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First Molecular Detection of *Anaplasma phagocytophilum* in *Ixodes ricinus* Ticks in Iran

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Lack of documented information and statistics about the prevalence of *Anaplasma phagocytophilum,* the causative agent of tick-borne Human Granulocytic Ehrlichiosis (HGE), in ticks, animals and also human beings in Iran, was the aim of this study to evaluate for the first time the infectivity rate of *A. phagocytophilum* in *Ixodes ricinus* ticks collected from a suburban woodland area in the eastern part of Ghaemshahr City in northern Iran. DNA was extracted from 98 unfed adult *I. ricinus* ticks (72 females and 26 males). Nested PCR Primers were designed based on *Ehrlichia* spp. 16S rDNA gene and PCR reaction was carried out. PCR product was detected using 254 nm UV Trans illuminator. For differentiation of *Ehrlichia* spp. RFLP technique was used. *A. phagocytophilum* 16S rDNA was detected in 5 (5.1%) of tested ticks. The positive ticks were 4 females and 1 male. The presence of *A. phagocytophilum* in the Iranian free-living *I. ricinus* ticks should alert our country to the possibility of HGE.

Key words: *Ixodes ricinus,* HGE, *Anaplasma phagocytophilum,* Iran

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INTRODUCTION

Anaplasma (*Ehrlichia*) *phagocytophilum, Ehrlichia chaffeensis* and *Neorickettsia* (*Ehrlichia*) *sennetsu,* the causative agents of Human Granulocytic Ehrlichiosis (HGE), Human Monocytic Ehrlichiosis (HME) and Sennetsu fever, respectively, which formerly belonged to the family Rickettsiaceae, were moved during a recent reclassification, based on phylogenetic analysis, into the family Anaplasmataceae, order Rickettsiales[1].

A. phagocytophilum and other *Ehrlichia* species which cause ehrlichioses in man and animals are gram-negative obligate intracellular bacteria. They vary from 0.2 to 1.5 μm in diameter and usually are coccoid or ellipsoid in shape. Common characteristics of *Ehrlichia* (*Anaplasma*) include their association with mammals and vectors (ticks) as part of their life cycle. *Ehrlichia* and *Anaplasma* are transmitted largely through the bite of infected ticks. *Ehrlichia* (*Anaplasma*) grow in membrane-lined vacuoles in host cells and compact clusters of growing micro-organisms comprise mulberry-shaped inclusions (morulae) that typify the genus. *Ehrlichia* (*Anaplasma*) have tropism for leucocytes[2,3]. Ehrlichioses are emerging Ixodid tick-transmitted, zoonotic infectious diseases. Human ehrlichioses include Sennetsu fever (which was identified in the 1950's)[2], HME (was first reported in 1987 and *E. chaffeensis* was isolated 4 years later)[4] and HGE (was identified in 1990 and reported in 1994)[5].

While the etiological agents of HGE and HME are distributed in the United States, Europe and Africa[6-12], the spreading of the agent of Sennetsu fever is limited to Japan and Malaysia[3]. Recently reports described the detection of *A. phagocytophilum* and *E. chaffeensis* in Asia in ticks, animals as well as humans[13-15]. The most common Ixodid ticks-vectors of HGE in the United States and Europe are *Ixodes scapularis* and *I. ricinus*, respectively[9,16].

The clinical manifestations of ehrlichioses in humans are nonspecific: fever, chills, headache, myalgia, nausea or vomiting, anorexia and weight loss[3]. The mentioned manifestations are often accompanied by following hematological and biochemical changes: pancytopenia, thrombocytopenia, increasing of ESR, CRP, ALT and AST[2].

Lack of documented information and statistics about the prevalence of *A. phagocytophilum* in ticks, animals and also human beings in Iran and observation of some farmers and stock-farmer inhabitants in suburban areas of Ghaemshahr City in northern Iran with similar clinical features and laboratory findings to HGE during recent years[17], made us study the probable infectivity rate of *I. ricinus* ticks with *A. phagocytophilum* and its role as HGE-vector for the first time in an eastern suburb of Ghaemshahr, which is a suitable biotope for *I. ricinus* tick.

MATERIALS AND METHODS

Sample collection: In spring 2003, 106 unfed adult *I. ricinus* ticks (78 females and 28 male) were collected by flagging vegetation. The collection site was a suburban forest area in the eastern part of Ghaemshahr (latitude 36°28`N and longitude 52°51`E). The tick specimens were preserved in ethanol until the DNA extraction procedure was initiated.

DNA extraction from ticks: Ticks were treated with acetic acid for 24 h and lysed with lysis buffer (150 mM Sucrose, 10 mM Tris, 5 mM $MgCl_2$, 1% Triton X-100). Reaction was centrifuged and supernatant was transferred to new tube and DNA was recovered by ethanol precipitation as described by Sparagano *et al.*[18].

PCR protocol: Nested PCR primers were designed based on *Ehrlichia* spp. 16S rDNA gene. Nest I primer: forward: Ehr1F5`-TAA CAC ATG CAA GTT GAA CGG-3` and reverse: Ehr1R 5`-CCA GTT TAT CAC TGG CAG TTT-3`.

