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EURASIAN *IXODES* TICK GENOTYPES, THEIR PROPERTIES AND VECTOR CAPACITY

ГЕНЕТИЧЕСКИЕ ТИПЫ КЛЕЩЕЙ РОДА *IXODES* ЕВРАЗИИ, ИХ ОСОБЕННОСТИ И ВЕКТОРНАЯ СПОСОБНОСТЬ

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Ключевые слова: клещи *Ixodes*, вирус клещевого энцефалита, *Borrelia*, *Ehrlichia*, *Babesia*, спирохеты, L-формы, генотипы, аномалии экзоскелета

ABSTRACT

To determine electrophoretypes based on malatdehydrogenase (MDH) allele structure, as well as exoskeleton anomalies, heavy metal ion content and tick-borne pathogen infection, over 7,000 *Ixodes persulcatus* Schulze and *Ixodes ricinus* (L.) adults and nymphs were collected by flagging in different parts of Eurasia, ranging from Denmark to the Far East of Russia, and studied.

A comparison of the distribution of *I. persulcatus* MDH-genotypes with areas of three genogroups of tick-borne encephalitis virus (TBEV) existing over Eurasia suggests that the prevalence of the second virus genogroup (Western, Neudoerfl one) correlates with the first genotype of ticks (alleles 1.1, based on MDH-enzyme detection), whereas the existence of the third (Urals-Siberian, Lesopark) and, partly, first (Far Eastern) virus genogroups depends on the location of tick genotype 4 (heterozygous, alleles 1.3 of MDH-enzyme).

Environmental pollution, the appearance of *Ixodes* ticks with exoskeleton anomalies which are cadmium ion-tolerant, their increasingly vast distribution in time and space, all this alters tick vector capacity and behavior, enhances their capability to be dually or triple infected, and most probably to increase the emergence of microscopically invisible forms (or cell wall-free, or L-form) of borreliae responsible for such dangerous consequences of Lyme disease as Parkinsonism, multiple sclerosis, neuritis etc. The difference in *Cd* content between the normal ticks and those with anomalous exoskeletons varies from 1 : 1.26 (Denmark) to 1 : 1.92 (Far East, Vladivostok Region, Russia), even up to 1 : 5.4 (Kalinigrad Region, near a highway).

Genetic (enzymological) and phenetic (hereditary) analyses of the heterogeneity of *Ixodes* population structure can serve as a powerful tool for the understanding of the intimate properties of *Ixodes* ticks determining their distribution, vector capacity and dangerousness.

РЕЗЮМЕ

Более 7 тысяч взрослых клещей и нимф *Ixodes persulcatus* и *I. ricinus*, собранных на флаг от Дании до Дальнего Востока России, было исследовано на наличие аномалий экзоскелета у имаго, состава электрофоретипов по различиям энзима НАД-мадатдегидрогеназа (МДГ), на содержание ионов тяжелых металлов и на зараженность клещевыми патогенами. Сравнение состава МДГ-генотипов популяций *I. persulcatus*, обитающих на территории распространения 3-х геногрупп клещевого энцефалита (КЭ), существующих в Евразии, позволило сформулировать следующую гипотезу. Преимущественное распространение 2-ой геногруппы вируса КЭ (Западной, Neudoerfl) коррелирует с преобладанием клещей 1-ого генотипа (аллель 1.1 по МДГ-генотипированию), в то время как распространение 3-ей (Урало-сибирской, Лесопарк) и частично 1-ой (Дальневосточной) геногрупп вируса КЭ зависит от наличия у клещей 4-ого генотипа (гетерозиготы, аллель 1.3 по МДГ-генотипированию).

Распространение боррелиоза на всей территории ареала *I. ricinus* и ограниченное распространение очагов КЭ в Западной Европе может быть объяснено только с учетом того обстоятельства, что боррелии (внеклеточные паразиты)

могут быть передаваемы клещами, принадлежащими к обеим МДГ-геногруппам, в то время как вирус КЭ (облигатный внутриклеточный паразит) может репродуцироваться только в клещах 1-й МДГ-геногруппы, которые редко встречаются в Дании. То есть ограниченное распространение КЭ в Западной Европе может быть объяснено ограниченным числом популяций клещей с достаточной долей особей 1-й МДГ-геногруппы. В условиях загрязнения внешней среды появляются толерантные к повышенному содержанию ионов *Cd* популяции клещей, меняется их векторная способность и их поведение, усиливается способность переносить и передавать двойные и тройные инфекции. В то же время, весьма вероятно, что это способствует появлению невидимых в световом микроскопе свободных от оболочки форм (или, как их еще называют, L-форм, или цист) боррелий, которые являются вероятной причиной появления таких опасных последствий болезни Лайма, как паркинсонизм, множественный склероз, невриты и т.п. Так, разница в концентрациях *Cd* в нормальных клещах и клещах с измененным экзоскелетом варьирует от 1:1.26 (Дания) до 1:1.92 (Дальний Восток, Владивосток). Выявление гетерогенности генетической (энзимологической) и фенетической (но наследуемой) структуры популяций *Ixodes* может служить мощным инструментом понимания интимных свойств клещей этого рода, от которых зависит распространение этих переносчиков, их векторная способность и опасность для людей.

INTRODUCTION

Eurasian ticks of the genus *Ixodes* have long been known to be capable of transmitting a wide range of pathogens of both medical and veterinary importance. Zilber [1939] revealed the role of the taiga tick, *Ixodes persulcatus* Schulze as vector of the Russia spring-summer encephalitis (RSSE). A little later, the same part was proven to be taken by *Ixodes ricinus* (L.) as vector of European strains of tick-borne encephalitis (TBEV). Nearly forty years later, the blacklegged tick, *Ixodes scapularis* Say was shown to be a vector of Lyme disease in the U.S.A. [Burgdorfer et al., 1982; Johnson et al., 1984]. Subsequent publications have demonstrated that American *Ixodes* ticks and the European *I. ricinus* can serve as vectors of human ehrlichiosis and, more rarely [Kjemtrup, Conrad, 2000; Duh et al., 2001], babesiosis as well. Our recent data suggest that multiple infections of *Ixodes* ticks are common.

Indeed, double or multiple infections with *Borrelia* spp., *Ehrlichia* spp., *Babesia* sp. and/or arthropod-borne viruses seem to be a rule rather than an exception [Alekseev et al., 2001a]. *Ehrlichia muris* discovered in the Baltic region of Russia [Alekseev et al., 1998] has been confirmed as an agent of monocytic ehrlichiosis (HME) in the Perm Region, Russia [Ravyn et al., 1999; Vorobyeva et al., 2001], where borreliosis and ehrlichiosis as well as tick-borne encephalitis and ehrlichiosis mixed infections have been verified [Vorobyeva et al., 2001]. *Babesia microti* together with RSSE virus [Alekseev et al., 2003] have recently been proved as having caused mixed infection in one patient from the Kemerovo Region, Russia [Semenov, Subbotin, 2003]. Representatives of the genus *Rickettsia* have also been detected in *I. persulcatus* [cf. Alekseev, 2003]. Some of the agents discovered in *I. ricinus*, such as *Rickettsia aeshlimanni* and *R. slovaca*, have become established as pathogenic to man [Raoult et al., 1997, 2002]. Borreliosis as one of the dominant tick-borne infections, whose distribution area in Eurasia ranges from the Atlantic to the Pacific Ocean, thus largely coinciding with that of *I. ricinus* (Europe) and *I. persulcatus* (Eurasia), is currently suspected as causing many other neuroinfections, such as amyotrophic lateral sclerosis, Parkinson disease, multiple sclerosis, neuritis etc. [Howenstine, 2004]. All these diseases are induced by invisible (or L-, or cell wall-deficient, or cystic) forms of borreliae at later stages of chronic borreliosis. This supports our recent data (Fig. 1) that invisible kinds of borreliae can coexist in one and the same infected tick and cause different forms of Lyme disease [Alekseev, 2001].

