Original Article

Molecular epidemiological updates on spotted fever rickettsioses in animal species and their hard ticks settling Egyptian desert

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ABSTRACT

Hard ticks are ectoparasites that infest animals prompting severe transmittable infections. It is aimed to identify then characterize spotted fever group (SFG) rickettsioses in ticks and their hosts from smallholdings and Bedouin communities in Egypt desert including; North West Coast, Western Desert Oasis, South East Coast, Suez Canal region and Sinai. The 5223 adult ixodid ticks were collected from 270 ruminants; 110 camels, 120 sheep, and 40 cattle, examined seasonally (4 times/year) from June 2014 to July 2016. The statistical analysis of infestation density of all species on each hosts was highly significant, but was not significant between different localities. The infestation density on cattle was higher (59 %) than camels (33.6%) and sheep (7.4%) regarding all 14 species of ticks identified. The adult stages (♀ & ♂) of *Boophilus annulatus*, *Hylomma dromedarii*, and *Rhipicephalus sanguineus* were the highest in density on cattle, camels, and sheep recording 56%, 24.7% and 5.9%, respectively. Nucleotides similarities of ticks' 18S rDNA and 16S rDNA genome markers were 99-100% against genbank records, which confirmed species identification by morphological key. Molecular screening for rickettsiosis was carried by multi-genes typing technology; where 16S rDNA was the primary target. Positive PCRs were then typed into Rickettsiae species by the *ompA* and *gltA* genes alignments. *Hylomma dromedarii* ticks were the most susceptible to *Rickettsia* (35.7%) than other tick species. The prevalent localities for ticks' rickettsiosis were Shalateen, Dakhla Oasis, Siwa Oasis, El Salloum, and Marsa Matrouh recording 17.8%, 11.8%, 11.2%, 9.6% and 9.2%, respectively. With regards to animal hosts, the SFG incidence was higher in camels than sheep recorded 64.7% and 29.4%, respectively. The hosts' rickettsiosis incidence recording 14.7%, 13.7%, 12.8%, 11.8% and 10.8% was in Bir El abed, Shalateen, Wadi Gharandal, Al hasna, Marsa Matruh and El Salloum, respectively. Additionally, *Rh. sanguineus* (dogs ticks) had high incidence for rickettsioses; collected from all infested animal species investigated during the study. Hence, the *Rh. sanguineus* PCR-positive *Rickettsia* collected from cattle (5.9%) is an incidence never recorded; therefore, needs experimental studies before being accepted. *Rickettsia sibirica mongolitimonae*-like was detected for the first time within Egyptian livestock's population. *Rickettsia aeschlimannii*-like, *R. africa*-likee, in addition to, unclassified *Rickettsia*-like were additionally detected within the investigated specimens. Still, the pathological impacts of freely moving rodents and migratory birds are underestimated in epidemiology of such exotic zoonosis. Moreover, the role of additional vector (rodents' soft ticks) and/or reservoirs (desert reptiles) not clearly identified yet; a hypothesis that needs comprehensive profounder studies to improve physicians, as well as, veterinarians' differential diagnosis of tick-borne fever due to rickettsioses in Egypt.

Keywords: *R. aeschlimannii, R. africae, R. sibirica mongolitimonae*, multi-genes sequence typing, Ixodidae, DNA markers, ruminants.

Introduction

Since its first description in 1914, spotted fever group (SFG) *Rickettsia* are still expanding [1]. The improvements in molecular technologies had significantly advanced our abilities to conduct genetic analyses and clearly indicated the proper phylogenetic positions of most of the fastidious bacterial species in the family *Rickettsiaceae*, order *Rickettsiales* [2, 3]. Tick-borne biohazards result in significant morbidity and thousands of human and animal deaths annually, hence, transmitting a great diversity of infectious agents than any other hematophagous arthropods [4, 5]. Ixodidae (Hard ticks) is a large family distributed all over the world [6, 7]. In Egypt, genera *Amblyomma, Hyalomma* and *Rhipicephalus* (*Boophilus*) include the most abundant ixodid ticks infesting animals [5, 8-15]. Taxonomical key of ticks is based mainly on the morphological criteria of adult's phase [16, 17]. Recently, the molecular characterization for taxonomic identification and

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phylogenetic purposes of ticks has been indispensable by a group of DNA markers with regards to nuclear and mitochondrial genes, respectively [18-21]. The 18S rDNA is best used for genera identification while the 16S rDNA is the most useful markers for ticks' taxonomy at the species level [22-24]. The role of vertebrates as reservoirs of some SFG rickettsiae has yet to be clarified [25, 26]. Since 1960s when the primary study on SFG in Egypt was published, other multiple traditional smear staining, serological, immunohistochemical as well as molecular studies had designated ticks, rodents, animals and human as being endemic with *Rickettsia* agents [5, 12-15, 27-32]. The public health measures including; the ecological conditions that influence the animal stocks production, as well as, the control of vectors and their diseases [12, 13, 15, 33-38] by the veterinary authorities within studied localities became fundamental [39, 40]. Updating the data concerning *Rickettsia* epidemiology in Egypt with regards to ticks' species susceptibility is essential [39, 40]. The identification of *Rickettsia* into species has been dependent on employing the multi-genes typing of five sequences 16S rRNA, *gltA, OmpA*, *OmpB*, and *sac4* genes [12, 13, 15, 33-38]. The outer membrane protein A of the cell surface antigen (*OmpA*) and citrate synthase (*gltA*) genes are characteristics of SFG Rickettsiae with powerful discrimination in between *Rickettsia* spp. due to their high variability among SFG [12, 13, 15, 33-38]. During the present investigation, it is aimed to identify and molecular characterize spotted fever group (SFG) rickettsioses in ticks and their domestic animals from smallholdings and Bedouin communities in Egypt settling the desert in North West Coast, Western desert Oasis, South East Coast, Suez Canal region and Sinai.

