Detection of *Anaplasma platys* and *Ehrlichia canis* in *Rhipicephalus sanguineus* ticks attached to dogs from Egypt; a public health concern

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1. Abstract

Anaplasma and *Ehrlichia* spp. are emerging tick-borne zoonotic bacteria with expanding reservoirs and vectors than ever. The current study aimed to molecularly detect and characterize the *Anaplasma* and *Ehrlichia* species in ticks of *Rhipicephalus sanguineus* sensu stricto (s.s.) attached to dogs in Egypt. For that purpose, ticks were collected from 156 dogs (72 household, 69 kenneled and 15 free-roaming). Ticks of *Rhipicephalus sanguineus* s.s. were identified morphologically then molecularly. *Anaplasma* and *Ehrlichia* spp. were detected in the collected tick pools using PCR targeted 16s rRNA of both genera, obtained bands were sequenced and analysed phylogenetically with other sequences recovered from humans and animals and infection rates were calculated. Out of the 156 collected tick pools, 151 were molecularly positive for *Rhipicephalus sanguineus*. PCR and sequencing for *Anaplasma* and *Ehrlichia* spp. revealed identification of two bacterial species; *A. platys* in two kenneled dogs tick pools (1.32%) and *E. canis* in two tick pools of household dogs and one recovered from the kenneled ones (1.98%). Moreover, the phylogenetic analysis of *E. canis* reveals genetic relatedness with those isolated from human cases.

Key words: Anaplasma; Ehrlichia; Rhipicephalus sanguineus; Dogs; Zoonoses; Egypt

2. Introduction

Despite being neglected from the developing countries compared to livestock zoonotic diseases, canine vector-borne zoonotic diseases have been flared up especially in the last few years due to explosion of dog population and their carried arthropods vectors in different human environments [27]. The brown dog tick, *Rhipicephalus sanguineus* s.s. [24] is a distinctive canine ectoparasite with a cosmopolitan distribution and a host-seeking behaviour enables it to seek its victims indoors and outdoors as well. Additionally, it has the adaptability to live in a wide range of ecological niches where the human dwellings are one of these niches. In fact, this tick is well survived in human dwellings where it can easily access for parasitism on humans [7]. *R. sanguineus* s.s. is a vector for many pathogens, some of which are solely a veterinary concern matter such as *Hepatozoon canis* while others represent public health significance such as *Leishmania infantum* and *Coxiella burnetii* [8,10]. Even so, human-biting cases from the brown dog tick do not occur very often, and certain factors such as dog ownership, occupationally daily handling of dogs and high environmental tick infestation increase its parasitism on humans. There is an on-going rise of human records bitten from *R. sanguineus* s.s. especially in countries where warm climatic conditions are available. Therefore, people in those countries are more liable to contract pathogenic agents carried by this species [8, 14]. *Anaplasma* and *Ehrlichia* spp. are obligate intracellular tick-borne bacteria belonging respectively to genus *Anaplasma* and *Ehrlichia* (Family Anaplasmataceae; order Rickettsiales) [11]. To date genus *Anaplasma* compromised seven species; *A. phagocytophilum*, *A. platys*, *A. marginale*, *A. bovis*, *A. ovis*, *A. capra*, and *A.*

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odocoilei, while species in genus *Ehrlichia*, on the other hand, are; *Ehrlichia canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, *E. ruminantium* and *E. minasensis* [34]. Many of these species have zoonotic potential causing human anaplasmosis and ehrlichiosis [16].

The clinical presentation of human anaplasmosis and ehrlichiosis ranges from asymptomatic, flu-like illness to multi-organ failure and deaths in some cases; furthermore, the last few years have witnessed a global expansion in their reservoirs and vectors which have attributed to increasing their human cases number [3,4]. Despite the public health burden of anaplasmosis and ehrlichiosis and detection of many zoonotic species of *Anaplasma* and *Ehrlichia* in *R. sanguineus* s.s. in different localities around the world [1,25], little attention on such issue is brought by the scientific community in Egypt. Therefore, the purpose of this study was to molecularly detect and characterize species of *Anaplasma* and *Ehrlichia* in *R. sanguineus* s.s. ticks attached to dogs in Egypt.

