

## Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt

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### Abstract

**Aim:** Rickettsioses have an epidemiological importance that includes pathogens, vectors, and hosts. The dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* play important roles as vectors and reservoirs of Rickettsiae. The aim of this study was to determine the prevalence of Rickettsiae in ixodid ticks species infesting dogs and camels in Egypt, in addition to, the morphological and molecular identification of *R. sanguineus* and *H. dromedarii*.

**Materials and Methods:** A total of 601 and 104 of ticks' specimens were collected from dogs and camels, respectively, in Cairo, Giza and Sinai provinces. Hemolymph staining technique and *OmpA* and *gltA* genes amplification were performed to estimate the prevalence rate of Rickettsiae in ticks. For morphological identification of tick species, light microscope (LM) and scanning electron microscope (SEM) were used. In addition to the phylogenetic analyses of 18S rDNA, Second internal transcript spacer, 12S rDNA, cytochrome c oxidase subunit-1, and 16S rDNA were performed for molecular identification of two tick species.

**Results:** The prevalence rate of Rickettsiae in ticks was 11.6% using hemolymph staining technique and 6.17% by *OmpA* and *gltA* genes amplification. Morphological identification revealed that 100% of dogs were infested by *R. sanguineus* while 91.9% of camels had been infested by *H. dromedarii*. The phylogenetic analyses of five DNA markers confirmed morphological identification by LM and SEM. The two tick species sequences analyses proved 96-100% sequences identities when compared with the reference data in Genbank records.

**Conclusion:** The present studies confirm the suitability of mitochondrial DNA markers for reliable identification of ticks at both intra- and inter-species level over the nuclear ones. In addition to, the detection of Rickettsiae in both ticks' species and establishment of the phylogenetic status of *R. sanguineus* and *H. dromedarii* would be useful in understanding the epidemiology of ticks and tick borne rickettsioses in Egypt.

**Keywords:** hard ticks, light microscope, phylogenetic analysis, polymerase chain reaction, Rickettsiae, scanning electron microscope.

### Introduction

Since 1960s, multiple traditional smear staining, serological, immunohistochemical as well as molecular studies had indicated that vectors, rodents, animals as well as human in Egypt are endemic with rickettsial agents [1-6]. Rickettsioses are recently worldwide recognized as emerging and reemerging arthropods-borne infectious diseases with zoonotic importance [7-13]. Bacteria of the order Rickettsiales were first described as obligatory intracellular short Gram-negative bacillary microorganisms that retained basic fuchsin when stained by the method

of Gimenez [14-16]. Despite its unpretentious, the hemolymph staining keeps ticks undamaged and the infected specimens can be used in further identification and/or isolation techniques of Rickettsiae [14]. Ticks are the main vectors of Rickettsiae, especially spotted fever group (SFG) that was transmitted through transstadial and transovarial transmission [17-19]. During the last decade, the taxonomy of Rickettsiae has undergone extensive reorganization [17,20]. Up to date, the order Rickettsiales includes two families; Anaplasmataceae and Rickettsiaceae, which necessitated advanced genetic guidelines for the reclassification of rickettsial isolates at the genus, group, and species levels, utilizing the multilocus sequencing of five genes including 16S ribosomal ribonucleic acid (16S rRNA), *gltA*, *ompA*, *ompB*, and *sac4* genes [21,22]. In the last years, the diagnosis of *Rickettsia* spp. was depending on the molecular tools. The outer membrane protein A of the cell surface antigen (*OmpA*) and

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citrate synthase (*gltA*) genes is only present in SFG Rickettsiae [23-26]. These two genes have high discrimination power to detection of *Rickettsia* spp. due to high variability within SFG [23,27].

In Egypt, *Rhipicephalus sanguineus* is the main dog infesting ticks [28-30]. While the most common ixodid ticks (order Parasitiformes of subclass Acari) infesting camels are *Hyalomma* species, especially *Hyalomma dromedarii*, *Hyalomma marginatum*, *Hyalomma excavatum*, and *Hyalomma impeltatum* [6,31,32]. Being hematophagous of biohazards danger and public health importance, they have been accused participant in emerging and/or reemerging tick-borne human and animal infectious diseases such as SFG, lyme disease, babesiosis; Q-fever and life-threatening arboviruses [6,17,33-36] through their two or three host life cycle [28,29]. The accurate taxonomy of tick species is a very important to control tick-borne diseases. The morphological identification using light microscope (LM) clarifies mainly the size and color of tick species besides other obvious characteristics such as mouth parts, the outline of body and scutum. However, scanning electron microscope (SEM) describes the fine characteristics which unclear by LM such as genital opening, punctuations, spiracles, and grooves [37]. Traditionally, taxonomical identification of ticks was based on the morphological criteria of adult ticks through LM or SEM, nonetheless, the applicability of this method is difficult when the specimens are engorged with blood, physically damaged, in immature stages; i.e. eggs, larvae, or nymphs [38,39] and very doubtful at subspecies and/or one group level; *R. sanguineus* group [40-46].

In recent years, the molecular characterization for taxonomic identification and phylogenetic purposes of ticks has been indispensable by DNA markers, including nuclear (18S rRNA) and mitochondrial (12S rRNA and 16S rRNA, and cytochrome c oxidase subunit-1 [CO1]) genes and nuclear regulatory non-translated stretches (second internal transcribed spacer [ITS2]) [44,47-50]. The 18S rRNA is best used for genera level identification [51] while the 16S rRNA, CO1, and ITS2 are the most useful markers for ticks taxonomy at species level [39,44,47-50,52-56]. The 12S rRNA is used as a tool for examining relationships among recently diverged branches of tick phylogenies [50,53,57].

In this study, the objectives were the determination of the prevalence of ticks-borne Rickettsiae with regard to ixodid ticks' species infestation in dogs and camels in Cairo, Giza and Sinai provinces in Egypt. In addition, to assess the ability of five DNA markers to confirm the identity of the most common tick species *R. sanguineus* and *H. dromedarii* at both species and genera levels besides the phylogenetic relationship between Egyptian tick species and others worldwide by sequences alignment with GenBank related records.

## Materials and Methods

### Ethical approval

Ethical approval of the study was obtained from the Ethics Committee of National Research Centre, Giza, Egypt.

### Animals

A total of 261 animals were investigated for tick infestation. The inspected animals were dogs that were collected from Cairo province and camels which were collected from Cairo, Giza, and Sinai provinces. These animals were examined for the presence of ticks in neck, chest area, and around the soft parts of their bodies such as the inner sides of the hind and forelegs, perineal area, inner surface of ears and udders [58].

### Ticks

A total of 705 adult ticks were collected (601 ticks from dogs and 104 ticks from camels) from Cairo, Giza, and Sinai provinces in Egypt. In addition, 9 nymphs were included in tick specimens. Ixodid ticks were collected during the period from March 2012 to October 2014. Ticks were detached from animals using strong forceps into plastic tubes covered by a piece of cloth and secured by rubber band. The field data of each sample such as date, locality, and number of examined animals were recorded [32]. Ticks were brought alive to the laboratory for further identification and investigation.

### Microscopical detection of Rickettsiae using hemolymph staining smears

All collected ticks were examined for Rickettsiae using hemolymph staining technique. According to Burgdorfer [59], hemolymph was impressed on slide following scissors amputating the distal portion of legs, fixed by air dry then stained with Gimenez stain [14]. The prepared hemolymph slides were examined under oil emersion lens using ordinary microscope (Zeiss).