Material and Methods

Geographical Scope of the Study in the Egyptian Desert:

Twelve sampling localities in 9 provinces from Egypt desert selected in the present study (Table 1).

Table 1: The coordinates of the studied regions and localities of the Egyptian deserts were the sampled animals' populations are settled.

Region/Province	City/Locality	Coordinates
North West Coast	Mariout	31° 9′ 11″ North, 29° 53′ 55″ East
	Marsa Matruh	31° 21′ 0″ North, 27° 14′ 0″ East
	El Salloum	31° 30′ 13″ North, 25° 6′ 54″ East
Ismailia	Qantara Shark	30° 51′ 0″ North, 32° 18′ 36″ East
	Ismalia	30° 36′ 0″ North, 32° 17′ 0″ East
Port Said	Tina Plain	31° 15′ 0″ North, 32° 17′ 0″ East
Southern Sinai	Wadi Gharandal	29° 30′ 0″ North, 33° 50′ 0″ East
Middle Sinai	Al hasna	30° 30′ 0″ North, 33° 36′ 0″ East
Northern Sinai	Bir Al abed	31° 0' 59" North, 33° 0' 38" East
Western Desert	Siwa Oasis	29° 11′ 0″ North, 25° 33′ 0″ East
New Valley	Dakhla Oasis	25° 29′ 29.6″ North,28° 58′ 45.2″ East
South East Coast	Shalateen	23° 7′ 54″ North, 35° 35′ 8″ East

Ethical Approval:

All procedures were in accordance with the ARRIVE guidelines which were in accordance to the European (EU) Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023 revised 1978). In addition, were in agreement with the adopted ethics guidelines of the ministry of higher education and scientific research (50/4/10) national research center (10120507); the funding organizations, and desert research center for the care and use of animals in Egypt.

Blood Samples of Investigated Animals Population:

A total number of 270 animals were investigated for tick infestations, which were examined/sampled seasonally (4 times/year) during the study period from June 2014 to July 2016. The inspected animals were; 110 camels, 120 sheep, and 40 cattle. EDTA-whole blood samples were collected from jugular veins [41] and used in preparing blood smears for staining technique and the remaining were stored at -80 °C until DNA was extracted for molecular studies. Additional 10 ml blood/host/trip was collected on plan tubes for additional serum samples analysis that were separated after centrifugation at 2000 rpm for 10 min.

Ticks Specimens and Taxonomic Classification by Stereo-Zoom Microscope:

A total of 5223 adult ticks were collected; other developmental stages were excluded. Ticks were captured from the host by forceps and orientated anticlockwise until the capitulum detaches from the host, then they were placed in polyethylene tube, 13 or 25 mm in diameter and 100 mm in height, sealed at one end by a mixture of gypsum and graphite at a ratio of 5:1. The tube was covered with a piece of muslin cloth securely held by a rubber band. The gypsum graphite was moistened to provide adequate humidity for the ticks during transportation to the laboratory [42]. Ticks were brought alive to the laboratory for morphological identification by stereomicroscope and identification keys. The collected ticks were counted and sorted to different genera, species and sex. Tick species were identified morphologically using taxonomic keys of [7, 8, 43]. The tick species were morphologically examined in details using stereo-zoom microscope, especially the dorsal and ventral surfaces of adult ticks. The adult ticks were photographed by a digital camera fixed on a stereomicroscope.

Detection of Rickettsiae in Stained Smears by Light Microscope:

All collected samples were examined for Rickettsiae using staining technique. According to Burgdorfer [44], hemolymph was impressed on slide following scissors amputating the distal portion of the legs, fixed by air dry. Both blood and hemolymph slides were stained with Gimenez stain [45]. Then, prepared slides were examined under oil emersion lens using an ordinary microscope (Zeiss).

DNA Purification:

Genomic DNA was extracted from camel, cattle, and sheep blood specimens using GF-1 Tissue Blood Combi DNA Extraction Kit (Vivantis) according to the manufacturer's instructions. Also, total DNA was extracted and purified from the tissues of adult ticks after dissection of each tick into quarters using high salt concentration protocol [46]. DNA concentration were measured by Nanodrop 2000c (Thermo Scientific), adjusted to 100 ng/µl genomic DNA working concentration.

