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Molecular Survey of Five Tick-Borne Pathogens (*Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Babesia microti*) in Egyptian Farmers

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Abstract: The close proximity of people with animal reservoir hosts and tick vectors constitutes a risk factor for the transmission of tick-borne zoonoses; therefore, farmers are at high risk. This study was intended to determine the occurrence of five tick-borne pathogens (*Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Babesia microti*) in Egyptian farmers. To execute this objective, a total of 67 EDTA-whole blood samples were collected from farmers residing villages of Nile Delta. Extracted DNA was used in nested PCR reactions targeting rRNA gene of *E. chaffeensis*, *E. ewingii* and *B. microti*. Detection of *A. phagocytophilum* and *B. burgdorferi* sensu lato was performed in standard PCR amplifications with specific primers for rRNA and *ospA* gene, respectively. All samples tested negative for *E. chaffeensis*, *E. ewingii* and *B. microti*. Five (7.5%) persons showed evidence of *A. phagocytophilum* infection, while DNA of *B. burgdorferi* was detected in 2 (3%) samples. One (1.5%) of the tested farmers was concurrently infected with both *A. phagocytophilum* and *B. burgdorferi* sensu lato at the same time. This report is the first molecular evidence of *B. burgdorferi* sensu lato and its coinfection with *A. phagocytophilum* in the country.

Key words: Human Ehrlichiosis · Human Granulocytic Anaplasmosis · Lyme Disease · Human Babesiosis · PCR

INTRODUCTION

Ehrlichia chaffeensis, *Ehrlichia ewingii* and *Anaplasma phagocytophilum* are emerging tick-borne pathogens and are the causative agents of human monocytic ehrlichiosis (HME), *E. ewingii* ehrlichiosis and human granulocytic anaplasmosis (HGA), respectively [1-3]. These infections are collectively referred to as human ehrlichiosis. These obligatory intracellular bacteria belong to family Anaplasmataceae and replicate within hematopoietic cells [4]. Previous field and laboratory studies demonstrated that these organisms utilize *Amblyomma americanum* or members of genus *Ixodes* (*I. scapularis*, *I. pacificus* and *I. ricinus*) as vectors [5-8]. White tailed deer as well as cattle, sheep, goats, horses, rodents and dogs are documented to be competent reservoirs for HME and HGA, respectively [9-11]. Clinically, HME and HGA are indistinguishable and characterized by fever, headache, myalgia, thrombocytopenia, leucopenia and elevated levels of liver enzymes. Most of cases are mild; however, life-threatening complications such as respiratory and renal failure can occur [12-14].

Three heterogonous species of *Borrelia burgdorferi* sensu lato complex (*B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*) are the causative agents of Lyme disease (LD), which is a multisystem infection with dermatologic, neurologic and rheumatologic manifestations. Ixodes ticks (*I. scapularis*, *I. pacificus* and *I. ricinus*) and rodents act as vectors and reservoirs for LD spirochetes [15-17].

Babesia microti, intraerythrocytic piroplasms, is a parasite of rodents and appears to be the sole agent responsible for cases of human babesiosis in the US [18-19]. Other babesia organisms like *B. divergens*, a cattle protozoan and unnamed species (WA1, CA1, MO1 and EU1) have been documented to cause human babesiosis as well [18-25]. In the US, the role of white footed mouse (*Peromyscus leucopus*) as rodent reservoir

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and *I. scapularis* as tick vector for *B. microti*, have been well established [26, 27]. Despite the fact that most infections are subclinical, severe disease is seen in immunocompromized and splenectomized individuals [28].

Ehrlichiosis, Lyme borreliosis and babesiosis all are diseases transmitted from animal hosts to humans through tick bite; therefore, the close proximity of people with tick vectors and animal reservoir hosts constitutes a risk factor that enhances disease transmission. Owing to the fact that ticks can harbor more than one disease-causing agent [29, 30]; patients can be concurrently infected with more than one pathogen, causing difficulty in both diagnosis and treatment. Several reports of human coinfection with *A. phagocytophilum*, *B. burgdorferi* or *B. microti* have been published [31, 32].