### Molecular identification of ticks-borne Rickettsiae by *OmpA* and *gltA* genes amplification

Polymerase chain reactions (PCRs) were performed to specifically amplify the *Rickettsia* spp. *OmpA* and *gltA* genes sequence by the appropriate primer set designed according to Roux *et al.* [24], Fournier *et al.* [25], Mediannikov *et al.* [26] (Table-1). *OmpA* primers, both the forward 190.70-F and the reverse 190.701-R primers, were designed to span the nucleotides positions from 70 to 90 and 701 to 681, respectively, as the predicted product size are ranged from 590 to 634 bp [60]. Moreover, primers CS2d-F and CSEnd-R were amplified the full-length of the *gltA* gene, as the predicted product size are ranged from 852 to 1265 bp, therefore; CS2d primer was designed to be completely homologous to the corresponding portion of the gene of *Rickettsia conorii* for only SFG [26].

The amplification reactions were performed in a PTC-100™ thermal cyler (MJ Research Inc., USA) under complete aseptic conditions. Each 25 µl total volumes of PCR mixture contained 25-50 ng/µl

**Table-1:** Primers utilized in amplification and sequencing of genes.

| DNA marker       | 5'- primers sequences-3'       | Amplified fragments | References |
|------------------|--------------------------------|---------------------|------------|
| <i>OmpA</i> gene |                                | 590-634 bp          | [25]       |
| 190.70-F         | 5'-ATGGCGAATATTTCTCCAAA-3'     |                     |            |
| 190.701-R        | 5'-GTTCCGTTAATGGCAGCATCT-3'    |                     |            |
| <i>gltA</i> gene |                                | 852-1265 bp         | [24,26]    |
| CS2d-F           | 5'-ATGACCAATGAAAATAATAAT-3'    |                     |            |
| CSEnd-R          | 5'-CTTATACTCTCTATGTACA-3'      |                     |            |
| 18S rRNA         |                                | 780 bp              | [49]       |
| 18S-F            | 5'-CATTAAATCAGTTATGGTTCC-3'    |                     |            |
| 18S-R            | 5'-CGCCGCAATACGAATGC-3'        |                     |            |
| ITS2             |                                | 1200-1600 bp        | [50]       |
| ITS2-F           | 5'-ACATTGCGCCTTGGGTCTT-3'      |                     |            |
| ITS2-R           | 5'-TCGCCTGATCTGAGGTCGAC-3'     |                     |            |
| 12S rRNA         |                                | 337-355 bp          | [57]       |
| T1B              | 5'-AAACTAGGATTAGATACCCT-3'     |                     |            |
| T2A              | 5'-AATGAGAGCGACGGGCGATGT-3'    |                     |            |
| CO1              |                                | 732-820 bp          | [54]       |
| CO1-F            | 5'-GGAACAATATATTTAATTTTTGG-3'  |                     |            |
| CO1-R            | 5'-ATCTATCCCTACTGTAAATATATG-3' |                     |            |
| 16SrRNA          |                                | 455 bp              | [49]       |
| 16S-F            | 5'-TTAAATTGCTGTRGTATT-3'       |                     |            |
| 16S-R            | 5'-CCGGTCTGAACTCASAWC-3'       |                     |            |

CO1=Cytochrome c oxidase subunit-1, 12S rRNA=12S ribosomal ribonucleic acid, ITS2=Second Internal transcribed spacer

genomic DNA, 10 pM/ $\mu$ l of each primers, 12.5  $\mu$ l of  $\times 2$  Dream Taq Green PCR master mix ( $\times 2$  buffer, 0.4 mM deoxynucleotide [dNTP] and 4 mM  $MgCl_2$ ; ThermoScientific, California, US), and 9  $\mu$ l nuclease free water (Qiagen) to complete the total volume of the reactions. 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, 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 [6]. A reagent blank was run simultaneously as a control with every PCR. Amplified products from the PCRs were electrophoresed in 1% agarose gels in Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3, Sigma-Aldrich) 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) [6].

#### Morphological identification of tick vectors by LM

The collected ticks were counted and sorted to different genera, species, and sex using dissecting microscope. Tick species were identified morphologically using taxonomic keys of Hoogstraal and Kaiser [28] and Estrada-Pena *et al.* [29]. The two most common tick species (*R. sanguineus* and *H. dromedarii*) were morphologically examined in details using LM, especially the dorsal and ventral surfaces of adult males and females. Male and female ticks were fixed on plasticine one dorsally and another one ventrally. The

adult ticks in these two positions were photographed by digital camera fixed on stereomicroscope [32].

#### Morphological identification of tick vectors by SEM

*R. sanguineus* and *H. dromedarii* were also examined morphologically in details using SEM. Ticks were prepared for SEM according to the method described by Abdel-Shafy [37]. Ticks individuals were cleaned by water-glycerol-potassium chloride solution, washed in tap water, taken through graded series of alcohol, glued by their dorsal and ventral surfaces to the SEM stub, dried by the dryer (Blazer Union, F1-9496 Blazer/Fürstentun Liechtenstein), mounted on SEM stubs, coated with gold using a S150A Sputter Coater and examined by SEM.

#### Molecular identification of tick vectors by PCR

To confirm the morphological classification proceeded on *R. sanguineus* and *H. dromedarii*, multi-locus sequence typing depending on five tick's genomic DNA markers amplified fragments was carried out which included; one nuclear gene 18S rRNA gene; and untranslated regulatory sequences of the second ITS2 and three mitochondrial genes 12S rRNA, CO1, and 16S rRNA (Table-1).

#### Primers design

Two pairs of primers were designed that were in accordance to GenBank records of nuclear sequences of the 18S rRNA gene (18S-F and 18S-R) and the nuclear ribosomal ITS2 (ITS2-F and ITS2-R) depending on previous publications in consequence to this subject [49,50], with predicted products sizes 780 and 1200-1600 bp, respectively (Table-1). Moreover, three pairs of primers were utilized that were complementary to fragments in three different mitochondrial DNA markers (Table-1). They were designed according to the complete mitochondrial genome sequence of

*Drosophila yakuba* (NC 001322.1) and modified to be complementary to wide divergent arthropods by aligning mitochondrial genes to DNA GenBank sequences' records from; *Anopheles gambiae*, *Daphnia pulex*, and *Apis mellifera*; genbank accession numbers: NC 002084.1, Z15015, and L06178, respectively. The 12S rRNA gene primers pair (T1B and T2A) with predicted product sizes approximately 337-355 bp [57]. The CO1 primers pair (CO1-F and CO1-R) was designed according to Chitimia *et al.* [54], while the 16S rRNA gene primers' set (16S-F and 16S-R) were designed according to Lv *et al.* [49], with predicted products sizes 732-820 bp and 455 bp, respectively (Table-1).