Molecular Confirmation of Ticks' Taxonomy:

Ten randomly selected adult ticks/species/locality were investigated by molecular techniques to confirm the previous morphological classification of ticks' into species. Two-locus sequences typing included 18S rDNA and 16S rDNA; the nuclear and mitochondrial genes, respectively, was carried out. The 18S-F and 18S-R (Vivantis) primers pair previously designed were used in 18S rDNA gene amplification [18, 19] with predicted products size 780 bp (Table 2). Moreover, the 16S rDNA gene primers' set (16S-F and 16S-R, Vivantis) were designed according to previous publications [18, 19], with predicted products size 455 bp, respectively (Table 2). Each PCR mixture contained 25-50 ng/µl genomic DNA, 10 pM/µl of each primer, 12.5 µl ×2 Dream Taq Green PCR master mix (×2 buffer, 0.4 mM dNTP and 4 mM MgCl2; Thermo Scientific), and 9 µl nuclease free water (Qiagen) to complete the total volume of the reactions. All amplifications were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA) utilizing the following cycling profile; one cycle at 94°C for 5 min of initial denaturation, then 30 cycles denaturation at 94°C for 1min, annealing at 45°C for 1 min and elongation at 72°C for 1 min, and the final elongation at 72°C for 10 min [18, 19, 47]. A reagent blank was run as control simultaneously with every PCR.

Table 2: Synthesized oligonucleotides primers used during PCR amplifications and sequencing of markers genes

Primers	Target Gene	5`-Sequence-3`	References
Ticks classification			
18S-F	18S rDNA	5`-CATTAAATCAGTTATGGTTCC-3`	[18, 19]
18S-R		5`-CGCCGCAATACGAATGC-3`	
16S-F	16S rDNA	5`-TTAAATTGCTGTRGTATT-3`	
16S-R		5`-CCGGTCTGAACTCASAWC-3`	
Internal quality control			
Act-F	B-actin	5`-TGGATCGGCGGCTCCATCCT-3`	[48, 49]
Act-R		5`-GAAGCACTTGCGGTGGACAATG-3`	
β-Glob-F	β-globin	5`-ACACAACTGTGTTCACTAGC-3`	
β-glob-R		5`-CAACTTCATCCACGTTCACC-3`	
Rickettsia spp. detection and classification			
wfD1	16S rDNA	5`-AGAGTTTGATCCTGGCTCAG-3`	[50]
Rc16S.452		5`-AACGTCATTATCTTCCTTGC-3`	
190.70-F	*OmpA*	5`-ATGGCGAATATTTCTCCAAAA-3`	[35, 37]
190.701-R		5`-GTTCCGTTAATGGCAGCATCT-3`	
CS2d-F	*gltA*	5`-ATGACCAATGAAAATAATAAT-3`	[34, 38]
CS2d-R		5`-CTTATACTCTCTATGTACA-3`	

Classification of Ticks-Borne Rickettsiae by Multi-Genes Amplification and Sequencing:

Rickettsiae 16S rDNA gene primers' set (wfD1 and Rc16S.452, Vivantis) were designed according to Ogo *et al.* [50], with predicted products size 426 bp, respectively (Table 2). The *OmpA* gene primers were designed to span the nucleotides positions from 70 to 90 and from 701 to 681 (Table 2), respectively, and the predicted product size ranged from 590-634 bp [35, 37, 51]. Moreover, primers CS2d-F and CSEnd-R could amplify the full-length of the *gltA* gene (Table 2), as the predicted product size ranged from 852 to 1265 bp, therefore; CS2d primer was designed to be completely homologous to the corresponding portion of the gene in *R. conorii* for only SFG [34, 38]. The amplification reactions were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA) under complete aseptic conditions. Each 25 µl total volumes of each PCR mixture contained 25-50 ng/µl genomic DNA (host or vector), 10 pM/µl of each primer, 12.5 µl of 2x Dream Taq Green PCR master mix (Thermo Scientific) and 9 µl nuclease free water (Qiagen) to complete the total volume of the reaction. All amplifications were performed utilizing the following cycling profile for *OmpA* primers; one cycle at 94 °C for 5 min (initial denaturation) followed by 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and elongation at 72 °C for 1 min, and the final elongation at 72 °C for 10 min [12]. While *gltA* protocol included one cycle at 94 °C for 5 min (initial denaturation) followed by 40 cycles of denaturation at 94 °C for 1.5 min, annealing at 52 °C for 1.5 min and elongation at 72 °C for 1.5 min, then the final elongation at 72 °C for 20 min [12]. A reagent blank was run simultaneously as negative control with each PCR.

Amplified products were electrophoresed in 1% agarose gels in TBE buffer then stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel. Gels photos were analyzed by Lab Image software (BioRad). Sequencing reactions were performed in an MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM®BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq®DNA polymerase (Applied Biosystems), following the protocols supplied by the manufacturer.

Internal Quality Control of Molecular Assays:

A semi-qualitative internal control to verify the efficiencies of the DNA isolation and the PCR assays were applied [48]. The primers used derivative from highly conserved regions in both the host species and ticks vectors genomes; β-globin and β-actin genes sequences according to Konnai *et al.* [49], (Table 2). All amplifications were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA) utilizing the following cycling profile; one cycle at 94°C for 5 min initial denaturation, then 30 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1.5 min and elongation at 72°C for 2 min, and the final elongation at 72°C for 10 min [48, 49].

Data Analyses by NCBI Blastn:

The obtained sequences were aligned, assembled and corrected using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia) and BioEdit sequence alignment editor (v. 7.0.9.0). Each fragment's sequence was analyzed using Blastn program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence homology searches against ticks' and pathogens GenBank

database. Multiple sequences alignments for evolutionary relationships in between new Egyptian records and Genbank reference isolates were inferred [51, 52].

