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PECULIARITIES OF CADMIUM-TOLERANT POPULATIONS OF *IXODES* TICKS: SPECIFICITY OF THEIR MICROBIOCENOSES, IMMUNITY AND VECTOR CAPACITY

ОСОБЕННОСТИ ТОЛЕРАНТНЫХ К КАДМИЮ ПОПУЛЯЦИЙ КЛЕЩЕЙ РОДА *IXODES*: СПЕЦИФИЧНОСТЬ ИХ МИКРОБИОЦЕНОЗОВ, ИММУНИТЕТА И ВЕКТОРНОЙ СПОСОБНОСТИ

A. N. Alekseev[1], H. V. Dubinina[1], P. A. Chirov[2], A. M Peterson[2], M. A. Turtseva[2]
А. Н. Алексеев[1], Е. В. Дубинина[1], П. А. Чиров[2], А. М. Петерсон[2], М. А. Турцева[2]

[1]Zoological Institute, Russian Academy of Sciences, 199034 St. Petersburg, Russia
[2] Saratov State University, 410026 Saratov, Russia
[1]Зоологический институт, Российская академия наук, 199034 Санкт-Петербург, Россия
[2]Саратовский Государственный Университет, 410026 Саратов, Россия

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ABSTRACT

The appearance and prevalence of exoskeleton anomalies in *Ixodes* ticks chosen as markers of anthropogenic press allows to reveal their distribution and the rate of occurrence in Russia and several Western European countries. Chemical analyses show the appearance of anomalies as being related to the accumulation of heavy metal ions, first of all cadmium which prevails in specimens and is known as changing tick metabolism. *Cd* concentrations are 1.5–2 times higher in anomalous ticks than in normal ones, cadmium supposedly suppressing tick immunity. To provide indirect evidence of this, microflora of anomalous and normal ticks has been compared, with 73 hungry tick females collected in April 2003 and 2004 in a focus of tick-borne diseases near St. Petersburg, Russia screened microbiologically. A study of "vulgar" microflora in both parts of the *Ixodes persulcatus* Schulze population shows the whole microbiocenosis of anomalous and normal ticks as being quite different. Microflora of Gram-negative and Gram-positive bacteria, bacilli, cocci and fungi appears to be much richer in anomalous ticks than in normal ones (47 versus 28 species). Only 14 (of 61) species representing the "vulgar" microflora are coincided in both groups of ticks. Not more than 4 species of microorganisms are present per normal tick, whereas one tick with exoskeleton anomalies can contain 5, 6 or even 7 species simultaneously. Only in anomalous ticks do the cocci of the genus *Staphylococcus* and the fungi of the genus *Penicillum* absolutely prevail. Staphylococci are known as active stimulants of lysozyme and defensin production, both the main tools of the tick immune system. Scarcity of these cocci and fungi in normal ticks and their abundance in anomalous ones, as well as the great prevalence of multi infections in anomalous ticks are accepted as markers of their immune system suppression. Revised (6,169 adult ticks screened using PCR; 1996–2000) data concerning the prevalence of tick-borne multi infections (such as mixtures of *Borrelia*, *Ehrlichia*, *Anaplasma*, *Babesia* or tick-borne encephalitis virus) confirm that exoskeleton anomalies represent external markers of cadmium accumulation, increased tick vector capacity and dangerousness. Analysis of anomalous tick microflora supports this observation as well, because only among anomalous tick numerous phyto- or zoopathogenic bacteria and, especially, fungi have been revealed, which can be very dangerous for man with an immune system suppressed by any factor or associated disease.

РЕЗЮМЕ

Полученные ранее авторами данные позволили связать загрязнение внешней среды ионами тяжелых металлов с наличием в популяциях клещей рода *Ixodes* особей с аномалиями экзоскелета. Установлено, что основной причиной этого феномена является

накопление в аномальных особях ионов кадмия, количество которого в них в 1.5–2 раза превышает таковое в нормальных особях. Встречаемость таких морфологически измененных особей в разных регионах России год от года растет; обнаружены они и в западной Европе. Более частая встречаемость в аномальных особях клещевых патогенов позволила предположить, что кадмий угнетает иммунную систему аномальных клещей. Для проверки этого положения косвенным путем было проведено микробиологическое исследование "вульгарной" микрофлоры в обеих группах клещей *Ixodes persulcatus* Schulze, собранных в апреле 2003 и 2004 гг. в очаге клещевых инфекций под Санкт-Петербургом (73 голодных самки). Микрофлора Грам-отрицательных и Грам-положительных неспорообразующих бактерий, бацилл и грибов оказалось гораздо более богатой именно у аномальных особей: 47 видов по сравнению с 28 — у нормальных. Только 14 из общего числа идентифицированных видов (61) оказались общими для обеих морфологических групп клещей. Ни в одной нормальной особи не было обнаружено более 4-х видов микроорганизмов одновременно, тогда как в аномальных клещах находили 5, 6 или даже 7 видов. Только в аномальных клещах абсолютно превалировали стафилококки и грибы рода *Penicillum*. Полученные данные свидетельствуют о подавленности иммунитета у аномальных, толерантных к кадмию представителей этой части популяции таежных клещей. Пересмотр накопленных за предыдущие годы данных о встречаемости множественного заражения *I. persulcatus* специфически клещевыми, вызывающими заболевания людей патогенами (за 1996–2000 гг. методом ПЦР исследовано 6,169 взрослых клещей) полностью подтвердило эту гипотезу. Встречаемость смешанных инфекций оказалась достоверно выше у аномальных клещей по сравнению с нормальными, что свидетельствует о том, что аномалии экзоскелета являются внешним маркером увеличения векторной способности представителей этой части популяции и ее опасности для людей. Анализ видового состава микробов и грибов у представителей аномальной части популяции показал, что значительное число этих микроорганизмов является потенциально патогенной как для растений, так и для животных и людей, особенно при наличии у них сопутствующих инфекций и явлений, характерных для иммунодефицита.

