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Molecular Identification and Characterization of Exotic Tick Species in Egypt: Livestock Ticks from Neighboring African Countries

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ABSTRACT

Out of the 44 hard tick species identified in Egypt, a minimum of ten species are classified as exotic. The current study was conducted to molecularly characterize the ticks that infest imported livestock animals. Ticks were obtained from livestock and camels in Cairo and Giza governorates that had been imported from other African countries. The study revealed the occurence of *Amblyomma gemma, Am. lepidum, Am. hebraeum, Am. variegatum, Hyalomma impressum*, and *Rhipicephalus decoloratus*. The collected ticks showed a significant similarity of *Am. gemma* to isolates from Kenya by 99.5%, *Am. hebraeum* to South African isolates by 98%, *Am. lepidum* to isolates from Kenya by 99%, *Am. variegatum* to Ethiopian isolates by 99.7%, *Hy. impressum* to isolates from Benin by 98.8%, and *Rh. decoloratus* to isolates from South Africa by 99%. Phylogenetically, the identified ticks have consistently clustered with their highest similarities showing high bootstrap values based on the sequences of 28S rRNA.

Keywords: Egypt, exotic, identification, livestock, molecular, ticks.

INTRODUCTION

Ticks (Acai: Ixodida) are familiar to the globe by their great medical and veterinary importance. Their damage comes directly through the hematophagy behavior on either human or animal blood, causing blood loss, anemia, inflammation, hypersensitivity, irritation, and skin wounds (Balashov, 1968; Raoult & Roux, 1997 and Senbill *et al.*, 2018). In addition, ticks are internationally ranked only second to mosquitoes by their ability to transmit a vast variety of pathogens, including viruses, protozoa, bacteria, and parasitic nematodes (Sonenshine & Mather, 1994; de la Fuente *et al.*, 2008; Düttmann *et al.*, 2016 and Senbill *et al.*, 2018). Based on the latest records, the hard ticks' family (Family: Ixodidae) includes the valid majority of the described tick species with 742 recognized members, including 138 of the genus *Amblyomma*, 85 of the genus *Rhipicephalus*, and 27 of the genus *Hyalomma* (Guglielmone *et al.*, 2020).

Out of the 44 ixodid tick species that are currently identified in Egypt, the genera *Hyalomma* and *Rhipicephalus* are altogether represent the majority of them with a total of 30 recognized species (Senbill *et al.*, 2022). In contrast, *Amblyomma* ticks are not established in Egypt; however, some of the genus members are introduced to the country from other African countries through importation of infested animals (Hoogstraal & Kaiser, 1958 and El-Kammah, 2001).

Egypt imports enormous numbers of live animals to overcome the market needs. As between 2016-2018, Egypt has imported 312,804 cattle and 669,222 camels from Sudan, 1,925 cattle and 3,403 camels from Somalia, 17,369 camels from Djibouti, and 9,816 camels from Ethiopia (COMESA, 2019). In view of unrestricted border check of imported animals, the country receives a number of exotic tick species comes attached with these imported animals, particularly from sub-Saharan countries (Hoogstraal & Kaiser, 1961; Hoogstraal *et al.*, 1963; Hoogstraal *et al.*, 1964; Galbraith *et al.*, 2014 and Senbill *et al.*, 2024).

Amblyomma gemma and *Am. lepidum* are Afrotropical species, infesting cattle, camels, equids, boars, and rhinoceroses (Guglielmone *et al.*, 2020), particularly distributed in the northeastern Tanzania, most of Kenya, western part of Somalia, and central and eastern part of Ethiopia (Clarke and Pretorius, 2005). Although this species has been experimentally confirmed to transmit the heartwater disease agent, *Ehrlichia ruminantium* (Ngumi *et al.*, 1997; Muramatsu *et al.*, 2005 and Omondi *et al.*, 2017), it has been found positive to *Theileria mutans* (Paling *et al.*, 1981), *R. aeschlimannii* (Koka *et al.*, 2017; Shuaib *et al.*, 2020