Statistical Analysis:

The *Chi*-square was carried out on the data collected in order to test homogeneity in the number of ticks collected according to; a) Infected hosts with *Rickettsia* in different localities and b) Infected different species of hard ticks with *Rickettsia* in different localities. This method analyzed using The FREQ Procedure Model of SAS [53] for Windows Evaluation Version. The method adopted was carried out according to Snedecor and Cochran [54]. Probability values (*P*-value) ≤ 0.05 were considered of statistical significant and ≤ 0.001 were considered of high statistical significant.

Results

Medical Inspection of Animals' Population:

The inspected animals were: 110 camels, 120 sheep, and 40 cattle. Ixodid ticks' collection began as a routine method of flocks' examination to detect the predilection sites of the ticks. It had begun by examining the head of the animal, then the outer and inner sides of ears, neck, lateral and medial aspects of the fore and hind limbs, abdomen, inguinal and anal regions as well as under the tail. The infestation sites examined were 14 on each animal inspected four times per year, each site on each host designated in 16 cm^2 area (4x4cm), in all species infestation were found of significant differences synergistic to the stage of infestation.

The epidemiological records (not shown) included: age, sex, breed, the purpose of rearing animal, production/reproduction records, external infestation, date of ticks' collection, clinical signs, suspected disease and time and type of treatments. The main clinical signs observed on infested animal hosts during sampling were fever, anorexia, lethargy, anemia, enlargement of superficial lymph nodes and emaciation, other than being apparently healthy in the majority of the inspected population. Older individuals were more susceptible for external infestation.

Morpho-Molecular Identification of Ticks Species:

Fourteen tick species were identified in the specimens collected from the 9 provinces of Egypt that were investigated during the present survey. The classified 5223 adult ticks were categorized into 1741 males and 3482 females. The classification was by both their taxonomic keys (Chart 1) then confirmed by their amplified and sequenced molecular contents. They were designated to 4 genera; *Amblyomma, Hyalomma, Rhipicephalus* and *Boophilus*, as the following: *Amblyomma gemma* (n=5), *A. lepidum* (n=6), *A. variegatum* (n=5), *B. annulatus* (n=2922), *H. albiparmatum* (n=25), *H. anatolicum excavatum* (n=127), *H. dromedarii* (n=1288), *H. impeltatum* (n=91), *H. marginatum marginatum* (n=105), *H. m. rufipes* (n=53), *H. truncatum* (n=90), *Rh. humeralis* (n=20), *Rh. pulchellus* (n=22) and *Rh. sanguineus* (n=307, only from animal spp. included in the study). The PCR screening utilizing the specific primers of 18S rDNA and 16S rDNA, proved the length of amplified fragments in all tick species of both genes were 780 and 455 bp, respectively (Fig 1: A & B). When the corrected sequences of each tick species were aligned against GenBank records, the identities of the Egyptian sequences confirmed the morphological classification of ticks to their designed species with similarities presents ranged from 97-100 % to records in the GenBank databases.

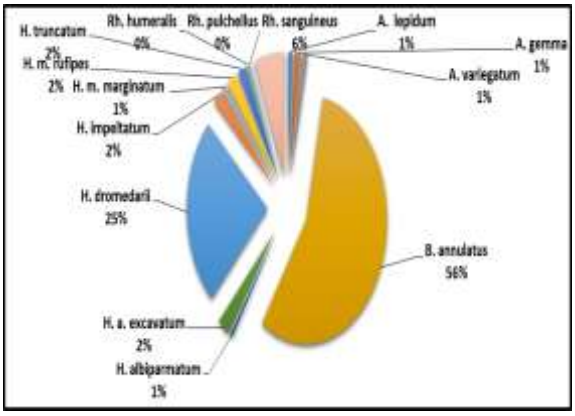

Chart 1: The infestation density of different ticks' species on all studied hosts in all destinations included in the present study.

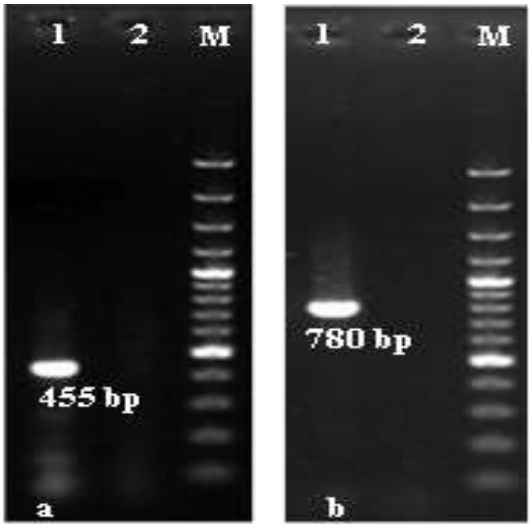

Figure 1. Molecular identification of tick species by PCR amplification of DNA markers detected in 1.5% agarose gels stained with ethidium bromide. A) lane 1: 100 bp DNA ladder, lane 2: Control negative, lane 3: 780 bp amplicon of 18S rDNA gene. B) lane 1: 100 bp DNA ladder, lane 2: Control negative, lane 3: 455 bp amplicon of 16S rDNA gene.

