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Integrated Characterization and Pathogen Detection of Ticks in Egyptian Cattle and Equines: Focus on *Rhipicephalus annulatus* and *Hyalomma excavatum*

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Abstract

IN EGYPT, the common tick species on cattle and equines are *Rhipicephalus annulatus* and *Hyalomma excavatum*, both vectors of tick-borne diseases (TBD). This study aimed to characterize of *R. annulatus* and *H. excavatum* using morphological, molecular, and immunological tools, and to investigate the tick-borne pathogens (TBP) associated with these species. A total of 186 adult ticks were collected from Giza, Sohag, New Valley, and Faiyum, including 28 ticks from 2 horses and 158 ticks from 104 cattle. The ticks were characterized through light microscopy (LM), scanning electron microscopy (SEM), and polymerase chain reaction (PCR) coupled with sequencing, along with molecular screening of associated pathogens. Blood samples were also collected from cattle and three calves as a negative control for immunological characterization. The integrated analyses confirmed *R. annulatus* as the dominant tick on cattle in all regions and on horses in Giza, while the tick *H. excavatum* was dominant on cattle in New Valley. Infection rates for anaplasmosis and borreliosis were 42.5% and 11.8%, respectively. Novel potential *Ehrlichia* sp. and *Borrelia theileri* genotypes were detected in both tick species. Western blotting analysis supported the development of a universal anti-tick vaccine, with detected bands reacting in the same molecular weight range (99–100.4 kDa) in both species. Combining different characterization methods shows promise for accurately diagnosing and controlling ticks and TBD and updating the understanding of native or exotic threats.

Keywords: *Borrelia theileri*, *Ehrlichia*, PCR, SEM, Western blotting**.**

Introduction

Ticks (Order: Ixodida) rank first as vectors of animal pathogens and second as vectors of human pathogens, just behind mosquitoes [1, 2]. Ticks are obligate hematophagous ectoparasites that are extensively distributed globally, infesting different vertebrate hosts, including humans [3-5]. Among the 900 tick species worldwide [4], the most widely distributed tick genera are *Rhipicephalus* and *Hyalomma*, which directly and indirectly affect the economy of cattle, buffalo, sheep, goat, camel, and horse industries in tropical and subtropical countries, including Egypt [6, 7]. Apart from severe anemia, weight loss, and damage to the host skin, they transmit viral, bacterial, and protozoal diseases to humans and animals [1, 8, 9].

Tick-borne diseases (TBD) have been emerging and re-emerging in many geographical areas, contributing a health burden to humans, livestock, companion animals, and wildlife [9]. Several factors have influenced TBD epidemiology and spread, including globalization, increased international trade, urbanization, travel, and animal mobility, consequently increasing human, and animal contact with ticks, posing unprecedented challenges to clinicians and veterinarians [10-12]. Furthermore, climatic changes have expanded the population of ticks, introducing exotic ticks and the spread of emerging TBD [13, 14]. In Egypt, various TBD such as anaplasmosis, borreliosis, bartonellosis, and rickettsiosis have been reported among livestock, especially cattle [7, 8, 15]. Previous studies have shown that the distribution of ticks and TBP in Egypt is influenced by these factors, leading to the arrival of new tick species and the spread of TBDs [8, 16].

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Identification of these ticks is crucial for the prevention and control of TBDs. While morphological identification is feasible for unengorged adult ticks, it becomes highly challenging for immature stages, engorged adults or nymphs, and damaged specimens [17]. Additionally, the declining number of entomologists capable of identifying ticks at different stages and species levels underscores the need for alternative identification techniques. Unfortunately, scanning electron microscopy (SEM) and matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) methods, which require specialized equipment, are not available to most laboratories [18-20]. Consequently, molecular tick identification using DNA markers, which are easily accessible in most laboratories, has become increasingly important for identifying all stages of ticks, particularly new invasive species [7, 21-23].

Western blotting (WB) is a reliable technique for antigen fractionation from a mixed protein extract. Antigens, being foreign elements, can trigger an immune response, making the resultant immunoblotting data highly sensitive and specific. This technique provides precise data from samples with evidence of exposure to an infectious agent. Moreover, WB offers a simpler procedure, faster identification, and clearer, more precise analysis of the results [24]. Characterizing proteins from different tick species and/or genera can preliminarily confirm successful protein expression and simultaneous immune response induction. Furthermore, the protein yield can reveal the tick–host relationship. Therefore, WB plays a fundamental role in accurately characterizing, diagnosing, and controlling tick and TBP, which is essential for epidemiological surveillance and control [25-28].

The overarching goals of this study were to 1) Conduct a detailed characterization of cattle ticks, *Rhipicephalus annulatus*, and *Hyalomma excavatum*, in Egypt using morphological, molecular, and immunological tools. This characterization is necessary to identify potential regional variations and novel traits that may influence disease transmission, which have not been thoroughly investigated in the Egyptian context; and 2) Investigate TBPs associated with these two tick species through molecular analyses to fill the existing knowledge gaps by providing detailed epidemiological mapping of ticks and TBDs in Egypt, thereby aiding in the development of effective control strategies.

Material and Methods

Study area and sample collection

From February 2019 to December 2019, a cross-sectional study employing a convenience sampling strategy was conducted to collect Ixodid ticks. A total of 186 adult ticks were gathered, comprising 28 ticks from 2 horses and 158 ticks from 104 cattle. These ticks were collected in four provinces of Egypt: Giza, Sohag, New Valley (including Al-Kharga and Al-Dakhla), and Faiyum (Fig. 1). First, ticks were detached from animals using strong forceps in plastic tubes covered by a piece of cloth and secured by a rubber band. The ticks were brought alive to the laboratory for initial identification using a stereomicroscope. The collected ticks were then preserved in 70% ethanol for further characterization via light microscope (LM), SEM, and polymerase chain reaction (PCR) coupled with sequencing, besides the molecular screening of the associated pathogens. Moreover, 2 mL of blood was collected from household cattle from which ticks were collected, in addition, three newly borne calves that hadn't been previously exposed to ticks were sampled as negative controls and subsequently added to the plain Vacutainer tubes for serum separation and immunological studies.