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and Getange *et al.,* 2021), and *Rickettsia africae* (Shuaib *et al.,* 2020 and Khogali *et al.,* 2024). Likewise, *Am. hebraeum* and *Am. variegatum* are considered as the main vectors of *Eh. ruminantium* pathogens in Africa (Uilenberg, 1983 and Wesonga *et al.,* 2001). As an Afrotropical species, *Hy. impressum* and *Rh. decoloratus* are primarily infesting as same as the host range of the aforementioned *Amblyomma* species (Guglielmone *et al.,* 2020). Unlike *Hy. impressum* that is left with no information regarding its vector competence, *Rh. decoloratus* transmits bovine anaplasmosis, babesiosis and borrelioses (Horak *et al.,* 2018).

Basically, morphological identification and characterization of ticks represents the backbone of tick systematics, along with other factors such as biological and ecological information (Klompen *et al.,* 1996 and McCoy *et al.,* 2013). However, a package of obstacles come with these basic methods, including damaging specimens, closely similar species, degree of engorgement, and lack of entomological background (Nava *et al.,* 2009). Recently, molecular techniques have offered several improvements in tick taxonomy and precise identification (Marrelli *et al.,* 2007; Livanova *et al.,* 2015 and Li *et al.,* 2017). Although *cox1* gene is widely used in tick identification and specification of evolutionary relationships between the species (Kamani, 2021 and Sándor *et al.,* 2021), some other genes left underestimated. Either 18S or 28S rRNA genes are not regularly used in molecular characterization at lower taxonomic purposes (Dabert *et al.,* 2010; Martin *et al.,* 2010; Glowska *et al.,* 2013; Lehmitz & Decker, 2017 and Hu *et al.,* 2019) due to their conserveness and subsequently, proper for identification at the higher taxonomic ranks. However, species identification at lower taxonomic ranks is apparently needs more attention.

In the view of recent findings that domains of D5, D6 and D8 of the 28S rRNA gene are suitable for molecular systematics of arthropods, we investigate the ability of this specific gene to determine exotic tick species found in Egypt.

MATERIALS AND METHODS

Tick collection:

A total of 617 ticks at the adult stage were collected using tweezers from exotic cattle and camels imported to Egypt from other African countries (Table 1). The collected specimens were from Cairo (30° 2' 39.912" N; 31° 14' 8.5632" E) and Giza (30° 0' 47.0016" N; 31° 12' 31.8708" E) governorates (Fig. 1). Adult ticks were manually picked out to separate vials that were labelled with collection date, host, and locality. The ticks were then preserved in 70% ethanol mixed with 30% glycerol for further investigation.

Morphological identification:

Thirty representative specimens (five/species) were run for morphological recognition following the taxonomical keys described by Hoogstraal (1956); Walker (2003) and Walker *et al.* (2014). The morphological examination was performed with the help of Motic SFC-11 stereoscope (Motic Asia, Kowloon, Hong Kong) at 40X magnification.

DNA extraction:

DNA was extracted using the ABT Genomic Mini Extraction Kit (Applied Biotechnology, Ismailia, Egypt) based on the protocol provided by the manufacturer from each tick species. Briefly, approximately 25-30 mg of each extract was homogenized with 700 µl of DNA lysis buffer, following by adding 0.2 ml of chloroform and vigorous vortexing. The mixture was centrifuged at 12,000 rpm for 5 minutes at room temperature. The aqueous phase was transferred into a fresh tube, followed by adding 700 µl of 70% ethanol and mixing through inversion 3 times. The mixture was then transferred into a spin-column tube AC and centrifuged at 12,000 rpm for 30 seconds at room temperature. Approximately 500 µl of washing buffer was added to the tube, followed by centrifuge at 12,000 rpm for 30 seconds. The last step was repeated to guarantee the maximum DNA yield. The spin- column tube was then centrifuged at 12,000 rpm for one minute to get rid of any remaining washing buffer. The spin-column was placed in 1.5 ml RNase-free centrifuge tube, followed by adding 50 µl of elution buffer and centrifuge at 12,000 rpm for one minute.

