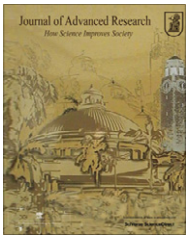
Journal of Advanced Research (2012) 3, 189–194

Cairo University

Journal of Advanced Research



SHORT COMMUNICATION

Prevalence and first molecular characterization of *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, in *Rhipicephalus sanguineus* ticks attached to dogs from Egypt

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Received 22 June 2011; revised 27 August 2011; accepted 30 August 2011
Available online 4 October 2011

KEYWORDS

Anaplasma phagocytophilum;
Rhipicephalus sanguineus;
PCR;
Prevalence;
Molecular characterization;
Egypt

Abstract PCR targeting 16S rRNA gene integrated with sequence analysis were performed to investigate the prevalence and the molecular identity of *Anaplasma phagocytophilum* in Egyptian *Rhipicephalus sanguineus* ticks attached to dogs. A total of 413 adult and nymphal *R. sanguineus* ticks were collected while attached to 72 free-roaming dogs from four locations (Imbaba, Boulaq, Haram, Monib) in Giza Governorate, Egypt. DNA was successfully extracted from 401 specimens (133 nymphs and 268 adults). The overall prevalence rate was 13.7% and adult ticks showed a significantly higher infection rate (16.4%) compared to nymphs (8.3%). Sequence comparisons of 218-bp showed that detected organism belongs to *A. phagocytophilum*. The sequence showed 99.1% similarity (2 nucleotide differences) with some strains described as human pathogens and with that detected in the established tick vectors. Phylogenetic analysis placed the bacteria on a separate branch with that found in *R. annulatus* from Egypt (DQ379972) (99.5% similarity). Our

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Peer review under responsibility of Cairo University.
doi:10.1016/j.jare.2011.08.002

Production and hosting by Elsevier

variant strain was designated as *A. phagocytophilum*-Ghafar-EGY (AB608266). This report is the first molecular characterization of *A. phagocytophilum* in *R. sanguineus* in Egypt, suggesting that this tick species may act as a competent vector for a variant strain of human granulocytic anaplasmosis agent.

Introduction

Human granulocytic anaplasmosis (HGA), an emerging tick-borne zoonosis, is a febrile systemic illness and its severity ranges from asymptomatic or non-specific flu-like symptoms to death. Headache, malaise, myalgia, lethargy, arthralgia, leucopenia, thrombocytopenia, and elevated levels of hepatic enzymes are the most encountered clinical and laboratory findings [1]. The first report of HGA came from United States in 1994 [2], and since that initial record, an increasing number of cases has been described in the US, Europe, and Asia [3–5]. The causative agent of HGA is *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), a Gram-negative obligatory intracellular bacterium, that replicates within neutrophilic granulocytes [6]. Recently, *A. phagocytophilum* has been designated after reorganization of order Rickettsiales, joining together the three previously characterized species, the agent of human granulocytic ehrlichiosis (HGE), *Ehrlichia phagocytophila* (the causative agent of tick-borne fever in cattle and sheep), and *Ehrlichia equi* (the causative agent of equine and canine granulocytic ehrlichiosis). This new designation was based on the similarities in 16S rRNA and *groESL* gene sequences as well as antigenic and biological characteristics [7]. However, genetic diversity among *A. phagocytophilum* strains has been described [8]. It is noteworthy to mention that, agents of HGA with different 16S rRNA sequence are associated with variable biological and ecological characteristics including pathogenicity and vector specificity [8,9]. Several members of genus *Ixodes* have been implicated in the natural transmission cycle of *A. phagocytophilum*; including *Ixodes scapularis* and *Ixodes pacificus* in the US [10,11], *I. ricinus* in Europe [12], and *Ixodes persulcatus* in Asia [13]. In Egypt, although no clinical cases of HGA have been reported, *A. phagocytophilum* DNA was detected in humans at risk who are occupationally exposed to ticks [14,15]. Nevertheless, the molecular identity of the recognized organism and its ecological cycle of transmission, including competent vectors and reservoirs, remains yet to be determined. We are hypothesizing that *Rhipicephalus sanguineus*, the brown dog tick, is a candidate competent vector for a genetically different *A. phagocytophilum* strain in the country. Testing this hypothesis is a multistep project, where its first initial experiment is to detect and identify the organism of concern in the suspected vector. Therefore, the objectives of this study were: (1) to detect and demonstrate the prevalence of *A. phagocytophilum* in *R. sanguineus* ticks, (2) to molecularly identify the detected organism.