Morphological characterization of the examined ticks

The collected ticks were subjected to morphological identification using the LM and SEM. The tick specimens were then mounted on glass slides by Canada balsam and photographed using a digital camera fixed on a Zeiss stereomicroscope according to the taxonomic key of Estrada-Peña, Bouattour [29] for identification. Some tick specimens were then subjected to further identification through the SEM. Thereafter, each tick was cleaned in water-glycerol-potassium chloride solution (composed of 96.6% (by weight) glycerol combined with 0.05% (by weight) of potassium chloride (KCL) and 3.35% (by weight) of distilled water), rinsed in tap water, and subsequently taken through a graded series of alcohol. The ticks' dorsal and ventral surfaces were then stuck into the SEM stub and dried using a dryer (Blazer Union, F1-9496 Blazer/Fürstentun Liechtenstein). Finally, the prepared tick specimens were fixed on the SEM stubs, coated with gold through an S15OA Sputter Coater, and subsequently examined by the SEM [30].

Molecular investigation

First, individual ticks were washed twice in distilled water, dried using sterile filter paper, and cut longitudinally into two halves. One-half was used as backup and stored at −20°C; another half was cut with a sterile scalpel into small pieces [31]. Subsequently, in a sterile 1.5-ml microcentrifuge tube, which contained 400 µl of lysis buffer and 10 µl of proteinase K (40 mg/µl; Simply), the cut half was put and incubated overnight at 65°C. After centrifugation at 4000 rpm for 5 min, the tick DNA

was extracted from 200 µl of the supernatant using a Tissue Genomic DNA Isolation Kit (Tissue; GeneDireX, Taiwan) following the manufacturer's protocols.

Molecular tick identification

The molecular characterization was performed to confirm the morphological identification of selected individual ticks using a mitochondrial marker cytochrome oxidase subunit 1 (CO1), designed by Chitimia, Lin [32] with a predicted product size of 750–850 bp (Table 1).

Molecular tick screening for TB pathogens

Tick DNA was screened for the presence of Anaplasmataceae [Anaplasma and Ehrlicha; 33], piroplasms [Theileria and Babesia; 34], *Borrelia* [35], *Bartonella* [36], and *Rickettsia* [37], using specific oligonucleotides for amplification (Table 1). Standard PCR reactions were performed in a Thermal Cycler (BIO-RAD, Singapore) using a 2× PCR master mix solution (OnePCR, GeneDireX, Taiwan) following the manufacturer's instructions. Notably, the negative (distilled water) and positive controls (MN625935 as *Anaplasma marginale*, MN625888 as *Theileria annulata*, MN621893 as *Borrelia theileri*, MW596416 as *Bartonella* sp., MN629893 as *Rickettsia africae*) were applied in each reaction. Lastly, the PCR products were visualized using a UV transilluminator on a 1.5% agarose gel electrophoresis stained with Red Safe (Intron). A 100-bp DNA Ladder (H3 RTU, GeneDireX, Taiwan) was used to estimate the PCR product size.

Sequence and phylogenetic analyses

All positive PCR products of ticks and associated TB pathogens were purified using the PCR Clean-Up & Gel Extraction Kit (GeneDireX, Taiwan), following the manufacturer's instructions. Subsequently, the bidirectional sequencing was applied to the purified product at "Macrogene Lab Technology, Korea" using the primers in Table 1. ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) was then used to assemble and correct the obtained sequences. The corrected good-quality sequences were analyzed for genotype diversity with nucleotide BLAST (Basic Local Alignment Search Tool) by NCBI and MEGA software version X and then submitted to GenBank. Furthermore, the phylogenetic analysis was conducted through the multiple sequence alignment of the corrected and reference sequences in GenBank using the CLUSTAL W in MEGA software version X [38]. Finally, the maximum-likelihood method based on the Tamura-Nei model with 1000 bootstrap replicates was applied for the tree construction [38, 39].

Immunological characterization

Antigen preparation

Ten semi-engorged preserved identified adult ticks for each species (*R. annulatus* and *H. excavatum*) were separately dissected. The cuticles of the ticks were then removed and discarded, and the internal organs were separately isolated in phosphate-buffered saline (PBS), pH 7.2, for the preparation of crude antigens of *R. annulatus* (CRA) and *H. excavatum* (CHE). Briefly, each antigen was homogenized in PBS, pH 7.2, sonicated, and centrifuged at 14,000 rpm for 30 min in a cooling centrifuge, according to Nikpay, Nabian [25]. Subsequently, the total protein content was estimated for both antigen preparations following Lowry, Rosebrough [40].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

First, 50 µg from crude antigens, CRA and CHE, were mixed with the sample buffer and fractionated as previously described using 10% SDS-PAGE under reducing conditions with a prestained molecular weight protein marker (Genedirex, USA) following Laemmli [41]. After the SDS-PAGE electrophoresis, one gel was stained with Coomassie Brilliant Blue R-250 dye, and three gels were transferred into 0.45 nitrocellulose membranes following Towbin, Staehelin [42]. After blocking, the membranes were then probed overnight against the negative and positive naturally infected *R. annulatus* and *H. excavatum* sera at a concentration of 1:50 in Tris-buffered saline (TBS), which contained 0.5% bovine serum albumin (BSA). The nitrocellulose membranes were incubated with protein A peroxidase conjugate, to probe and detect IgG antibodies bound to the blotted antigens on the membranes, at 1:2500 in 0.5% BSA/TBS buffer for 1 h. Furthermore, the substrate solution (1-chloronaphthol [Sigma-Aldrich, USA], one tablet [30 mg/1 ml methanol] added to 10 ml of methanol, 39 ml of TBS, and 30 µl of 30% H_2O_2) was used during the 10-min incubation of the blots to develop the immune-reactive bands. Finally, the gel and blotted membranes were visualized, and the detected bands were analyzed using a gel documentation system (Bio-Rad, Hercules, USA).

Statistical analyses

Significant differences were considered at values of $P < 0.05$ regarding the infection rate with *Anaplasma* and *Borrelia* of the investigated ticks calculated using the chi-square test (χ^2) through the Statistical Package for Social Sciences software (v.20.0).