Epidemiological Analyses of Infestation Incidence and Density on Hosts with regards to Geographical Distribution:

All animals (n=270) were found infested around the whole year during the study. The infestation density of all ticks' species on cattle was the highest with 3083 adult ticks (59.03%), then camels with 1756 adult ticks (33.62%), followed by sheep with 384 adult ticks (7.35%). Statistically, the infestation density of

different species of ticks on different hosts was highly significant ($P < 0.001$). During the period of study, the adult *B. annulatus* [Say (1821)] was the highest density abundant among species on cattle; 2922 adult ticks (55.94%), followed by *H. dromedarii* [Koch (1844)] on camels; 1288 adult ticks (24.66%), then *Rh. sanguineus* [Latreille (1806)] on sheep; 307 adult ticks (5.88%), (Chart 2: A, B & C). In comparison, *B. annulatus* the most common species on cattle (55.94%) was higher than *H. dromedarii* species on camels (24.66%), (Chart 2: A, B & C). According to hosts susceptibility for ticks' infestation; with regards to all ticks species, the infested camels and cattle percentage recorded 33.62% & 59.03%, respectively, were higher than infested sheep recording 7.35%, (Chart 2: A, B & C).

Rickettsiosis Incidence with regards to Microscopical Detection by Stained Smears Examination:

Gimenez staining of hemolymph smears was a successful traditional tool for primary detection of *Rickettsia* infections in ticks but was not successful on examination of ruminants blood smear (Fig 2: A & B). Therefore, prevalence of *Rickettsia* spp. in animal hosts recorded 0%, while their tick species revealed 9% using Gimenez staining technique. These results triggered the importance of molecular identification of infection in both specimens type by PCR.

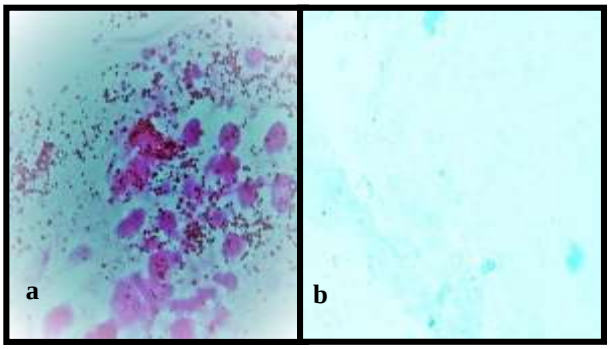

Figure 2: Ticks haemolymph smears stained with Gimenez showing: A) Positive slides containing Rickettsiae (red cocci) inside or around green haemocytes, and B) Negative slide containing green haemocytes, (Magnification Power = 1000x).

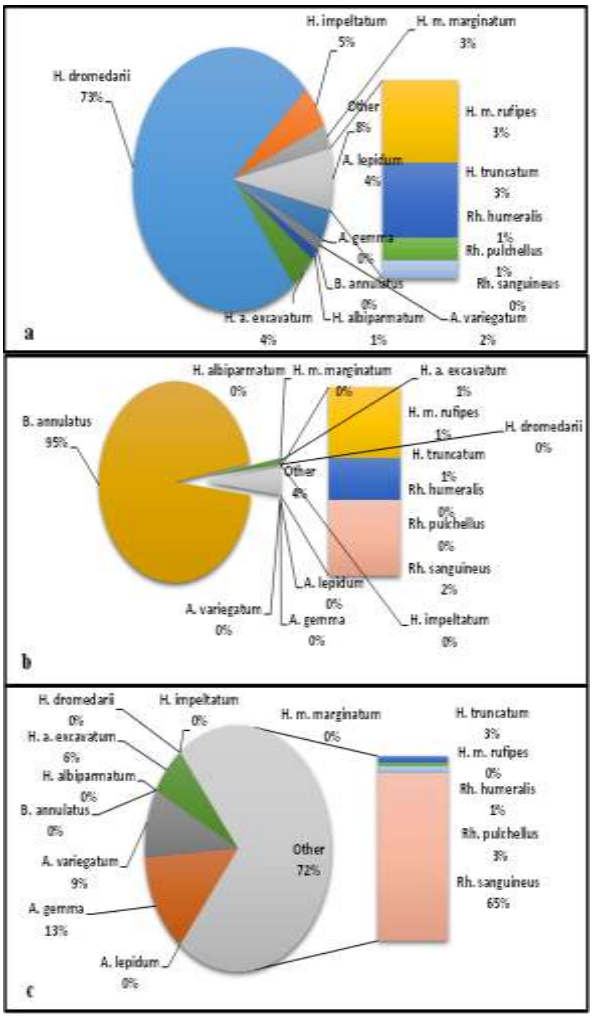

Chart 2: The ticks' infestation density on camel (a), cattle (b), and sheep (c) investigated during the study with regards to ticks' species.