In Egypt, in 2005, DNA of *A. phagocytophilum* has been detected in blood of a 42-year shepherd using nested PCR technology [33]; however, to date and to the best of our knowledge, no molecular surveys have been executed to demonstrate the occurrence of HME, *E. ewingii* ehrlichiosis, Lyme disease and human babesiosis in the country. This study was intended to molecularly detect *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, *B. burgdorferi* sensu lato and *B. microti* in Egyptian farmers who are occupationally exposed to ticks and in close contact with animals known to be reservoirs for these pathogens.

MATERIALS AND METHODS

Blood Samples: A total of 67 EDTA-whole blood samples were collected from Egyptian farmers residing villages of Nile Delta. Information regarding age, tick bite, tick exposure, contacted animals and clinical symptoms were recorded. Blood samples were stored at-20°C until DNA extraction.

DNA Extraction: Total DNA was extracted from 200µl blood samples using QIAamp DNA Blood Mini Kit (QIAGEN Inc., CA, USA), according to the manufacturer's protocol. A negative control for the extraction (distilled water) was included with every 10 samples. DNA concentration and purity were assessed spectrophotometrically and DNA samples were stored at-20°C till used in PCR.

PCR Assay: Standard PCR routines and inclusion of negative (PCR-grade water) and positive controls were applied throughout the experiments to control contaminations and false-negative amplification results. All PCR reagents and DNA polymerase were obtained from the Jena Bioscience (Jena Bioscience, GmbH, Germany) and used as recommended by the supplier. All amplification reactions were executed in an automated thermocycler (Techne TC512, USA). Oligonucleotide sequences used to detect pathogens of this study are presented in Table 1.

PCR for Detection of *E. chaffeensis* and *E. ewingii*: The 16S-rRNA of ehrlichiae was amplified in a nested PCR reaction. For the initial amplification, 10µl of template DNA were amplified in a 50µl reaction mixture containing 20 pmoles of primers ECB and ECC, which amplify all known *Ehrlichia* species [34]. Reactions consisted of an initial 2-min denaturation at 95°C, 40 cycles (each consisting of a 1-min denaturation at 94°C, a 2-min annealing at 45°C and a 30-s extension at 72°C) and a 5-min final extension at 72°C. For the nested amplification, 5µl of each initial reaction product was used as a template in another two separate 50µl reaction mixture containing 20 pmoles of *E. chaffeensis*-specific primers (HE1 and HE3) [35] and *E. ewingii*-specific primers (HE3 and EE5) [36]. Reactions with species-specific primers consisted of

Table 1: Oligonucleotide sequences of primers used to detect pathogens in this study

Primer designation	Length (nt)	Oligonucleotide sequence (5' to 3')
ECC	23	AGA ACG AAC GCT GGC GGC AAG CC
ECB	22	CGT ATT ACC GCG GCT GCT GGC A
HE1	29	CAA TTG CTT ATA ACC TTT TGG TTA TAA AT
HE3	27	TAT AGG TAC CGT CAT TAT CTT CCC TAT
EE5	29	CAA TTC CTA AAT AGT CTC TGA CTA TTT AG
E1	24	GGC ATG TAG GCG GTT CGG TAA GTT
E2	25	CCC CAC ATT CAG CAC TCA TCG TTT A
SL-Forward	27	AAT AGG TCT AAT AAT AGC CTT AAT AGC
SL-Reverse	27	CTA GTG TTT TGC CAT CTT CTT TGA AAA
Bab1	23	CTT AGT ATA AGC TTT TAT ACA GC
Bab2	25	GTT ATA GTT TAT TTG ATG TTC GTT T
Bab3	20	AAG CCA TGC GAT TCG CTA AT
Bab4	25	ATA GGT CAG AAA CTT GAA TGA TAC A

an initial 2-min denaturation at 95°C, 40 cycles (each consisting of a 1-min denaturation at 94°C, a 2-min annealing at 55°C and a 30-s extension at 72°C) and a 5-min final extension at 72°C. Positive result was indicated by the generation of ~500-bp band for outer common reaction and 389-bp for the nested species-specific amplifications.