The DNA concentration was measured using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The extracted DNA was divided into aliquots of 10 µl and kept at -80ºC until the PCR conduction.

Polymerase Chain Reaction (PCR):

The 28S rRNA gene of the collected ticks were amplified using universal Primers 28S R1/1 (5′-TTCTATGCTTAAATTCAGGGGGT-3′) and 28SRLR2 (5′- TCTCGCCTGATGTTAGGT-3′) as described by Wesson *et al.* (1993) and McLain *et al.* (1995), producing approximately 800-900 bp amplicons. The PCR reaction mixtures contained 2 µl of 300–500 ng/ µl genomic DNA (gDNA) in 50 µl reaction mixture mixed with 2 µl of each primer concentrated to 10 pmol (forward and reverse); 2 µl of dNTP mixture (2.5 mM); 5 µl of ABT 2X Red Mix (Applied Biotechnology, Ismailia, Egypt); and 39 µl of distilled water. The GeneAmp PCR System 9700 thermal cycler

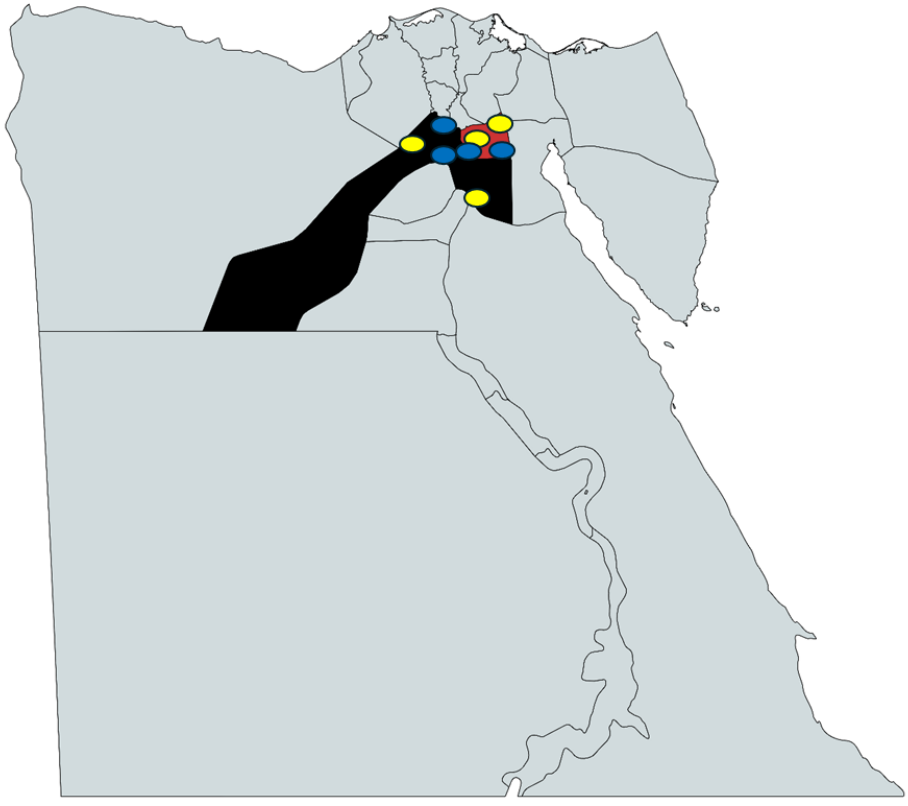

Fig. 1. A map of Egypt showing the tick collection localities in Cairo (red) and Giza (Black) Governorates. The yellow dots refer to camel hosts and the blue dots refer to cattle hosts

(Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to amplify the desired gene. The obtained PCR products were run by agarose 1% gel electrophoresis later, and every pathogen group sample was loaded into the gel. One-kb DNA marker was used as ladder (Applied Biotechnology, Ismailia, Egypt). The Alpha Innotech AlphaImager EP Gel Imaging System (Bio-Techne, Connecticut, USA) was used for visualization.