INTRODUCTION

Multiyear monitoring of the populations of *Ixodes persulcatus* Schulze in the vicinities of St. Petersburg (since 1992), later (2002–2004) both extended over the whole area of the tick's distribution in Russia and accompanied by stereomicroscopic studies, allowed to reveal and describe some anomalies of tick exoskeleton structure [Alekseev, Dubinina, 1993, 1994, 1996; Dubinina, Alekseev, 1994]. Then both the prevalence and abundance of anomalous ticks were shown as tending to increase from year to year along with a growing rate of environment pollution, first of all in relation to traffic intensity [Alekseev, Dubinina, 2002]. The same was observed when, since 1995, monitoring of *Ixodes ricinus* (L.) had started on the Curonian Spit, Kaliningrad Region, Russia. Later some additional observations were made in several Western European countries. As a result, ticks with exoskeleton anomalies appear to be widely distributed all over Russia from the Kaliningrad Region to the Far East. The same concerns Western Europe, such as England, Denmark and, very probably, also Germany. The prevalence of anomalous ticks ranges from 12 to 50% in different regions and, as mentioned above, it grows from year to year all over the territories monitored.

In *I. persulcatus*, seven agents pathogenic to man have hitherto been revealed: *Borrelia afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto, the agents of different kinds of borreliosis; *Ehrlichia muris*, the agent of monocytic ehrlichiosis; *Anaplasma phagocytophilum*, the agent that causes granulocytic ehrlichiosis; *Babesia microti*, the agent of babesiosis; and tick-borne encephalitis virus [Alekseev et al., 2003]. Nearly the same set of pathogens, with the exception of *Babesia microti*, has been found in *I. ricinus* from the Kaliningrad Region [Alekseev et al., 2001].

As similar exoskeleton deformations had been observed in experiments with heavy metal ion accumulations affecting the oribatid mite *Opiella nova* (Jacot) [Dubinina, Alekseev, 1994], the exoskeleton anomalies observed in *I. persulcatus* and *I. ricinus* were suggested as having the same roots [Alekseev, Dubinina, 1996]. Modified flame chromatography used for estimating the content of heavy metal ions in normal and anomalous ticks confirm the above assumption [Dubinina et al., 2004]. The ratios of *Cd* ion content in anomalous specimens to normal ones are as follows (mg/kg): Denmark (Zealand), 6.93/5.52; Kaliningrad Region (Curonian Spit), 19.44/3.6; Novgorod, 5.6/

2.93; St. Petersburg, 5.56/3.29; Siberia, Irkutsk, 7.59/4.44 and Listvyanka, 40.6/23.64; Far East, Vladivostok, 5.29/3.63. These figures show that the amount of *Cd* in anomalous ticks is 1.5–2 times higher than that in normal ones. The capability of *Cd* ion accumulation is inherited in the progeny of *I. ricinus* parents with exoskeleton anomalies. Both normal and anomalous specimens of F_1 show high levels of *Cd* contamination even when reared in clean laboratory conditions. The greater the content of *Cd* in the parent, the greater the progeny with exoskeleton defects. For example, F_1 of one such female embodied 93% of anomalous specimens and only 7% of F_1 specimens did not have them. The body of their mother after egg-laying still contained 16.91 mg/kg of cadmium.

It is noteworthy that about 30% of F_1 of the normal parents also show exoskeleton anomalies. And the rate of such specimens is the greatest among the "children", whose mothers contained the greater amounts of this metal in their bodies. Only normal progeny of normal parents are free from detectable amounts of *Cd*.

This shows that *Cd* affects the mechanism of tick heredity, most probably the transfer of tolerance being a recessive character [Dubinina et al., 2004]. Hence, cadmium accumulation unequivocally changes tick heredity, metabolism, vector capacity and immunity. Tick vector capacity can be estimated by comparing the prevalence of dual or triple infection among anomalous and normal ticks. This task was partly achieved by Semenov [2003], who analyzed material obtained in 2000; it will be revised here on the basis of a more representative dataset. To prove that tick immunity has altered, an indirect method has been applied, with tick microflora analyzed for this purpose.

Studies on tick immunity mechanisms enjoy a long history. Hindle & Duncan [1925] and, later, Duncan [1925] were the first to continue Flemming's [1922] pioneer work concerning the lysozyme bacteriolytic properties of the gut content in the soft tick, *Argas persicus* (Oken). Anigstein et al. [1950] dissected the guts of some Ixodidae and confirmed the data that Hindle & Duncan [1925] had obtained using Argasidae. The biochemistry of gut lysozymes both of argasid and ixodid ticks has been deciphered by Podboronov [2004]. Precise characteristics of the gut lysozyme of the soft tick, *Ornithodoros moubata* (Murr.) are available in Kopacek et al. [1999]. Nakajima et al. [2002] show that antibacterial peptid defensin is involved in the midgut immunity of *O. moubata.*

To prove that increased amounts of hemocytes and soluble proteins are indicator of the tick immune system's response, most of the authors study the immune mechanisms of ticks with the use of injections of various materials. These include absolutely foreign substrates or microorganisms, such as *Escherichia coli* or *Pseudomonas aeruginosa* [e.g. Johns et al., 1998]. Earlier, however, Kryuchechnikov [1991] showed the abundance of hemocytes to increase following a *Rickettsia conori* inoculation in the hemocoel of soft ticks. Ceraul et al. [2002] suggest that also encapsulation/nodulation can be an important component of the immune response in ticks, but they, too, inoculated such bacteria foreign to hard ticks as *Escherichia coli.*

According to Johns et al. [2000], only increased amounts of hemocytes and soluble proteins play a real role in the deactivation of *Borrelia* in the hemolymph and other tissues of the American tick, *Dermacentor variabilis* (Say), which is incapable of transmitting the borreliosis agent. Being destroyed in the hemolymph, salivary glands and ovaries, *B. burgdorferi* B31 are unable to colonize saliva and to be transmitted by this tick. Nevertheless, according to Rudakova et al. [2004], *Dermacentor reticulatus* (Fabr.) in West Siberia might play a role in borreliosis affection, as both transovarial and transphasic pathways of transmission of *B. afzelii* do exist. According to Johns et al. [2001], there is a great contrast observed in tick innate immune responses to *B. burgdorferi* challenge. Thus, immunotolerance existing in *Ixodes scapularis* Say contrasts immunocompetence in *Dermacentor variabilis.* These observations contradict those of Rudakova et al. [2004] obtained for *D. reticulatus,* but this can be a result of differences in the biology and ecology of the American and Eurasian ticks, even though both are congeners. Not only hemocytarian reactions ensure tick immunity, but also defensins, the specific peptides present in the gut of hungry ticks; defensin activity and abundance grow after bacterial absorption or injection. The greatest reaction has been observed following a contact with *Staphylococcus aureus* [Nakajima et al., 2002]. Data concerning the "normal", or "vulgar", microflora of hungry ticks are very scarce.