The increasing anthropogenic impact, pollution and global change bring about certain qualitative as well as quantitative changes in some biotic components. *Ixodes* tick populations are no exception from this common rule. Due to a detailed study of the morphological variation in Eurasian *Ixodes* populations, new populations have recently appeared [Alekseev, Dubinina, 1993] that show some new properties as vector of tick-borne pathogens [Alekseev, Dubinina, 2000; Dubinina et al., 2004]. For example, such ticks contain more heavy metal ions in their organisms and they are probably more capable of supporting invisible L-forms of borreliae. Morphologically modified *I. persulcatus* ticks tend to be more often infected by a mixture of some tick-borne pathogens, more usually being dually or even triple infected [Alekseev et al., 2002]. Many

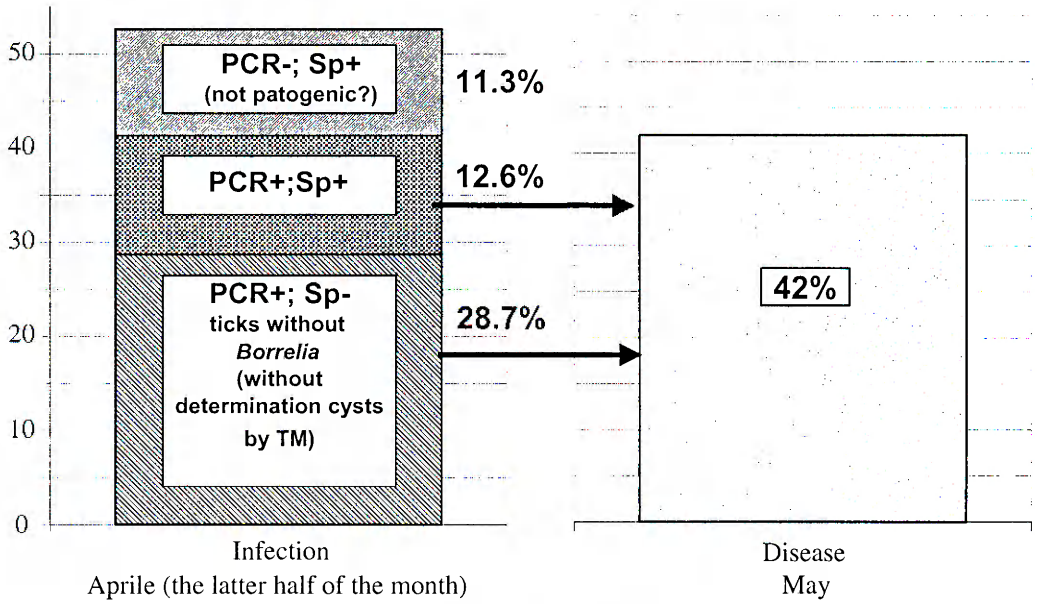

Fig. 1. Coincidence of morbidity and the proportion of invisible forms of borreliae in *Ixodes persulcatus* ticks. Prevalence of the detectable and invisible forms of pathogen were evaluated for the second half of April (original data), the morbidity was calculated according to clinical data [Uskov, 2003].

Рис.1. Совпадение величин заболеваемости и встречаемости невидимых форм боррелий в клещах *Ixodes persulcatus*. Встречаемость видимых и невидимых форм возбудителей определена во второй половине апреля (собственные данные), заболеваемость подсчитана в соответствии с клиническими данными [Усков, 2003].

of the dually and triple infected vectors contain TBEV as one of the typical tick-borne pathogens, coupled with borreliae.

The distribution of Eurasian tick-borne encephalitis viruses is strictly associated with its *Ixodes* vectors. Votyakov et al. [2002] provided maps of the foci of tick-borne encephalitis, quoting Savitsky who had correctly subdivided the whole TBE area into three provinces, i.e. European, Eurasian and Far Eastern, and one European subprovince. Such a regionalization was made on the basis of the differences in mammalian faunas with their specific centers of biodiversity. Zlobin and Gorin [1996] and, then, Votyakov et al. [2002] referred all three genogroups (most probably species) of TBEV to these provinces: the Western, or Neudoerfl, group in Europe, including northwestern Russia; the Lesopark group (third, according to Zlobin and Gorin's classification), or Urals-Siberian, occupying the middle of Russia; and the Far Eastern genogroup spread over the territory of the Amur-Sakhalin "country" according to Kucheruk's classification [after Votyakov et al., 2002]. Currently, there is no reason to question these three types (species) and patterns of TBEV. The distribution of the genotypes, in particular group 2 (Western, or Neuedoerfl), group 3 (Urals-Siberian) and group 1 (Far Eastern), largely coincides [Votyakov et al., 2002] with that of *I. ricinus* (Southwest Europe) and *I. persulcatus* (Eurasia). The clinical peculiarities of the disease also show some typical symptoms related to TBEV type as well as vector. For example, there are virtually no lethal cases entailed by the Western (Neudoerfl) strains of the virus. TBE type meningitis is typical there, whereas nodal forms are very rare. In the Far Eastern strain, the lethality rate sometimes reaches 35%, the nodal forms being typical (40–60%), with meningoencephalitis as the main form of the disease.

The clinic caused by the third type of the virus (Lesopark, Aina) is close to but not the same as the one caused by Neuedoerfl, the nodal forms occurring twice as often, and the rate of lethality as high as 2.4% [Votyakov et al., 2002].

There seems to be no contradiction between the opinions that the main roles in TBE distribution are played by mammalian fauna, climatic conditions and vegetation cover, while the intimate structure of *Ixodes* tick populations is more or less irrelevant to the subject. But if so, how is one to understand that only some *I. persulcatus* populations (or even part of some of them) are intensively infected by the virus of any type and are responsible for TBE disease epidemics, but not a virus prevalence *per se*? This puzzling phenomenon has been emphasized in some publications [Kovalevsky, Korenberg, 1987; Kovalevsky et al., 1988].

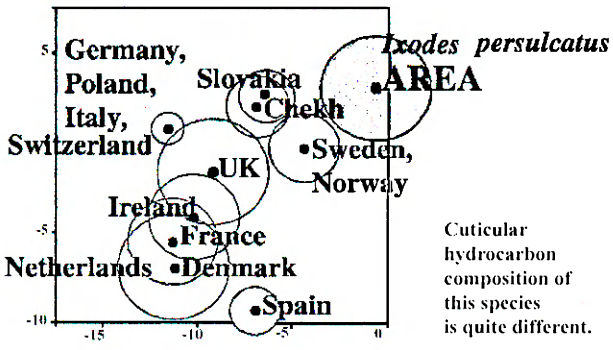

Fig. 2. Phenotypic relationships of *Ixodes ricinus* populations based on cuticular hydrocarbon composition diversity.
Рис.2. Встречаемость различных фенотипов в популяциях *Ixodes ricinus*, различающихся по содержанию углеводородов в их кутикуле.

How is it so that *Borrelia burgdorferi* sensu lato, the agents of ehrlichiosis and, most probably, also of babesiosis are transmitted by the same (and only two) *Ixodes* species from Spain to Japan? The first attempt to solve the problem of *Borrelia* transmission in Europe by taking into account the intimate structure of *Ixodes* tick populations seems to be that by Estrada-Peña et al. [1996]. These authors tried to compare and classify *I. ricinus* populations according to tick chitin properties or the diversity of cuticular hydrocarbon composition. The results, with small changes though, are reproduced in Fig. 2. Relating the diversity of analyzed tick populations to their ability to transmit different species of borreliosis agents has largely failed, as only Spanish populations appear to be responsible for *Borrelia lusitaniae* transmission in Portugal and Spain. So this kind of typification has only reconfirmed the vector capacity of Iberian populations of *I. ricinus* to disperse *Borrelia lusitaniae.*

This result is hardly surprising, because migratory birds are known to transfer representatives of different tick populations over the whole *Ixodes ricinus* area. According to Olsén et al. [1995] and our recent data, ticks transferred by birds can contain lots of tick-borne human pathogens, i.e. at least three species of *Borrelia* and two species of *Ehrlichia* [Alekseev et al., 2001b; Dubinina, Alekseev, 2003].

Much more productive and profitable for evaluating the tick vector capacity proves to be the approach suggested by Jensen et al. [1999]. This implies an electrophoretic analysis of the various fractions' molecular weight of the enzyme malathdehydrogenase as determined by different alleles. They represent different genotypes inside the *Ixodes* tick population analyzed. In general, the use of allozymes for determining the genetic heterogeneity of morphologically poorly discernible organisms is well known [Korochkin et al., 1977].