Results

Morphological characterization the examined ticks

The ticks were morphologically identified into two main ixodid tick species (*Rhipicephalus* sp. and *Hyalomma* sp.). First, all *Rhipicephalus* spp. were adult females collected from cattle in Sohag, New Valley (including Al-Kharga and Al-Dakhla), and Faiyum and from horses located in Giza. Based on the LM and SEM, the inner margin of the first palp article in all adult females was long, slightly concave, and without a protuberance bearing a seta on their inner margins (Fig. 2), identifying *Rhipicephalus* spp. as *R. annulatus*.

Conversely, concerning the *Hyalomma* sp., they were all collected from the cattle in New Valley (including Al-Kharga and Al-Dakhla). The *Hyalomma* males were identified as *H. excavatum*, showing the dorsal and ventral views of male characteristics with taxonomic importance. In addition, the *H. excavatum* male had a distinct depressed area in the posterior scutum and a characteristic form of the festoons in which the paracentral festoons are joined anteriorly, forming an arch. The posterior margins of the adanal shields were also truncated (Fig. 3). However, the adult female *H. excavatum* had steep sides and curved margins (Fig. 4). Moreover, the mouthparts, fovea, coxae, and genital opening of the *H. excavatum* female were similar to those in *Hyalomma anatolicum*. Therefore, it is necessary to describe the males to confirm their identity and to conduct additional investigations such as molecular characterization.

Molecular characterization of the examined ticks

Based on the CO1 gene, the standard PCR and sequencing successfully identified the *Rhipicephalus* sp. and *Hyalomma* sp. as *R. annulatus* and *H. excavatum*, respectively. BLAST analysis of *R. annulatus* sequences identified four different novel genotypes of *R. annulatus*. These potential novel genotypes (GenBank: MZ505527-MZ505530) were collected from cattle in Sohag, Faiyum, and Al-Kharga; however, the last one was from horses in Giza, with 99% (802/807) similarity to the *R. annulatus* from cattle in the United States (GenBank: KX228534). In addition, three different potential novel genotypes of *H. excavatum* were identified from four individual ticks (GenBank: MZ505537-MZ505539) that were collected from cattle in Al-Dakhla and Al-Kharga, with 99% (715/721) similarity to the *H. excavatum* from a camel in Tunisia (GenBank: MT108550). Figure 5 presents the phylogenetic tree of these novel tick genotypes.

Tick-borne pathogens

Tick DNA was screened for TB pathogen DNA (Anaplasmataceae, Piroplasm sp., *Borrelia* sp., *Bartonella* sp., and *Rickettsia* sp.). Standard PCR coupled with sequencing showed that the DNA of *Anaplasma* and *Borrelia* sp. were in *R. annulatus*, and *H. excavatum* and the two tick species were free from piroplasm species., *Bartonella* sp., and *Rickettsia* sp. DNA. Notably, there were significant differences ($P < 0.01$) between the infection proportions with *Borrelia* and Anaplasmataceae in ticks collected from the horses and cattle from Giza and Al-Dakhla, New Valley (Table 2).

Anaplasmataceae DNA was detected in 79 of the 186 ticks (42.5%) collected from all localities, with infection rates ranging between 9.7% and 91.7%, including 50% in *R. annulatus* (51/102) and 51.2% in *H. excavatum* (43/84) (Table 2). Regarding BLAST analysis, we identified two genotypes of a potential novel *Ehrlichia* sp., one in *R. annulatus* (GenBank: ON738716) with 99% (465/466) similarity to those of the uncultured *Ehrlichia* sp. detected in *R. annulatus* in Egypt (GenBank: OL721671) and the other in *Rhipicephalus microplus* in China (GenBank: OL838198), the bacterium in *H. excavatum* (GenBank: ON738717) had 100% (475/475) identity with the same references. Furthermore, these genotypes clustered in a well-supported branch (bootstrap value 88) with other *Ehrlichia* sp. references and *E. chaffeensis* in the phylogenetic tree (Fig. 6).

Borrelia DNA was detected in 22 of the 186 ticks (11.8%), including 7.1% in *R. annulatus* (2/28) from Giza, 50% in *R. annulatus* (6/12) from Sohag, and 19.7% in *H. excavatum* (14/71) from Al-Dakhla Oasis, New Valley (Table 2). However, the ticks in the other regions were free from *Borrelia* DNA. The 16S rRNA gene successfully sequenced *Borrelia* in *R. annulatus* but failed in *H. excavatum*. Furthermore, from the BLAST research, we identified a novel potential *B. theileri* (GenBank: MZ491201) with 96% (458/475) similarity to those of *B. theileri* found in cattle blood from Egypt (GenBank: MW562684) and in *Rhipicephalus geigyi* in Mali (GenBank: KF569941). Finally, the phylogenetic analysis showed that our genotype clustered in the same clade with other *B. theileri* genotypes (bootstrap value 83; Fig. 7).

Immunological characterization

The electrophoretic and Western blotting patterns of the CRA and CHE antigens were resolved. The SDS-PAGE resultant data confirmed the slight variations in the protein bands of both antigens. The CRA antigen showed five protein bands at molecular weights of 270, 107, 100.7, 41.1, and 22.6 kDa. However, the CHE antigen exhibited eight protein bands: four with high molecular weights at > 270 kDa and the other four with molecular weights of 107, 94.9, 41.4, and 21.1 kDa (Fig. 8-a). Conversely, the Western blotting profiles showed two immunogenic bands at

molecular weights of 100 and 100.4 kDa generated while blotting the CRA and CHE antigens against the positive naturally infested *R. annulatus* bovine serum, respectively (Fig. 8-b). In addition, two immunogenic bands were found at 99 and 99.9 kDa molecular weights with the same antigens when blotted against the positive naturally infected *H. excavatum* bovine serum, respectively (Fig. 8-c). However, no detected band developed when using the negative bovine serum against the CRA and CHE antigens (Fig. 8-d).