Material and methods

Tick collection

Adult and nymphal ticks were collected while attached to 72 free-roaming dogs from four locations (Imbaba, Boulaq, Haram, and Monib) in Giza Governorate (30°1′0″N, 31°13′0″E), Egypt. Tick larvae were excluded during sampling as well as recovered ticks were morphologically identified [16] and preserved in 70% ethanol till nucleic acid extraction.

DNA extraction from ticks

Total DNA of individual ticks was extracted using the QIAamp DNA Mini kit (QIAGEN Inc., CA, USA) according to the manufacturer's protocols and stored at −20 °C until PCR. A negative control for the extraction (distilled water) was included with every 10 samples. The efficiency of the DNA extraction was validated by PCR using a primer set designated as MJH3 and MJH4. These primers were designed to amplify the 16S mitochondrial rRNA gene of five tick genera (*Rhipicephalus*, *Ixodes*, *Dermacentor*, *Haemaphysalis*, and *Argas*) and correspond to the published *Ixodes ricinus* sequence [17].

PCR and electrophoresis

Only successfully extracted templates were used in PCR and downstream analysis. To avoid contamination, standard PCR routines were implemented. "NO DNA" negative controls (PCR-grade water) and positive controls (extracted DNA from blood sample of dog confirmed to be positive for *A. phagocytophilum* by PCR) were included in each experiment to control contaminations and false-negative amplification results. All PCR reagents and enzyme were obtained from Jena Bioscience (Jena Bioscience, GmbH, Germany) and used as recommended by the supplier. Twenty pmoles of oligonucleotide primers, E1 (5′-GGC ATG TAG GCG GTT CGG TAA GTT-3′) and E2 (5′-CCC CAC ATT CAG CAC TCA TCG TTT A-3′), that target specific sequences in the 16S rRNA gene of the phagocytophila genogroup [18] were used in a standard PCR reaction. The thermocycler program involved initial denaturation (94 °C for 2 min), followed by 30 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s) and then final extension at 72 °C for 5 min. Generation of 262 bp amplicons during analysis, as assessed by agarose gel electrophoresis, is considered positive results.

Sequencing of PCR products

Double-stranded PCR products were purified from excised gel bands by using the commercial Agarose Gel Extraction Kit (Jena Bioscience GmbH, Germany) and subjected for bidirectional sequencing using Jena Bioscience facilities. Cycle sequencing reactions were performed using an ABI Prism Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3130 DNA Sequencer, according to the manufacturer's instructions. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB608266.

Sequence analysis

A BLAST search was performed (http://www.ncbi.nlm.nih.gov/BLAST) with the consensus sequence of this study. The obtained sequences were aligned separately and manually using MacClade v.4. The unalignable and gap-containing sites were deleted so that 218 bp were left for the analysis. Genetic analysis was performed using the PAUP* 4.0b10 software [19] by heuristic searches with the TBR branch swapping and 10 random taxon additions. A tree was constructed using the neighbor-joining (NJ) method [20] with distance option of Tajima-Nei. Bootstrap resampling with 2000 replications was performed to statistically support the reliabilities of the nodes on the tree [21]. The 16S rRNA gene from *Neorickettsia risticii*, *N. sennetsu*, *N. helminthoeca* (accession numbers M21290, M73225 and U12457, respectively) was used to root the tree.

Results

Tick identification and PCR

A total of 413 adult and nymphal ticks of variable degrees of engorgement were collected while attached to 72 free-roaming dogs. All recovered ticks were morphologically identified as *R. sanguineus* and DNA was successfully extracted from 401 specimens (133 nymphs and 268 adults). Detailed PCR results for nymphs and adults from different sampling sites are summarized in Table 1. PCR positivity was indicated by the generation of a single band of the appropriate size (Fig. 1). The infection rate in adult (16.4%), was significantly higher than that in nymphs (8.3%) ($\chi^2 = 4.99$, degrees of freedom [df] = 1, $P < 0.05$). The difference in infection rates among sampling locations in Giza Governorate was not significant ($\chi^2 = 2.19$, df = 3, $P > 0.05$).