Malatdehydrogenase is one of the highly important components of the Krebs cycle, the ticks that contain the allele responsible for the production of the molecular heaviest enzyme showing a greater vector capacity [Semenov, Alekseev, 2000]. This method proves useful for both parts of any *Ixodes* tick population analyzed, i.e. with or without exoskeleton anomalies that appeared as a result of selection under heavy anthropogenic press, most probably as an aftereffect of heavy metal ion accumulation in the soil, vegetation and the blood of ticks' vertebrate hosts.

The present study is an attempt to reveal the intimate structure of different, mostly highly remote *Ixodes* tick populations that makes them efficiently infected by different pathogens, often simultaneously. To achieve this objective, ticks were collected from many territories across Denmark to the Russian Far East.

MATERIAL AND METHODS

Ticks

Adults and nymphs of *I. ricinus* ticks were collected in forests mainly in suburbs or near highways in Denmark (Zealand, Grib Scov), Germany (Bonn) and the Kaliningrad Region of Russia (Curonian Spit). *Ixodes persulcatus* were obtained from the Velikiy Novgorod Region, the vicinity of St. Petersburg, from two districts in the Vologda Region, from near Novosibirsk, two places near Irkutsk and from Vladivostok Region, Far East, Russia. All ticks were collected by flagging within their local activity time (mainly from April to June). At Morskaya, Lisy Nos, which is a recreation zone for St. Petersburg forests, ticks were collected from 1992 to 2004. The area has long been known as a focus of TBEV, but currently [Alekseev et al., 2003] this place has proved to be a focus of all three borreliosis agents, of both ehrichiosis agents, of babesiosis and probably of some rickettsiosis as well. During April and July 2000, collections were made at ten days intervals to study tick anomalies, MDH-genotypes, the type of infection and the content of heavy metal ions. Each collection consisted of 2 h of flagging, followed by a 1 h pause, repeated over 24 h. Sometimes flagging was repeated during the daytime to take at least 100 specimens in every 10-day period. A total of 1,391 adults and 19 nymphs were taken during this season, 1,282 were investigated using PCR for seven pathogens.

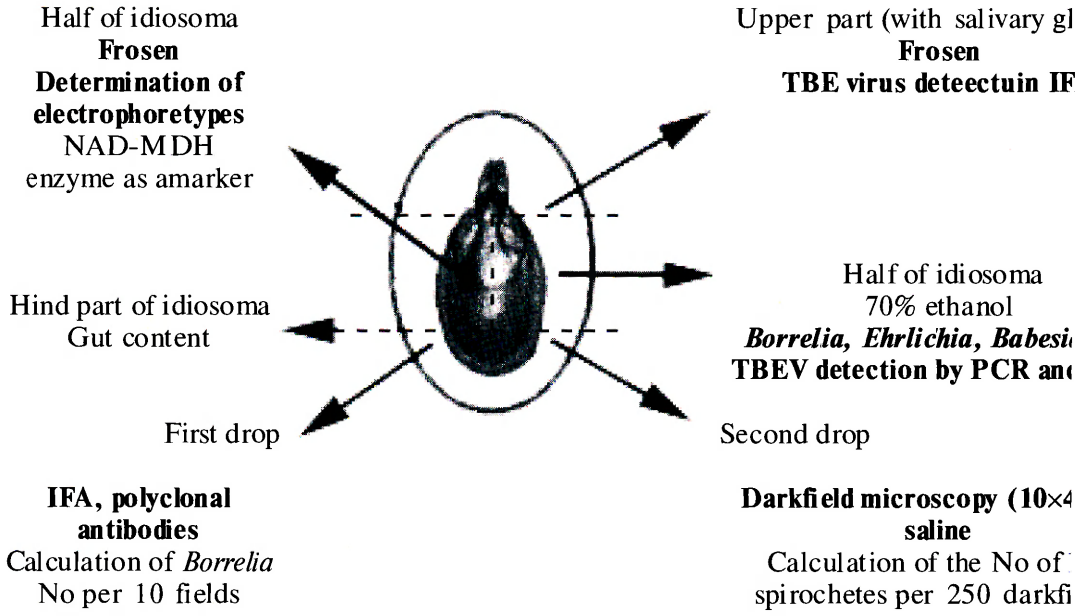

Fig. 3. Methods of genotype and pathogen content determination in one specimen of *Ixodes* tick.
Рис. 3. Методы определения генотипов и содержания патогенов в одной особи клеща рода *Ixodes*.

In other places within other seasons, smaller numbers of ticks were collected but nymphs have never been studied in amounts lesser than one hundred.

Tick study order

All collected live adults and some of the nymphs were studied under a stereomicroscope to spot anomalies of the exoskeleton. These were classified according to a key diagram featured in our former communication [Alekseev, Dubinina, 1996]. The number of *I. persulcatus* and *I. ricinus* used for the detection of anomalies amounted to a total of 6,825.

The locomotor activity of some ticks was evaluated using inclined ticksdrom, a technique described earlier [Alekseev et al., 2000].

Ticks obtained from our collaborators in 70% ethanol were first studied for exoskeleton anomalies and then for heavy metal content. Live specimens or their half after pathogen detection were also analyzed for heavy metal ions. The number of investigated nymphs totaled 800, of adults 5,374.

Ticks collected by us in the Curonian Spit or at St. Petersburg as well as those obtained as live specimens from the Velikiy Novgorod and Novosibirsk Regions were screened for their microflora (3,204). Different methods were used for this purpose: detection of live spirochetes in the dark field of the microscope (DF method), the IFA method with specific antibodies as well as PCR with specific primers. Half of the tick idiosoma was frozen for MDH-genotype determination, the latter made using 3,102 specimens. A scheme summarizing the experimental work with individual ticks is presented in Fig. 3.

Sample preparation for the determination of microorganisms

Adult ticks were rinsed in 70% alcohol, then dried, frozen in liquid nitrogen and crushed. To every sample 200 ml of sterile H_2O, 3 ml of tRNA solution and 200 ml 4M guanidine thiocyanate were added, followed by 10 min incubation at 0° C. After incubation, 200 ml of a phenol:chloroform: isoamyl (25:24:1) solution was 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 75% ethanol, dried and redissolved in 150 ml H_2O. Samples were used for PCR either immediately or following storage at –70° C.

PCR assay

The primers were used as described in special literature [Marconi, Garon, 1992; Persing et al., 1992; Armstrong et al., 1998; Chu, 1998; Belikov et al., 2002]. LD1-HME-3R were supplied by DNA

Technology ApS (Aarchus, Denmark), the primers BAB I-TBEVf and PIRO — A-B were supplied by ASB-Viriology (St. Petersburg, Russia); dNTP, Taq-polymerase, RT-set, and buffers were obtained from "DNA-Technology" and MBI "Fermentas", Russia. PCR analyses were performed in accordance with the usual techniques as described elsewhere [Alekseev et al., 2003]; only the method of RSSE virus determination, being original, is described below in detail.

Reverse transcription PCR (RT-PCR) tests were used to detect RSSEV (Russian Spring-Summer Encephalitis Virus, i.e. a Russian strain of TBEV). The first step was a reverse transcription reaction: 8 ml of template RNA and 2 ml of reverse primer were incubated at 70° C for 5 min, then at 0° C for 10 min. Each 15 ml of RT mix contained 200 mM each of dNTP, 20U of RNAsin, 10U of AMV-revertase. RT mix was incubated at 42° C for 1 h. Each PCR mix contained 200 mM each of dNTP, 2U *Taq* polymerase and 0.5 mM of each primer. A three-step PCR cycling program was used as the 2nd step: an initial 5 min denaturation at 94° C; 35 cycles of a 30 s denaturation at 94° C, 40 s annealing at 55° C and 40 s polymerization at 72° C; 5 min extension at 72° C.

PCR products were analyzed by electrophoresis on a 1.5% agarose horizontal gel, visualized by staining with ethidium bromide and photographed.

