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Detection of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and Spotted Fever Group Rickettsiae in ticks from the region of Sofia, Bulgaria (Acari: Parasitiformes: Ixodidae)

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Abstract: The aim of this study is to determine the prevalence of a number of bacterial pathogens in ticks from Sofia region. The data on prevalence for *Borrelia*, *Anaplasma* and *Rickettsia* in ticks can be used to assess the risk for human health of tick-borne diseases. Up to now, only a few surveys on the presence of *Borrelia* and *Anaplasma* in ticks from Bulgaria exist. Detection of *Rickettsia* spp. in ticks corresponds to the risk of tick-borne rickettsioses, because of existence of pathogenic and apathogenic rickettsiae. The high prevalence of tick-borne pathogens found revealed many cases of co-infections. Our data showed that about half of the males and one third of the tick females were simultaneously infected with two or three pathogens. Furthermore, the risk for humans to be infected becomes very high after a long stay of the tick in the skin.

Key words: *Ixodes*, *Rickettsia* spp., tick-borne diseases, co-infections

Introduction

Nowadays, tick-borne diseases are of great interest to the medical science. Lyme borreliosis is the most common tick-borne disease in the Northern Hemisphere. The etiological agent, *Borrelia burgdorferi* sensu lato, is transmitted by *Ixodes ricinus* Latreille, 1795 ticks in Europe. The complex *B. burgdorferi* sensu lato, has been divided into a number of genospecies: *B. burgdorferi* sensu stricto, *B. afzelii* Canica *et al.*, 1994 and *B. garini* Baranton *et al*., 1992 (Baranton 1992, Canica 1993, Johnson 1984). Some other species with still-questionable pathogenicity have been found in European *I. ricinus* ticks (Wang 1997, Lefleche 1997). The anaplasmosis had been a well known disease of domestic animals until 1980, but later it became associated with human infection as well. There are many reports of granulocytic anaplasmae-infected *I. ricinus* ticks – the main vector of the disease and some polymerase chain reaction (PCR) -proved cases of HGA have been reported in patients (Karlsson 2001, Tylewska-Wierzbanowska 2001).

The etiological agents of rickettsioses belong to the genus *Rickettsia* divided into two groups: the typhus group and the spotted fever group. Mediterranean spotted fever is transmitted mainly by *Rhipicephalus sanguineus* Latreille, 1806 ticks, and presents itself with tache noire, high fever, rash, headache, myalgia and arthralgia. Prevalence data for *Rickettsia* in ticks can be used to assess the risk of tick-borne disease for public health, because of existence of pathogenic and

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apathogenic rickettsiae. Up to now, only a few surveys on *Borrelia, Anaplasma* and *Rickettsia* prevalence in ticks from Bulgaria exist. The aim of this study is to determine the prevalence of the number of bacterial pathogens in ticks from the Sofia region.

Materials and Methods

The ticks were collected by flagging vegetation in the wooded area of the Sofia region in May 2005. The ticks were determined by sex and stage: 96 females, 70 males, 80 nymphs. The DNA was extracted using phenol-chloroform as described previously (Christova 2001). The ticks were mechanically homogenized in lysing buffer consisting of 10 mM Tris, 1 mM EDTA, 100 µg/ml proteinase K and 0.5% Soium dodecyl sulfate. After 1h of incubation at 60ºC and 10 min of boiling, 5 mM NaCl and 5 mM CTAB were added, and the samples were incubated at 65ºC for 20 min. DNA was precipitated with isopropanol, washed with 70% ethanol, air dried, and dissolved in 10 mM Tris, pH8. All DNA extracts were stored at -20ºC until usage. Two microliter aliquots of the tick extracts were amplified in 25 µl PCRs using *B. burgdorferi* sensu lato specific primers: LD primers (Marconi 1992), *Anaplasma* specific primers LA1/LA6 (Walls 2000) and *Rickettsia* specific primers Rick 16S For and B-Rick 16S Rev (Christova 2003b). Each PCR run included samples containing DNA of the various species as positive controls. Each PCR run also included negative controls containing PCR mix with water added instead of DNA extract. For typing of the complex *B. burgdorferi* sensu lato primers for genospecies were used: *B. burgdorfer* senso stricto BB1/BB2; *B. garinii* – BG1, BG2; *B. afzelii* – BA1/BA2. All ticks were studied for presence of *Borrelia* using dark field microscopy (D.F.M.). A detailed description of the method and its estimation was published previously (Tasseva 1999)

Results and Discussion

A total of 246 *I. ricinus* ticks were examined. The largest number of ticks harbouring borreliae was found among the females – 29% (28/96), followed by the males – 19% (13/70) (Table 1). It was lowest among the nymphs – 10% (8/80). This correlated with the data from other areas in Bulgaria (Atova 1993, Georgieva 1995) and confirmed the presence of transstadial transmission of borreliae in ticks. The largest number of *B. burgdorferi* sensu lato RCR-positive ticks was found among the males – 40% (28/70). The prevalence of the *B. burgdorferi* sensu lato complex was 35% (34/96) in females and the least – 14% (11/80) in nymphs. *B. afzelii* was the predominant species in the adults with prevalence of 19% (31/166). In the second place was *B. burgdorferi* sensu stricto species which was detected in 11% (18/166) of the adult ticks and in 3% (2/80) of the nymphs. Approximately 2% of the adult ticks and 1% of the nymphs carried simultaneously more than one *B. burgdorferi* species. Nineteen percent (32/166) of the adult ticks and 4% (3/80) of the nymphs harboured *Anaplasma phagocytophilum*.