Discussion

The first aim of this study was to comprehensively characterize *R. annulatus* and *H. excavatum* using LM, SEM, and molecular analyses. This characterization focused on differentiating these species from their closely related ticks, *R. microplus* and *H. anatolicum*. Additionally, immunological studies were conducted to identify antigenic sites in specific ticks, which could inform the development of a universal vaccine against these ticks. The second aim was to detect TBDs associated with these tick species, in order to understand the epidemiology of TBDs and develop effective control strategies for both ticks and TBDs.

The morphological identification of ticks is fundamental for the epidemiological survey of TBD. This step identified the tick species in four Egyptian provinces: Giza, Sohag, New Valley (including Al-Kharga and Al-Dakhla), and Faiyum. The cattle tick *R. annulatus* was the most dominant tick since it was found on horses and cattle in all regions, while the tick *H. excavatum* was found on the cattle at New Valley (including Al-Dakhla and Al-Kharga Oasis) but is more widely spread in Al-Dakhla than in Al-Kharga. The cattle tick *R. annulatus* is a unique species from the genus *Rhipicephalus* (formerly *Boophilus*), which spreads in Egypt majorly on cattle and may be found on equines and buffaloes [7, 43, 44]. In addition, the genus *Rhipicephalus* (formerly *Boophilus*) includes four species: *R. annulatus* (Koch 1844), *R. microplus* (Canestrini 1888), *R. decoloratus* (Koch 1844), and *R. geigyi* (Aeschliman and Morel 1965). The first palp article is the main characterized part, which is used to differentiate between these four species according to the description of Walker [45]. They reported that *R. annulatus* was identical to *R. microplus*; however, both species are without a protuberant bearing a seta on the inner margin of the first palp articles. Therefore, this differentiates them from *Rhipicephalus decoloratus* and *R. geigyi*. He added that the inner margin of the first palp article of *R. annulatus* was long and slightly concave; however, this margin in *R. microplus* was short and deeply concave. Briefly, *R. annulatus* remains a unique boophilid tick species, with no other boophilid imported and/or adapted in Egypt. Moreover, *H. excavatum* was closely related to *H. anatolicum*, particularly the females. However, the distributions of these two species overlap in some areas. Therefore, it is crucial to examine both females and males of *H. excavatum* to accurately differentiate them in detail by the SEM. Furthermore, when we collected the tick specimens from the cattle in New Valley, we expected them to be *H. excavatum* since they had large size, were robust, and were dark-colored. The mouthparts, fovea, coxae, and genital opening of *H. excavatum* females were similar to those in *H. anatolicum* based on the description of Walker [45]. Therefore, it is necessary to describe the male to confirm the identification. The posterior margins of the adanal shields were truncated. These male characteristics aligned with the description of Walker [45].

In Egypt, *Rhipicephalus* and *Hyalomma* ixodids are the most prevalent ticks infesting cattle and horses [7, 44]. In this study, the molecular and phylogenetic analysis of the CO1 gene confirmed that cattle were infested by *R. annulatus* (four potential novel genotypes) and *H. excavatum* (three potential novel genotypes); however, *R. annulatus* infested the horses. Consistent with our findings, previous studies reported that the major ixodid ticks infesting cattle in Egypt were *R. annulatus* [6, 43, 44] and *H. excavatum* [6], in addition to *R. annulatus* in horses [7]. These results showed that *R. annulatus* remains a unique boophilid tick species in Egypt and no invasive boophilid tick species have emerged in Egypt due to climatic changes [16]. Furthermore, our findings agreed with the expediency of CO1 mitochondrial DNA markers for molecularly identifying ticks and overcoming the morphological identification's limitations [6, 7, 21, 32].

Furthermore, regarding TB pathogen detection, we identified *Anaplasma* and *Borrelia* DNA in both *R. annulatus* and *H. excavatum*. The infection rates of anaplasmosis were 50% and 51.2% in *R. annulatus* and *H. excavatum*, respectively. In addition, the phylogenetic analysis revealed a novel potential *Ehrlichia* sp. in both tick species and was reported in all investigated localities. This potential new *Ehrlichia* sp. was clustered in a well-supported branch (bootstrap value 46) with other *Ehrlichia* sp. references and *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, a serious disease affecting humans in the USA [46, 47]. This finding highlights the need for physicians to consider this disease in their diagnoses and treatment plans. In Egypt, *Ehrlichia* sp. was recently detected in *R. annulatus* and *H. excavatum*, which were collected from cattle [6], and in *R. annulatus* from horses and donkeys [7]. Later, a new *Ehrlichia* sp. was found in different tick species such as *Amblyomma variegatum*, *Hyalomma truncatum*, and *R. microplus* [47-50]. Moreover, the infection rate of borreliosis in both

tick species was 11.8% and was identified as the new *B. theileri* genotypes. Consistent with this finding, some studies in Egypt reported *B. theileri* in *R. annulatus* [7, 51], in addition to cattle and sheep [8, 15]. Globally, *B. theileri* has been reported in *R. annulatus* [52]. However, *B. theileri* was found in other tick species rather than *R. annulatus*, such as *R. geigyi*, *R. microplus*, *Rhipicephalus turanicus*, *A. variegatum*, *H. truncatum*, and *H. marginatum* [48, 53-55]. Finally, none of the tick species were positive for Piroplasm, *Bartonella* sp., and *Rickettsia* sp. DNA. Recent studies in Egypt reported that the DNA of *R. annulatus* was negative for Piroplasm sp., *Bartonella* sp., and *Rickettsia* sp., supporting these findings [8, 15, 56]. Consequently, *R. annulatus* and *H. excavatum* may serve as vectors for Anaplasmataceae and borreliosis in Egypt, and therefore, further studies are needed to examine the transmission risk of other emerging TB pathogens through these two tick species in Egypt.