The first successful attempt to detect and describe conditionally pathogenic and saprophytic microorganisms, by which *Dermacentor* ticks (as well as horseflies) are spontaneously infected, was made quite recently (Chirov et al., 2003, 2004) in the Povolzh'e region (region of lower riches of the Volga river, Russia). The authors showed up the complex of associations of bacteria belonging to the different genera.

Nevertheless most of the authors look for and describe the bacteria and/or fungi that could be used in the biological control of ticks as vectors of various diseases [Cherepanova, 1964; Zhioua et al., 1999]. No-one seems to have ever tried to investigate the common microflora of intact hungry ticks in order to estimate their immunocompetence, especially in the specific conditions when new subpopulations of ticks appeaı under the heavy anthropogenic press. The objective of this study is to see if, together with the other differences expressed by anomalous ticks in determining the varied vector capacities of both parts of the tick population and mirroring their immunocompetence, their microflora proves different as well.

MATERIAL AND METHODS

Ticks. *Ixodes persulcatus* adult ticks were taken in the St. Petersburg recreation zone long known as a tick-borne encephalitis and borreliosis focus (forests near Morskaya, Lisiy Nos) from April to July in 1996 to 2000 to study them using the polymerase chain reaction (PCR) technique in order to detect tick-borne encephalitis virus (TBEV), pathogenic borreliae, ehrlichiae and *Babesia*. A total of 6,169 adult ticks were collected and screened. Seventy-three females were collected in April 2003 and 2004 to study all kinds of their supposedly non-pathogenic microflora: bacteria, bacilli, cocci and fungi.

Pathogen detection. *Sample preparation for the detection of agents pathogenic to man.* Adult ticks were rinsed in 70% alcohol, then dried, frozen in liquid nitrogen and crushed. To every sample, 200 μl of sterile H_2O, 3 μl of a tRNA solution and 200 μl 4M guanidine thiocyanate were added, followed by 10 min incubation at 0° C. After incubation, 200 μl of a phenol:chloroform:isoamyl (25:24:1) solution were added. Samples were frozen at –30° C and centrifuged for 4–5 min at 10,000 rpm. The supernatant was transferred into a polypropylene tube and mixed both with 1/10 vol of 2M sodium acetate (pH 4.5) and an equal volume of isopropyl alcohol. Samples were frozen in liquid nitrogen, then thawed, and centrifuged for 10 min at 15,000 rpm. The supernatant was removed carefully. RNA/DNA pellets were washed with 0.5 ml of a 75% PCR assay.

The primers LD1–HME–3R were supplied by DNA Technology ApS (Aarchus, Denmark), the primers BAB I–TBEVf and PIRO–A–B by ASB–Viriology (St. Petersburg, Russia); dNTP, Taq-polymerase, RT-set, and buffers were obtained from "DNA-Technology" and MBI "Fermentas", Russia. The specific primers and PCR techniques have been described in due detail elsewhere [Alekseev et al., 2003].

Sample preparation for the detection of conditionally pathogenic agents and saprophytic microorganisms. All females were first rinsed in 70% alcohol, then twice in sterile bi-distilled water and then, still alive, crushed individually in sterile porcelain vials, with 0.5 ml of a saline solution added. This dilution was accepted as 1:10, because this experiment used only one half of the tick body, the other half was kept in 70° ethanol to further detect in the very same specimens the agents pathogenic to man. The suspension obtained was titrated using the tenfold dilution until 10^{-9} and then 0.1 ml of every dilution was used as seeding material for insemination of agar in Petri dishes. Different culture media were used: meat-peptone agar (GRM-AGAR, Obolensk, Russia), meat-peptone glucose glycerine agar (GRM-AGAR + 1% glucose + 2% of glycerine), Endo-Agar-GRM (Obolensk, Russia), for fungi the Saburo medium. All seeds were incubated at 28° and 37° C. Both species-specificity and pathogenicity of the so-called "vulgar" microflora were determined using the methodology recommended by "Bergey's Manual of Systematic Bacteriology"([1997], improved by Holt et al. [1997]; Dobler, Braveny [2003] and "Change-over in taxonomy…", [2004]) and the "Guide to Clinically Significant Fungi" [Sutton et al., 2001].

RESULTS

As mentioned above in the "Material and methods" part, material for an analysis of non-pathogenic flora was taken during April 2003 and 2004. The use of only April samples was deemed representative enough, since, according to the previous observations, anomalous ticks were distributed in the spring and summer more or less alike, whereas the seasonal abundance of *I. ricinus* was quite distinctive (Table 1).

The results of the microbiological studies are presented in Tables 2–4.

As can be seen from Table 2, the number of ticks, and of species of Gram-negative bacteria, is very similar in both groups of *I. persulcatus*, but only three cases contained the same species of bacteria: *Enterobacter agglomerans* (3 and 5), *Pseudomonas pseudoalcaligens* (3 and 1) and *Pantoea dispersa* (1 and 2). Gram-positive spore negative

Table 1

Prevalence of two species of *Ixodes* ticks with exoskeleton anomalies in different periods of activity

Таблица 1

Встречаемость двух видов клещей рода *Ixodes* с аномалиями наружного скелета в разные периоды их активности

Time of tick collection (periods of tick activity))	Prevalence, %							
	Ixodes persulcatus (St.Petersburg Region, 1988–2000)				*Ixodes ricinus* (Kaliningrad Region, 1995–2004)			
	No of ticks	min	max	average	No of ticks	min*	max*	average
Spring (April, May)	1643	23.9	37.2	28.7±2.1	1862	10.4	41.6	21.3±5.9
Summer (June, July)	749	17.8	40.0	28.1±3.1	n.d.	–		
Autumn (September)	–**	–			682	16.3	54.8	29.2±5.7
Value of *p*	–	0.86			–	0.046		
Statistical significance	–	Insignificant				Significant, $p<0.05$		

n.d. — no data, minimal abundance of *I. ricinus*; * minimal prevalence of anomalies in 1995–1996, maximal in 2004; ** *Ixodes persulcatus* virtually absent in autumn.