Sequence analysis

Alignment of the partial 16S rRNA gene sequences showed that the anaplasmal 16S rRNA gene from *R. sanguineus* belongs to the *A. phagocytophilum*. Phylogenetic analysis using selected sequences from the GenBank (Fig. 2) placed our strain on a separate branch with that detected in *R. annulatus* from Egypt (DQ379972-99.5% similarity) and in the clade (99.1% similarity) as the strains described as human pathogens (U02521, U23038, AF093788, AF093789, AY886761) and that detected in established tick vectors in the US (EF123258, AF036645), in Europe (GU734324, FJ172530), and in Asia (HM366579, AF205140, AF470701). The percent identities for other selected anaplasmas were 97.3 for *A. bovis* (U03775) and 95.9 for *A. centrale* (AF283007), *A. ovis* (AY262124), and *A. marginale* (M60313). Sequence similarities to other organisms used in the tree were 91.7%, 91.7%, 90.7%, 91.7%, 91.3%, 86.3%, 78.8%, 79.7%, and 78.4% for *Ehrlichia canis* (M73221), *E. chaffeensis* (M73222), *E. muris* (U15527), *E. ewingii* (M73227), *E. ruminantium* (U03777), *Wolbachia pipientis* (AF179630), *N. risticii* (M21290), *N. sennetsu* (M73225), and *N. helminthoeca* (U12457), respectively. Nucleotide and some epidemiological aspect differences between present strain and other selected ones used in the phylogenetic tree are summarized in Table 2.

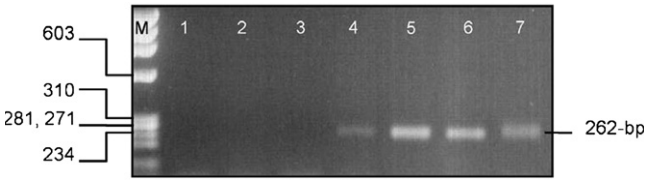

Fig. 1 Agarose gel electrophoresis of PCR products obtained by amplification of DNA of some individual *R. sanguineus* tick with the *A. phagocytophilum*-specific primers. Lane M, molecular size standard marker, ∑X174 DNA-*Hae* III Digest (bp). Generation of a fragment of 262-bp (lanes 4–7) indicate positive result.

Discussion

The present study aimed to detect and molecularly identify *A. phagocytophilum* in the suspected tick vector, *R. sanguineus*, as a crucial initial step in vectorial competence studies. Proposing *R. sanguineus* as a candidate competent vector for the agent of HGA in Egypt is based on the following considerations: (1) *R. sanguineus* is widely distributed in Egypt [22]. (2) *R. sanguineus* is well adapted to human dwellings [23] and was found to occasionally attack humans [24], thus increasing the risk of human exposure to zoonotic tick-borne HGA. (3) *R. sanguineus* is the main dog tick in Egypt [25], and a genomic evidence of *A. phagocytophilum* was reported in Egyptian dogs [14]. (4) *R. sanguineus* ticks parasitizing Egyptian dogs were found to harbor the nucleic acids of *A. phagocytophilum*; however, the molecular identity of the organism was not revealed [14]. (5) *R. sanguineus*, in the country, was found to parasitize sheep and goats [26] and these hosts were known to be global competent reservoirs for *A. phagocytophilum* [27,28]. (6) Egyptian sheep that could be parasitized by *R. sanguineus* were found to contain *A. phagocytophilum* DNA in their blood [15]. (7) Absence of the established tick vectors of HGA agent (*I. scapularis*, *I. pacificus*, and *I. persulcatus*) from the Egyptian tick fauna, suggests the presence of possible alternative vectors.

Table 1 Results of PCR for the identification of *A. phagocytophilum* in *R. sanguineus* ticks from four locations at Giza Governorate, Egypt.

Location	Dogs participated	Ticks collected	Successfully extracted DNA	PCR results Positive/tested (%)		
				Nymph	Adult	Total
Imbaba	17	96	95	2/33 (6.1)	8/62 (12.9)	10/95 (10.5)
Boulaq	21	114	108	1/29 (3.5)	12/79 (15.2)	13/108 (12)
Haram	18	99	98	2/16 (12.5)	13/82 (15.9)	15/98 (15.3)
Monib	16	104	100	6/55 (10.9)	11/45 (24.4)	17/100 (17)
Total	72	413	401	11/133 (8.3)	44/268 (16.4)	55/401 (13.7)