Therefore, it is crucial to define the immunogenic fractions in the characterization of different tick species and/or genera to highlight the events in acquiring and expressing tick–host–pathogen interaction. Western blotting provides possible avenues to show basic information about the antigens extracted from *R. annulatus* and *H. excavatum* ticks. The pattern of immune-reactive bands that were recognized by both positive naturally infested bovine sera collected from infested cattle with these species could be a useful prospect in subsequent antigen characterization studies and to understand antigenic sites in specific tick tissues, which could be the common vaccine against these two genera and/or improve the diagnostic assays useful for detecting TB pathogens. In this study, the reactivity pattern supports the hypothesis for developing universal anti-tick vaccines where the detected bands reacted at the range of molecular weights (99–100.4 kDa). However, minor differences may occur among molecular weights of the detected bands due to protein denaturing, cleavage, and high temperature, which are vital for breaking the disulfide bonds of the polypeptide chains. Subsequently, these factors may lead to the slow migration of some fractionated polypeptides. Furthermore, in this study, our resultant data were consistent with those of many investigators who have studied the different fractionated tick tissue extracts in either characterization or diagnosis and control of different tick genera or species [25-28].

Conclusion

The LM, SEM, and molecular characterization revealed that the cattle tick *R. annulatus* is the most prevalent tick on cattle in all investigated locations of Egypt. Furthermore, the tick *H. excavatum* is the dominant tick species in cattle in New Valley province, especially in the Al-Dakhla region. Novel potential *Ehrlichia* sp. and *B. theileri* genotypes were detected in *R. annulatus* and *H. excavatum*. Immunoblotting, a qualitative and analytical technique, identified immunogenic bands resulting from tick–host immune interaction. These reaction bands require further characterization and identification. Therefore, integrating various characterization methods in the clinical field is necessary to identify potential regional variations and novel traits that may influence disease transmission. This approach promises prospects for accurately diagnosing and controlling ticks and TBDs. However, these findings necessitate further investigation in large areas with diverse animal populations and different arthropod species.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was approved by the Medical and Veterinary Research Ethics Committee at the National Research Centre, Egypt, under the number 19147. The animal's owners provided their verbal approval for the samples to be collected, and the authors attest that the owners were informed and aware of the study's purpose and benefits. In addition, all the methods were performed following relevant guidelines and regulations.

Author contribution

Hend H.A.M. Abdullah and Sobhy Abdel-Shafy shared in the design of this study. Sobhy Abdel-Shafy and Mohamed A. Mahmoud collected ticks and blood from animals. Sobhy Abdel-Shafy and Heba M.Z. Ashry identified ticks morphologically by LM and SEM. Hend H.A.M. Abdullah, Seham H.M. Hendawy, and Sobhy Abdel-Shafy extracted DNA from ticks and conducted PCR protocols. Sobhy Abdel-Shafy and Seham H.M. Hendawy extracted tick proteins and conducted the immunological investigation. Hend H.A.M. Abdullah purified PCR products, corrected DNA sequences, and constructed phylogenetic trees. Hend H.A.M. Abdullah, Seham H.M. Hendawy, and Sobhy Abdel-Shafy analyzed the data and drafted the manuscript. All authors revised and approved the final version of the manuscript.

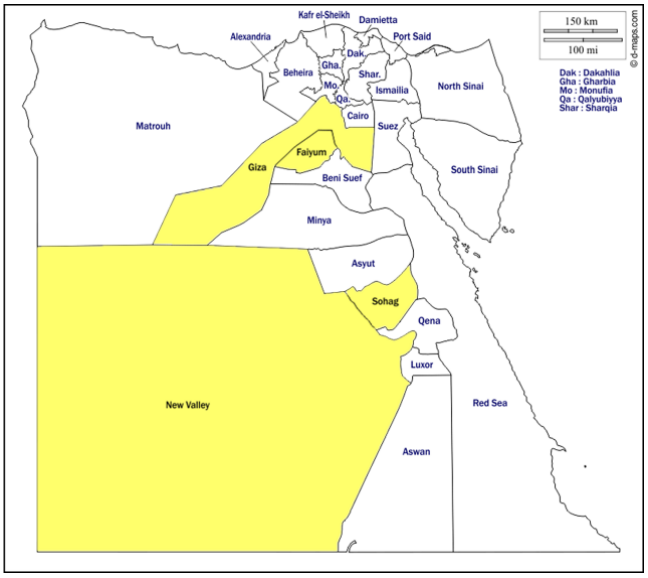

Fig. 1. Map of Egyptian provinces, highlighting in yellow the four provinces where blood samples were collected.

TABLE 1. Oligonucleotides were used for standard PCR and sequencing.

Organisms	Targeted gene	Primers F & R (5'-3')	Tm (°C)	References
Ticks identification	CO1 (750-850 bp)	CO1F-GGAACAATATATTTAATTTTTGG CO1R-ATCTATCCCTACTGTAAATATATG	46	**[32]**
Anaplasma sp.	16S rRNA (500 bp)	ECB-CGTATTACCGCGGCTGCTGGCA ECC-AGAACGAACGCTGGCGGCAAGC	65	**[33]**
Piroplasmids	18S rRNA (969 bp)	piro18S-F1-GCGAATGGCTCATTAIAACA piro18S-F4-CACATCTAAGGAAGGCAGCA	58	**[34]**
Borrelia sp.	16S rRNA (1200 bp)	16S-F-GCTGGCAGTGCGTCTTAAGC 16S-R-GCTTCGGGTATCCTCAACTC	57	**[35]**
Bartonella sp.	Barto ITS (750 bp)	Urbarto1-CTTCGTTTCTCTTTCTTCA Urbarto2-CTTCTCTTCACAATTTCAAT	50	**[36]**
Rickettsia sp.	*gltA* (1200 bp)	CS2D-ATGACCAATGAAAATAATAAT CSEnd-CTTATACTCTCTATGTACA	50	**[37]**