Sequencing:

The purified PCR products were sent to Macrogen, Inc in Seoul, South Korea for sequencing using the Sanger sequencing method, also known as dideoxy sequencing or chain termination method (Sanger *et al.*, 1977).

The 28S rRNA tick nucleotide sequences were initially constructed using the CAP3 online program (http://doua.prabi.fr/software/cap3) provided by the Pôle Rhône–Alpes de Bioinformatique Site Doua (PRABI–Doua) in Auvergne–Rhône–Alpes, Lyon, France. The consensus contigs were subjected to a BLASTn analysis on the National Centre for Biotechnology Information (NCBI) using the Megablast option to determine the similarity between the obtained sequences and existing sequences in the NCBI database through multiple alignment. Subsequently, the contigs were uploaded to the GenBank database using the BankIt submission tool provided by the National Centre for Biotechnology Information (NCBI) in Bethesda, Maryland, USA. The GenBank database can be accessed at https://www.ncbi.nlm.nih.gov/genbank/. The data were concurrently provided to the European Nucleotide Archive (ENA) located in Hinxton, Cambridgeshire, UK, and to the DNA Data Bank of Japan (DDBJ) situated in Shizuoka, Japan. Subsequently, accession numbers were acquired for 28S rRNA tick sequences.

Phylogenetic analysis:

The sequences were aligned using the DNASTAR tool (Wisconsin, USA) with default parameter values, against sequences of several tick species taken from the NCBI database. Neighbor-Joining (NJ) trees were generated using 1000 bootstrap replicates. The trees were produced based on the alignments of 28S rRNA sequences with the help of MEGA X software developed by Pennsylvania State University, USA (Kumar *et al.*, 2018). The

evolutionary distances were calculated using the Kimura 2–parameter approach (Kimura, 1980) and are expressed in terms of the number of base substitutions per site. All instances of unclear places were eliminated for each pair of sequences, and the phylogenetic trees were subsequently generated using the identical gene sequences from other tick species that were available for each individual tree.

RESULTS

Molecular characteristics of ticks:

The sequencing process has confirmed the prior morphological identification to reveal the presence of *Amblyomma gemma*, *Am. lepidum*, *Am. hebraeum*, *Am. variegatum*, *Hyalomma impressum*, and *Rhipicephalus decoloratus* as exotic ticks in Egypt. The produced amplicons of the ticks' 28S rRNA genes were approximately 730 bp in size. Except for *Am. gemma* AT-rich 69.58%), all other tick species under this study were almost neutral (GC= 50%). The obtained accession numbers of the ticks' 28S rRNA gene are presented in Table (1).

The partial sequences of 28S rRNA gene of *Rh. decoloratus* showed over 97% homology with South African isolates of the species (KY457485). Similarly, sequences of *Hy. impressum* found in Egypt shared 98.8% similarity to other isolates of the same species from Benin (KU130521). Both *Am. gemma* and *Am. lepidum* showed high similarities (99-99.5%) to isolates from Kenya (OQ566202 and OQ566211). The sequences of *Am. hebraeum* were found homologous to the South African isolates of the species (KY457490) by 98%, while sequences of *Am. variegatum* were highly similar to isolates of the species from Ethiopia (AF120308) by 99.7%.

Phylogenetic analysis:

The constructed Neighbor–Joining phylogenetic trees were built based on the multiple alignment of 28S rRNA sequences of the tick species under this study along with sequences of other tick species available at the GenBank (https://www.ncbi.nlm.nih.gov/). The ixodid tick, *Ixodes pavlovskyi* (JQ085392) was used as an out group. The exotic *Rh. decoloratus* found in Egypt clustered with South African isolate (KY457485), and close to the Texas cattle tick, *Rh. annulatus* (MF946470) and a sister clade to other cattle ticks *viz. Rh. microplus* (KC769640), *Rh. geigyi* (KC769638), and *Rh. australis* (KC769635). Exotic specimens of *Hy. impressum* found in Egypt clustered with isolate from Benin (KU130521) and close to *Hy. somalicum* (KU130556). *Amblyomma* ticks found in this study are clearly clustering with isolates from African countries, including Kenyan *Am. gemma* (OQ566202) and *Am. lepidum* (OQ566211), while to South African *Am. hebraeum* (KY457490) and Ethiopian *Am. variegatum* (AF120308) (Fig. 2).