Table 2

Prevalence of various species of bacteria and bacilli in *Ixodes persulcatus* ticks with (A) or without (N) exoskeleton anomalies

Таблица 2

Встречаемость разных видов бактерий и бацилл в клещах *Ixodes persulcatus* при наличии (A) или отсутствии (N) аномалий наружного скелета

Characteristic of the microorganism group								
Gram-negative bacteria			Gram-positive spore negative bacteria			Bacilli		
Species	No of cases		Species	No of cases		Species	No of cases	
	A	N		A	N		A	N
Acetobacter pasterianus	–	1	*Arcanobacterium* sp.[4)]	1	–	*Bacillus alcaligens*	–	1
Alcaligenes xylosoxidans	1	–	*Arthrobacter globiformis*	2	1	*Bacillus brevis*	2	–
Alcaligenes fecalis [1)]	–	2	*Arthrobacter* sp.	1	1	*Bacillus cereus*	1	–
Budvicia aqutica	–	4	*Aureobacterium barkeri*	–	7	*Bacillus circulans*	2	–
Enterobacter agglomerans [1)]	3	5	*Cellulomonas gelida*	–	2	*Bacillus coagulans*	–	3
Francisella novicida [2)]	3	–	*Curtobacterium plantarum* [3?)]	1	–	*Bacillus firmus*	2	–
Pantoea dispersa [1)]	1	2	*Curtobacterium pussilum* [3?)]	1	–	*Bacillus marinus*	1	–
Pseudomonas cichorii [3)]	4	–	*Lactobacillus bulgaricus*	2	–	*Bacillus mycoides*	1	1
Pseudomonas pseudoalcaligens [3)]	3	1	*Lactobacillus casei tolerans*	2	–	*Bacillus pumilis*	2	–
Pseudomonas sp.	1	–	*Microbacterium lacticum*	2	2	*Bacillus subtilis*	1	1
Weeksella sp.	1	–	*Mycobacterium komossense*	1	–	*Bacillus sphericus*	1	–
Gram-negative trivial bacteria gen.sp.	–	1						
Total No of species	12			11			11	
Total No of ticks with various species of microorganisms	17 in 8	16 in 7		13 in 9	13 in 5		13 in 9	6 in 4
Coincidence (No of cases)	3			3			2	

bacteria were revealed in anomalous ticks 13 times with 9 different species of microorganisms, whereas the same number of normal specimens contained only 5 species. Both types of tick contained the same species of bacteria only 3 times: *Microbacterium lacticum* (2 and 2), *Arthrobacter globiformis* (2 and 1), and *Arthrobacter* sp. (1 and 1). The difference in the type of anomalous versus normal tick infection by the microbial genus *Bacillus* was much greater than that in two previous cases. Thirteen anomalous ticks were infected by 9 species of bacilli, whereas only 6 normal specimens by 3 species of these microorganisms. A coincidence (infection by *Bacillus mycoides* and *Bacillus subtilis*) was observed only 2 times (in both cases 1 and 1). Among Gram-negative bacteria, *Francisella novicida,* known to infect laboratory animals, was discovered 3 times, only in anomalous ticks though. The identified species of the bacterial genus *Pseudomonas*, which presumably are phytopathogenic, occurred in anomalous ticks more often (7 versus 1). *Alcaligenes fecalis*, *Enterobacter agglomerans* and *Pantoea dispersa*, which sometimes cause opportunistic infections, were detected alike in both groups of ticks.

Despite the numerous ticks analyzed, in which different kinds of cocci were revealed in anomalous (47) or normal (36) specimens, not all of them contained at least one species of cocci (Table 3). For example, of the total of 73 ticks investigated, only 35 were normal. This means that most of the ticks contained more than one species of these microorganisms. Often enough in one 2, 3 or even 4 species were discovered. It is of interest that *Micrococcus luteus* occurred in 16 normal specimens, more than 2 times more often than in anomalous ticks (7). In case of a coccal infection, the number of coincidences of their prevalence in both kinds of ticks was greater than that in cases of infection by other microorganisms (5 versus 1, 2, 3). It is necessary to emphasize that *Staphylococcus* infections mainly occurred among anomalous ticks: 8 times by 4 species of these cocci. In one case, an anomalous specimen contained simultaneously two species of these microorganisms known as inciters of defensins, the peptides that represent one of the elements of the immune system in arthropods. Some species of *Staphylococcus*, e.g. *S. cohnii*, can cause human or animal opportunistic infections, some can eject extracellular toxins; 3 anomalous ticks contained this coccal species, but none of the normal specimens.

Among 11 species of fungi revealed in the ticks, only one species, *Penicillum citrinum*, occurred both in anomalous and normal specimens. *P. citrinum,* being known as the agent of keratitis, also involved in cases affecting the urinary track, was revealed among anomalous ticks 15 times more often and detected among normal specimens only once. Species of the fungal genus *Aspergillus* were discovered in anomalous ticks 8 times, versus once among normal ticks. All *Aspergillus* are agents of one or another human disease or at least causing secondary infections from among patients with different kinds of health affections. *Penicillum* fungi identified to the species level were discovered 19 times versus 2, also largely amongst ticks with exoskeleton anomalies. *Aspergillus nidulans, Asp. niger, Asp. restrictus, Cladosporium* sp., *Ochroconis humicola, Penicillum citrinum, P. janthenellum* were revealed only among anomalous ticks.

As many as 23 of 30 (56.7%) anomalous ticks were infected by zoo- or phytopathogenic Gram-negative or Gram-positive bacteria, whereas only 10 normal specimens of 29 (34.5%) contained the same microorganisms. The difference in the rate of infection by fungi pathogenic to man or animals was much greater: 79.3% (23 of 29) were revealed among anomalous ticks and only 20% (1 of 5) were detected in normal ones.

The greatest commonality was observed in the cases involving anomalous ticks: from 63.6% (fungi) to 87.5% (cocci). The maximal similarity index for the microorganism species in the case of normal ticks amounted to only 58.3 % (Table 4), being observed among Gram-negative bacteria.

The coincidences of infection in both types of ticks (Fig. 1) range between 31.3% (cocci) and 9.1% (fungi). It is noteworthy that, of the microorganism species totaling 61, the number of coincided ones amounted to some 14, whereas of the differing ones to as many as 47.

In most of the ticks, several species of microorganisms were detected. Only once it was not

Notes for Table 2:

1) — sometimes can play a role as a source of opportunistic infections and can be discovered in blood, urine and gleeting wounds;

2) — can cause experimental animals' infection in the laboratory;

3) — phytopathogenic bacteria;

3?) — possible phytopathogenic bacteria;

4) — man and livestock obligate pharynx parasites, sometimes agents of pharynx or skin inflammation.