3. Materials and Methods

2.1. Collection of samples

From November 2018 to September 2019, a total of 909 ticks were collected from 156 dogs (72 household, 69 kenneled and15 free-roaming) residing Cairo and Giza Governorates, ticks were collected from every single dog as a pool where the pool contains no more than fifteen ticks per single dog (Table 1). In order to collect ticks alive and undamaged for morphology; a medium-sized steel forceps with blunt points and serrated inner surfaces was laid against dog skin where ticks were pulled directly outward, placed in strong well-ventilated tubes with a piece of moistened paper and cooled over ice during the period of transportation to the laboratory [12].

2.2. Morphological identification

R. sanguineus .s.s. species were identified morphologically using light and steromicroscopes [6,9,32]. Other tick species in the collected pools were excluded. Identified ticks were preserved at -40 °C till DNA extraction.

2.3. DNA extraction

Prior DNA extraction, ticks were washed by vortexing three times in sterile phosphate-buffered saline then left for air dryness on sterile dry filter paper under laminar flow. After dryness ticks were longitudinally dissected into two halves in sterile petri-dish using disposable sterile scalpel. One tick half was stored at -40 °C and the other was used for DNA extraction. DNA extraction was carried out on the processed tick pools using DNeasy blood and tissue extraction kit (Qiagen, Germany) according to the manufacturer's instructions with overnight incubation in the lysis buffers and elution in 60 ul. Eluted DNA was preserved at -40°C for further molecular processes.

2.4. PCR amplification

In order to validate the extracted DNA for further molecular processes; a PCR targeted 400bp of 12S rRNA mitochondrial gene of *R. sanguineus* tick group was performed using primers AAA CTA GGA TTA GAT ACC CTA TTA TTT TAG and CTA TGT AAC GAC TTA TCT TAA TAA AGA GTG [31]. The reaction was conducted using 2.5 ul ticks DNA, 12.5 ul EmeraldAmp GT PCR master mix (Takara, Japan), 0.75 ul of 10 uM of each forward and reverse primer (Metabion, Germany), and nuclease-free water (Qiagen, Germany) up to final volume of 25 ul. DNA of positively detected *R. sanguineus* were used for detection of *Anaplasma* and *Ehrlichia* spp using primers PER1 (TTTATCGCTATTAGATGAGCCTATG) and PER2 (CTCTACACTAGGAATTCCGCTAT) which amplify 451 bp of 16S rRNA gene of all known *Anaplasma* and *Ehrlichia* species[24]. Amplification was carried out in 50 ul volume containing 5 ul ticks DNA, 25 ul EmeraldAmp GT PCR master mix (Takara, Japan), and 1 ul of 10 uM of each forward and reverse primer (Metabion, Germany), and 18 ul nuclease-free water (Qiagen, Germany).

Additional negative controls contained 5 ul nuclease-free water (Qiagen, Germany) instead of template DNA were run simultaneously in all performed reactions.

All PCR amplifications were performed in (Techne TC-512, UK) thermal cycler with the following conditions. For *R. sanguineus* tick

group 12S DNA amplification; one single step of initial denaturation at 94°C for 3 min, 35 cycles of 45 sec denaturation at 94°C, 45sec annealing at 57°C, 45sec extension at 72°C and a final extension step at 72 °C for 5 min. Whereas, conditions for *Anaplasma* and *Ehrlichia* species16S PCR detection were: one step of initial denaturation at 95 °C for 5 min, 40 cycles of 95°C denaturation for 30 s, 55 °C annealing for 30 s, and 72 °C extension for 30 s, and a final extension at 72 °C for 5 min. Subsequently, 10 ul of all PCR products including those of the negative controls were analysed by 1.5 % agarose-TAE gel electrophoresis and visualized by UV transillumination.