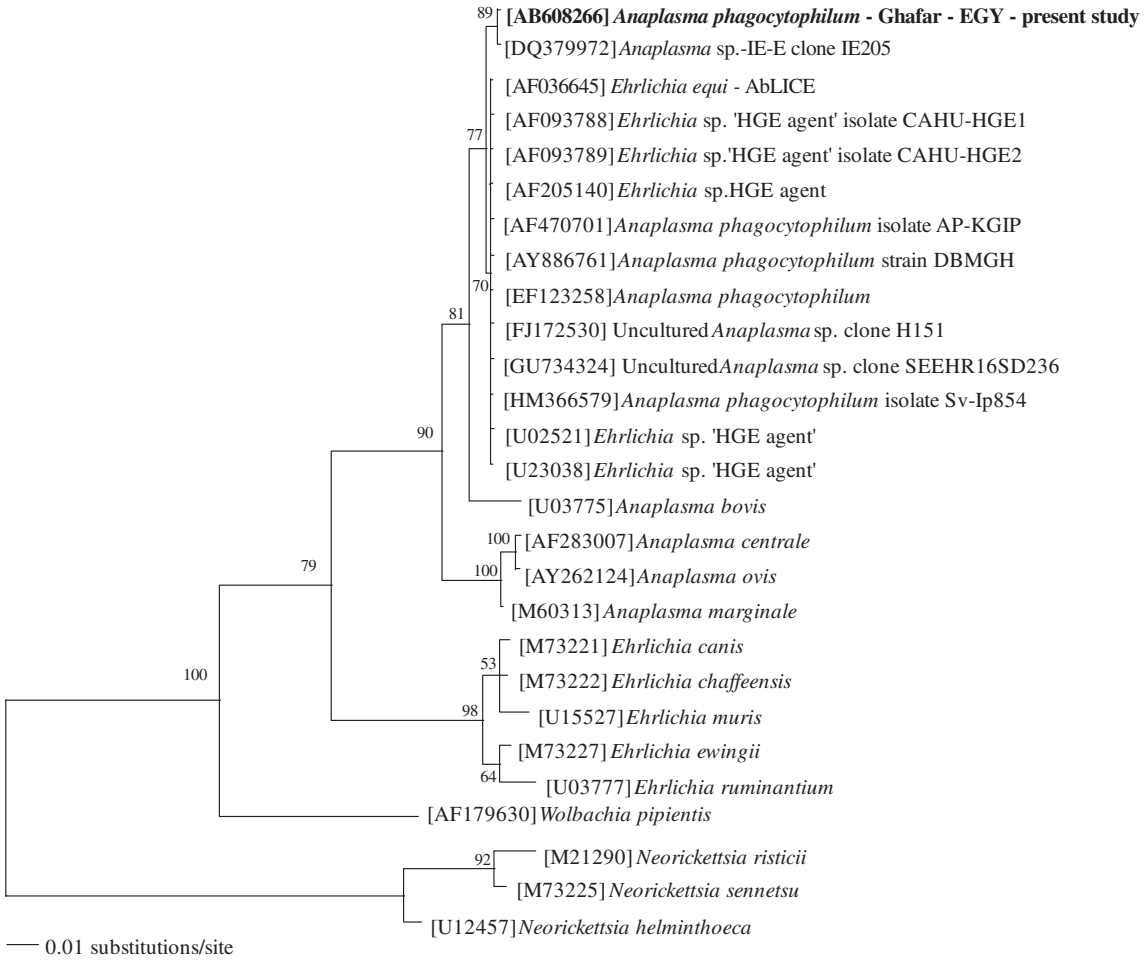

Fig. 2 Neighbor-joining tree based on partial (218-bp) 16S rRNA sequences obtained with distance option of Tajima-Nei and bootstrap analysis of 2000 replicates. Numbers on branches indicate percent of replicates that reproduced the topology for each clade. Parentheses enclose GenBank accession numbers of the sequences used in the analysis. The scale bar represents 1% differences.

Table 2 Comparison of partial 16S rRNA gene sequences of *A. phagocytophilum* detected in Egyptian *R. sanguineus* tick with selected published sequences used in the phylogenetic tree analysis.

Biological host	Geographic origin	Nucleotide difference at position[a]		GenBank accession No.
		37	76	
Human	USA	A	A	U02521
Human	USA	A	A	U23038
Human	USA	A	A	AY886761
Human	USA	A	A	AF093789
Human	USA	A	A	AF093788
I. scapularis	USA	A	A	EF123258
I. pacificus	USA	A	A	AF036645
I. ricinus	Turkey	A	A	FJ172530
I. ricinus	France	A	A	GU734324
I. persulcatus	Russia	A	A	HM366579
I. persulcatus	Republic of Korea	A	A	AF470701
I. persulcatus	China	A	A	AF205140
R. annulatus	Egypt	A	C	DQ379972
R. sanguineus	Egypt	–[b]	C	AB608266

[a] The position of the nucleotide relative to the 16S rRNA sequence of the agent of human granulocytic ehrlichiosis (HGE).
[b] Indicate no nucleotide corresponds to HGE agent; a gap was required at this position to align the adjacent sequence.