Table 3

Prevalence of various species of cocci and fungi in *Ixodes persulcatus* ticks with (A) or without (N) exoskeleton anomalies

Таблица 3

Встречаемость разных видов кокков и грибов в клещах *Ixodes persulcatus* при наличии (A) или отсутствии (N) аномалий экзоскелета

Characteristic of the microorganism group					
Cocci			Fungi		
Species	No of cases		Species	No of cases	
	A	N		A	N
Deinococcus radiophilus	1	–	*Aspergillus nidulans* [2]	3	–
Deinococcus sp.	2	1	*Aspergillus niger* [3]	1	–
Dermacoccus nishinomiyaensis	–	1	*Aspergillus restrictus* [4]	4	–
Kocuria kristinae	10	–	*Aspergillus* sp.	–	1
Kocuria rosea	8	9	*Cladosporium* sp.	1	–
Kytococcus sedentarius	3	–	*Ochroconis humicola*	1	–
Marinococcus halophilus	1	4	*Penicillum chrisogenum*	–	1
Micrococcus agilis	1	–	*Penicillum citrinum* [5]	15	1
Micrococcus luteus	7	16	*Penicillum janthenellum*	4	–
Micrococcus varians	3	3	*Penicillum* sp.	–	1
Staphylococcus cohnii [1]	3	–	*Ulocladium* sp.	–	1
Staphylococcus caprae	1	–			
Staphylococcus delphini	–	2			
Staphylococcus saprophyticus	3	–			
Staphylococcus xylosus	1	–			
Cocci gen.sp.(20, 21, 113)	3	–			
Total No of species	16			11	
Total No of ticks with various species of microorganisms	47 in 14	36 in 7		29 in 7	5 in 5
Coincidence (No of cases)	5			1	

[1] — some species of *Staphylococcus* can cause human or animal opportunistic infections, some can eject extracellular toxins;
[2] — various infections of man and animals are known, including 4 well documented cases of deep mycosis from among patients with chronic granulomatosis;
[3] — most often this species is the agent of secondary infections from among patients with pulmonic diseases and an attenuated immune system;
[4] — this species is very often associated with endocarditis, onchomycosis and affections of the lungs;
[5] — identified in one case of pulmonic infection, in several cases of keratitis and at affections of the urinary track.

possible to reveal any representative of "vulgar" microflora either in anomalous or normal specimen of *I. persulcatus.* Figure 2 illustrates examples of the difference between the types of multi infection of normal and anomalous ticks. Three-four microorganisms were met with in both types of ticks virtually with the same frequency, whereas specimens with 5–7 species of microorganisms were only revealed amongst the anomalous part of the study tick groups. Based on these data, it can hardly seem strange that the previously obtained results of the prevalence of multi infections in anomalous ticks by tick-borne agents pathogenic to man was significantly greater than that among normal ticks.

During five years of research (1996–2000), the PCR techniques used have allowed to build up a data bank concerning the prevalence and types of multi infection anomalous and of normal ticks in the *I. persulcatus* population in the St. Petersburg focus of tick-borne infection. Both types of microorganisms pathogenic to man, extracellular as well as obligate intracellular parasites of the tick vector, appear to occur in couples or triples mainly in anomalous specimens (Table 5). The difference is statistically significant.

As can be seen from Table 5, not only numerous representatives of the so-called "vulgar" microflora prevailed in anomalous specimens, but also extra- and intracellular parasites pathogenic to man, which can provoke different tick-borne diseases. Combinations of multi infections were discovered 2.2 times more often, when anomalous

Table 4

Commonality of microbes occurring in different groups of ticks

Таблица 4

Соответствие видов микробов в разных группах клещей

Microorganisms			Characteristic of ticks		
Group	Values	Total No of species	Anomalous	Normal	Anomalous and normal together
Gram-negative bacteria	Abs	12	8	7	3
	%	–	66.7	58.3	25.0
Gram-positive spore negative bacteria	Abs	11	9	5	3
	%	–	81.8	45.5	27.3
Bacilli	Abs	11	9	4	2
	%	–	81.8	36.4	18.2
Cocci	Abs	16	14	7	5
	%	–	87.5	43.8	31.3
Fungi	Abs	11	7	5	1
	%	–	63.6	45.5	9.1
TOTAL		61	47	28	14

Table 5

Prevalence of multi (dual or triple) infections in *Ixodes persulcatus* ticks with or without exoskeleton anomalies (PCR techniques of pathogen detection, 1996–2000)

Таблица 5

Встречаемость множественных форм заражения (как двойных, так и тройных) в клещах *Ixodes persulcatus* с аномалиями наружного скелета и без них (патогены определены методом ПЦР, 1996–2000 гг.)

Year of study	Types of parasite cooperation							
	Extracellular parasites (pathogenic borreliae only)				Intracellular parasites with extracellular ones (ehrlichiae and borreliae, babesiae and borreliae, TBE virus in any kind of combination)			
	ticks with anomalies		tick without anomalies		ticks with anomalies		tick without anomalies	
	abs*	%	abs*	%	abs*	%	abs*	%
1996	1/23	4.3	2/77	2.6	–	–	–	–
1997	4/30	13.3	5/62	8.1	–	–	–	–
1998	15/150	10.0	27/466	5.8	2/150	1.3	5/466	1.1
	–	–	–	–	–	–	3/466	0.6
	–	–	–	–	–	–	1/466	0.2
1999	2/137	1.5	2/351	0.6	–	–	1/351	0.3
	–	–	–	–	–	–	1/351	0.3
	–	–	–	–	–	–	1/351	0.3
2000	25/439	5.7	38/952	3.9	4/439	0.9	4/952	0.4
	5/439	0.7	3/952	0.3	10/439	2.3	27/952	2.8
	–	–	–	–	4/439	0.9	5/952	0.5
	–	–	–	–	2/339	0.8	5/806	0.6
	–	–	–	–	5/439	1.1	1/952	0.1
	–	–	–	–	7/439	1.6	3/952	0.3
Total No of ticks	758	–	1908	–	928	–	2575	–
Mean values of prevalence	–	5.9±2.0	–	3.6±1.2	–	1.3±0.2	–	0.6±0.2
p value	<0.05				<0.05			

* Different numerators with one and the same denominator mean that, in the same group of ticks analyzed, different combinations of pathogens occurred, e.g. *B. afzelii* or *B. garinii* with one or another species of *Ehrlichia, Borrelia* with *Babesia* or with TBE virus etc.