Table 1. Accession numbers of the collected exotic ticks found in Egypt and their reference similarities to other African countries

Tick species	Hosts	Accession numbers	Reference acc. numbers	Reference country	% Similarity
Amblyomma gemma	*Bos taurus*	PP331836 PP331837	OQ566202	Kenya	99.5
Amblyomma hebraeum	*Bos taurus*	PP320505 PP320506 PP320507	KY457490	South Africa	98
Amblyomma lepidum	*Camelus dromedarius*	PP337788	OQ566211	Kenya	99
Amblyomma variegatum	*Bos taurus*	PP320508 PP320565 PP320566	AF120308	Ethiopia	99.7
Hyalomma impressum	*Bos taurus*	PP320570 PP320572 PP320573	KU130521	Benin	98.8
Rhipicephalus decoloratus	*Bos taurus*	PP321322 PP321338 PP330394	KY457485	South Africa	99

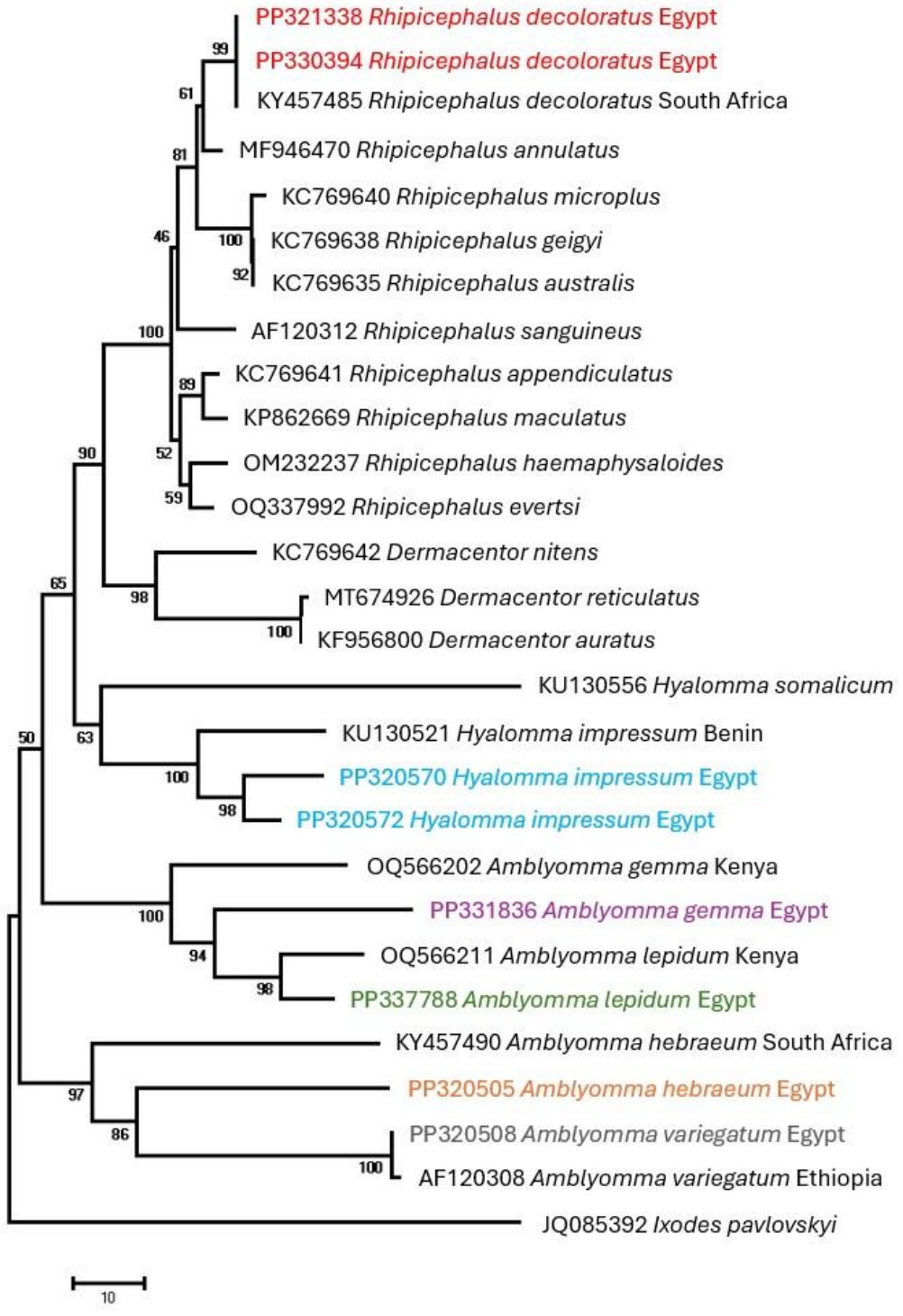

Fig. 2. Phylogenetic relationships between the exotic tick species found in Egypt and other tick species based on the sequences of 28S rRNA gene. *Ixodes pavlovskyi* was used as an outgroup

DISCUSSION

Egypt has natural strategic location that made it connecting almost all the continents' trading routes. In the view of the increasing population and shortage of livestock production to meet this rapid growth, the Egyptian authorities have decided to import live animals from neighboring African countries, particularly Djibouti, Somalia, Sudan, Ethiopia, and Chad (FAO, 2020 and USDA, 2023). The importation of African animals that are naturally infested by exotic ticks over the barrier of sub-Saharan countries plays an important role in the introduction of these tick species into the country, particularly in the absence of border inspection (Hoogstraal & Kaiser, 1961; Hoogstraal *et al.,* 1963; Hoogstraal *et al.,* 1964 and Galbraith *et al.,* 2014).

With the global increasing reports regarding the geographical expansion of several tick species (Léger *et al.,* 2013; Nyangiwe, 2017; Gasmi *et al.,* 2018; Sonenshine, 2018 and Raghavan *et al.,* 2021), regular tick surveillance has become crucial to keep our status updated. Despite that morphological basics are still represent the backbone of tick identification, the modern tick systematics are currently counting on molecular techniques to identify, classify, and phylogenetically analyze different tick species (Klompen *et al.,* 2000; Beati & Keirans, 2001; Beati *et al.,* 2008 and Nava *et al.,* 2009). Generally, the tick systematics and phylogenetics are primarily improved by our understanding to the molecular techniques and its role in studying the tick taxonomy and evolution. Although the nuclear ribosomal gene sequences give better resolution at the family and subfamily levels (Black *et al.,* 1997; Dobson & Barker, 1999; Fukunaga *et al.,* 2000; Klompen *et al.,* 2000; Beati *et al.,* 2008 and Mans *et al.,* 2011), the expansion segments D2 and D3 of the nuclear 28S rRNA could be amplified through single PCR reaction to contain unique variations across family, genus, and species levels (Gillespie *et al.,* 2003 and Schulmeister, 2003).