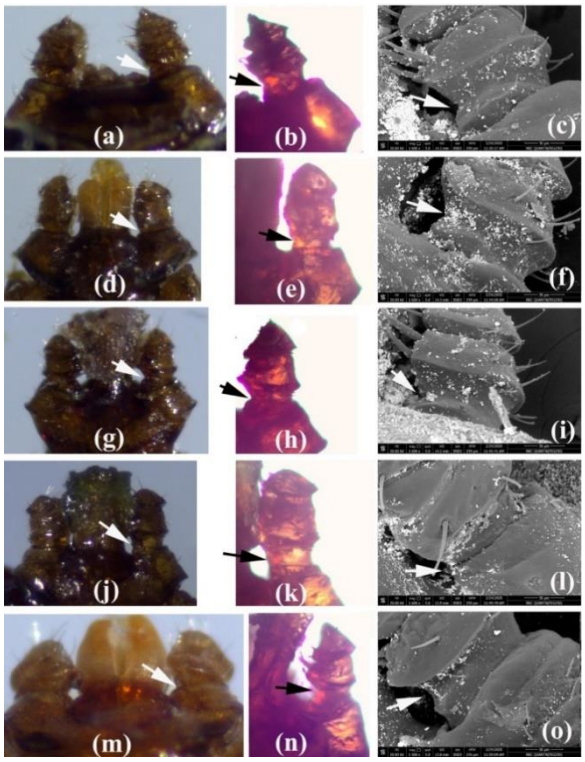

Fig. 2. The cattle tick *Rhipicephalus annulatus*: the left column shows the mouthparts photographed using a stereomicroscope (50×), the middle column shows the palpi photographed using a light microscope (100×), and the right column shows the first segment of palpi photographed using a scanning electron microscope (1500–1600×), a–c: *R. annulatus* collected from cattle in Al-Kharga (New Valley), d–f: *R. annulatus* collected from horses in Giza, g–i: *R. annulatus* collected from cattle in El-Dakhla (New Valley), j–l: *R. annulatus* collected from cattle in Sohag, m–o: *R. annulatus* collected from cattle in Faiyum.

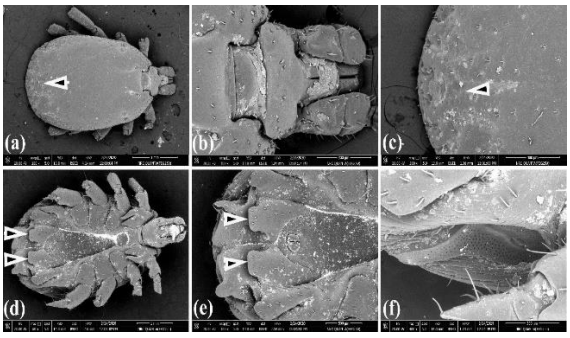

Fig. 3. The adult male of the tick *Hyalomma excavatum*: a) dorsal view of the whole body, b) dorsal view of the mouthparts, c) caudal area, d) ventral view of the whole body, e) anal and subanal shields, f) spiracular plate.

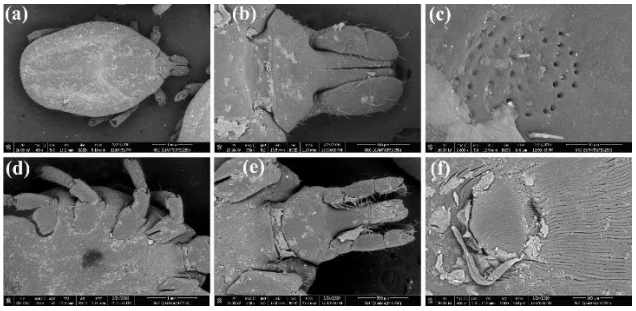

Fig. 4. The adult female of the tick *Hyalomma excavatum*: a) dorsal view of the entire body, b) dorsal view of the mouthparts, c) fovea, d) ventral view including the coxae, e) ventral view of mouthparts, f) genital opening.

Fig. 5. The CO1-based phylogenetic analysis of tick genotypes identified in this study. The phylogenetic tree highlights the positions of *R. annulatus* and *H. excavatum* in this study (Bold). A total of 721 positions were in the final dataset. The scale bar represents a 1% nucleotide sequence divergence.

Table 2. Infection rate of pathogens (*Anaplasma* spp. and *Borrelia* spp.) according to location and tick species.

Locality (Geographic coordination)	Tick species (No.)	Infested animals (No.)	*Anaplasma* Infection rate (%)	*Borrelia* Infection rate (%)	χ^2	*P value*
Giza 29˚ 58’ 27.00" N, 31˚ 08’ 2.21" E	*R. annulatus* (28)	Horse (2)	16 (57.1)	2 (7.1)	10.89	0.001
Sohag 26° 32' 59.9964" N, 31° 42' 0.0036" E	*R. annulatus* (12)	Cow (8)	11 (91.7)	6 (50.0)	1.471	0.225
El-Dakhla Oasis 25° 30' 59.99" N, 29° 09' 60.00" E	*H. excavatum* (71) *R. annulatus* (9)	Cow (54)	41 (57.7) 6 (66.7)	14 (19.7) 0	13.255	<0.001
El-Kharga Oasis 25° 26' 10.79" N, 30° 33' 17.99" E	*H. excavatum* (13) *R. annulatus* (22)	Cow (23)	2 (15.4) 15 (68.2)	0 0		
Faiyum 29° 18' 35.82" N, 30° 50' 30.48" E	*R. annulatus* (31)	Cow (19)	3 (9.7)	0		
Total	186	106	79 (42.5)	22 (11.8)		