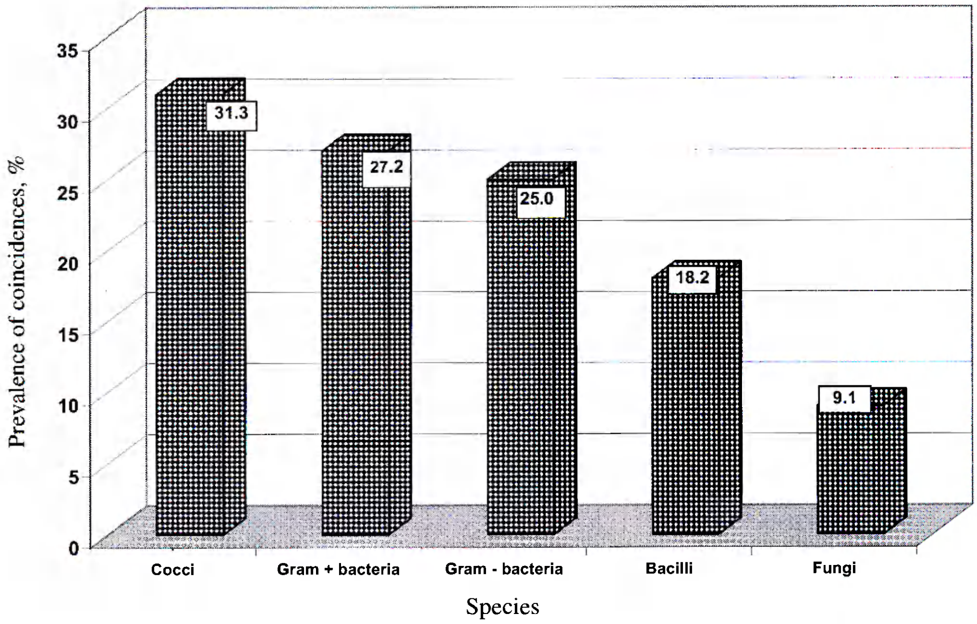

Fig. 1. Proportions of microorganism species detected simultaneously in anomalous and normal ticks.
Рис. 1. Доли встречающихся микроорганизмов, которых обнаруживали одновременно в клещах с аномалиями наружного скелета и без них.

specimens were infected by intracellular parasites together with extracellular ones simultaneously: ehrlichiae and borreliae or babesiae and borreliae, TBEV and any kind of mixtures of other pathogens.

The difference in the prevalence of combinations of three *Borrelia* species, which occurred in anomalous and normal specimens was more modest, 1.6 times, yet statistically significant.

DISCUSSION

An analysis of all previously obtained evidence and the data presented in this communication allows to conclude that the immune system of anomalous ticks tolerant to cadmium is suppressed. According to Jollés [1964], lysozyme as one of the main tools of the tick defense system is active against Gram-positive bacteria, being destructive for its cell membrane by hydrolyzing its peptidoglycan layer, but not active against Gram-negative bacteria. The lesser activity of this enzyme in anomalous ticks might account for the number of species of Gram-positive bacteria in normal ticks being smaller than among anomalous ones (5 versus 9). Gram-negative bacteria dominated the microflora of anomalous specimens (17 cases versus 16, and 8 species of bacteria versus 7), but the difference was not great (Table 2). It is noteworthy that Gram-positive staphylococci, against which lysozyme of *I. persulcatus* is especially active [Podboronov, 2004], occurred 4 times more often among anomalous ticks (8 versus 2). The diversity of this group of pathogens was likewise much richer in these ticks: 4 species against 1. In the normal ticks, only *Staphylococcus delphini* has been detected. This very species failed to occur in anomalous specimens. Staphylococci are known as inciters of defensins [Nakajima et al., 2002]. For example, *S. aureus* pathogenic to man was used in experiments as a stimulator of defensin production. Thus, the appreciable difference in the prevalence of staphylococci in anomalous ticks suggests that their immune system is much more asthenical than that of normal ones. It is noteworthy that it was many years ago when the lysozyme of *I. persulcatus* ticks was shown to be very active in vitro against staphylococci and micrococci [Podboronov, 1990].

A similar conclusion can be drawn on the basis of the analysis of the number and kind of species of fungi discovered in the two groups of ticks. Only anomalous ticks contained fungi of the genus *Aspergillus* identified to the species level. *Penicillum* likewise infected anomalous ticks much more often than normal ones (19 versus 3). Some species/strains of this genus, e.g. *P. insectivorum*, are known to be pathogenic to arthropods, including *Ixodes* ticks [Cherepanova, 1964]. The coincidence values of microflora shared by both types of ticks also emphasize their different susceptibility to different microorganisms (Fig. 1). It was maximal among cocci, the most abundant complex occurring in both groups of ticks (47 and 36; Table 1), but minimal among fungi (only 9.1%).

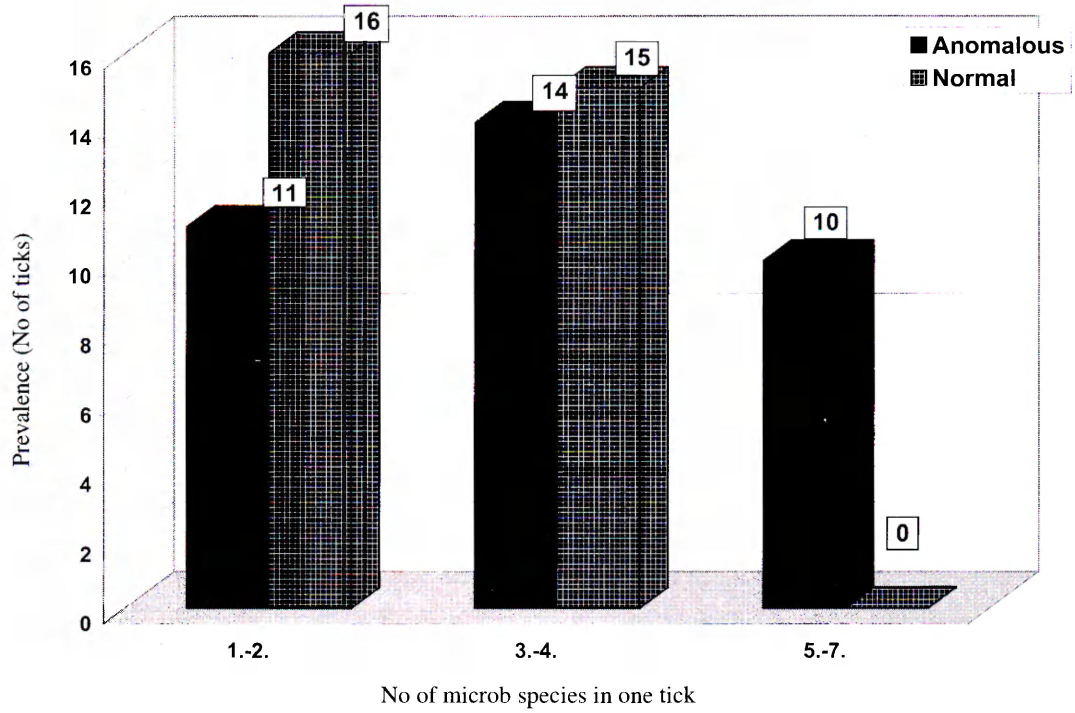

Fig. 2. Prevalence of normal or anomalous ticks with a varying number of microorganism species.
Рис. 2. Встречаемость клещей с разным числом видов микроорганизмов среди особей с аномалиями наружного скелета и без них.