#### *Ticks genomic DNA purification*

Total DNA was extracted and purified from the complete body of adult ticks of each tick species after dissection into quarters. Before dissection, each sample was washed in different decreasing concentrations of alcohol (70%, 50%, 30%, 10%) for 1 h per each concentration and finally in double distilled water for 1 h. DNA isolation procedures were performed using high salt concentration protocol [61]. 20 mg from each tick tissues were incubated with lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 3.4 mM SDS and 20 mM NaCl pH 8.0, Sigma-Aldrich), in addition to 1 M DTT (Sigma-Aldrich) and proteinase K (20 mg/ml) (Stratagin) at 56°C overnight, then the supernatant was transferred to new collecting tube (Coaster) to proceed in DNA isolation. Purity and concentration of isolated genomic DNA was measured by nanodrop 2000c (Thermo Scientific). Samples recording <1.5 ratio at 260/280 wavelength were proceeded to second phase of DNA isolation by shaking in phenol/chloroform/isoamyl alcohol (25:24:1, Sigma-Aldrich) and precipitated by adding 2 volumes of ice-cold ethanol for 4-6 h [62]. After centrifugation (at 14,000 rpm for 5 min), the pellet was resuspended in 50 µl Tris-EDTA buffer (10 mM Tris, pH 8.0 and 1 mM EDTA), and the DNA was used as template for PCR and the remaining DNA was stored at -20°C.

#### *PCR amplification of target sequences*

The amplification reactions were performed in 25 µl total volumes under complete aseptic conditions. Each PCR mixture contained 25-50 ng/µl genomic DNA, 10 pM/µl of each primers, 12.5 µl of ×2 Dream Taq Green PCR master mix (×2 buffer, 0.4 mM dNTP and 4 mM MgCl<sub>2</sub>; ThermoScientific, California, US), 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 (initial denaturation) for all five markers. The PCR protocol of 18S rDNA, CO1, and 16S rDNA were amplified according to the following: 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 [49,54]. While the ITS2 protocol included 30 cycles of denaturation at 94°C for 1.5 min, annealing at 55°C for 1.5 min and elongation at 72°C for 2 min, then the final elongation at 72°C for 10 min [50]. In addition to 12S rDNA was 5 cycles of denaturation at 94°C for 15 s, annealing at 51°C for 30 s and elongation at 68°C for 30 s, followed by 25 cycles denaturation at 94°C for 15 s, annealing at 53°C for 30 s and elongation at 70°C for 30 s, and the final elongation at 70°C for 5 min [57]. A reagent blank was run as control simultaneously with every PCR. Amplification success rate was measured based on the proportion of samples that produced sequences of the appropriate length. These sequences were further evaluated for their utility in species identification.

The PCR products were inspected by electrophoresis in 1.5% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3, Sigma-Aldrich) and stained with ethidium bromide (Sigma-Aldrich). A gene ruler 100 bp plus DNA ladder (Thermo Scientific, California, USA) was used with each gel. Gels photos were analyzed by Lab Image software (BioRad) [6].

#### **Phylogenetic analysis of amplified sequences**

##### *Sequencing of the obtained PCR products*

PCR products were purified for sequencing using ExoSAP-IT PCR Product Cleanup Kit (Affymetrix, Ohio, USA) according to manufacturer's recommendation. 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 (FS enzyme; Applied Biosystems), following the protocols supplied by the manufacturer. Each sequencing reaction was repeated at least three times in both the forward and reverse directions before being accepted for analysis [6]. The utilized primers sets for PCRs were used for sequencing of designated amplicons (Table-1).

##### *Data submission in GenBank*

The sequences of 18S rDNA, ITS2, 12S rDNA, CO1, and 16S rDNA of each tick species were aligned assembled and corrected using ChromasPro 1.49 beta (Technelysium Pty. Ltd., Tewantin, QLD, Australia), then the corrected ticks' sequences were submitted in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) to record each sequence with accession number.

##### *Tree construction*

Amplified sequences of each fragment were aligned using BLASTN program of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) for sequence homology searches against ticks' GenBank database. Multiple sequences alignments for evolutionary relationships between new Egyptian records and other ticks reference isolates were inferred using the ClustalW 1.8® program [63] after modification of sequences length by BioEdit sequence alignment editor (v. 7.0.9.0). Two

phylogenetic trees were constructed with the neighbor-joining method (NJ) [64], and the unweighted pair group method with arithmetic mean (UPGMA) [65]. The evolutionary distances were calculated by the maximum composite likelihood method [66]. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [67,68]. Phylogenetic analyses were conducted in MEGA4 [69].

## Results

### Prevalence of ticks and tick-borne Rickettsiae on dogs and camels

The tick-borne Rickettsiae infections rates were calculated in all tick species collected from camels and dogs in the three Egyptian provinces investigated. Hemolymph staining of tick specimens by Gimenez stain was successful for preliminary detection of Rickettsiae in ticks estimating the prevalence rate 11.89% in dog ticks and 10.1% in camel ticks (Table-2).

Ticks which were positive by hemolymph staining technique were screened molecularly by *OmpA* and *gltA* genes with the fragment product 600 and

1200 bp, respectively (Figure-1). Five ticks were positive with *Rickettsia* spp. four of which obtained from *R. sanguineus* from Cairo province and one from *H. marginatum* from Sinai province. The prevalence rate of *Rickettsia* spp. was 6.17% depending on molecular identification of tick specimens by *OmpA* and *gltA* genes amplification (Table-2).

The six collected tick species from Cairo, Giza, and Sinai provinces were identified according to the developmental stage into adults and nymph; furthermore the adults were classified according to sex into females and males (Table-3). The brown dog tick *R. sanguineus* is the unique tick species that was found on dogs recording 100% infestation (Table-2). However, the camels tick *H. dromedarii* was the most abundant tick species found on camels recording 91.9% (Table-2). Lower percentages of infestation of other tick species were also recorded on camels such as *H. marginatum*, *H. impeltatum*, *H. excavatum* and *H. rufipes* (Table-2).

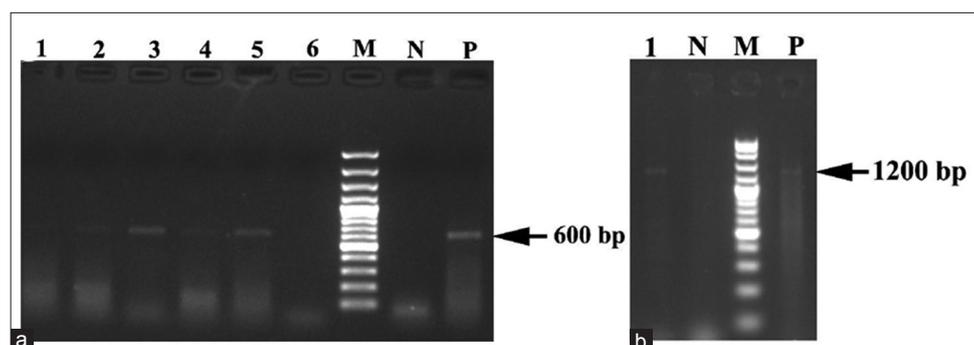
### Morphological identification

Both male and female specimens which classified into *R. sanguineus* and *H. dromedarii* were photographed by LM and SEM for morphological description (Figures-2-5).