Our findings presented the first nuclear 28S rRNA sequences for several exotic tick species and their phylogenies in Egypt. The study revealed the presence of several *Amblyomma* ticks, including *Am. gemma*, *Am. lepidum*, *Am. hebraeum*, *Am. variegatum*, in addition to *Hy. impressum* and *Rh. decoloratus*. To the best of our knowledge, these recorded tick species are not actually belonging to the Egyptian tick fauna and introduced to the country attached to imported livestock animals from other African countries. *Amblyomma gemma* is native to eastern Ethiopia, northern and southern Somalia, Kenya, and north-eastern Tanzania. *Amblyomma hebraeum* is endemic in South Africa, Swaziland, southern Mozambique, eastern Botswana, southern and eastern Zimbabwe. *Amblyomma lepidum* is well-distributed in central and eastern Sudan, Ethiopia, southern Somalia, eastern Uganda, Kenya, and the northern region of central Tanzania. *Amblyomma variegatum* exists in high abundance in Zambia, northeastern Botswana, the Caprivi Strip of Namibia, northwestern Zimbabwe, and central and northern Mozambique. *Hyalomma impressum* invades West African countries, particularly Sudan and Ethiopia. *Rhipicephalus decoloratus* is widely distributed in Namibia, South Africa, and Botswana (Walker *et al.,* 2003).

Apparently, cattle are the highly infested animal by ticks from different tick species as we found in our study, followed by camels. These specific hosts are undoubtedly lies within the host ranges of the collected tick species (Walker *et al.,* 2003). Phylogenetically, *Rh. decoloratus* and *Am. hebraeum* clustered with isolates from South Africa, a native country of the species, while *Am. gemma* and *Am. lepidum* clustered with Kenyan isolates. *Hyalomma impressum* clustered with isolates of the species from Benin. *Amblyomma variegatum* clustered with Ethiopian isolates. According to the Common Market for Eastern and Southern Africa (COMESA), Egypt imports livestock from Sudan, Djibouti, Ethiopia, Somalia, and other sub-Saharan African countries close to 150,000 beef cattle and 240,000 camels per year. Without proper border inspection for the imported animals coming to Egypt, new tick species might have the opportunity to establish in our environment and spread more diseases to the Egyptian population.

Although the collected tick species in this study have been reported in Egypt in earlier studies, including *Am. gemma*, *Am. variegatum*, *Am. lepidum*, *Am. hebraeum*, *Rh. decoloratus*, *Hy. impressum* (Hoogstraal & Kaiser, 1958 and Ashour *et al.,* 2023), this study provides the genetic information of the 28S rRNA genes of the reported ticks for the first time in Egypt. In the view of the limited information about ticks and their harbored pathogens in Egypt, it is hard to set a proper plan to effectively control these arthropods. The solutions proposed to overcome these obstacles included the expansion of laboratory diagnostic capabilities in the concerned authorities and providing the full details to the disease prevention policymakers (Senbill *et al.,* 2024).

CONCLUSION

Ticks resemble a huge risk to the Egyptian economy through the influence on both human and animal lives. To overcome the risk of establishing new tick species to the country, the Egyptian authorities should adopt several strategies to prevent the introduction of these

exotic ticks. The laboratory diagnostic capability in the concerned authorities should be redesigned to permit regular preparation of tick checklists, boost diagnosis, and inform disease prevention policymaking.

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الملخص العربي

التعرف الجزيئي وتوصيف أنواع القراد الأجنبية في جمهورية مصر العربية: قراد الماشية من الدول الأفريقية المجاورة

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من بين ٤٤ نوعًا من القراد الجامد التي تم تعريفها في مصر، تم تصنيف ما لا يقل عن عشرةً منها على أنها أنواعاً أجنبية. تهدف الدراسة الحالية إلى إجراء تحليل جزيئي لتوصيف القراد الذي يصيب الثروة الحيوانية المستوردة بجمهورية مصر العربية. تم جمع القراد من الماشية والإبل في محافظتي القاهرة والجيزة والتي تم استيرادها من عدة دول أفريقية .حددت الدراسة وجود *Amblyomma gemma* و *Am. lepidum* و*Am. hebraeum* و *Am. variegatum* و *Hyalomma impressum* و *Rhipicephalus decoloratus* . أظهرت أنواع القراد التي تم جمعها تشابهاً كبيراً يتراوح من ٩٨٪ إلى ١٠٠٪، مع العزلات من جنوب إفريقيا وكينيا وبنين وذلك بناءً على تسلسل 28S rRNA.