blood

Whole blood samples (5 ml each) were collected from the jugular veins to prepare blood smears for Gimenez staining technique and the remaining were stored at -80 °C until DNA was extracted for molecular studies.

2.3. Staining technique for detection of *Ehrlichia* spp.

All collected samples were subjected to Gimenez stain technique.[11] Both blood and tick hemolymph were smeared on glass slides. The obtained smears were stained and analyzed microscopically for the presence of intracellular bacteria.

2.4. Extraction of DNA

Ticks were individually dissected and homogenized under sterile conditions. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiaqgen, Cat No. 69506, Germany) following the manufacturing`s protocol. DNA samples were stored at -20 °C and later used as templates for the PCR amplification.

2.5. Amplification of tick-borne pathogen DNA

Detection of *Ehrlichia*spp. Was performed ,depending on two genes; 16S rRNA and *p28* with specific primers are listed in **Table 1**. The primer set 16S8FE and B-GA1B was designed based on the 16S rRNA gene sequence belonging to Eubacteria, nonetheless, genus *Ehrlichia*specific annealing to nucleotide positions 8–27and 476–456, amplifying 500bp of the gene. DNA amplification was done according to the previous study.[12] for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Table 1: Synthesized primers used during PCR amplifications of *Ehrlichia*species-specific marker genes.

Primers	Target Gene	5`-Sequence-3`
***Ehrlichia*species-specific genes**		
16S8FE **B-GA1B**	**16S rRNA gene**	**5`-GGAATTCAGAGTTGGATCATGGCTCAG-3`** **5`-CGGGATCCCGAGTTTGCCGGGACTTCTTCT-3`**
p28f159 **p28f263 p28r1336**	**28kDa OMP gene (p28)**	**5`-ACTTCTACTATTGTTAATTTATTGTC-3`** **5`-AGTATCATTTTCCGACCCAGCAGGTAG-3`** **5`-GCTGTTGTGTAACTGTAGACTGGT-3`**

The second PCR was targeting the gene of *p28;* the 28-kDa immunodominant outer membrane protein described as a major immunodominant antigen recognized early in the immune response of *E. chaffeensis*. The *p28*-based hemi-nested PCR assay (**Figure 1**); p28f159, p28f263 and p28r1336 amplifying 850 bp of *p28* **(Table 1)** was used to amplify the *p28* gene. PCR amplification was performed according to previously described [13]for 30 cycles at 95°C for 2 min, 55°C for 1 min, and 72°C for 2 min.

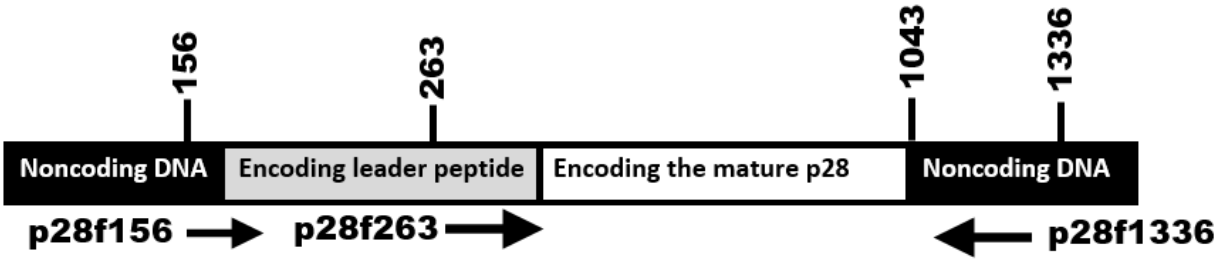

Fig 1: Diagram of PCR amplification of the *p28* gene. Arrows indicate the directions of primers.

2.6. Data Analyses by NCBI Blastn

DNA sequencing, analysis, and editing were accomplished by using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia) and BioEdit sequence alignment editor (v. 7.0.9.0). Using BLASTN, ver. 2.2.10 (http://www.ncbi.nlm.nih.gov/blast /Blast.cgi). Finally, edited sequence data was compared with genetic sequence from characterized examples of *Ehrlichia* spp. published in Genbank.

2.7. Statistical Analysis

The Chi-square was carried out to test the number homogeneity of collecting ticks depending on infected hosts with *Ehrlichia* in different localities and different species of hard ticks that infected with *Ehrlichia* by using the FREQ Procedure Model of SAS[14] for Windows Evaluation Version. The approved method was applied according to Snedecor and Cochran.[15] Probability values (P-value) < 0.05 were considered statistically significant and < 0.001 were judged as highly significant.

2.8. Ethical Approval

All procedures were agreed with the ethical guidelines adopted by the Ministry of Higher Education and Scientific Research (50/4/10).

3. RESULT

3.1. Incidence of Ticks-Borne Ehrlichiosis

A total 416 adult hard ticks were collected and then analyzed morphologically , six species were identified as shown in Table 2; encompassing *Hyalommadromedarii* (*H. dromedarii*) (23.4%), *Ambyommavariegatum* (*A. variegatum*) (17.9%), *Hyalommaanatolicum excavatum* (*H. a. excavatum*) (17.1%), *Rhipicephalus sanguineus* (*Rh. sanguineus*) (14.6%), *Boophilusannulatus* (*B. annulatus*) (10.7%), and *Rhipicephalus humeralis* (*Rh.humeralis)*(9.4%).