Ticks-Borne Rickettsiosis Incidence with regards to Geographical Distribution Utilizing Multi-Genes Typing in the Studied Hosts and their Ticks Species:

Hosts and ticks' samples were both screened molecularly by 16S rDNA, *OmpA* and *gltA* genes primers sets in two steps molecular detections (additional primers sets of 6 genes were applied but results are not shown in this study) considering the representation of different localities. Samples with amplified fragments of 455, 600 and 1200 bp recorded for amplified 16S rDNA, *OmpA* and *gltA* genes were considered *Rickettsia* positive after alignments of products' sequences against GenBank database, respectively (Fig. 3: A & B). The localities investigated with positive results for *Rickettsia* spp. as well as the incidence and number of positive ticks and their host with are statistically analyzed in (Tables 3 & 4). The hard ticks of *H. dromedarii* species were the highest susceptible vectors to *Rickettsia* (35.67%) than other species (Table 3). The most prevalent localities with infected hard tick species; vector for SGF *Rickettsia*, were Shalateen, Dakhla and Siwa Oasis recording 17.83 & 11.78 & 11.15%, respectively, followed by El-Salloum and Marsa Matruh recording 9.55 & 9.24%, respectively (Table 3). The highest

infection percentage was in camels recorded 9.8% in both Shalateen and Wadi Gharandal districts which belong to the Red Sea and Southern Sinai Governorates, respectively. Followed by Marsa Matrouh district which belongs to Marsa Matruh Governorate with infection percentage recorded 7.84% (Table 4). Moreover, the highest infection incidence in sheep was 7.84% in Bir El abed district that belongs to Northern Sinai Governorate (Table 4). Generally, the highest incidence for rickettsiosis was in Bir El abed, Shalateen and Wadi Gharandal recording 14.71, 13.73 & 12.75%, respectively, followed by Al hasna, Marsa Matruh and El Salloum recording 11.76% and 10.78%, respectively (Table 4). Camels were proved the most susceptible to rickettsiosis (64.71%) than other animals' species studied (even dogs which were not shown in this study) (Table 4). On the other hand, cattle rickettsiosis was documented in 5.88% of studied population; 1.96% of cattle populations in Dakhla Oasis in the Western desert, and Qantara Shark and Tina Plain in the Suez Canal region, which a result that needs further confirmation and explanation, hence, *Rickettsia* infections are not recorded in cattle previously (Table 4).

The alignment of *OmpA* and *gltA* genes sequences against previously recorded isolates in GenBank indicate novel isolates that have less than 95% sequences similarity to the previously identified *Rickettsia* isolates in Sinai in Egypt; which was molecularly characterized to *R. aeschlimannii*-like*;* in all ticks species except *A. variegatum, H. albiparmatum*, *Rh. sanguineus* and *Rh. humeralis*, while *R. africae*-like*;* in all ticks species except *Rh. sanguineus*. Moreover, the obtained sequences of amplified genes indicated the existence of new species identified *R. sibrica mongolitimonae*-like. In continence, further molecular investigations to fully characterize these species/isolates was urgent (additional 6 genes are not shown in this study). The similarity percent of identity matrix of Egyptian *Rickettsia* sequences was based on multiple alignments. The *R. africae*-like and *R. aeschlimannii*-like isolate from *H. anatolicum excavatum, H. dromedarii, H. impeltatum, H. marginatum marginatum, H. m. rufipes* were clustered in a separate clade from other *R. africae* reference strains with a bootstrap value ranged from 85-99 (trees are not shown in this study), which indicated a novel strain of *R. africae*-like within ticks picked from camel from Sinai provinces, Shalateen and Western Desert Oasis. Moreover, *R. sibrica mongolitimonae*-like is the first detection in Saini and Shalateen provinces in ticks; *A. variegatum, H. m. rufipes*, *Rh. sanguineus* and *Rh. pulchellus*, respectively, collected from sheep (as far as this publication is released).

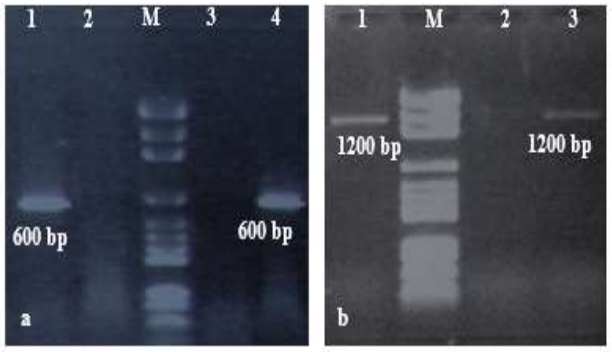

Figure 3. Molecular identification of tick-borne Rickettsiae by PCR amplification of the *OmpA* (A) and *gltA* (B) genes detected in *Rickettsia* positive samples of both hosts and/or vectors in 1.5% agarose gels stained with Ethidium bromide.

Lane M: 100 bp DNA ladder, lane 2: Negative control, (A): lanes 1 & 4 *OmpA* positives with molecular-sized bands of 600 bp, whereas, (B): lanes 1& 3 *gltA* positives with molecular size band of 1200 bp.

Table 3. *Rickettsia* incidence within hard ticks species in the investigated localities of egyptian desert.