Thirty one percent (52/166) of the adult ticks and 16% (13/80) of the nymphs were found to carry *Rickettsia* species. Our data showed good correlation between positive results from two methods: D.F.M. and PCR. The adults were more infected than nymphs. The differences were due to the different sensitivity and specificity of the two methods.

Three kinds of co-infections were found in *I. ricinus*: *Borrelia* + *Rickettsia, Anaplasma* + *Rickettsia*, and *Borrelia* + *Anaplasma*. *Borrelia* and *Anaplasma* co-infections in ticks have been reported by a number of authors (Schouls 1999, Jenkins 2001, Baumgarten 1999). Co-infections with these pathogens in patients have been confirmed by studies in the USA, Europe (Tissot-Dupont 1994, Nadelman 1997). Nineteen percent (18/96) of triple infections with agents were found in the females, 27% (19/70) - in the males, and 1% (1/80) - in the nymphs. Up to now only a few surveys on *Borrelia* and *Anaplasma* prevalence in ticks from Bulgaria have been carried

Table 1. Distribution of *Borrelia, Anaplasma and Rickettsia* species in *Ixodes ricinus* ticks.

	No (%)	of	ticks					
Positive results:	Female (n=96)		Males (n=70)		Adults (n=166)		Nymphs (n=80)	
D.F.M. *Borrelia*	28	(29)	13	(19)	41	(25)	8	(10)
PCR *Borrelia burgdorferi* sensu lato	34	(35)	28	(40)	62	(37)	11	(14)
PCR *Borrelia burgdorferi* sensu stricto	8		10		18		2	
PCR *Borrelia afzelii*	18		13		31		4	
PCR *Borrelia garinii*	5				5		5	
PCR *Borrelia* unspeciated	3		5		8			
PCR, ticks, infected with two *Borrelia* species	3				3		1	
PCR *Anaplasma phagocytophilum*	25	(26)	7	(10)	32	(19)	3	(4)
PCR *Rickettsia* species	23	(24)	29	(41)	52	(31)	13	(16)
PCR *Borrelia* + *Rickettsia*	8	(8)	7	(10)	15	(9)	2	(3)
PCR *Anaplasma* + *Rickettsia*	4	(4)	10	(14)	14	(8)	2	(3)
PCR *Borrelia* + *Anaplasma*	3	(3)	1	(1)	4	(2)		
PCR *Borrelia* + *Rickettsia* + *Anaplasma*	18	(19)	19	(27)	37	(22)	1	(1)

out (Christova 2001, 2003b). The high prevalence of tick-borne pathogens found revealed many cases of co-infections. Our data showed that about half of the males and one third of the females were infected simultaneously with two or three pathogens. Furthermore, the risk of human infection is very high due to the long stay of ticks in the skin.

However, co-infections with three microorganisms – *Borrelia, Anaplasma* and *Rickettsia* were found more frequently than co-infections with two microorganisms (Christova 2003a). There was a remarkable difference between prevalence rates established for the different sexes of the adult ticks. It is unclear what is causing these changes and whether they have any biological significance. Changes in prevalence are probably determined by many factors: animal reservoirs, temperature, humidity, etc. *B. afzelii* was the most common *Borrelia* species in all ticks that correlates with the most common clinical manifestation of late Lyme borreliosis in Bulgaria - neuroborreliosis (Stoilov 1995). Some of the *Ixodes* ticks were co-infected with different genera and/or species of the same genus. These ticks may be infected with multiple tick-borne pathogens which predetermines a possibility of simultaneous transmission during a single tick bite. The results of this

study show that many tick-borne diseases are probably endemic in Bulgaria. Further investigations based on molecular-biological methods will be useful to reveal the regional characteristics of these microorganisms.

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Установяване на *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* и рикетсии от групата на петнистите трески в кърлежи (Acari: Parasitiformes: Ixodidae) от района на град София (България)

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(Резюме)

Целта на проучването е да установи разпространението на някои бактериални патогени в кърлежи от района на град София. Данните за наличието на *Borrelia*, *Anaplasma* и *Rickettsia* в тях са от голямо значение при оценката на риска от заразяване на човека със съответната инфекция, предавана чрез кърлежите. До момента проучвания за заразеността на кърлежите с *Borrelia* и *Anaplasma* в страната са доста оскъдни. Установяването на видове от род *Rickettsia* съответства на риска от причинените от кърлежи рикетсиози. Големият брой патогени показва наличието на взаимно заразяване. Резултатите показват още, че половината от мъжките и една трета от женските кърлежи са заразени с два или три инфекциозни агента едновременно. Особено висок е рискът за заразяване на човека при дълъг престой на кърлежа върху кожата.