Highly interesting and important is a comparison between the number of microorganism species occurring in normal and anomalous ticks (Fig. 2). In the former group, specimens with 1–2 species of microorganisms absolutely prevailed, with none of the normal ticks containing more than 4 microbial species. In contrast, anomalous ticks can serve as a reservoir of 5, 6 or 7 microorganisms simultaneously.

Such a capacity allows to suggest why it is the cadmium-tolerant specimens with the external signs (anomalous exoskeleton) of their immune system suppression that are much more often infected by tick-borne pathogens dangerous to man: TBEV, *Borrelia*, *Ehrlichia, Anaplasma* and *Babesia* in different combinations. TBEV can be mixed with any *Borrelia* species, sometimes with two of them simultaneously [Alekseev et al., 2003]. Sometimes TBEV co-occurs with one *Borrelia* species and with one of the other intracellular pathogens. *Ehrlichia muris* coexists with borreliae and TBEV, but never with *A. phagocytophilum* or *Babesia microti*. *Ehrlichia* has been revealed together with neither *Anaplasma* nor *Babesia. Anaplasma phagocytophilum* co-occurs only with *B. afzelii* or *B. garinii*, but never with other extra- or intracellular pathogens [Alekseev et al., 2003]. As emphasized elsewhere [Alekseev et al., 2004], not more than 3 tick-borne agents pathogenic to man have ever been discovered in one infected tick. As can be seen from Table 5, it is among the anomalous ticks that species of the genus *Borrelia* as extracellular parasites are to be revealed most often (5.9±2.0% of cases), whereas amongst normal ones they occur 1.6 times less often (3.6±1.2%). Even though the other combinations appear 4.5 times more rarely (1.3±0.2%), multi infections concern anomalous ticks 2.2 times more often. Dual or triple infection of normal ticks has only been revealed in 0.6±0.2% of cases.

Eventually, 1.6–2.2 times of predominance of dual and triple infected anomalous specimens could seem not too important when less than 10% of the whole population show exoskeleton defects, but when the proportion of anomalous ticks increases to 25 or even 50% such a difference can play highly important roles in tick-borne diseases' incidence.

However, the dangerousness of this part of the population, which has appeared in response to the growing anthropogenic press and environmental pollution by heavy metal ions, is not exhausted by the increased capability of transmitting the known tick-borne infections. Anomalous tick microflora contains lots of agents potentially zoo- or phytopathogenic.

The discovery of *Francisella novicida* is hardly striking, because *Ixodes* ticks are known as carriers, more rarely as vectors, of such intracellular parasites as *Francisella tularense*, the tularemia agent [Petrov, 1959, 1968]. It is of interest that, in all three cases of *F. novicida* detection (Table 2), this agent pathogenic to laboratory animals has been revealed in anomalous ticks, but never in normal ones. Similarly, the bacterial genus *Pseudomonas*, presumably phytopathogenic, occurs among anomalous ticks more often (7 versus 1). A transmission of phytopathogens by ticks is no exoticism, because *Ixodes* spp. have long been observed to compensate for their water deficit by sucking the juices of some plants during hot and dry periods of their activity.

Furthermore, anomalous specimens, even when free from obligate tick-borne pathogens, remain more dangerous, because they much more often serve as reservoirs of pathogens such as the fungal genus *Aspergillus* known as agents of deep mycosis and the cause of opportunistic infections. Some of the bacteria revealed mainly in anomalous ticks can also play negative roles as associates of diseases accompanying immunity suppression. They also occurred more often amongst anomalous ticks.

Altogether, 23 of 30 (56.7%) anomalous ticks were infected by zoo- or phytopathogenic Gram-negative or Gram-positive bacteria, whereas only 10 normal specimens of 29 (34.5%) contained same microbes. The difference in the rate of infection by fungi pathogenic to man or animals was much greater: 79.3% (23 of 29) revealed among anomalous ticks versus only 20% (1 of 5) in normal ones.

All these data allow to conclude that both the appearance and the increasing distribution of anomalous *Cd*-tolerant ticks mark not only a more capable vector of the known tick-borne multi infections, but they also mean additional dangerousness and challenge.

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REFERENCES

Alekseev A.N., Dubinina E.V. 1993. Abnormalities in *Ixodes* ticks (Ixodoidea, Ixodinae). *Acarina*, 1 (1): 73–85.

Alekseev A.N., Dubinina H.V. 1996. Some aspects of mite (Oppiidae) and tick (Ixodidae) pathology as a result of anthropogenic pressure. *In*: R. Mitchell, D.J. Horn, G.R. Needham, W.C. Welbourn (Eds). Acarology IX. Columbus, Ohio. Proceeding. Vol. 1, p. 117–120.

Alekseev A.N., Dubinina H.V. 2001. Heterogeneity of *Ixodes persulcatus* Schulze (Acarina: Ixodidae) population determines the qualities of infected specimen as a host and vector of parasite. *Acarina*, 9 (2): 277–298.

Alekseev A.N., Dubinina H.V. 2002. *Stability of parasitic systems under conditions of anthropogenic pressure*. Contributions from the Zoological Institute, No. 6. St. Petersburg. 43 pp.

Alekseev A.N., Dubinina H.V. 2004. Eurasian *Ixodes* tick types, their properties and vector capacity. *Acarina*, 12 (2): 121–139.

Alekseev A.N., Dubinina H.V., Van de Pol I., Schouls L.M. 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. *Journal of Clinical Microbiology,* 39 (6): 2237–2242.

Anigstein L., Whithey D., Micks D. 1950. Antibacterial activity of a substance present in ticks (Ixodidae) of Texas. *Nature*, 4212: 141–143.

Ceraul S.M., Sonenshine D.E., Hynes W.L. 2002. Resistance of the tick *Dermacentor variabilis* (Acari: Ixodidae) following challenge with the bacterium *Escherichia coli* (Enterobacteriales: Enterobacteriaceae). *Journal of Medical Entomology*, 39 (2): 376–383.