2.5. Sequencing, analysis, and phylogeny.

All *Anaplasma* and *Ehrlichia* positive bands were excised from the gel, purified using gel purification kit (Qiagen, Germany) and subjected to bidirectional Sanger sequencing in ABI 3500 Genetic Analyzer (Applied Biosystems, USA) using Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, USA) according to manufacture protocol. Obtained sequences were analysed for similarity using BLAST. In order to evaluate our sequences for their zoonotic potentiality, they were analysed with selected NCBI sequences obtained from animals and humans. Alignment was performed using MUSCLE algorithm of "Geneious Prime 2020 software (https://www.geneious.com) "and the evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [17] using MEGAX software [19] with 1000 bootstrap replicates.

2.6. Nucleotide sequence accession numbers

Sequences obtained in this study were deposited in the GenBank under the following accession numbers: MT020422.1, MT044313.1, MT066093.1, MT053461.1, and MT066094.1

2.7. Statistics

Pools infection rates were calculated using maximum likelihood estimate (MLE) and minimum infection rate (MIR). Calculations were adjusted based upon the type of participated dogs (household, kenneled, free-roaming) and carried out using PooledInfRate software (https://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html) provided by the Centers for Disease Control and Prevention. Both MLE and MIR were used together since using MIR alone is not accurate, as it can underestimate the infection rate [15].

4. Results

3.1. Identification of ticks

R. sanguineus s.s. was the only species identified morphologically in 145 out of the 156 collected tick pools. While the other remaining 11 tick pools (8 from household dogs, 2 from kennelled dogs and one from free-roaming dogs) were encompassed other tick species, nevertheless, *R. sanguineus* s.s. was still also the most common tick species found in those pools with percentage ranged from 66 to 90.

Moreover, the molecular detection of *R. sanguineus* tick group 12S rRNA gene revealed identification of that gene in 151 tick pools among the 156 collected ones (Table 1).

3.2. Identification of *Anaplasma* and *Ehrlichia* spp.

16srRNA of *Anaplasma* and *Ehrlichia* spp. was identified in 5 (3.31 %) pools; 2 (2.94%) recovered from household dogs and 3(4.41%) recovered from the kenneled ones, whereas no signal was detected in any of the free-roaming dogs pools (Table 1, Figure 1). Upon sequencing, *A. platys* was identified in two kenneled dogs tick pools (1.32%) and *E. canis* in the two tick pools of the household dogs and the other remaining recovered from the kenneled ones (1.98%) (Table 1).

3.3. Statistics

The estimated MIR for *Anaplasma* and *Ehrlichia* spp. in tick pools of household dogs was 0.49% (95% CI: 0.00, 1.17), while this rate was 0.93% (95% CI: 0.00, 1.98) in tick pools of the kennel ones. On the other hand the MLE in tick pools of household and kennel dogs were 0.49% (95% CI: 0.09, 1.60%) and 0.94% (95% CI: 0.25, 2.54%) respectively.

5. Discussion

E. canis and *A. platys* are causative agents of canine monocytic ehrlichiosis (CME) and canine infectious cyclic thrombocytopenia in