PCR for Detection of *A. phagocytophilum*: Twenty pmoles of oligonucleotide primers, E1 and E2, that target specific sequences in the 16S rRNA gene of the phagocytophila genogroup [37] and 10µl of extracted template 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 30s, annealing at 58°C for 30s and extension at 72°C for 30s) and then final extension at 72°C for 5 min. Generation of 262 bp amplicons during analysis by electrophoresis indicates positive results.

PCR for Detection of *B. burgdorferi* sensu lato: SL-Forward and SL-Reverse primers designed to amplify *OspA*-specific target sequences of all three pathogenic genospecies of *B. burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii*) [38] were used in a standard PCR. Amplification was performed as previously described with slight modifications. Ten microliters of each extracted DNA template were amplified in a 50µl reaction mixture containing 20 pmoles each primer. The reaction mixture was subjected to 35 cycles of amplification; each cycle involved heating to 93°C for 1 min (DNA denaturation), cooling to 60°C for 1 min (primer annealing) and again heating to 72°C for 1 min (primer extension). The amplification was concluded with an extension reaction at 72°C for 5 min. A positive result was considered a clear band at 307-bp.

PCR for Detection of *B. microti*: For specific detection of *B. microti*, nested PCR was performed using outer primers Bab1 and Bab4 and inner primers Bab2 and Bab3. These primer sets target a fragment of 18S rRNA gene that is specific for the organism [39]. The oligonucleotides amplify 238-bp and 154-bp fragments, respectively. In a nested reaction, extracted template DNA was amplified in 50µl reaction mixture containing 20 pmoles of each primer. Thermocycler program included initial denaturation (1 min. at 94°C) followed by 35 rounds of temperature cycling (94°C for 1 min, 60°C for 1 min and 72°C for 2 min). The amplification was concluded with additional final extension at 72°C for 5 min.

Agarose Gel Electrophoresis: PCR products were analyzed by electrophoresis on agarose gels and visualized under ultraviolet (UV) transilluminator. A positive result was considered a clear band of expected fragment.

RESULTS

The median age of tested participants was 39.5 year. Although all showed tick exposure, none of them reported tick bites. All individuals reported frequent direct and indirect contact with cattle, buffaloes, sheep, goats, donkeys, dogs and rats. None of participants reported typical clinical signs of the corresponding diseases. Results of PCR experiments executed in this study are shown in figures 1,2 and Table 2.

Table 2: PCR results for different pathogens detected in this study

	PCR result No. positive (%)					
No. tested	*E. chaffeensis*	*E. ewingii*	*A. phagocytophilum*	*B. burgdorferi*	*B. microti*	*A. phagocytophilum* and *B. burgdorferi*
67	0 (0)	0 (0)	5 (7.5)	2 (3)	0 (0)	1 (1.5)

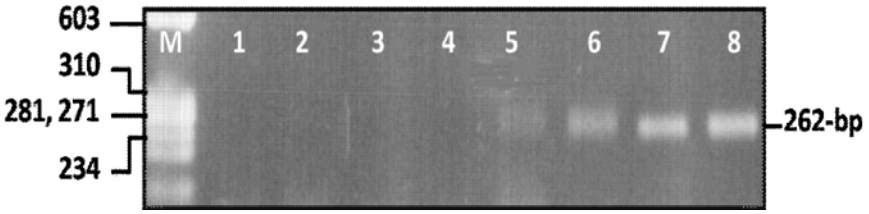

Fig. 1: Agarose gel electrophoresis of PCR products of amplified 16S rRNA of *A. phagocytophilum*. Lane M, molecular size standard marker, □X174 DNA-*Hae* III Digest (bp). Lanes 1-4, negative samples; lanes 5-8, positive samples for *A. phagocytophilum* as indicated by the presence of 262-bp fragment