The localities showed positive results for *Ehrlichia*spp. together with the incidence and number of positive ticks and their host were statistically analyzed (**Tables 2 & 3**).Overall statistics for each identified tick species or animal in terms of infected counts versus total infected samples.

Themostareas that contain infected tick species with *Ehrlichia* spp. wereShalateen,Siwa Oasis, and Kharga Oases recording: 4.6%, 4.3%, and 3.8%, respectively, **(Table2)** while the lowest percentage of incidence was recorded in Gharandal valley (0.5%). From another point of view, the detection of infection in ruminant animals revealed that thecamelswerethe most susceptible animal toehrlichiosis(14.8%) followed by cattleandsheepwhere they recorded 9.9% and 7.7%, respectively. The highest infection in camels and sheep was in Shalateenwith record 18.9% and 14.3%, respectively, while the highest infectionincattle was in Qantara Shark (12.1%). Moreover, The lowest ratio of infection was in sheep (7.7%) and was established in two localities; MarsaMatrouh (4.9%) and Siwa oasis (4.8%). It should be noted from the obtained results that the examined camels in all localities, infection was documented in different ratios as mentioned before while there was no infection recorded in cattle in MarsaMatrouh and Siwa oasis, and in sheep in QantaraSharq, Kharga oasis and Gharandelvally. As illustrated in **(Table 3)**.

3.2. Molecular detection of *Ehrlichia*detection

All collected ticks, after subsequent morphological analysis, were subjected to amplification and molecular sequence to confirm the identification and alignments of their DNA sequences against GenBank database, 6 species and 4 genera (*Amblyomma, Hyalomma, Rhipicephalus* and *Boophilus)* were obtained. On the other hand, a total of 324 animals (135 camel, 168sheep and 71 cattle) was examined for tick infestation and ehrlichiosis. The hemi-nested PCR technique was employed for the detection of *Ehrlichia*spp via amplification of 500 bp fragment 16S rDNA gene and 850 bp fragment P28 gene (**Fig. 2: A & B**) in 71 ticks (32 *H. dromedarii*, 13 *H. a. excavatum*,9 *B. annulatus*,7*A. variegatum*, 7 *Rh. sanguineus*,and 3 *Rh. humeralis)*and 40 animals (20 camels, 13 sheep and 7cattle) as shown in (**Table 2 and 3)**.

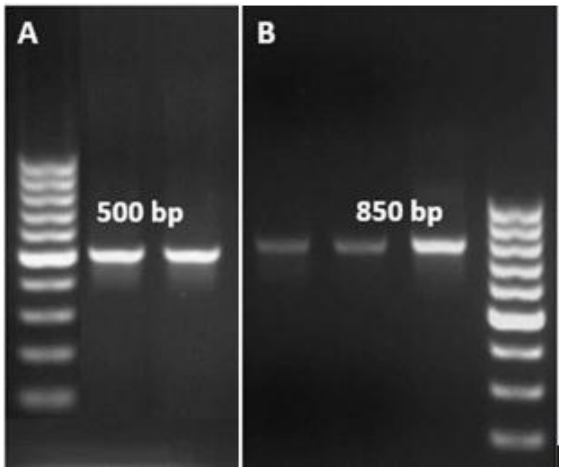

Figure 2: Molecular identification of Tick borne Ehrlichiacea by PCR products of 16S rRNA and *p28*genesdetected in 1.5% agarose gels stained with ethidium bromide.

(A): Lane 1:100bp DNAladder, Lane2 and 3:500bpampliconof 16S rRNAgene.**(B)**: Lane 4:100bp DNAladder. Lane 1, 2 and 3: 850bpampliconof *p28* gene.

3.3. Sequencing of extracting DNA

The isolated DNAs were sequenced, and their origin was determined by using NCBI-BLAST software which yield that *Ehrlichia*spp. Sequences were identical in ratio 100% to those reported from Iran and Africa with GenBank Accession numbers as AF311968.1 and JN626225.1 respectively.

Table 2: Prevalence of *Ehrlichia* spp. Within hard tick's species in selected localities in Egypt.