Isoenzyme analysis

The frozen ticks or their part (see Fig. 3) were studied. The buffer used for extraction of the enzyme was based on a buffer made from magnesium chloride (10 mM), sodiumhydrogen carbonate (10 mM), sodium EDTA (0.1 mM) and Trisma base (20 mM), and adjusted to pH: 8.00 by the addition of hydrochloric acid (HCl). The extraction buffer was prepared by mixing the base buffer (100 ml) with â-mercaptanol (78 ml), concentrated Triton X-100 (100 ml) and sucrose 10 g. Ticks (adults or nymphs) were homogenized in Eppendorf tubes. The homogenate was suspended with 10îl buffer and centrifuged. Three îl of the supernatant were applied to the polyacrylamide gel wells in preparation for electrophoresis. Cooled vertical electrophoresis was performed using the equipment "Mighty Small" from Hoefer Scientific Instruments (San Francisco, USA). Then this method described by Jensen et al. [1999] was slightly modified [Semenov et al., 2001]. Following electrophoresis, the gels were stained with a 0.5 M Tris-Malat buffer (pH 8.0) containing 0.03% NAD, 0.03% NBT, 0.018% PMS; then fixed in 1% acetic acid during 24 h. The gels were scanned and analyzed. Gels could be stored in 50% glycerol at +4°C up to 10 months for scannings to be rechecked. The number of Russian *I. persulcatus* ticks whose genotypes were determined totaled 2,536, that of *I. ricinus* samples from the Curonian Spit, Kaliningrad Region amounted to 439 specimens, whereas 157 live nymphs collected in Denmark were studied elsewhere [Jensen et al., 1999].

Methodology of analyzing the tick samples for the content of heavy metals: *Zn, Cd, Pb, Cu*

Each sample was first placed into a quartz glass and then dried in a thermostat at 105°C to a constant weight. After that the sample was subjected to dry mineralisation in the electric furnace which temperature gradually changed increasing by 50° C every 30 minutes from 150° to 450° C.

After mineralisation (before obtaining white ciders), the sediment dissolved in distilled water with addition of 1.0 cm^3 hydrochloric acid in 6.0 m/l concentration was transferred to a measuring flask with a volume of 25 ml, with addition of the background solution.

The content of *Zn*, *Cd*, *Pb* and *Cu* ions in the prepared sample solution was determined using a SVA-IBM device with the method of inversion voltamperometry with linear potential scanning This method is based on the electrochemical accumulation of metals on a rotated glass-carbon electrode at the constant potential, subsequently metals were dissolved at a determined speed of changing of the electrode potential.

At the measuring stage, the changing of current was registered in correlation with potential, which agreed with voltamperogramme. In the presence of metal ions the current peaks appear on the voltamperogramme. The position of peaks on the potential axis is a qualitative description of the metal; the height of the peak depends of the metal concentration and therefore serves as a quantitative description. The concentration of metal ions in the sample is determined by a comparison of the height of the peak in the analyzing solution with the height of the peak after addition to the sample of a determined quantity of standard solution with certain concentration of the given metal. The number of samples totaled 141 (the minimal number of one sample of adult tick was equal to 20, of nymphs to 100).

RESULTS

The distribution of different genotypes in the various populations of *I. persulcatus* collected over Russia demonstrates a visible trend to increasing

Table 1.
Variation in MDH-genotypes of *Ixodes persulcatus* ticks from different regions and the tick-borne encephalitis virus proportion.
Таблица 1.
Вариации МДГ генотипов клещей *Ixodes persulcatus* из разных регионов и экстенсивность заражения клещей вирусом клещевого энцефалита в этих регионах.

MDH-genogroups and genotypes	Regions					
	North-West of European Russia			Western Siberia	Eastern Siberia	Far East of Russia
	Velikiy Novgorod Region (n=44)	Vicinity of St Petersburg* (n=1,803)	VologdaRegion (n=189)	Vicinity of Novosibirsk** (n=131)	Irkutsk Region (n=154)	Vicinity of Vladivostok (n=187)
First genogroup with allele 1						
1(1.1)	68.20	64.30	36.0	11.45	39.0	35.0
4(1.3)	13.60	21.18	35.0	36.64	38.0	47.0
6(1.2)	13.60	3.42	11.0	22.14	11.0	6.0
Second genogroup without allele 1						
2(2.2)	2.30	3.42	8.0	8.40	4.0	3.0
3(3.3)	0	4.26	2.0	16.03	3.0	4.0
5(2.3)	2.30	3.42	8.0	5.34	5.0	4.0
TBEV or RSSE virus proportion (%)	0.5 (7 of 1,330)	1.2 (35 of 2,826)	2.3 (5 of 221)	4.5 (4 of 106)	1.5-10.0	3.6***
Methods of virus detection	Pools of 10 ticks tested on suckling mice [Fedorova et al., 1984]	IFA and PCR Original data 1992–2000 seasons of tick collection	IFA [Rybakova, personal communication] 2001–2003	IFA Original data 2000 season	IFA and PCR [Zlobin, Gorin, 1996]	IFA and PCR [Bolotin, 2004]

* virus detected only in the first genogroup [genotype 1(1.1) in 90%, genotype 4(1.3) in 10%];
** virus detected only in the first genogroup and only in the 4th genotype (1.3) in 100%;
*** near Vladivostok; n — number of genotyped ticks, to make a precise comparison only ticks collected in the same periods of the season were used.

the ratio of a heterozygous genotype 4(1.3) (Table 1) from east to west of the tick borne encephalitis focal territories.

The abundance of representatives of the first genotype decreased from east to the western Siberian region and then gradually increased. It is noteworthy that the abundance of the second genogroup was always much less than that of the first one (on the average 8 times in the northwestern part of TBE area; and on the average 5 times in western Siberia and the Far East).

As one can see from Tables 2–4, borreliae being extracellular parasites occurred in all genotypes in both of the study populations, i.e. St. Petersburg and Novosibirsk ones. For example, during the 1998 season 636 adults, 74 nymphs and two larvae of *I. persulcatus* were collected. Of the 613 adults analyzed using the IFA method, only four ticks (0.65%) were TBEV-infected. The virus was also revealed in one occasionally screened nymph. During the 1998 season, only DF-positive specimens were analyzed for pathogenic *Borrelia* species using the PCR techniques. Genotypes of all DF and IFA positive ticks were determined.

As can be seen from Tables 2 and 3, the distribution of *Borrelia* positive ticks (according to IFA and DF+ data) among the *I. persulcatus* genotypes analyzed is very similar, whereas virus-containing specimens were only detected among representatives of the first genogroup (1, 4 and 6).

Table 2.
Prevalence of MDH genotypes of *Ixodes persulcatus* ticks (St. Petersburg Region, 1998) and levels of *Borrelia* and TBEV infection of them.
Таблица 2.
Встречаемость МДГ генотипов клещей *Ixodes persulcatus* (С. Петербург, 1998) и их зараженность боррелиями и вирусом клещевого энцефалита.

Proportion of analyzed genotypes			Borrelia positive tick detected by:				TBE virus-positive ticks	
			IFA		DF			
Number	Abs	%	Abs	%	abs	%	Abs	%
First genogroup with allele 1								
1(1.1)	171	64.1	79	67.5	61	64.8	2*	50.0
4(1.3)	46	17.2	23	19.6	19	20.2	1*	25.0
6(1.2)	20	7.5	4	3.4	4	4.3	1**	25.0
Second genogroup without allele 1								
2(2.2)	6	2.2	5	4.3	4	4.3	0	–
3(3.3)	12	4.5	3	2.6	2	2.1	0	–
5(2.3)	12	4.5	3	2.6	4	4.3	0	–
Total No of studied ticks	267	100	117	100	94	100	4	100

* virus detected with IFA: in three cases the virus was revealed in ticks, but no spirochetes detected using either IFA or DF;
** in this one case, dual infection was recorded: TBEV and *B. garinii*.

In the Novosibirsk Region, Bbsl-positive specimens occurred in both genogroups, but they absolutely prevailed in the first one. They were detected in genotype 1(1.1) in 6.7% cases, in genotype 4(1.3) only in 2.1%, whereas the maximum (24.1%) was detected in genotype 6(1.2). In the second genogroup (geterozygous genotype 5(2.3)) they occurred only once.

All RSSE virus positive specimens (4) were detected only in the first genogroup in genotype 4 (1.3), which is the most abundant in the western Siberian region (Table 1). It is to be emphasized that most of the intracellular parasites (virus, *Babesia* and *Ehrlichia*) mainly occurred among ticks of the first genogroup (Table 3), with only two exceptions from this rule: *Ehrlichia muris* was met with in the genotypes of the second genogroup, but only together with pathogenic borreliae in a dual or even triple combination. In the single case when HME was tested for in the so-called "singletons" group (Table 3), it was not a real monoinfection, because this pathogen occurred in combination with a non-pathogenic, live, DF-detected spirochete (borrelia?) not belonging to the *B. burgdorferi* sensu lato group.