[Change-over in taxonomy and nomenclature of bacteria]. *Klinicheskaya microbiologiya i antimikrobnaya khemoterapiya,* 2004. 6 (1): 4–9. [In Russian]

Cherepanova N.P. 1964. [Fungi on ticks]. *Botanichesky Zhurnal*, 49 (5): 696–699. [In Russian]

Chirov P.A., Peterson A.M., Turtseva M.A. 2003. [Native microbial association of gastrointestinal tract of horseflies *Tabanus bovinus* and *Hybomitra ciureai*]. *Entomologicheskie i parasitologicheskie issledovaniya v Povolzh'e*. Saratov, 2: 95–105. [In Russian]

Chirov P.A., Turtseva M.A., Peterson A.M. 2004. [Comparative characteristics of associations of symbiotic microorganisms revealed from two species of ticks of the genus *Dermacentor Koch.*] *In*: Yu.S. Balashov (Ed.) VIII Vserossiyskoe akarologicheskoe soveshchanie (Saint-Petersburg, November 30 – December 2, 2004). Publisher: Zoological Institute of Russian Academy of Sciences, St. Petersburg, pp. 144–145. [In Russian]

Dobler G., Braveny I. 2003. Recent taxonomic changes and update of nomenclature for bacteria identified in clinical material. *European Journal of Clinical Microbiology and Infectious Disease,* 22: 643–646.

Dubinina H.V., Alekseev A.N. 1994. Skeleton changes in the oribatid mites under the influence of the heavy metal ions accumulation. *Acarina*, 2 (1–2): 81–93.

Dubinina H.V., Alekseev A.N., Svetashova E.S. 2004. New *Ixodes* tick populations appearing as a result of, and tolerant to, cadmium contamination. *Acarina*, 12 (2): 141–149.

Duncan J.T. 1925. On a bactericidal principle present in the alimentary canal of insects and arachnids. *Parasitology*, 18 (2): 238–252.

Flemming A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. *Proceeding of Royal Society*, 93: 306–317.

Hindle E., Duncan J.T. 1925. The viability of bacteria in *Argas persicus*. *Parasitology*, 17 (2): 434–446.

Holt J.G., Krieg N.R., Sneath H.A., Staley J.T. Williams S.T. 1997. [*Bergey's Manual of Determinative Bacteriology*]. Ninth edition. Publisher: Mir, Moscow. Ò. 1: 430 pp.; Ò. 2: 800 pp. [In Russian]

Johns R., Ohnishi J., Broadwater A., Sonenshine D.E., de Silva A.M., Hynes W.L. 2001. Contrasts in tick innate immune responses to *Borrelia burgdorferi* challenge: Immunotolerance in *Ixodes scapularis* versus immunocompetence in *Dermacentor variabilis* (Acari: Ixodidae). *Journal of Medical Entomology*, 38 (1): 99–107.

Johns R., Sonenshine D.E., Hynes W.L. 1998. Control of bacterial infections in the hard tick *Dermacentor variabilis* (Acari: Ixodidae): Evidence for the existence of antimicrobial proteins in tick hemolymph. *Journal of Medical Entomology*, 35 (4): 458–464.

Johns R., Sonenshine D.E., Hynes W.L. 2000. Response of the tick *Dermacentor variabilis* (Acari: Ixodidae) to hemocoelic inoculation of Borrelia burgdorferi (Spirochetales). *Journal of Medical Entomology*, 37 (2): 265–270.

Jollés P. 1964. Neuere Untersuchungen an Lysozymen. *Angewandte Chemie*, 76: 20–28. [In German]

Kopacek P., Vogt R., Jindrak L., Weise C., Safarik I. 1999. Purification and characterization of the lysozyme from the gut of the soft tick *Ornithodoros mubata. Insect Biochemistry and Molecular Biology*, 29: 989–997.

Kryuchechnikov V.N. 1991. Protective responses of Ixodoidea hemocytes. *In*: F. Dusbabek and V. Bukva (Eds.) Modern acarology. SPB Academic, Prague, p. 331–334.

Nakajima Y., van der Goes van Naters-Yasui A., Taylor D., Yamakawa M. 2002. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *Insect Molecular Biology*, 11: 611–618.

Petrov V.G. 1959. [Summing-up of *Ixodes* tick study as of tularemia vectors]. *In*: E.N. Pavlovsky (Ed.). X soveshchanie po parazitologicheskim problemam i prirodnoochagovym boleznyam (22–29 October 1959). Proceedings. Publisher: Academy of Sciences USSR, Moscow, Leningrad, pp. 160–162. [In Russian]

Petrov V.G. 1968. [Comparative study of the ticks *Dermacentor marginatus* Sulz. and *Ixodes ricinus* L. as reservoirs of causative agent of tularemia]. *Parazitologiya*, 2 (6): 424–429. [In Russian]

Podboronov V.M. 1990. [Lysozyme from *Ixodes persulcatus* ticks]. *Meditsinskaya parazitologiya i parazitarnye bolezni,* 3: 21–23. [In Russian]

Podboronov V.M. 2004. [*The Pathogens of Human and Animal Diseases and the Ticks*].–Publisher: Meditsina, Moscow, 224 pp. [In Russian]

Rudakova S.A., Matuscenko E.V., Yakimenko V.V., Tokarevich N.K., Andreychuk Yu.V. 2004. [Experimental study of participation of the tick *Dermacentor reticulates* in the circulation of borreliae in natural foci of tick-borne borrelioses]. *In*: Yu.S. Balashov (Ed.) VIII Vserossiyskoe akarologicheskoe soveshchanie (Saint-Petersburg, November 30 – December 2, 2004). Publisher: Zoological Institute of Russian Academy of Sciences, St. Petersburg, pp. 90–93. [In Russian]

Semenov A.V. 2003. [Multiformity of infection by pathogens of the population of *Ixodes persulcatus* ticks on the territory with hard anthropogenic pressure]. Abstract of the Candidate of Biological Sciences thesis. Moscow. 21 pp. [In Russian]

Sutton D.A., Fothergill A.W., Rinaldi M.G. 2001. [*Guide to Clinically Significant Fungi*]. Publisher: Mir, Moscow, 468 pp. [In Russian]

Zhioua E., Ginsberg H.S., Humber R.A., Lebrun R.A. 1999. Preliminary survey for entomopathogenic fungi associated with *Ixodes scapularis* (Acari: Ixodidae) in Southern New York and New England, USA. *Journal of Medical Entomology*, 36 (5): 635–637.