**Table-2:** The prevalence of Rickettsiae in ixodid tick species infesting dogs and camels by hemolymph staining technique with Gimenez stain and PCR using *OmpA* and *gltA* genes.

| Ticks species        | Tick number | Prevalence of tick species |        | Prevalence of Rickettsiae |                               |
|----------------------|-------------|----------------------------|--------|---------------------------|-------------------------------|
|                      |             | Camels %                   | Dogs % | Hemolymph staining        | PCR using ompA and gltA genes |
|                      |             |                            |        | N (%)                     | N (%)                         |
| <i>R. sanguineus</i> | 597/597     | 0                          | 100    | 71/597 (11.89)            | 4/71 (5.6)                    |
| <i>Hyalomma</i> spp. | 99/99       | 100                        | 0      | 10/99 (10.1)              | 1/10 (10)                     |
| <i>H. dromedarii</i> | 91/99       | 91.9                       | 0      | 8/91 (8.79)               | 0 (0)                         |
| <i>H. marginatum</i> | 5/99        | 5.05                       | 0      | 1/5 (20)                  | 1/1 (100)                     |
| <i>H. excavatum</i>  | 1/99        | 1.01                       | 0      | 0 (0)                     | 0 (0)                         |
| <i>H. impeltatum</i> | 1/99        | 1.01                       | 0      | 1/1 (100)                 | 0 (0)                         |
| <i>H. rufipes</i>    | 1/99        | 1.01                       | 0      | 0 (0)                     | 0 (0)                         |
| Total                | 696         | 14.22                      | 85.78  | 81/696 (11.6)             | 5/81 (6.17)                   |

*H. dromedarii*=*Hyalomma dromedarii*, *R. sanguineus*=*Rhipicephalus sanguineus*, *H. marginatum*=*Hyalomma marginatum*, *H. excavatum*=*Hyalomma excavatum*, *H. impeltatum*=*Hyalomma impeltatum*, *H. rufipes*=*Hyalomma rufipes*, PCR=Polymerase chain reactions

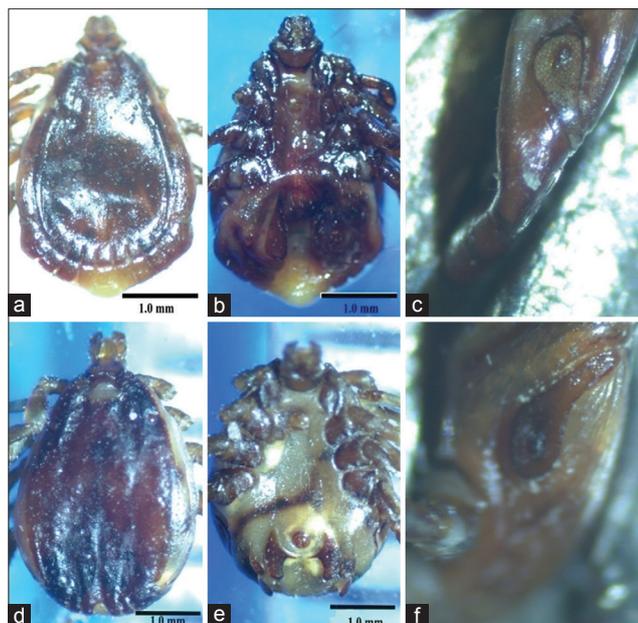


**Figure-1:** Molecular identification of tick-borne Rickettsiae by polymerase chain reaction products of the *OmpA* (a) and *gltA* (b) genes detected in *Rhipicephalus sanguineus* and *Hyalomma dromedarii* species in 1.5% agarose gels stained with ethidium bromide. Lane M: 100 bp DNA ladder, Lane N: Control negative, Lane P: Control positive and Lane 1 and 6 are negative tick samples, while tick samples on Lanes 2 to 5 are *OmpA* positive (a) with molecular sized ranged from 600 bp. Whereas, Lane 1 (b) are *gltA* positive with molecular size 1200 bp.

**Table-3:** The prevalence of tick species collected from camels and dogs in three provinces of Egypt.

| Governorates | Localities of collection | Ticks species        | Ticks fauna |     |       |     |                |
|--------------|--------------------------|----------------------|-------------|-----|-------|-----|----------------|
|              |                          |                      | ♂           | ♀   | Nymph | No. | Prevalence (%) |
| Cairo        | 3                        | 3                    | 276         | 353 | 9     | 638 |                |
|              | El-Abbasia               | <i>R. sanguineus</i> | 257         | 338 | 4     | 599 | 93.88          |
|              | El-Dowaika               | <i>R. sanguineus</i> | 0           | 2   | 0     | 2   | 0.313          |
|              | Gisr El-Swiss            | <i>H. dromedarii</i> | 19          | 11  | 5     | 35  | 5.49           |
| Giza         | 1                        | 4                    | 5           | 7   | 0     | 12  |                |
|              | EL Haram                 | <i>H. dromedarii</i> | 5           | 4   | 0     | 9   | 75             |
|              |                          | <i>H. marginatum</i> | 0           | 1   | 0     | 1   | 8.33           |
|              |                          | <i>H. excavatum</i>  | 0           | 1   | 0     | 1   | 8.33           |
|              |                          | <i>H. rufipes</i>    | 0           | 1   | 0     | 1   | 8.33           |
| Sinai        | 1                        | 3                    | 38          | 17  | 0     | 55  |                |
|              | Raas Sidr                | <i>H. dromedarii</i> | 37          | 15  | 0     | 52  | 94.55          |
|              |                          | <i>H. marginatum</i> | 1           | 1   | 0     | 2   | 3.64           |
|              |                          | <i>H. impeltatum</i> | 0           | 1   | 0     | 1   | 1.82           |

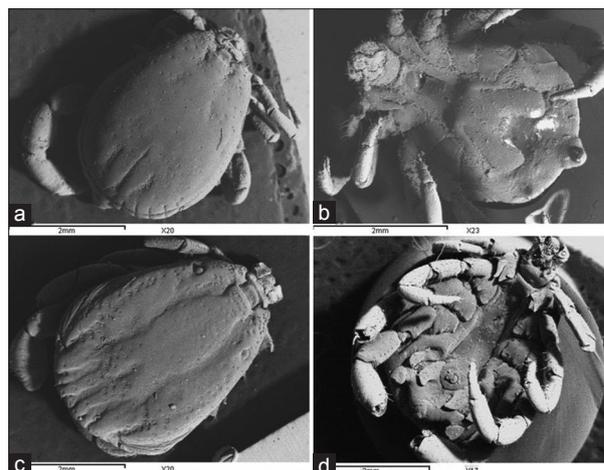
*H. dromedarii*=*Hyalomma dromedarii*, *R. sanguineus*=*Rhipicephalus sanguineus*, *H. marginatum*=*Hyalomma marginatum*, *H. excavatum*=*Hyalomma excavatum*, *H. impeltatum*=*Hyalomma impeltatum*, *H. rufipes*=*Hyalomma rufipes*



**Figure-2:** The adult males of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* photographed by light microscope: (a) Dorsal view of *R. sanguineus*, (b) ventral view of *R. sanguineus*, (c) spiracular plate of *R. sanguineus*, (d) dorsal view of *H. dromedarii*, (e) ventral view of *H. dromedarii*, (f) spiracular plate of *H. dromedarii*.