Locality	Total collected hard tick species (n=416)						
	***H. a. excavatum* (n=76)**	***H. dromedarii* (n=137)**	***A. variegatum* (n=39)**	***B. annulatus* (n=84)**	***Rh. humeralis* (n=32)**	***Rh. sanguineus* (n=48)**	**Total infected ticks/collected**
	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)	+ve n (%)
Qantara Shark	0 (0%)	3 (2.2%)	0 (0%)	1 (1.2%)	0 (0%)	3 (6.3%)	7 (1.7%)
MarsaMatrouh	3 (3.9%)	5 (3.6%)	0 (0%)	0 (0%)	0 (0%)	1 (2.1%)	9 (2.2%)
Siwa Oasis	2 (2.63%)	11 (8%)	3 (7.7%)	1 (1.2%)	0 (0%)	1 (2.1%)	18 (4.3%)
Kharga Oases	1 (1.3%)	4 (2.9%)	1 (2.6%)	7 (8.3%)	1 (3.1%)	2 (4.2%)	16 (3.8%)
Shalateen	7 (9.2%)	7 (5.1%)	3 (7.7%)	0 (0%)	2 (6.2%)	0 (0%)	19 (4.6%)
Gharandal Valley	0 (0%)	2 (1.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (0.5%)
Total infected ticks/species	13 (17.1%)	32 (23.4%)	7 (17.9%)	9 (10.7%)	3 (9.4%)	7 (14.6%)	71 (17.1%)
DF	**25**	**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)	**46.37**						
Probability	**						

Table 3: Prevalence of *Ehrlichia* spp. Within domestic ruminants in selected 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. (%)
Qantara Shark	11	0 (0%)	33	4 (12.1%)	11	0 (0%)	55	4 (7.3%)
MarsaMatrouh	20	3 (15%)	7	0 (0%)	61	3 (4.9%)	88	6 (6.8%)
Siwa Oasis	18	2 (11.1%)	3	0 (0%)	21	1 (4.8%)	42	3 (7.1%)
Kharga Oasis	11	1 (9%)	28	3 (10.7%)	7	0 (0%)	46	4 (8.7%)
Shalateen	58	11 (18.9%)	0	0 (0%)	63	9 (14.3%)	121	20 (16.5%)
Gharandal Valley	17	3 (17.6%)	0	0 (0%)	5	0 (0%)	22	3 (13.6%)
Total examined	135		71		168		374	
Total infected ticks/species	20 (14.8%)		7 (9.9%)		13 (7.7%)		40 (10.7%)	
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)	**38.08**							
Prob.	**							

4. DISCUSSION

Universally, ticks are important vectors of human and animal pathogens, and a variety of tick-borne infections are of medical interest where it has negative feedback on the health and productivity of the livestock.[9,16] Worldwide, The Ixodidae family (hard tick) are the major vectors of *Ehrlichia* spp.[17,18] Among Ixodidae family, *Hyalomma*, *Boophilus*and *Rhipicephalus* spp. Are considered the most abundant and important species in Egypt.[19]

Direct detection of *Ehrlichia* spp. In ticks and mammalian hosts would be valuable and necessary for experimental and epidemiological studies, interactions between the parasites and their vertebrate and invertebrate hosts, diagnosis and monitoring of infections and treatment. In this respect, all investigated samples (host blood and tick hemolymph)by a Gimenez staining technique were negative, may be attributed to their low levels in host blood, even during the acute phase of the disease and are probably cleared from the blood by the action of immune system.Thus, the commonly used assays for detection of *Ehrlichia* spp. Are serological tests that based on detection of antibodies by immunofluorescence or by enzyme-linked immunosorbent assay (ELISA), which are also limited to apply in vertebrates because they refer to exposure rather than active infection.[20] So, molecular techniques (including PCR and sequencing) are presently the most reliable and accurate approach for detection and identification of ticks-borne infections with improved sensitivity and specificity of the diagnosis.[21,22] Sothat,theproper diagnostic tools such as molecular techniques were designed dependent on specificity of genes which primers complementary to improve the sensitivity and specificity of diagnosis.[23,24]

The fact that *Ehrlichia* spp. Contained multiple copies of the *p28* gene increased the chances for their detection when it was targeted,[25] whereas, the 16S rRNA is the highly conserved mitochondrial gene, but the fact that only one copy of the gene is present diminished the success in the detection of this species.[26,27]

The sequencing results of infected tick species revealed the significant presence of *Ehrlichia* spp. With high

incidence (23.4%) in *Hy. Dromedarii*and with considerable percentages in others ticks infest cattle and sheep ranging from 9.4% up to 17.9%. This finding highlighted the subclinical infectivity in mammalian hosts, especially in camels (69.1%) and cattle (23.35%) confirm the belief that camels were the most susceptible to *Ehrlichia* spp. Infection followed by cattle and both are being lifelong reservoirs that means they susceptible to disease development.

Despite the previous detection of *Ehrlichiaspp*.by molecular means, little is known about the epidemiology of tick-borne ehrlichiosis in Egyptin both animals and humans.[28] Thus, the present study not only confirmed the endemic status of the infection, but also gave an alert for potential future epidemics by new species that were not clearly classified. In addition, they adapted them to three animal hosts studied in this study; camels, cattle and sheep.[29] Moreover, ehrlichiosis can be lethal if the disease is unchecked. Up till now the extent of the infection and the loss of livestock productivity remain poorly understood.[30]

To conclude, ehrlichiosis seems to be endemic with zoonotic potentials in the examined regions. Moreover, host specificity and transmission, including tick carriers, are requiring further researches and should not be neglected. Critical forms of infection can be motivated within infected animals when they are stressed by other factors, especially co-infections with other pathogens.

5. FUNDING

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