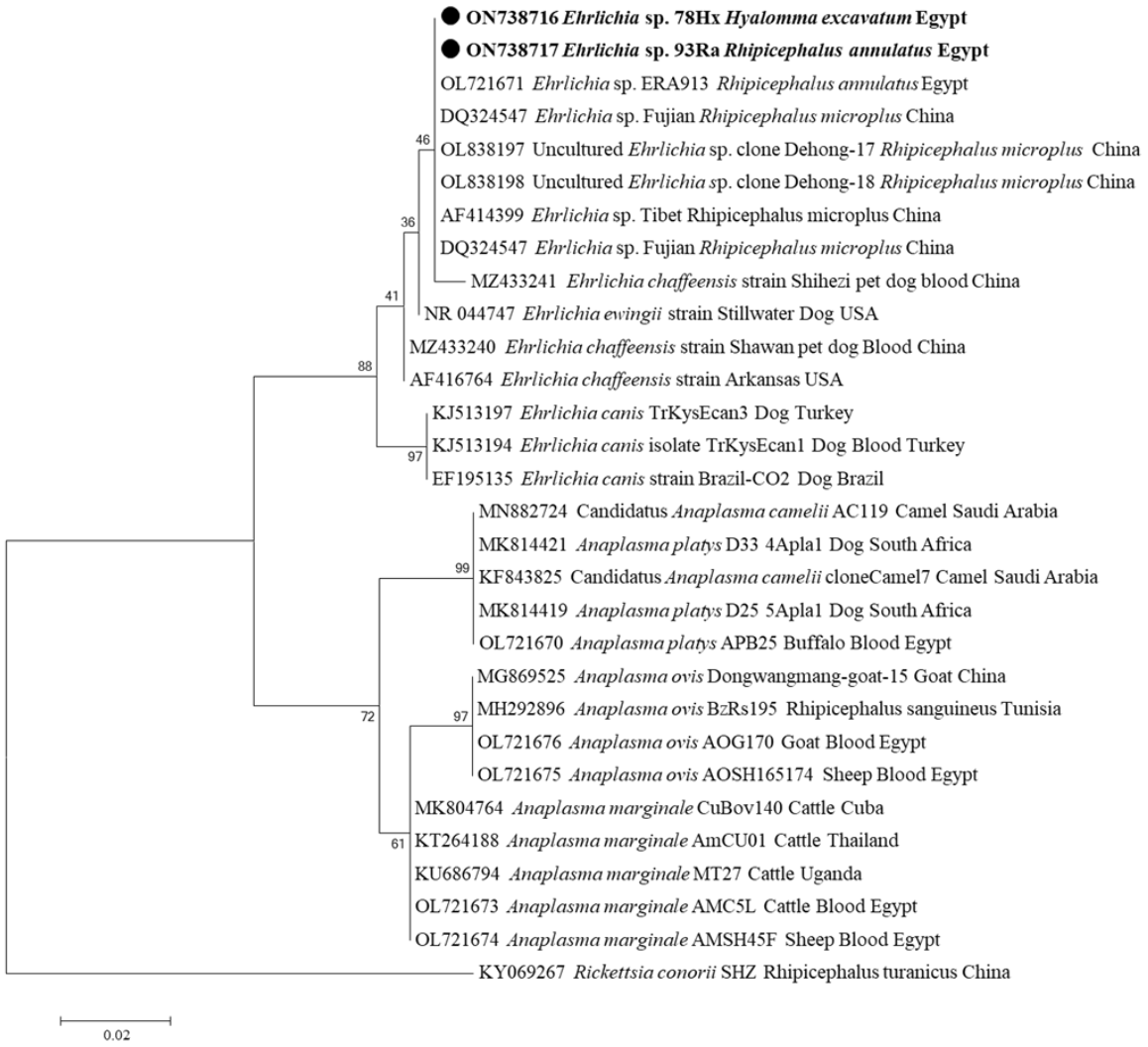

Fig. 6. The 16S rRNA gene-based phylogenetic analysis of genotypes identified in this study. The phylogenetic tree highlights the position of *Ehrlichia* sp. in this study (Bold) related to other *Ehrlichia* and *Anaplasma* spp. A total of 401 positions were in the final dataset. The scale bar represents a 2% nucleotide sequence divergence.

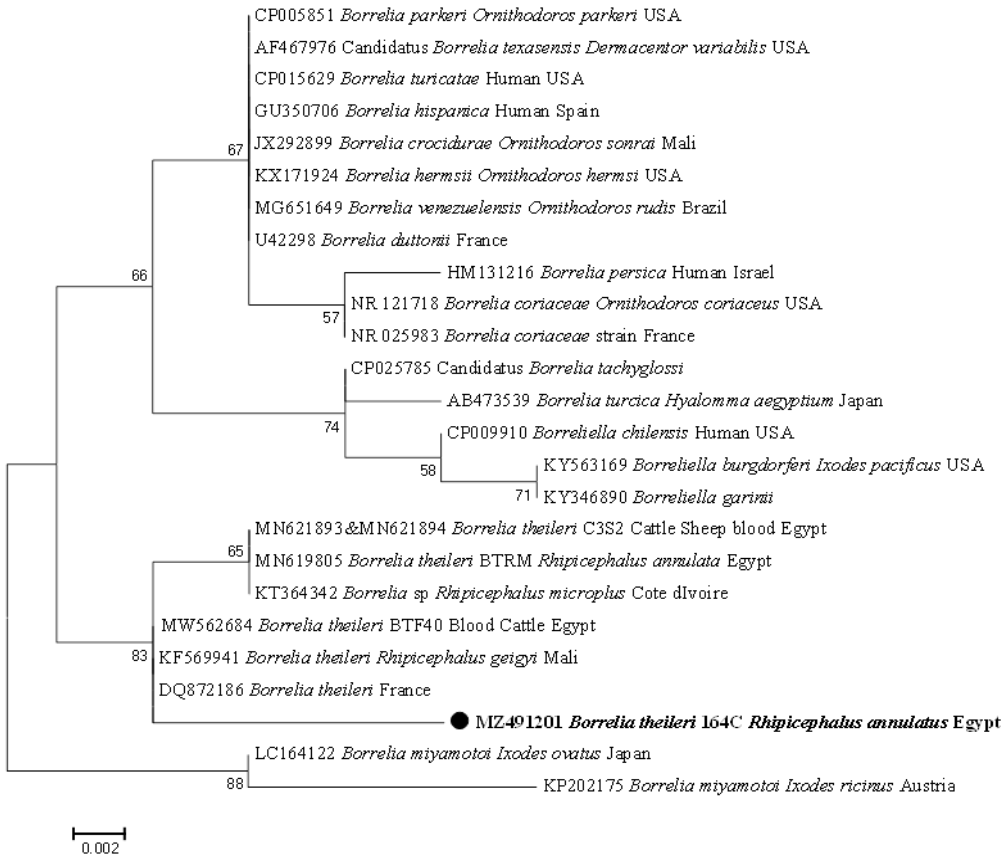

Fig. 7. The 16S rRNA gene-based phylogenetic analysis of genotypes identified in this study. The phylogenetic tree highlights the position of *Borrelia* sp. in this study (Bold) associated with other *Borrelia* spp. In addition, 700 positions were in the final dataset. The scale bar represents a 2% nucleotide sequence divergence.