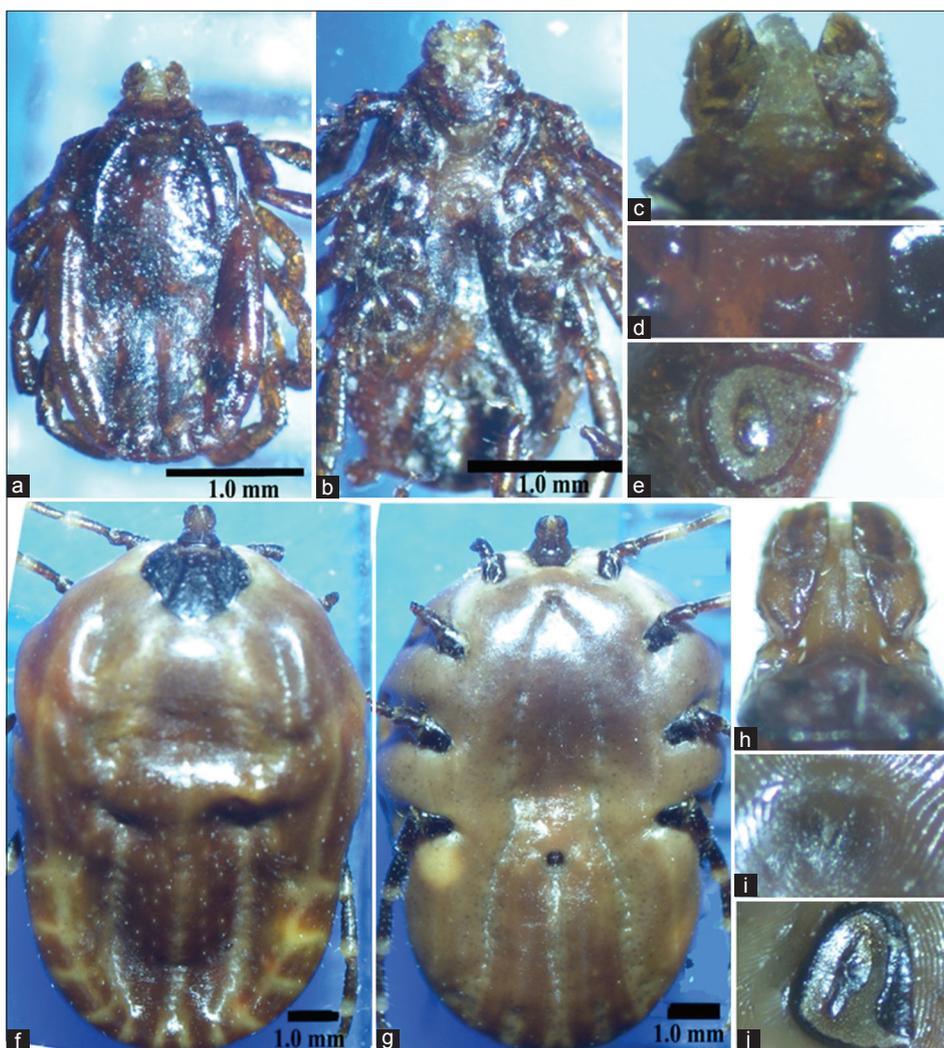
### Males

LM showed that the color of the two tick species is similar (dark brown), while the size is different (*H. dromedarii* is larger than *R. sanguineus*). Additional characteristics are illustrated obviously by the LM such as grooves on the scutum, parma, adanal shields, subanal shields, and spiracular plates (Figure-2a-f). Lateral grooves are deep, narrow, and long (extending from eyes to the festoons) in *R. sanguineus*. They are deep and short (limited to the posterior third of the scutum) in *H. dromedarii*. Parma has a white color in both tick species while it protrudes beyond the festoons in *R. sanguineus* and at the level

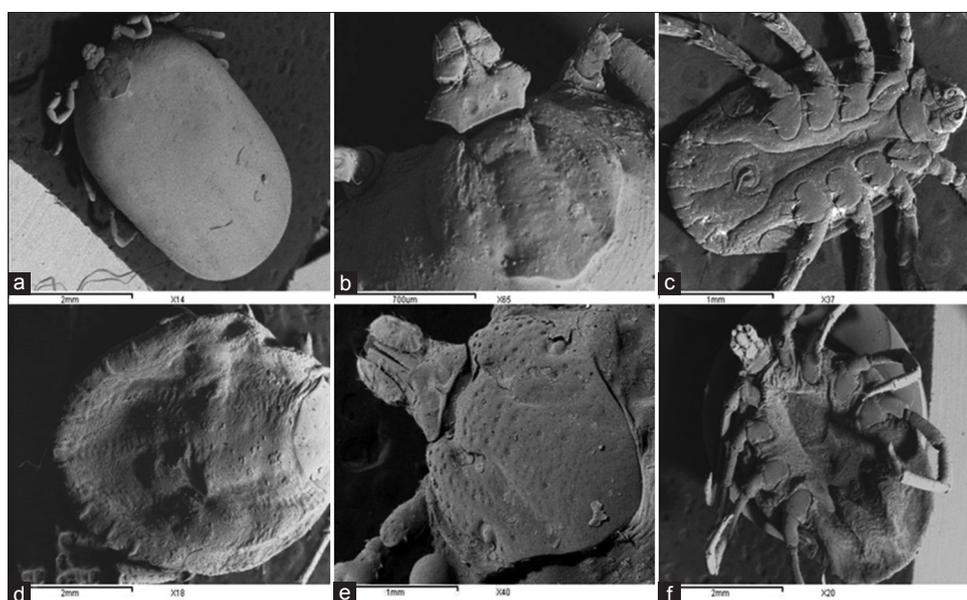


**Figure-3:** The adult males of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* photographed by scanning electron microscope: (a) Dorsal view of *R. sanguineus*, (b) ventral view of *R. sanguineus*, (c) dorsal view of *H. dromedarii*, (d) ventral view of *H. dromedarii*.

of festoons in *H. dromedarii*. Adanal shields have subtriangular shape rounded posteriorly in *R. sanguineus*. These shields have long margins strongly curved in *H. dromedarii*. Subanal shields are absent in *R. sanguineus* and present exteriorly of the axis of the adanal shields in *H. dromedarii*. Spiracular plates have long tails which are narrow less than the width of the adjacent festoon in *R. sanguineus*. In *H. dromedarii*, spiracular plates resemble those in *R. sanguineus* but their tails may be wider. The most previous characteristics in males of the two tick species were confirmed by SEM. Additional features were well distinguished by this instrument as posteromedian groove, paramedian grooves, caudal area of the scutum, and scutal punctuations (Figure-3a-d). The posteromedian groove is deep in both *R. sanguineus* and *H. dromedarii* but it is wider in *H. dromedarii*. The paramedian grooves are equal and shorter than posteromedian groove in the two tick species, while they are wider in *H. dromedarii*



**Figure-4:** The adult females of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* photographed by light microscope: (a) Dorsal view of *R. sanguineus*; (b) ventral view of *R. sanguineus*; (c) gnathosoma of *R. sanguineus*; (d) genital opening of *R. sanguineus*; (e) spiracular plate of *R. sanguineus*; (f) dorsal view of *H. dromedarii*; (g) ventral view of *H. dromedarii*; (h) gnathosoma of *H. dromedarii*; (i) genital opening of *H. dromedarii*; (j) spiracular plate of *H. dromedarii*.



**Figure-5:** The adult females of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* photographed by scanning electron microscope: (a and b) Dorsal view of *R. sanguineus*; (c) ventral view of *R. sanguineus*; (d and e) dorsal view of *H. dromedarii*; (f) ventral view of *H. dromedarii*.

than *R. sanguineus*. In general, the caudal area of the scutum is more depressed in *H. dromedarii*. Scutal punctuations consist of four irregular rows of large punctuations in *R. sanguineus* and few scattered large punctuations in *H. dromedarii*.