The tick-borne encephalitis agent, RNA virus, an obligate intracellular and even intrachromosomal parasite [Votyakov et al., 2002], was revealed exclusively among representatives of the first genogroup: in the St. Petersburg population mainly (90%) in a homozygous genotype 1(1.1), and in 10% cases in a heterozygous genotype 4(1.3). In the Novosibirsk Region, the virus was only detected in genotype 4(1.3). These data (Table 5) suggest that genotype 1 and genogroup 2 of TBE viruses (Neudoerfl type) coincide with the northwestern territories of Russia (covariance correlation: 1(1.1) MDH — Neudoerfl 42.750), whereas the heterozygous genotype 4(1.3) mainly matches with the third TBE virus genogroup (Lesopark) over the Siberian and Far Eastern territories (covariance correlation: 4(1.3) MDH — Lesopark 72.635).

Only *I. ricinus* is distributed over the territories of Western and, partly, Eastern European TBE foci. A comparison of the genotypes in both study populations of *I. ricinus* shows that, in the Curonian Spit, Kalinigrad Region, Russia and, especially, in Denmark, representatives of the second genogroup absolutely prevailed (Table 6).

In the Curonian Spit, where the first genogroup only contained 38.9% ticks, 0.5% were infected by TBE (most probably Neudoerfl) virus. Nevertheless, the relatively high proportion of genotype 4(1.3) ticks (21.9%) makes it clear, why Zlobin and Gorin [1996] detected there one case of the Far Eastern virus (group one, strain Pregolia). Over the territory of Denmark, where genogroup 1 is minor (8.4%), TBEV has hitherto remained unknown.

Table 3.

Prevalence of various tick-borne pathogens (%) among different genotypes of *Ixodes persulcatus* ticks (St. Petersburg, 2000).

Таблица 3.

Встречаемость разных клещевых патогенов (%) среди клещей *Ixodes persulcatus* различных генотипов (С. Петербург, 2000).

MDH-genogroups and genotypes	Naive (n=865)	Pathogens									
		Singletons					Multiinfected				
		Extra-cellular parasites		Intracellular parasites			Extra-cellular parasites	Combinations of extra-and intracellular parasites			
		Borrelia afzelii (Ba) (n=175)	*Borrelia garinii* (Bg) (n=57)	TBEV (n=4)	HGE (n=4)	HME** (n=16)	Ba, Bg, *Borrelia burgdorferi* s.str. in different combination (n=259)	TBEV and other (n=16)	HGE and borreliae (n=8)	HME* and other (n=46)	*Babesia* sp. and other (n=7)
First genogroup with allele 1											
1(1.1)	63	58	55	100	50	66	64	90	67	65	72
4(1.3)	23	25	28	0	50	27	24	10	33	25	14
6(1.2)	3	5	4	0	0	0	3	0	0	0	14
Second genogroup without allele 1											
2(2.2)	4	3	4	0	0	0	0	0	0	2	0
3(3.3)	4	6	6	0	0	0	6	0	0	3	0
5(2.3)	3	3	3	0	0	7	3	0	0	5	0

* ME (*Ehrlichia muris*) occurred in the second genogroup only together with borreliae: with *B. afzelii* (in genotypes 2 and 5) or, in genotype 3, with *B. garinii* and *B. burgdorferi* s.str. in triple infected ticks;

** in a single case when HME was checked for in the so-called "Singletons" group, it was no real monoinfection, because *Eh. muris* occurred in combination with a non-pathogenic, DF-detected, live spirochete (borrelia?) not belonging to the *B. burgdorferi* sensu lato group.

Table 4.
Prevalence of Bbsl-positive (tested by IFA) *Ixodes persulcatus* ticks (Novosibirsk Region, 2000) belonging to different genotypes.
Таблица 4.
Встречаемость клещей *Ixodes persulcatus* разных генотипов зараженных боррелиями, определяемыми методом РНИФ (Новосибирская область, 2000).

Genotype	No of ticks of this genotype		Bbsl-positive ticks		TBE (RSSE) virus-positive ticks*	
	Abs	%	Abs	%	abs	%
First genogroup with allele 1						
1(1.1)	15	11.5	1/15	6.7	0	–
4(1.3)	48	36.7	6/48	2.1	4/48**	8.3
6(1.2)	29	22.1	7/29	24.1	0	–
Second genogroup without allele 1						
2(2.0)	11	8.4	0	–	0	–
3(3.3)	21	16.0	0	–	0	–
5(2.3)	7	5.3	1/7	14.3	0	–
Total No of studied ticks	131		99		48	

* tick-borne encephalitis virus determined using IFA and PCR methods;
** virus-infected specimens only revealed among IFA negative specimens.

Table 5.
Comparison of the genetic composition of *Ixodes persulcatus* populations (based on MDH-marker) and the genetic heterogeneity of the TBEvirus groups (species) over the territory of Russia.
Таблица 5.
Сравнение генетического состава популяций *Ixodes persulcatus* (по МДГ маркеру) и генетической гетерогенности разных групп (видов) клещевого энцефалита на территории России.

Genotypes of ticks and viruses	Prevalence in different regions:		Differentiation in prevalence (No of times)	Covariance correlation
	Northeastern*	Siberian-Far Eastern*		
1(1.1) MDH-genotype	50.8 (2031)	28.3 (472)	<1.8	1(1.1) MDH - Neudoerfl 42.750
Virus group 2 (Western, Neudoerfl)	7.1 (28)	3.3 (89)	<2.1	
4(1.3) MDH-genotype	21.2 (2031)	41.1 (472)	>1.94	4(1.3) MDH - Lesopark 72.635
Virus group 3 (Urals-Siberian, Lesopark)	35.7 (28)	43.0 (89)	>1.2	

* In brackets — the number of analyzed ticks based on MDH-marker (original data) and data on the frequency of virus genotype identification according to Zlobin and Gorin [1996].

Most probably it will not be discovered there in the future either.

Thus, an increase in 4(1.3) genotype proportion from west to east (Fig. 4) is most likely to be related to west-east differences in virus genotype distribution in the following way: second type — Neudoerfl, then Urals-Siberian type — Lesopark, and then Urals-Siberian together with First one, Far Eastern — Sofyin. This trend is associated with an enhanced virus prevalence among infected ticks. At the same time, the proportion of this genotype in *I. persulcatus* populations is well correlated with the anomalous tick prevalence over the study territories (Table 7).

Table 6.

Variation in MDH-genotypes of *Ixodes ricinus* prevalence from different regions of Europe and the tick-borne encephalitis virus proportion.

Таблица 6.

Вариации встречаемости различных МДГ-генотипов клещей *Ixodes ricinus* из разных регионов Европы и их зараженность вирусом клещевого энцефалита.

MDH-genogroups and genotypes	North-West of Europe	
	Denmark (Zealand)* (n=157)	Kalinigrad Region (Curo Spit, Russia) (n=439
First genogroup with allele 1		
1(1.1)	3.3 (6)	7.0 (31)
4(1.3)	0.6 (1)	21.9 (96)
6(1.2)	4.5 (7)	10.0 (44)
Abs No of ticks	(14)	(171)
Second genogroup without allele 1		
2(2.2)	63.7 (100)	13.0 (57)
3(3.3)	0.6 (1)	20.0 (88)
5(2.3)	26.8 (42)	28.1 (123)
Abs No of ticks	(143)	(268)
TBEV virus proportion (%)	0	0.5**
Methods of virus detection	IFA (2000–2004)	IFA Original data (1998)

* genotype determination according to Jensen et al. [1999];