domestic dogs respectively [29]. For long period, these two species have been known for their strict disease role in dogs, although, over the last few years several human infections have been emerged due to these pathogens [2,22,28]. Here in this study, we report the occurrence of *E. canis* and *A. platys* among *R. sanguineus* s.s. collected from household and kenneled dogs in Egypt. Results of the current study revealed detection of *E. canis* and *A. platys* with overall (1.98 and 1.32%), such results are comparable with those obtained by [21,34] who reported their occurrence by (0.6, 1.8%) and (0.7, 2.9%) respectively. Moreover, our results came lower for *E. canis* than those obtained from [33] (4.2%) but, yet higher for *A. platys* than those recorded in the same study (0.6%).Interestingly, sequence analyses of all positive tick pools of household dogs were confirmed to be *E. canis*, whilst pools of kenneled dogs contained both *E. canis* and *A. platys*. Unexpectedly, we could not able to detect any *Anaplasma* or *Ehrlichia* spp. in tick pools of free-roaming dogs, this result was on the contrary to [13] who was able to detect *A. phagocytophilum* in 13.7% of *R. sanguineus* s.s. ticks collected from free-roaming dogs and [18] who has also tested the same tick in free-roaming dogs and reported occurrence of *A. phagocytophilum* and *E. canis* by 51.5% and 3.0% respectively, this discrepancy between our results could be attributable to differences in sampling approach where collected ticks were individually tested in the aforementioned studies. We could not able to detect any *Ehrlichia* spp. in *R. sanguineus* s.s. other than *E. canis* and this is expected since this tick is globally known as a vector for *E. canis* [5,20,23]; however, its role for *A. platys* has not been fulfilled [30], yet presence of *A.platys* DNA in *R. sanguineus* s.s. in our study supports that this tick may be also vector for *A. platys* as well. Indeed, the phylogenetic analysis of our *A. platys* strains grouped them in the same clade with other strains from different geographical areas, moreover, the evolutionary analysis of our *E. canis* strains placed them in separate clade but yet share the same cluster that encompasses both human and animals strains (Figure 2 and 3). The overall MIR and MLE for *E. canis* and *A. platys* were 0.61 % (95% CI: 0.08, 1.13%) and 0.61% (95% CI: 0.23, 1.35%) respectively. Although, this rate is relatively low; they still represent a public health alarm for dog pet owners, kennel workers and all other people who come in contact with different dogs in Egyptian community particularly that Egypt conjoins factors enhancing the zoonotic transmission of *E. canis* and *A. platys* by *R. sanguineus* s.s. these include; the steady outgrowth of dog population, the wide disruption of *R. sanguineus* s.s. in Egyptian dogs and warm climatic conditions nearly throughout the entire year.

6. Conclusion

The results of this current study are to set up a flash warning signal for all veterinarians and other public health community to such significant neglected pathogens and to pave ways to other upcoming studies which should be warranted on the same track in order to accurately evaluate the burden of these bacteria on public health.

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able1: PCR, sequencing results of collected tick pools in relation to type of participated dog.

Types of participated dog	No of dogs	No of ticks collected	No of pools	No of successfully extracted pools (No of +ve identified *R.sanguineus)*	No of +ve (%) *E. canis* and *A. platys*	
					E. canis	*A. platys*
House hold	72	407	72	68	2/68 (2.94 %)	-
Kenneled	69	322	69	68	1/68 (1.47 %)	2/68 (2.94 %)
Free roaming	15	180	15	15	-	-
Total	156	909	156	151	3/151 (1.98 %)	2/151 (1.32 %)

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Fig.1. Molecular detection of *Anaplasma* and *Ehrlichia* spp among examined tick pools Lane M; DNA ladder 100 bp; lane 1-5 represented the positive samples with specific bands at 451 bp; lane 6 represented the negative control.

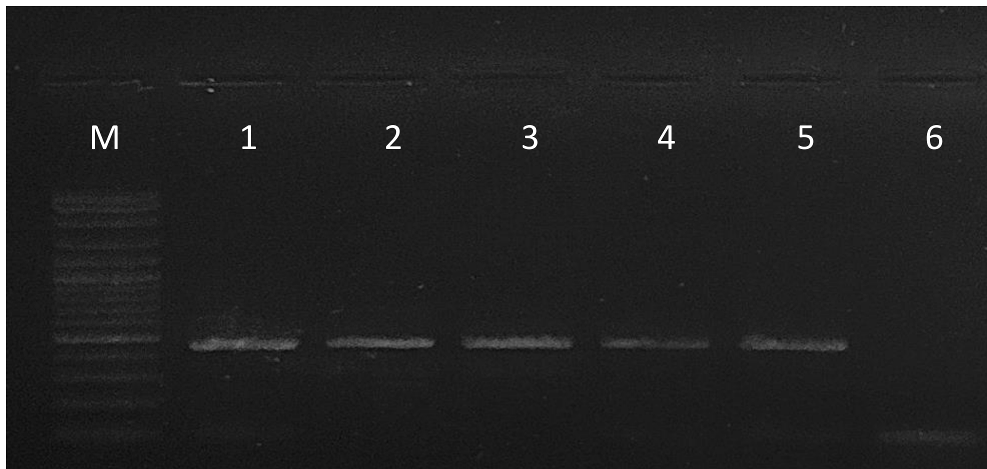

Fig.2. Phylogenetic analysis of *Anaplasma spp* based on partial sequences of 16S rRNA show evolutionary relatedness with other obtained sequences from the GenBank based on the Maximum Likelihood statistical method and Kimura 2-parameter model using MEGAX software with 1000 bootstrap replicates.