#### Females

Most features in females of the two tick species can be distinguished by LM such as color, size, gnathosoma (mouth parts), genital aperture, and spiracular plates (Figure-4a-j). Some of these features are clearer with SEM beside additional characteristics as the outline of scutum and its punctuations (Figure-5a-f). The color of females in the two tick species is similar (dark brown) resembling males. The size of *H. dromedarii* female is larger than that of *R. sanguineus*. The dorsal view of gnathosoma in Figure-4c and h represents females and males in the two tick species. The dorsal surface of gnathosoma consists of basis capitulum and palpi. Basis capitulum has hexagonal shape with sharper lateral angles in *R. sanguineus* and tetragonal shape with blunt angles in *H. dromedarii*. Palpi are longer in *H. dromedarii* than in *R. sanguineus*. Genital aperture has broad U-shape in *R. sanguineus* and V-shape which in profile slopes gradually in *H. dromedarii*. Spiracular plates have small narrow tails in the two tick species, but the tails are slightly curved in *R. sanguineus*. Posterolateral margins of scutum are undulated in *R. sanguineus* and slightly convex in *H. dromedarii*. Scutum has large punctuations which scattered in *R. sanguineus*

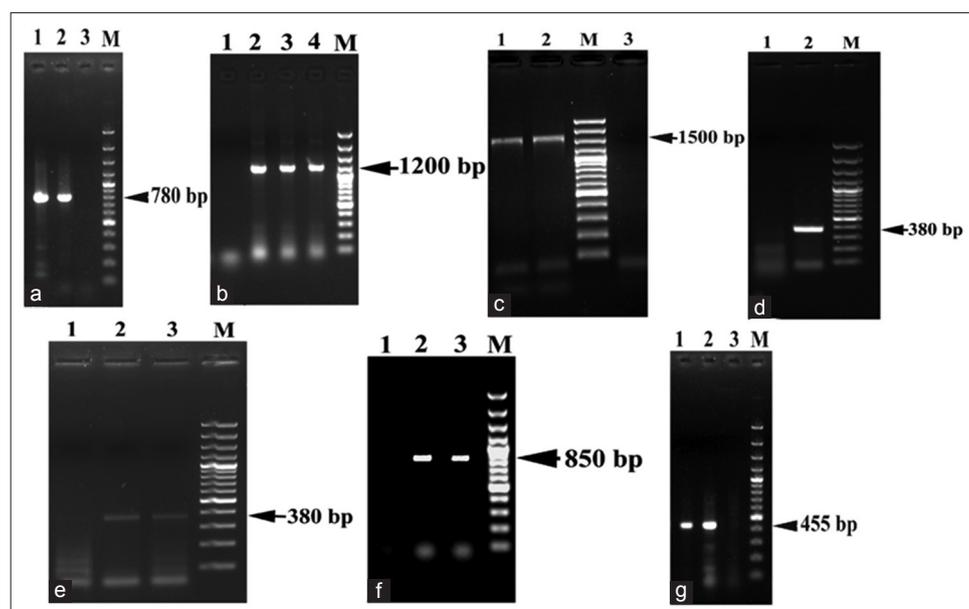
and denser over the central field and scapular fields in *H. dromedarii*.

#### Molecular identification of tick genome by DNA markers

*R. sanguineus* and *H. dromedarii* were screened by PCR using five specific primers of DNA markers; 18S rDNA, ITS2, 12S rDNA, CO1 and 16S rDNA, in accordance, the documented length of amplifications fragments in both tick species of 18S rDNA, 12S rDNA, CO1, and 16S rDNA were 780, 380, 850, 455 bp, respectively, while in ITS2 it was 1200 bp in *R. sanguineus* and 1500 bp in *H. dromedarii* (Figure-6a-g).

#### Sequences analyses

The obtained sequences of the two tick species (*R. sanguineus* and *H. dromedarii*) were submitted in GenBank. The length of the obtained sequences varied from 355 to 1500 bp. Accession numbers of the sequences of the Egyptian tick amplified genes and other details as tick species, tick sexes, and localities of collection are listed in Table-4. Moreover, the identities of the Egyptian obtained sequences to other reference tick strains were ranged from 97% to 100% (Table-4). The partial sequence of 18S rDNA of *R. sanguineus* showed 99% identity with *R. sanguineus* (KF958435.1), while 18S rDNA of *H. dromedarii* showed 100% similarity to *H. dromedarii* (L76348.1). The identities of ITS2 were 99% and 96% of *R. sanguineus* and *H. dromedarii* obtained with reference strains *R. sanguineus* (JQ625707.1) and *H. dromedarii* (JQ733570.1), respectively. The sequence identities of partial sequence of 12S rDNA



**Figure-6:** Molecular identification of tick species (*Rhipicephalus sanguineus* and *Hyalomma dromedarii*) by polymerase chain reaction products of the five DNA markers detected in 1.5% agarose gels stained with ethidium bromide. In all figures Lane M: 100 bp DNA ladder, Lane 1 or 3: Control negative. (a) Lane 1 present 780 bp amplicon of 18S rRNA gene of *R. sanguineus*, while Lane 2 present 780 bp amplicon of 18S ribosomal ribonucleic acid (rRNA) gene of *H. dromedarii*, (b) 1200 bp amplicons of Second Internal transcribed spacer (ITS2) of *R. sanguineus*, (c) 1500 bp amplicons of ITS2 of *H. dromedarii*, (d and e) 380 bp amplicon of 12S rRNA gene of both *R. sanguineus* and *H. dromedarii*, (f) Lane 2 present 850 bp amplicon of cytochrome c oxidase subunit-1 (CO1) gene of *R. sanguineus*, while Lane 3 present 850 bp amplicon of CO1 gene of *H. dromedarii*, (g) Lane 1 present 455 bp amplicon of 16S rRNA gene of *R. sanguineus*, while Lane 2 present 455 bp amplicon of 16S rRNA gene of *H. dromedarii*.

**Table-4:** GenBank accession numbers of sequenced genes were amplified from genomes of *R. sanguineus* and *H. dromedarii* and their identities with other reference strains.

| DNA markers | Ticks                |     | Governorates of ticks specimens | GenBank No. | Similarity with recorded tick species in GenBank |              |                                   |
|-------------|----------------------|-----|---------------------------------|-------------|--|--------------|-----------------------------------|
|             | Species              | Sex |                                 |             | Identity (%)                                     | Covering (%) | Reference strains of tick species |
| 18S rRNA    | <i>R. sanguineus</i> | ♀   | Cairo                           | KU198407    | 99   | 93           | KF958435.1                        |
|             | <i>H. dromedarii</i> | ♀   | Giza, Sinai                     | KU198408    | 100  | 99           | L76348.1                          |
| ITS2        | <i>R. sanguineus</i> | ♀   | Cairo                           | KU198406    | 99   | 100          | JQ625707.1                        |
|             | <i>H. dromedarii</i> | ♀   | Giza, Sinai                     | KU214593    | 96   | 100          | JQ733570.1                        |
| 12S rRNA    | <i>R. sanguineus</i> | ♂   | Cairo                           | KU198403    | 99   | 98           | JQ480844.1                        |
|             | <i>H. dromedarii</i> | ♂   | Cairo                           | KU963224    | 97   | 85           | U95874.1                          |
| CO1         | <i>R. sanguineus</i> | ♂   | Cairo                           | KU214592    | 98   | 99           | KM494916.1                        |
|             | <i>H. dromedarii</i> | ♀   | Giza, Sinai                     | KU323789    | 99   | 75           | KM235697.1                        |
| 16S rRNA    | <i>R. sanguineus</i> | ♀   | Cairo                           | KU198404    | 99   | 99           | KR870984.1                        |
|             | <i>H. dromedarii</i> | ♀   | Giza, Sinai                     | KU198405    | 98   | 99           | L34306.1                          |