** genotypes of infected ticks not determined.

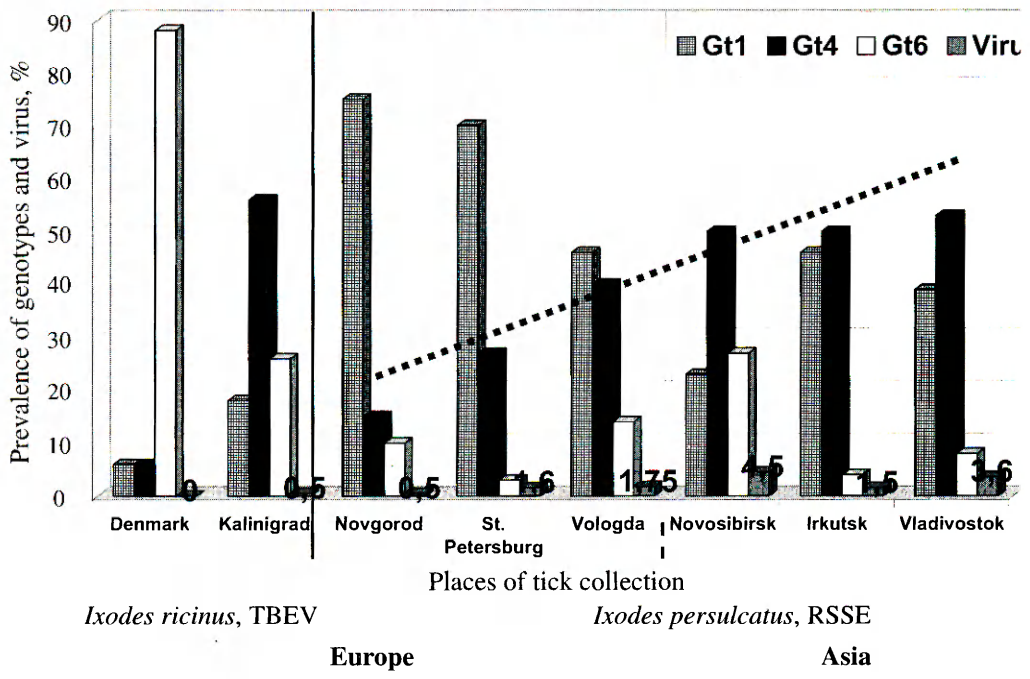

Fig. 4. Proportions of genotypes (first genogroup) and of tick-borne encephalitis virus in Eurasian *Ixodes* ticks.

Рис. 4. Встречаемость генотипов (первой геногруппы) и вируса клещевого энцефалита в клещах рода *Ixodes* Евразии.

Table 7.

Variation in anomalous tick ratios and the proportions of the fourth MDH-genotype in *Ixodes persulcatus* populations from different TBE regions of Russia.

Таблица 7.

Вариации встречаемости клещей *Ixodes persulcatus* 4-го МДГ-генотипа с аномалиями экзоскелета на различающихся по эндемичности клещевого энцефалита территориях России.

Geography of TBE regions							
Northeastern				Siberian-Far Eastern			
Territory of investigation	Years of collection	Prevalence of the 4th MDH-genotype*	Proportion of ticks with anomalies*	Territory of investigation	Years of collection	Prevalence of the 4th MDH-genotype*	Proportion of ticks with anomalies*
St.Petersburg	1998	17 (252)	24 (616)	Novosibirsk	2000	38 (131)	39 (133)
Velikiy Novgorod	1999	13 (49)	29 (49)	Irkutsk	2002	38 (154)	41 (190)
St.Petersburg	1999	18 (287)	28 (488)	Vladivostok	2003	47 (187)	45 (197)
St.Petersburg	2000	28 (1,264)	32 (1,369)	Total No of analyzed ticks		(472)	(992)
Vologda	2002	35 (179)	50 (309)				
Total No of analyzed ticks		(2,031)	(2,276)				
Correlation (r)		0.879		Correlation (r)		0.945	
Statistical significance (*p*)		<0.05		Statistical significance (*p*)		<0.05	

* In brackets — the number of ticks that served as the basis for proportion calculations.

Table 8.

Prevalence of different MDH genotypes of *Ixodes persulcatus* (St. Petersburg Region) in different years.

Таблица 8.

Изменения генотипического состава Петербургской популяции *Ixodes persulcatus* в разные годы наблюдений.

Genotype	1998		1999		2000	
	abs	%	abs	%	abs	%
First genogroup with allele 1						
1 (1.1)	171	64.0	192	66.2	795	62.7
4 (1.3)	46	17.3	52	18.0	301	23.8
6 (1.2)	20	7.5	16	5.5	37	2.9
Total No	237	**88.8**	260	**89.7**	1133	**89.4**
Second genogroups without allele 1						
2 (2.0)	6	2.2	12	4.1	40	3.2
3 (3.3)	12	4.5	11	3.8	55	4.3
5 (2.3)	12	4.5	7	2.4	39	3.1
Total No	30	**11.2**	30	**10.3**	134	**10.6**
Total No of analyzed ticks	267		290		1,267	

In the Curonian Spit (1998 season), where the prevalence of genotype 4(1.3) in *I. ricinus* was high enough, 21.9% (Table 5), and where 0.5% ticks were TBEV-infected, anomalous ticks amounted to 11.3%, whereas in Denmark, instead of a small share of genotype 4(1.3) (0.6%), the proportion of abnormalities in Zealand's *I. ricinus* reached 15% [Alekseev et al., 2000]. This means that, in *I. ricinus* populations, the proportion of anomalies is most probably not correlated with the share of genotype 4(1.3).

Table 9.

Proportion (%) of anomalous *Ixodes persulcatus* ticks in different territories during several years of observation.

Таблица 9.

Встречаемость (%) аномальных особей клещей *Ixodes persulcatus* на разных территориях по годам.

Years of study	*Ixodes ricinus*	*Ixodes persulcatus*					
	Curonian Spit	Velikiy Novgorod Region	Vicinity of St. Petersburg	Vologda Region	Vicinity of Novosibirsk	Irkutsk Region	Vladivostok Region
1998	12.7	–	24.6	–	–	–	–
1999	24.1	26.6	27.5	–	–	–	–
2000	–	33.1	31.1	–	33.8	–	–
2001	–	–	37.1	–	–	–	–
2002	35.5	45.2	44.6	50.3	–	40.7	–
2003	42.3	46.0	44.9	51.0	–	–	46.2
Total No of analyzed ticks	1,546	317	3,592	285	133	189	982

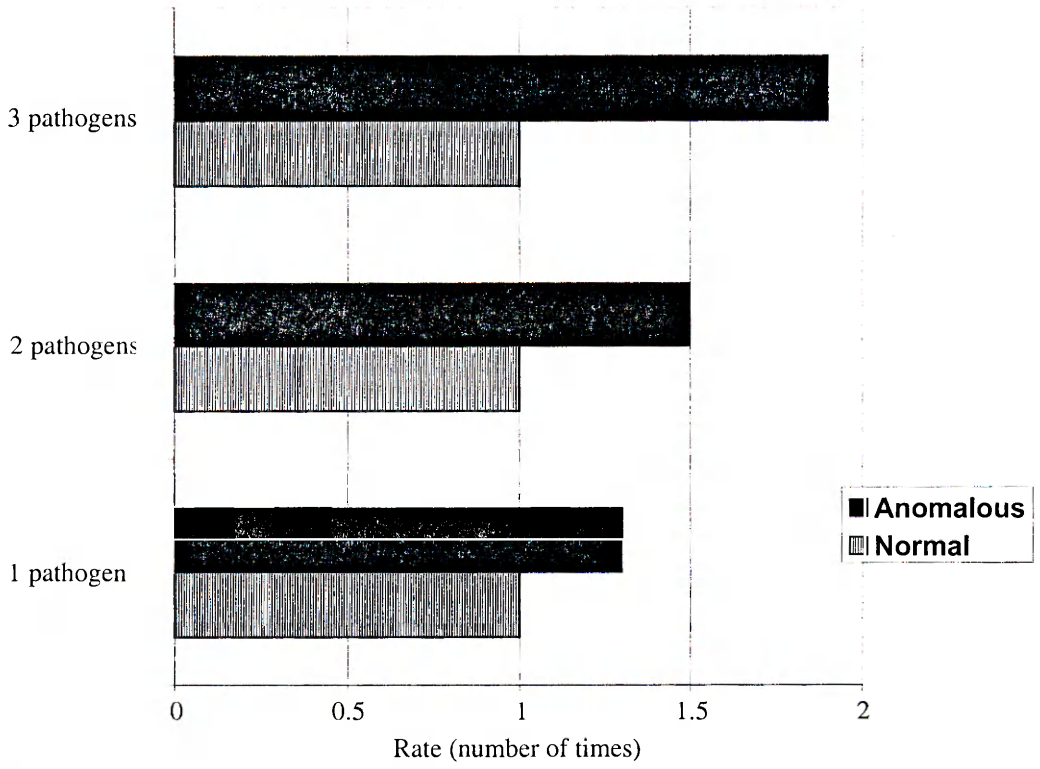

Fig. 5. Peculiarities of the vector capacity of ticks without (normal) and with exoskeleton deformations.