Locality / Ticks Species	Bir El abed %	Dakhla Oasis %	Wadi Gharandal %	Ismailia %	Mariout %	Marsa Matruh %	Qantara Shark %	El Salloum %	Shalateen %	Siwa Oasis %	Tina Plain %	Al hasna %	Infected No.	Total No.	(2) Infected each species%
A. gemma	0.00	0.64	0.00	0.00	0.32	0.96	0.00	0.64	1.58	0.64	0.00	0.00	15	51	4.78
A. lepidum	0.00	0.96	0.00	0.00	0.32	1.27	0.00	1.26	1.58	0.96	0.00	0.00	20	67	6.37
A. variegatum	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.96	0.32	0.00	0.00	5	55	1.59
B. annulatus	0.00	0.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64	0.00	5	2922	1.59
H. albiparmatum	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	1.26	0.32	0.00	0.00	6	25	1.91
H. a. excavatum	0.96	0.64	0.96	0.32	0.32	0.32	0.64	0.32	1.26	0.32	0.64	0.96	24	127	7.64
H. dromedarii	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17	0.64	3.17	112	1288	35.67
H. impeltatum	0.64	0.96	0.64	0.32	0.32	0.64	0.32	0.96	0.96	0.96	0.32	0.64	24	91	7.64
H. m. rufipes	0.64	0.64	0.96	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.32	24	53	7.64
H. m. marginatum	0.96	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.32	24	105	7.64
H. truncatum	0.00	0.96	0.00	0.00	0.00	0.00	0.00	0.00	2.55	1.58	0.00	0.00	16	90	5.10
Rh. humeralis	0.00	0.32	0.00	0.00	0.00	0.32	0.00	0.64	0.96	0.32	0.00	0.00	8	20	2.55
Rh. pulchellus	0.00	0.64	0.00	0.00	0.00	0.64	0.00	0.64	1.27	0.64	0.00	0.00	12	22	3.82
Rh. sanguineus	0.96	0.64	0.96	0.00	0.00	0.64	0.00	0.64	0.96	0.64	0.00	0.64	19	307	6.05
Total infected No.	23	37	23	16	18	29	17	30	56	35	11	19	314	5223	
(1) Infection%	7.33	11.81	7.33	5.09	5.73	9.24	5.41	9.55	17.79	11.15	3.52	6.05			

DF =143, *Chi*-Square (X) = 162.45, Prob.= n (Non Significant), (1) Infection% = infection percentage for each locality regardless of tick species, (2) Infected each species % = infected each species percentage with regards to total infected species number within all localities, Total No.= Total numbers of collected tick species within each locality, Infected No.= Number of each infected tick species regardless to locality, Total infected No.= Total number of infected ticks in each locality.

Table 4. *Rickettsia* incidence within animal species in the investigated localities of egyptian desert

Hosts Species		Camel (n= 120)			Cattle (n= 40)			Sheep (n= 110)			Total (n= 270)		
Governorate	Locality	No.	Infected		No.	Infected		No.	Infected		No.	(1)Infected Hosts	
			No.	%		No.	%		No.	%		No.	%
North West Coast	Mariout	15	4	3.92	0	0	0.00	10	0	0.00	25	4	3.92
	Marsa Matruh	14	8	7.84	0	0	0.00	10	3	2.94	24	11	10.78
Ismailia	El Salloum	10	7	6.86	0	0	0.00	10	3	2.94	20	10	9.80
	Qantara Shark	0	0	0.00	10	2	1.96	5	0	0.00	15	2	1.96
	Ismailia	7	3	2.94	0	0	0.00	5	0	0.00	12	3	2.94
Port said	Tina Plain	0	0	0.00	10	2	1.96	10	0	0.00	20	2	1.96
Southern Sinai	Wadi Gharandal	12	10	9.80	0	0	0.00	10	3	2.94	22	13	12.75
Middle Sinai	Al hasna	10	6	5.88	0	0	0.00	10	6	5.88	20	12	11.76
Northern Sinai	Bir Al abed	12	7	6.86	0	0	0.00	10	8	7.84	22	15	14.71
Western Desert	Siwa Oasis	10	6	5.88	0	0	0.00	10	2	1.96	20	8	7.84
New Valley	Dakhla Oasis	10	5	4.90	20	2	1.96	10	1	0.98	40	8	7.84
South East Coast	Shalateen	20	10	9.80	0	0	0.00	10	4	3.92	30	14	13.73
(2)Infected host%			66	64.71		6	5.88		30	29.41		102	

DF =22, *Chi*-Square (X) = 73.33, Prob. = ** highly Significant, (1) Infection% = infection percentage for each locality regardless of host species, (2) Infected each species % = infected each host percentage with regards to total Infected hosts number within all localities, Total No. = Total numbers of collected infected host within each locality, Infected No.= Number of each infected host regardless to locality, Total infected No.= Total number of infected hosts in each locality.

Discussion

No doubt, ticks' infestation hampered the growth of the livestock sector and imposed serious constraints on the health and productivity of domesticated animals settling Egyptian deserts [13-15, 41, 42]. In Egypt, the ethnic most important and abundant hard ticks included *Hyalomma*, *Boophilus* and *Rhipicephalus* spp. [6, 7]. Statistically, the distribution of infestation density of tick's species on hosts was highly significant ($P < 0.001$). Still, camels had demonstrated a substantial load of hard ticks' infestation; precisely *H. dromedarii* species, which is in agreement with previous reports [8, 12, 13, 15, 42]. Other tick species reported to infest camels included *H. impeltatum, H. marginatum, H. excavatum*, *H. rufipes* and *H. anatolicum* which were also in agreement with previous investigations [5, 8, 12, 13-15, 42]. However, partially disagreed with published results of Diab *et al.* [55], El Kammah *et al.* [8] and Abdel-Shafy *et al.* [12, 42] who illustrated that grazing animal species are parasitized mostly by genus *Hyalomma*, hence, the most dominant species were *H. dromedarii, H. impeltatum*, *H. excavatum, H. anatolicum, H. truncatum, H. marginatum, H. rufipes, H. turanicum, H. schulzei and H. impressum*. On the other hand, *B. annulatus* density was recorded higher than *Rh. sanguineus*. This is in accordance with the findings of Gabaj *et al.* [56] and Yassin et al. [15] who found that *B. annulatus*; despite its specificity to cattle, was quite common on the coastal belts [57]. This might be attributed to suitability of climatic and other environmental factors to these tick species and their main animal hosts [8, 14, 15, 55-60].