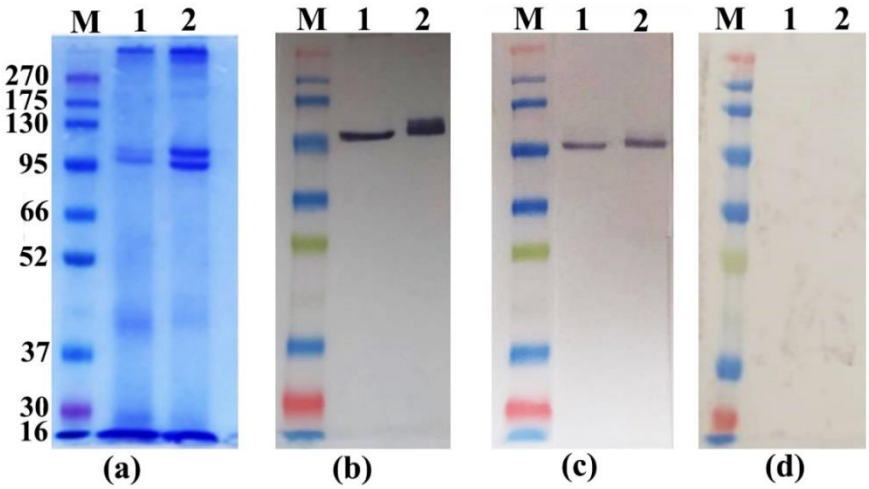

Fig. 8. a) SDS-PAGE protein profile of *R. annulatus* antigen (Lane 1), *H. excavatum* antigen (Lane 2), and BLUltra prestained protein marker, Genedirex (Lane M, 16–270 kDa), b) Immunogenic reactivity pattern of *R. annulatus* antigen (Lane 1), *H. excavatum* antigen (Lane 2) generated against the positive naturally infected *R. annulatus* serum, and BLUltra prestained protein marker, Genedirex (Lane M, 16–270 kDa), c) Immunogenic reactivity pattern of *R. annulatus* antigen (Lane 1), *H. excavatum* antigen (Lane 2) generated against the positive naturally infected *H. excavatum* bovine serum, and BLUltra prestained protein marker, Genedirex (Lane M, 16–270 kDa), d) Immunogenic reactivity profile of *R. annulatus* antigen (Lane 1), *H. excavatum* antigen (Lane 2) against the negative bovine serum, and BLUltra prestained protein marker, Genedirex (Lane M, 16–270 kDa.

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التوصيف المتكامل والكشف عن مسببات الأمراض فى القراد في الماشية والخيول المصرية: التركيز على قراد *الريبيسفلاس أنيولاتس* و*هيالموما إكسكافاتم*

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[2] وحدة أبحاث القراد والأمراض المنقولة به، معهد البحوث البيطرية، المركز القومى للبحوث، الدقى، الجيزة، جمهورية مصر العربية.

الملخص

يعد أكثر الأنواع الشائعة من القراد على الماشية والخيول ،فى مصر، هي قراد *الريبيسفلاس أنيولاتس* (*Rhipicephalus annulatus*) وقراد *هيالموما إكسكافاتم* (*Hyalomma excavatum*)، وكلاهما يعد ناقلا للأمراض المنقولة بالقراد(TBD). هدفت هذه الدراسة إلى توصيف كل من قراد الريبيسفلاس أنيولاتس وقراد هيالموما إكسكافاتم باستخدام التوصيفات المورفولوجية والجزيئية والمناعية والتحقق من مسببات الأمراض المنقولة بالقراد (TBP) المرتبطة بهذه الأنواع. تم تجميع عدد ١٨٦ قرادة بالغة من الجيزة وسوهاج والوادي الجديد والفيوم، بما في ذلك عدد ٢٨ قرادة من حصانين و١٥٨ قرادة من ١٠٤ من الماشية. وقد تم توصيف القراد من خلال الفحص المجهري الضوئي(LM) ، والفحص المجهري الإلكتروني الماسح(SEM) ، وتفاعل البلمرة المتسلسل (PCR) بالإضافة إلى التتابع النيوكليتيدى المتسلسل، وكذلك الفحص الجزيئي لمسببات الأمراض المرتبطة بها. كما تم جمع عينات دم من الماشية وثلاثة عجول كدلائل تحكم سلبي للتوصيف المناعي. أكدت نتائج التوصيف المتكامل أن قراد *الريبيسفلاس أنيولاتس* هو القراد الشائع المتواجد على الماشية في جميع المناطق وعلى الخيول في الجيزة، بينما كان قراد *هيالموما إكسكافاتم* هو الشائع انتشاره على الماشية في الوادي الجديد. وبلغت معدلات الإصابة بمرضى الأنابلازما والبوريليا ٤٢.٥ % و١١.٨ % على التوالي. تم الكشف عن تواجد عترات جينية جديدة محتملة للإرليخيا والبوريليا ثيليري في كلا النوعين من القراد. وقد دعم التوصيف باستخدام تقنية الطبع المناعى الفرصة نحو إيجاد لقاح شامل مضاد للقراد، حيث تفاعلت الحزم البروتينية في نفس نطاق الوزن الجزيئي (٩٩-١٠٠.٤ كيلو دالتون) في كلا النوعين. وكذلك الجمع بين طرق التوصيف المختلفة يعتبر نظاما واعدا في التشخيص الدقيق للقراد ومكافحته ومكافحة القراد والمسببات المرضية المنقولة عن طريقه، وكذلك تحديث الفهم العميق لتواجد المسببات المرضية الخطرة المحلية أو الدخيلة .

الكلمات الدالة: بوريليا ثيليري، الإرليخيا، تفاعل البلمرة المتسلسل، الفحص المجهري الإلكتروني الماسح، الطبع المناعى.