Рис. 5. Различия в способности быть переносчиками у клещей без изменений экзоскелета (нормальных) и клещей с изменениями покровов.

The proportion of different genotypes varies between years (Table 8), but not very considerably so, whereas the share of anomalies is increased from year to year (Table 9). This is typical not only of the St. Petersburg populations, where the prevalence of abnormal ticks rose from 24.4% in 1998 to 44.9% in 2003, but of the other populations as well.

This process is nowise harmless. As can be seen from Fig. 5, abnormal ticks were much more often infected by *Borrelia* and other pathogens, especially when they were dually or triple infected.

Monoinfections involving any of the pathogens discovered in 2000 occurred amongst normal ticks in 27.9% cases, whereas they amounted to 36.1% (1.3

Table 10.

Proportions of mono, dual and triple infected *Ixodes persulcatus* ticks (St. Petersburg Region, 2000) with or without exoskeleton anomalies in relation to visible (DF+, spirochetes) and invisible forms of borreliae (DF-, L-forms, cysts) .

Таблица 10.

Встречаемость аномальных и нормальных клещей *Ixodes persulcatus*, содержащих 1, 2 или 3 патогена одновременно, и соотношение этих величин с встречаемостью видимых под микроскопом живых боррелий и их невидимых форм (DF-, L-форм, цист).

Existence of anomalies	Total No of ticks with pathogenic borreliae*	Type of infection*		Type of infected ticks detected with DF		Rate I:II
				Spirochete-positive I	Undetected II	
Without exoskeleton anomalies	277	*B. afzelii* or *B. garinii*	Singletons	59	116	1:1.98
		Borreliae in mixture with other pathogens	Dual	29	61	1:2.01
			Triple	2	10	1:5.0
With exoskeleton anomalies	116	*B. afzelii* or *B. garinii*	Singletons	30	31	1:1.03
		Borreliae in mixture with other pathogens	Dual	14	31	1:2.21
			Triple	1	9	1:9.0

* Borreliae detected using darkfield microscopy (DF) and PCR. Other pathogens revealed with PCR only.

times more frequently, $p < 0.05$) among anomalous ticks. The proportion of dually infected normal ticks reached 14.5%, of abnormal ones, 21.7% (1.5 times more frequently, $p < 0.01$). The share of triple infected normal specimens was up to 3.1%, whereas that of anomalous ticks nearly twice as great, 5.9% ($p < 0.01$). What seems noteworthy is, that often enough anomalous ticks were infected by invisible (or L-, or cell wall-deficient) forms of borreliae (Table 10).

Dual and triple infections among ticks with anomalies occurred more often that among normal ticks: 39 vs 32%, and 9 vs 4%. Triple infected anomalous ticks were met with 2 times more frequently, with no visible live spirochetes detected in their bodies. Just the opposite, borreliae in normal tick singletons were revealed 2 times more often, with invisible (or L-, or cell wall-deficient) forms of spirochetes involved.

Invisible forms of borreliae occurred among anomalous dually infected ticks only slightly more frequently than among normal ones. In contrast, the vector capacity of triple infected ticks with anomalies was considerable enough to contain invisible forms.

All the above peculiarities of abnormal ticks suggest that they are a result of the same external factor. According to our long-term observations, this factor is selection of new *Ixodes* populations that appeared as an aftereffect of heavy metal pollution. The members of each new population that coexist with the main population of normal ticks are able to accumulate, and to be much more tolerant to, increasing quantities of *Cd* in their organism. This process proves similar over all study territories polluted by heavy metal ions (Table 11).

The capability to accumulate *Cd* and to be tolerant to it has been proved as hereditary [Dubinina et al., 2004].

DISCUSSION

Analysis of different *Ixodes* tick populations over the territory of Eurasia was made according to their genetic composition, using some of their properties as markers. Analyzing the isoenzymological heterogeneity (NAD — malatdehydrogenase alleles as markers), the morphological heterogeneity (visible hereditary exoskeleton anomalies) and the capability to accumulate *Cd* as well as using different methods of pathogen detection allow to arrive at some inferences concerning the vector capacity of *I. ricinus* and *I. persulcatus* within their distribution areas.

Table 11.

Proportion of anomalies in *Ixodes* ticks and the *Cd* content in anomalous and normal specimens belonging to different populations.

Таблица 11.

Встречаемость аномалий у клещей *Ixodes* разных родов, и содержание ионов *Cd* в особях из разных популяций.

Territories	Species of tick	Proportion* of ticks with exoskeleton anomalies (%)	Content of *Cd* (mg/kg) per test*:		Ratio of anomalous to normal
			Nymphs**	Adults (anomalous / normal)***	
Denmark (Zealand, Grib Skov)	*Ixodes ricinus*	15.0	10.73	6.93/5.52	1.26:1
Germany (Bonn region)		–	14.51	–	–
Russia (Kalinigrad Region, Curonian Spit)		38.9	7.94	9.25/3.74	2.47:1
Velikiy Novgorod Region	*Ixodes persulcatus*	41.4	–	14.36/7.88	1.82:1
Vicinity of St. Petersburg		39.4	11.27	7.82/4.77	1.64:1
Vologda Region		50.7	–	12.82/8.75	1.47:1
Irkutsk Region		40.7	–	16.34/9.76	1.67:1
Vladivostok Region		46.2	–	7.44/3.87	1.92:1

* mean values, data of 2000–2003;
** 100 per test;
*** min 20 ticks per test.

Despite the considerable amount of tick samples analyzed, the material hardly permits us to draw final conclusions. However, it does provide a nice opportunity to put forth some working hypotheses deemed fruitful for future investigations.

The first observation. The distribution of TBE viruses and their species [or genogroups: the first — Far Eastern, the second — Western European (Noedoerfl), and the third — Urals-Siberian (Lesopark)] depends not only on environmental conditions such as climate and vegetation cover [Randolph, 2000], altitutde [Jouda et al., 2004] as well as vertebrate host fauna [Savitsky, quoted after Votyakov et al., 2002], but also on the intimate structure of *Ixodes* tick populations. As demonstrated above, differences in MDH-marker suggest that TBE virus infection is related to ticks belonging to the first genogroup, that is, to the owners of the first, molecularly heaviest allele either homozygous or heterozygous in form.

Ixodes persulcatus ticks distributed over the northwestern part of RSSE virus area are mostly possessors of the first genotype (alleles 1.1). Further to the east, in the Vologda Region, the *I. persulcatus* population is represented by genotypes 1(1.1) and 4(1.3). It is from this area that the Lesopark type of virus begins to prevail. In western and eastern Siberia, the fourth genotype absolutely dominates the first MDH-genogroup.

The genotypic analyses of intimate tick population properties allows to eliminate three main contradictions heretofore noted in TBE epidemiology:

1. Why are all three main focal territories [according to Kucheruk, quoted after Votyakov et al., 2002] dominated by the same third genotype (Lesopark)?

An answer might be the prevalence of genotype 4(1.3) over all of these territories.

2. Why do highly infected ticks (the minority of an infected population, according to the data of Kovalevsky and Korenberg [1987]) appear and prevail in some years?