KF958435.1=*R. sanguineus* isolate 39\_S\_C4 18S rRNA gene, partial sequence, L76348.1=*H. dromedarii* 18S rRNA gene, JQ625707.1=*R. sanguineus* isolate 3464 5.8S ribosomal RNA gene, partial sequence; ITS2, complete sequence; and 28S rRNA gene, partial sequence, JQ733570.1=*H. dromedarii* 5.8S ribosomal RNA gene, partial sequence; ITS2, complete sequence; and 28S rRNA gene, partial sequence, JQ480844.1=*R. sanguineus* isolate 252\_12s\_SP6 12S rRNA gene, partial sequence; mitochondrial, U95874.1=*H. dromedarii* isolate Hydr6 12S rRNA gene, partial sequence; mitochondrial, KM494916.1=*R. sanguineus* isolate Gilan-e Gharb CO1 gene, complete cds; mitochondrial, KM235697.1=*H. dromedarii* voucher INHM: TC1314 CO1 gene, partial cds; mitochondrial, KR870984.1=*R. sanguineus* isolate Orkun-RS314 small subunit ribosomal RNA gene, partial sequence; mitochondrial, L34306.1=Mitochondrion *H. dromedarii* 16S rRNA gene. *H. dromedarii*=*Hyalomma dromedarii*, *R. sanguineus*=*Rhipicephalus sanguineus*, CO1=Cytochrome oxidase subunit-1, 12S rRNA=12S ribosomal ribonucleic acid, ITS2=Internal transcribed spacer 2, 28S rRNA=28S ribosomal ribonucleic acid

of *R. sanguineus* and *H. dromedarii* were 99% and 97% compared to *R. sanguineus* (JQ480844.1) and *H. dromedarii* (U95874.1). The similarity was 98-99% of CO1 gene of *R. sanguineus* and *H. dromedarii* compared with reference strains *R. sanguineus* (KM494916.1) and *H. dromedarii* (KM235697.1), respectively. Finally, the partial sequence of 16S rDNA of *R. sanguineus* showed 99% identity with *R. sanguineus* (KR870984.1), while 16S rDNA of *H. dromedarii* showed 98% similarity to *H. dromedarii* (L34306.1).

#### Phylogenetic analysis

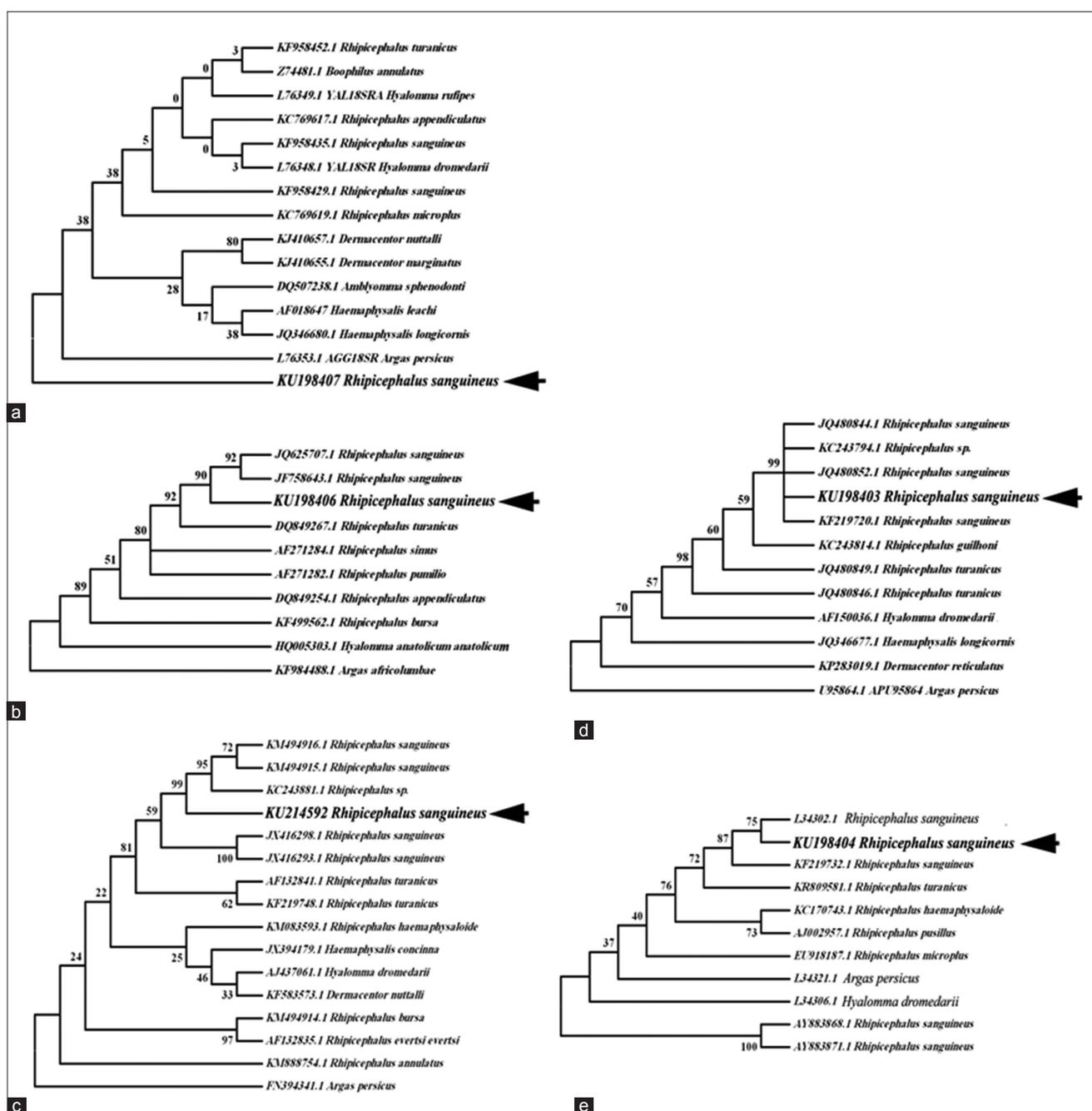
The phylogenetic tree of the two tick species (*R. sanguineus* and *H. dromedarii*) was constructed for each marker (Five DNA markers) based on Clustal W multiple alignments using two methods UPGMA and NJ method. Therefore, the only NJ method was discussed (Figures-7a-e and 8a-e). The phylogenetical analyses of five DNA markers of the two tick species sequences (*R. sanguineus* and *H. dromedarii*) were agreed with their morphological identification. The NJ trees of *R. sanguineus* were performed based on alignment of five DNA markers sequences using *Argas* spp. as outgroup inferred that *R. sanguineus* strains of this study were branched out or clustered together with *R. sanguineus* reference strains (Table-4) with a bootstrap value ranged from 87 to 99 (Figure-7a-e), except 18S rDNA tree *R. sanguineus* deposit in separate clade (Figure-7a). In *H. dromedarii*, the NJ trees were constructed based on alignment of five DNA markers sequences using *Argas persicus* or *Ornithodoros* spp. (soft ticks) as outgroup indicated that *H. dromedarii* strains of this study in all five markers were branched out or clustered together

with *H. dromedarii* reference strains (Table-4) with a bootstrap value ranged from 51 to 100 (Figure-8a-e).