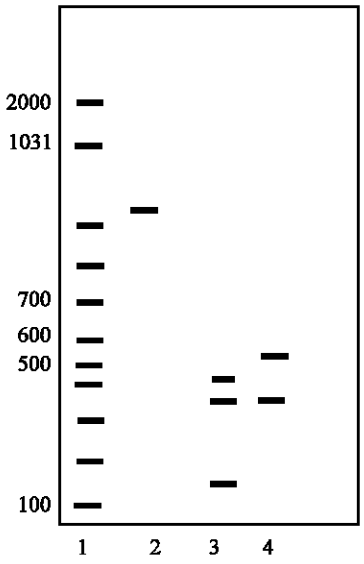

Fig. 1: RFLP by Bcn I restriction enzyme
Lane 1: 100 bp DNA ladder marker
Lane 2: 941 bp PCR product
Lane 3: Digested PCR product of *Anaplasma phagocytophilum* by Bcn I enzyme (156, 366 and 418 bp)
Lane 4: Digested PCR products of *Ehrlichia* ovis, *E. chaffeensis* and *E.* ovin (366 and 574 bp)

Nest II primers: forward: Ehr2 F 5`-GTT AGT GGC AGA CGG GTG-3` and reverse: Ehr 2 R 5`-TCA CGA CAC GAG CTG ACG-3`. The Nest I PCR reaction contained 3 μL of extracted DNA (app. 50 ng), 2.5 μL of 10X PCR buffer, 0.1 mM dNTP, 20 Pico mol each of forward and reverse primers, 1 unit of Taq DNA polymerase and deionized water up to 25 μL. A 1092 bp fragment of 16S rDNA gene was amplified. Nested II PCR reaction was done with 2 μL from PCR product as template. So a 940 bp fragment of that gene was amplified. The cycling parameters were as follows: initial pre-denaturation at 94°C for 5 min, 30 cycles consisting of denaturing at 94°C for 30 sec, annealing temperature at 64°C for 30 sec (external and internal primers) and primer extension at 72°C for 30 sec and the final extension at 72°C for 5 min as described by Newton and Graham[19].

Agarose gel electrophoresis: PCR product was electrophoresed on 1% agarose gel, stained by ethidium bromide and DNA bands were visualized at 254 nm by UV Trans illuminator.

RFLP: The PCR products of *A. phagocytophilum* were determined using restriction enzymes (Fig.1).

RESULTS AND DISCUSSION

The PCR analysis targeting the 16S rDNA gene from *Ehrlichia* spp. was carried out on 106 *I. ricinus* ticks collected in a suburban forest area in the eastern part of Ghaemshahr. DNA was successfully extracted from 98 ticks (72 females and 26 males) and solely these ticks were further investigated by PCR for the presence of *Ehrlichia* spp. 16S rDNA gene. After RFLP analysis *A. phagocytophilum* DNA was detected in 5 (5.1%) of tested ticks. The positive ticks were 4 females and 1 male (Table 1).

Wide-spreading in the world, having 3 different hosts during the developmental stage, transmission ability of some harmful agents of Arthropod-borne diseases, coexistence of multiple tick-borne pathogens in it and also sometimes being as a reservoir for numerous pathogens have all contributed a high medical importance to *I. ricinus* tick. Hence, the presence of the above tick in any location-as a contributing formative factor in some natural foci of infectious diseases-could be considered a potential hazard in nature for the health of man and animals.

Wide-leaved and mixed forests in Mazandaran province in northern Iran-similarly seen in the flora of Central Europe-make the most favorable situation for *I. ricinus* ticks to live on.

Table 1: Infectious prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks collected in a suburban woodland area of Ghaemshahr city

	Examined ticks	*A. phagocytophilum* positive	
I. ricinus gender	n	n	%
Female	72	4	5.5
Male	26	1	3.8
Total	98	5	5.1

PCR analysis of DNA extracted from tick samples has emerged as an important method for determining the presence and abundance of *A. phagocytophilum* in nature.

This study, demonstrated for the first time the infection of *A. phagocytophilum*, the causative agent of HGE, in unfed adult *I. ricinus* ticks collected from northern Iran. This finding is surprising since *A. phagocytophilum* had not already been detected in ticks in Iran.

The 5.1% infectivity of examined ticks with *A. phagocytophilum* detected in our survey is comparable to results obtained in northwestern Poland (4.5%)[20] and in Germany (4.1%)[21], but is higher than those obtained in Slovenia (3.2%)[22] and Switzerland (1.7%)[23].The studies on the prevalence of *A. phagocytophilum* in *I. ricinus* ticks in Bulgaria (34%)[24], in northeastern Poland (19.5%)[10] and in eastern Slovakia (8.3%)[25] show a higher infectivity rate than that achieved in our survey. Surprisingly, the result we obtained was identical to the study performed in Austria (5.1%)[26].

This may indicate different enzootic cycles of *A. phagocytophilum* in reservoir hosts from different geographic areas.

It may be concluded that *A. phagocytophilum* is present in northern Iran and it should alert our country to the possibility of HGE.

The infectivity of ticks to *A. phagocytophilum* confirm with a high probability the HGE in those groups of farmers and stock-farmer patients from Ghaemshahr suburban areas which had been observed with similar clinical and laboratory findings to HGE in recent years[17].

The finding of *A. phagocytophilum* in unfed adult *I. ricinus* ticks in our investigation and also horizontal transmission of this pathogen in ticks indicates the previous contamination of ticks-especially in nymphal stage-after blood sucking of reservoir hosts. On the other hand, the molecular detection of *A. phagocytophilum* in some of the surveyed livestocks from above mentioned area-that we will publish soon-confirms definitely the presence of enzootic sites of this pathogen in northern Iran; which is certainly a fact that should not be disregarded.

This finding should be noted by physicians, especially in suspected HGE patients with a history of tick-bite, similar clinical symptoms and hematological and biochemical changes due to HGE but with a negative microscopic evidence of the pathogen. Further studies are needed surely to detect *A. phagocytophilum* from humans, wild and domestic animals and also ticks in several parts of Iran to confirm its presence and capacity to cause disease.

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