From the topology of Egypt's map, urbanization and international animal mobility either legal from countries at the western and southern borders including Sudan, Somalia, Ethiopia, and Libya and/or illegal in Sinai eastern borders from Gaza strip through tunnels are factors that led to rapid extension of exotic ticks as well as emerging of novel SFG rickettsioses [15, 56]. Camels are the main animal-structure in the Bedouin desert communities, in addition to, horses, sheep, goats, and dogs, hence, animal grazing is the main activity practiced (shepherds) by the citizens at eastern, western and southern borders, and Sinai. On the other hand, agriculture is the main practice by the smallholdings farmers at Western Oasis (southwestern border) and Suez Canal region; therefore, cattle were the main animal-structure, in addition, to donkeys, sheep, camels and dogs. Over and above, the burden of infested small mammals and/or migratory birds [61-63] holding and/or spreading the immature stages of ixodid ticks was obvious yet needed more clarification. Either passage from place to place as rodents or wandering across countries as birds which consumed an important role in the distribution of ticks' species, hence, the present detection of mutant isolates (*R. africae*-like and *R. aeschlimannii*-like), and novel SFG species (*R. sibrica mongolitimonae*-like) inducing rickettsioses [63, 64]. The obtained results could be justified by the socio-ecological characteristics of Egyptian society, the hard ticks' fauna, and structure of the animal population which influenced the zoogeographical range, as well as, the molecular epidemiology, and clinical aspects of SFG rickettsioses in the governorates included in the present investigation [8, 14]. Therefore, the present study not only confirmed the endemic status of the *R. africae* and *R. aeschlimannii* infections, but also, declared a poll of *Rickettsia* spp. that had biodiversity form previously detected ones [39]. Nevertheless, emphasize the possibility of future epidemics due to novel species not clearly classified; *R. sibrica mongolitimonae*-like, in addition to, unclassified *Rickettsia* spp. Nonetheless, *R. sibrica mongolitimonae* was previously detected in specimens of travelers coming back from southern Egypt (Shalateen) to France [65], moreover, in hard ticks collected from a camel in Israel [66-68].

Despite the previous detection of *Rickettsia* spp., little is known about the epidemiology of tick-borne rickettsioses in Egypt in both animals and humans [12, 31, 32, 68]. The three animal hosts investigated in the present study highlighted the persistent infectivity in mammalian hosts; confirmed the hypothesis that

camels' is being reservoirs [12-14, 31, 32, 68], illustrated sheep increased susceptibility [15], yet cattle rickettsioses needs more justification. In addition, the results highlighted queries about the equines and canines long in contact with grazing herd and/or inside the stables. Moreover, illustrated novel pathological role of *Rh. sanguineus* in the adaptation of rickettsioses in cattle; which is a hypothesis that needs fundamental studies before being accepted. Until now, the role of other insect species in vector-borne SFG rickettsioses transmission is a strongly suggested hypothesis that needs future investigations taking in consideration equines rickettsioses [12-15, 40, 68]. So far, the fatality of SFG rickettsioses in grazing animals and their owners' remains poorly understood. Unfortunately, the loss of livestock productivity has been associated with stress factors; hot weather, vaccination, dehorning/deworming, heavy infestation, long-distance transportation and animal movement [13, 42, 68]. Additionally, the expanded geography of infections into new governorates; which were not included in previous studies that are characterized by diverse socio-ecological structures was a dangerous alarm [12-16, 42, 68]. Furthermore, co-infections with more than one *Rickettsia* species and/or other tick-borne pathogens are the scenarios that have been deserted in numerous tick-borne epidemics [39, 42, 68]. Unquestionably, a phenomenon that had previously prompted disastrous mutations in all recorded zoogeographical infections, hence, shift the pathogen's infectivity and virulence, in turn, pathogenicity and fatality [40, 42, 68].

Conclusion

Still, the mitochondrial DNA markers annotation is reliable identification technology of ticks at both intra and inter-species level. A novel pathological role of *Rh. sanguineus* in the adaptation of rickettsioses in cattle; which is a hypothesis that needs fundamental studies before being accepted. Until now, the role of other insect species in vector-borne SFG rickettsioses transmission is a strongly suggested that needs future investigations.

DNAs of *R. aeschlimannii*-like, *R. africae*-like, in addition to, unclassified *Rickettsia* spp. were detected. Moreover, *R. sibirica mongolitimonae*-like was detected for the first time within Egyptian livestock's population. Of priority, the urgent need for comprehensive molecular characterization of obtained novel *Rickettsia* species detected in camels, cattle and sheep and their tick vectors which will help in better understanding epidemiology of tick-borne rickettsioses in Egypt.

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