#### Discussion

Nowadays, the studies on emerging and reemerging infectious diseases as Rickettsioses are increased due to their public health implication, zoonotic importance in addition to, increasing the contact between wild and domestic animals, animals and human, as well as animals, human and vectors [11,12,70-72]. So that the proper diagnostic tools, such as molecular techniques, are needed to improve the sensitivity and specificity of the diagnosis of Rickettsiae. Therefore, this study was designed to achieve two goals. The first goal is to determine the ability of ixodid tick species infesting dogs and camels to carry Rickettsiae using Gimenez stain and PCR. The second goal is to illustrate the accurate taxonomic status of the two most common ticks, the brown dog tick *R. sanguineus*, and the camel tick *H. dromedarii* using a combination of LM, SEM and molecular analysis.

Hemolymph staining technique of collected ticks revealed that the prevalence rate of *Rickettsia* spp. was 11.89% in dog ticks and 10.1% in camel ticks (Table-2). The total prevalence rate in ticks was 11.6% (Table-2). While the prevalence rate of *Rickettsia* spp. depending on molecular identification of tick specimens by *OmpA* and *gltA* genes amplification was 6.17% (Table-2 and Figure-1). The low prevalence rate of Rickettsiae in ticks in this study may retain to the low numbers of *Rickettsia* spp. circulated in the blood of the host even during the acute phase of the disease [73,74]. Furthermore, the *Rickettsia* DNA in the host blood and their vectors (ticks) can be detected only during



**Figure-7:** Phylogenetic trees of *Rhipicephalus sanguineus* based on the five DNA markers. All sequences of each marker were aligned and Neighbor-joining trees were constructed. (a) 18S rDNA, (b) Second internal transcribed spacer, (c) Cytochrome c oxidase subunit-1, (d) 12S rDNA,, (e)16S rDNA.

rickettsia (recent infection) [75]. Moreover, the infection rates of *Rickettsia* spp. in ticks under natural condition were tend to be low (<1%) due to the lethal effect of Rickettsiae on ticks [76,77].

In Egypt, there were few reports documented the presence of *Rickettsia* spp. in *R. sanguineus* [5], while many studies in the surrounding countries had detected *Rickettsia* spp. in *R. sanguineus* as Algeria, Tunisia and Israel [78-81]. Moreover, the previous reports detected the presence *R. aeschlimannii*, *R. africae*, and other *Rickettsia* spp. by PCR in *H. dromedarii* from Egypt [3,5,6] and in other adjacent countries as Tunisia, Algeria, Israel and the United Arab Emirates [82-86]. Subsequently, this work

confirms the role of both tick spp. (*R. sanguineus* and *H. dromedarii*) in transmission of *Rickettsia* spp. in Egypt and the importance of domestic dogs and camels as potential infection amplifiers. This finding agree with Piranda *et al.* [87] and Kamani *et al.* [88] who found Rickettsiae in both dogs and camels.

The brown dog tick *R. sanguineus* is the unique tick species that was found on dogs recording 100% infestation (Tables-2 and 3). The result confirms the fact that *R. sanguineus* is still the main dog tick in Egypt which agrees with previous reports [28,30]. In camels, *H. dromedarii* was the most abundant tick species found on camels recording 91.9%, meanwhile lower percentages of infestation of other tick species



confirming morphological identification. Moreover, the presented phylogenetic analyses in this study were pioneer ones, hence, no earlier report of similar molecular taxonomy and evolutionary analyses based on the five DNA markers in Egypt.

In this study, the utilized five DNA markers; nuclear (18S rDNA and ITS2) and mitochondrial (12S rDNA, CO1 and 16S rDNA), revealed that the documented length of amplified fragments of 18S rDNA, 12S rDNA, CO1 and 16S rDNA were 780, 380, 850, and 455 bp, respectively, are the same in length within both tick species. While ITS2 was different 1200 bp in *R. sanguineus* and 1500 bp in *H. dromedarii* (Figure-6a-g).

In *R. sanguineus*, phylogenetic trees based on the five DNA markers showed that the Egyptian ticks species (KU198407, KU198406, KU198403, KU214592 and KU198404) were close to the reference counterparts (KF958435.1, JQ625707.1, JQ480844.1, KM494916.1 and KR870984.1) which obtained from the surrounding geographical countries as Israel, Iran and Turkey (the temperate strain group) (Figure-7a-e). These results agree with Dantas-Torres *et al.* [42], Moraes-Filho *et al.* [92], Sanches *et al.* [93] who divided the *R. sanguineus* group into tropical and temperate strains. On the other hand, the phylogenetic tree of 18S rDNA gene sequence (KU198407), placed the Egyptian brown dog tick in a separate clad that may return to 18S rRNA identified at genera level (KF958435.1) [51], subsequently, doubted the usefulness and applicability of such gene in molecular taxonomy of ticks (Figure-7a). In *H. dromedarii*, the evolutionary relationship is more clear, sharp, and easier to be interpreted since the phylogenetic trees based on the five DNA markers (KU198408, KU214593, KU963224, KU323789 and KU198405) placed the Egyptian species together with the reference ones (L76348.1, JQ733570.1, U95874.1, KM235697.1 and L34306.1) derived from different geographical countries as Iraq, Israel, India and America (Figure-8a-e).

On the other hand, it is obvious that mitochondrial markers have several advantages over the nuclear genes during this study. The sequence length of both 12S rDNA and 16S rDNA are <450 bp, even the 850 bp length of CO1 can be obtained in one reaction in contrast to the length of 18S rDNA and ITS2 fragments. Moreover, the evolutionary rate and sequence alignment of mitochondrial genes is faster than that calculated for nuclear genes [94,95]. In addition to, the reference database of complete and partial mitochondrial genomes of several tick species are more available than nuclear genes [44,54-57,95-97]. This is return to the fact that mitochondrial genes have strict and simple maternal inheritance compared to nuclear DNA [98].

## Conclusion

The detection of Rickettsiae in ixodid tick species and establishment of the pylogenetic status of

*R. sanguineus* and *H. dromedarii* would be useful in studying the geographical distribution and understanding the epidemiology of ticks and tick-borne Rickettsioses in Egypt which consequently help in the control strategy against the disease. Moreover, this study confirms the suitability of the mitochondrial genes as DNA markers for reliable identification of ticks at both intra and interspecies level over the nuclear ones.

## Authors' Contributions

AE, FAS, NATA, AAG and SA designed and supervised the experiments. HHAMA collected tick samples from dogs and camels. All authors shared in protocols of staining technique, LM, SEM, PCR, sequences, GenBank submission and phylogenetic trees. All authors contributed in draft and revision of the manuscript. All authors read and approved the final manuscript.

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## Competing Interests

The authors declare that they have no competing interests.

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