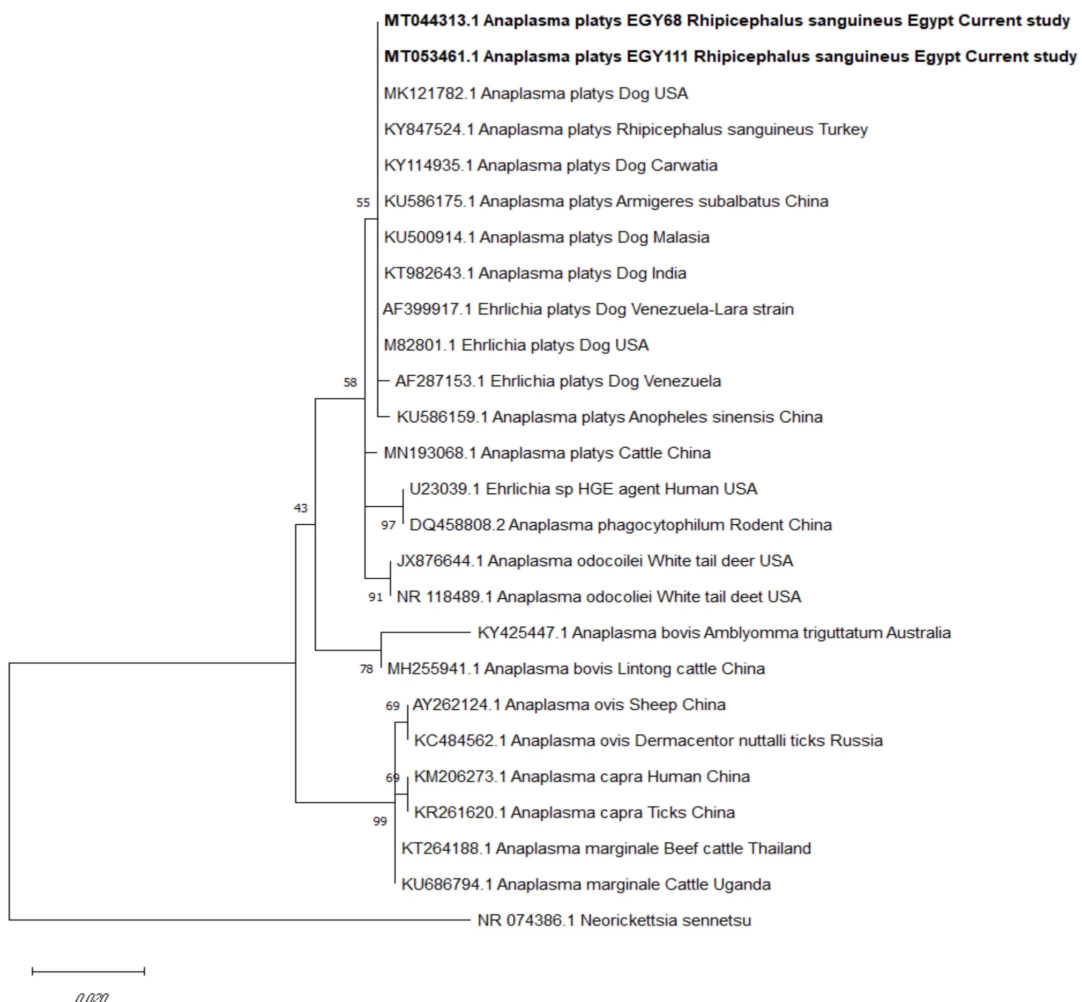

Fig.3. Phylogenetic analysis of *Ehrlichia* spp based on partial sequences of 16S rRNA show evolutionary relatedness with other obtained sequences from the GenBank based on the Maximum Likelihood statistical method and Kimura 2-parameter model using MEGAX software with 1000 bootstrap replicates.