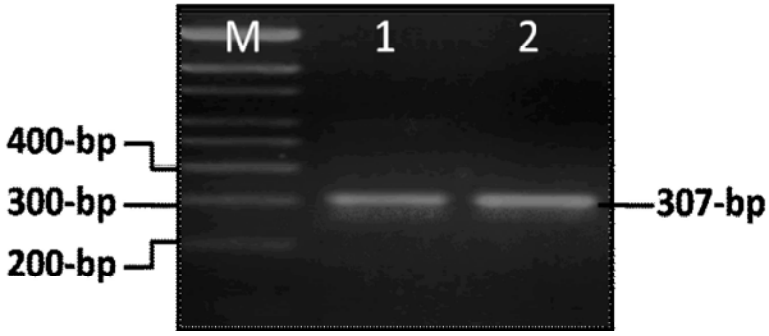

Fig. 2: Agarose gel electrophoresis of PCR products using *B. burgdorferi* sensu lato-specific primers. Positive result was indicated by generation of 307-bp fragment. Lane M, molecular size marker, 100-bp DNA ladder; lanes 1 and 2, positive human samples

DISCUSSION

In Egypt, to date and to the best of our knowledge, no work of any kind was executed to demonstrate the occurrence of *E. chaffeensis* and *E. ewingii* in both animal and human hosts. With regard to *A. phagocytophilum*, in 2004, the first preliminary molecular report addressing existence of this pathogen has recorded its occurrence in dogs and in brown dog tick (*Rhipicephalus sanguineus*) [40]. One year later, the organism has been detected in sheep and in 42-year shepherd using the highly sensitive and specific nested PCR technology [33]. Few surveys have detected the presence of anti-*B. burgdorferi* antibodies in Egyptian people [41-44] and only one molecular survey recorded its presence in acarine hosts [45]. Regarding *B. microti*, to date, 2 human cases of human babesiosis have been detected microscopically [46, 47]. In addition, the pathogen was verified by both microscopic examination and PCR in rodents [48, 49]. Farmers are occupationally exposed to tick-borne pathogens; therefore, the herein study was conducted in a trial to address occurrence of *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, *B. burgdorferi* and *B. microti* and the possibility of their coinfection in Egyptian farmers.

The high median age of human samples (39.5 year) could be attributed to the fact that this study was focused on certain occupation that requires adult persons. Although all participants showed tick exposure, none of them reported tick bite. Because of the small size of the blood sucking larval and nymphal tick stages to the level they cannot be detected and carelessness, tick bite should not be excluded.

Negative incidence of both *E. chaffeensis* and *E. ewingii* could be explained by the assumption that these *Ehrlichia* species are not present in the country or its occurrence is very rare. Although presence of *B. microti* in Egyptian rodents has been confirmed molecularly [49], all participants of this study tested negative. This could be ascribed to the fact that most cases of babesiosis occur in splenectomized and immunosuppressed people [28].

Five out of the 67 tested farmers were positive for *A. phagocytophilum*. This relatively high prevalence rate (7.5%) was expected as this pathogen was recorded before in dogs, sheep, ticks and humans in the country [33, 40]. In addition, tested farmers were in contact to many animals that could act as competent reservoirs to this organism [9-11, 15-17, 26]. Two farmers (3%) were positive for *B. burgdorferi* sensu lato while one (1.5%) showed coinfection with *B. burgdorferi* sensu lato and *A. phagocytophilum*. Two plausible explanations could account for the occurrence of concurrent infection. The first, most likely, explanation is that this person was bitten by one tick species that was coinfected and act as competent vector for these pathogens. The second, unlikely, explanation is that this farmer was bitten by two different tick species where each of them is a vector for one pathogen.

All tested farmers, even the positive ones, did not report typical clinical symptoms of the corresponding diseases. This could be attributed to that detected pathogens were variant strains that were less virulent. Therefore, comparative genomic studies should be performed with strains documented to be human pathogens in other parts of the world.

The lack of reported human cases of tick-borne infections in the country may be due to low medical awareness of these infections and the absence of well-evaluated diagnostic assays for routine detection in diagnostic laboratories. Many questions remain to be answered, such as whether the amplified sequences are the same as that detected in Egypt before in both animal and human hosts and whether the detected agents have the same genetic profile as that causing human disease.

This report revealed further evidence of *A. phagocytophilum* infection in people exposed to ticks; in addition, it recorded the occurrence of *B. burgdorferi* and its coincidence with *A. phagocytophilum* in Egyptian farmers for the first time.

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