Exclusion of tick larval stages during sampling is attributed the fact that HGA agent is transstadially, but not transovarially, transmitted by tick vectors [29]. We have utilized 16S rRNA gene in our PCR, sequencing, and phylogenetic analysis experiments. Targeting this gene was based on the relatively conserved nature of this gene on the evolutionary scale [30].

Our samples contained ticks of variable degrees of engorgement, meaning that they contained canine host blood. Therefore, there are two possible sources of *A. phagocytophilum* in a positive PCR sample, either the tick or the dog. Given that not all semiengorged and fully engorged ticks collected on the same dog showed evidence of *A. phagocytophilum* DNA, it is suggested that the *R. sanguineus* may be a vector of the agent. However, examination of unfed tick stages and other vectorial competence experiments should be performed.

The infection rate in adult *R. sanguineus* (16.4%) was significantly higher than that in nymphs (8.3%). This result could be explained by the fact that *R. sanguineus* is a typical three-host tick; therefore, adult ticks are more exposed to more infected hosts than nymphs. The overall detection rate of *A. phagocytophilum* in this study was 13.7%, which is remarkably higher than that (5.3%) previously reported in the country by Ghafar [14]. This discrepancy in positive rates could be attributable to differences in sampling approach and the way in which infection rate was expressed; where in the previous study, ticks including larvae were pooled and the minimum infection rate (MIR) was recorded. Given the very close relationship between dogs and their owners, the fact that *R. sanguineus* is a three-host tick (meaning that it spends most of its lifetime in the environment), and the fact that *R. sanguineus* is very well adapted to human dwellings in both urban and rural areas [23], our reported high infection rate is considered a flashing warning signal for the risky role played by *R. sanguineus* in human infections. Nevertheless, an extensive molecular survey testing the currently suspected tick vector collected from different ecological niches all over the country is needed to assess the precise prevalence rate and geographical distribution of HGA agent in Egypt.

Our sequence comparisons suggest that the amplicons derived from *R. sanguineus* in this study are true *A. phagocytophilum* species. Phylogenetic analysis revealed that this organism constituted a separate branch in the *A. phagocytophilum* cluster group with one recently described *Anaplasma* sp. (DQ379972) from *R annulatus* ticks collected in Egypt [31] (Fig. 2). These two sequences were 99.5% identical but differed from *A. phagocytophilum* cluster group sequences (99.1% identity). Therefore, the detected organism in this study could represent a distinct strain designated as *A. phagocytophilum*-Ghafar-EGY (AB608266).

Given the close relatedness of these two organisms, the same geographic area (Egypt) of occurrence, and the same tick genus (*Rhipicephalus*) as biological origin; it is suggested that members of genus *Rhipicephalus* may act as natural vectors for a genetically different strain of *A. phagocytophilum* in the country.

The variant strain detected in this study has only 2 nucleotide differences at position 37 and 76 with selected strains described as human pathogens in the US and those recoded in established tick vectors of HGA in the US (*I. scapularis* and *I. pacificus*), in Europe (*I. ricinus*), and in Asia (*I. persulcatus*) (Table 2). This variation in the short sequenced fragment (218-bp) may be of a great impact on ecological and pathological properties of the present strain, especially when it is associated with other genetic differences in protein coding genes. However, full length 16S rRNA and other immunodominant protein genes should be sequenced and comparatively analyzed to reveal both genetic and antigenic profiles.

Given the previous information, we cannot conclude that *A. phagocytophilum*-Ghafar-EGY strain can cause human infections. Therefore, comparative genomic studies with strains causing clinical HGA in the country should be performed. Absence of clinical reports of HGA in Egypt could be attributable to unawareness of clinicians, lacking of the diagnostic tools, and or causation by less virulent strain.

Conclusion

Although being the second molecular detection, this study is considered the first molecular characterization of *A. phagocytophilum* in *R. sanguineus* in Egypt. Detection of HGA agent in brown dog tick does not confirm that this tick species is a competent vector for this pathogen; however, this work is a crucial initial step in vectorial competence studies. Identifying the competent vectors utilized by *A. phagocytophilum* in Egypt will help understanding the global epidemiology of the disease as well as designing and execution of efficient prevention and control measures.

Acknowledgements

We are indebted to Dr. Magdy Ghoneim (Former Head of Biotechnology Center for Services and Research, BCSR, College of Veterinary Medicine, Cairo University, Egypt) for continuous scientific help and providing us with the opportunity to using BCSR facilities and property. We also thank Dr. Yassin Al-Sodany (Biology Department, College of Science, Taif University, KSA) for doing statistical analysis of this work.

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