The most feasible explanation seems to be the existence of genotype share fluctuations from year

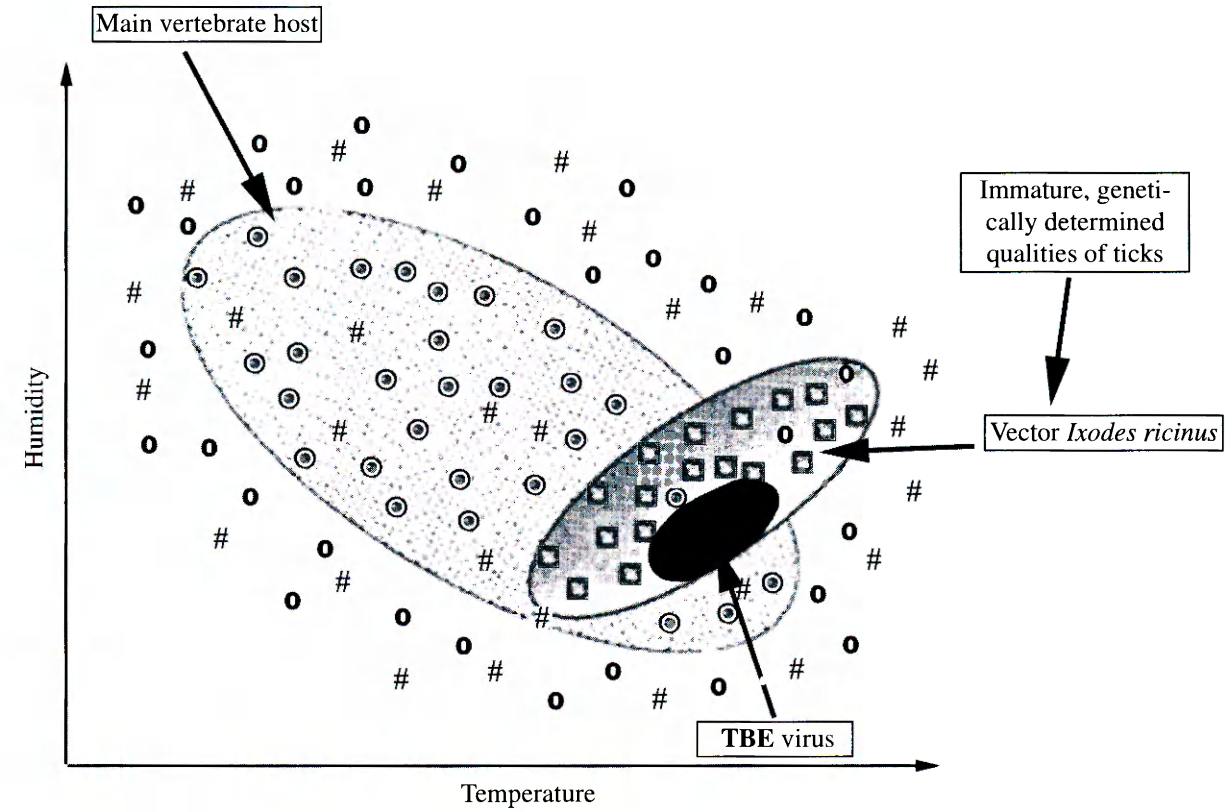

Fig. 6. An improved chart of TBE focus determination (after Randolph, Rogers [2002], with changes).
Рис. 6. Усовершенствованная схема, предсказывающая возможность существования очагов клещевого энцефалита (по Randolph, Rogers [2002], с изменениями).

to year [Table 9: an increasing genotype 4(1.3), decreasing genotypes 1(1.1) and, especially, 6(1.2)].

3. Why does the tick *I. ricinus* distributed over entire Western Europe flourish and successfully transmit all bacterial and protozoon infections (borreliosis, ehrlichiosis and most probably babesiosis as well), whereas the tick-borne encephalitis focal territories are so strictly limited?

As Tables 2 and 3 show, *Borrelia* can be detected not only among genotypes of the first genogroup, but among all genotypes of the second one. *Ehrlichia muris*, revealed for the first time in *I. ricinus* in Europe [Kalinigrad Region, Alekseev et al., 1998], has since been detected in *I. persulcatus* together with *Borrelia* in multiinfected ticks or, in the so-called monoinfected specimens, together with non-pathogenic spirochetes (Table 3). *Ixodes persulcatus* sympatric with *I. ricinus* not only at St. Petersburg and adjacent parts, where both the genogroups of TBE viruses have been documented (second, Neudoerfl and third, Lesopark), is mainly represented there by MDH-genotype 1(1.1). Such a pattern of tick prevalence correlates with the Neudoerfl type of TBE virus proportion (covariance correlation index 42.75, see Table 5). Just the opposite, the share of MDH 4(1.3) heterozygous genotype correlates with the Urals-Siberian genogroup of virus (covariance correlation index 72.635, i.e. 1.7 times greater). The TBE virus proportion in the population from the vicinity of St. Petersburg varies from 1.2% (original data, Table 1) to 2% (epidemiological service data). In the Kaliningrad Region, where only *I. ricinus* occurs naturally, the TBE virus share reaches 0.5%, the proportion of the MDH 1(1.1) genotype being not more than 7%. However, the fourth genotype (1.3) amounted to 21.9%. Such a high share could have led to the discovery in the Kaliningrad Region of the Pregolia strain [Zlobin, Gorin, 1996], which belongs to the first, Far Eastern genogroup of RSSE virus. In Denmark, the first MDH genogroup totals 8.4%. So it is hardly surprising that TBE virus has hitherto not been detected in Denmark. Most likely, it will not be found there in the future as well. The absolute prevalence of the second MDH genogroup genotype, especially of 2(2.2), alone amounting to 63.7%, and of MDH 5(2.3), can well account for the discovery of *B. garinii* and *B. afzelii* in Denmark [Jensen, 1998].

All the above suggests that *I. ricinus* immature, genetically codetermined properties must be added to the chart of Randolph and Rogers [2002] (Fig. 6).

It seems feasible that MDH-analysis will reveal the concentration of *I. ricinus* belonging to the first MDH genogroup, most probably even to the first genotype (1.1) Quite possibly the ticks belonging to this genotype will be detected either in the middle of the TBE area as currently known or in new places as predicted with the use of the satellite forecasting technique.

The second observation confirms that new populations of Eurasian *Ixodes* ticks have appeared. These populations consist of specimens tolerant to cadmium and rich in ions of this heavy metal. The growing environmental pollution results in increasingly higher proportions of such specimens both in time and space. This still ongoing process has been documented as ranging from the Atlantic (Denmark) to the Pacific (Vladivostok, Russian Far East) (Table 9).

This trend leads to an increase in the prevalence of a heterozygous genotype 4(1.3) in *I. persulcatus* populations (Table 7), heterozygosis being typical of ongoing selection [Korochkin et al., 1977]. This is of importance to the distribution and proportions of the different virus genotypes and this can result in increasing the number of specimens whose ability to reproduce TBE virus is much higher than that of normal specimens belonging to other representatives of the same genogroup. The same process can be observed in Western Europe, where abnormal ticks have also been detected as well as their high capability to accumulate *Cd* (Denmark and Germany — Table 11). It is of interest that the ratio of *Cd* content in normal and anomalous ticks is only very rarely greater than 1 to 2, being very similar in populations from different territories.

The locomotor activity of anomalous ticks appears to be higher when these are *Borrelia*-infected [Alekseev et al., 2000; Zharkov et al., 2000]. In addition, such ticks are more frequently capable of containing two or even three tick-borne pathogens simultaneously. Neither cadmium (*Cd*) nor lead (*Pb*) is known to be incorporated into any enzyme system, instead being active suppressors of the immune system. So it is hardly surprising that such ticks can be multiinfected more often.

It still remains quite unclear why the invisible forms of borreliae occur more often among anomalous ticks than among normal ones (Table 10). According to some data, they appear *in vivo* either under the action of antibiotics or as the result of a sufficiently strong immunological reaction [Malawista et al., 1994; Schaller, Neubert, 1994; Kazragis et al., 1996; Mouritsen et al., 1996; Branigan et al., 1997]. Cystic (or invisible, or L-, or cell wall-deficient) forms of *Borrelia* emerge under such adverse conditions as low *pH* values, cold or protein impoverishment [Brorson, Brorson, 1998a, b; Alban et al., 2000]. As noted above, *Cd* and *Pb* suppress a live organism's immunity, but it is also possible that high concentrations of *Cd* in abnormal tick organisms behave as other adverse factors like they act *in vivo*. This matter requires a more thorough study in the future.

All the above facts and hypotheses are to revive keen interest to the genetic peculiarities of *Ixodes* tick populations because, according to our observations, their vector capacity, distribution and ability to serve as reservoirs and vectors of different tick-borne pathogens and other microorganisms depends a great deal on the intimate properties of the organism in ticks belonging to different genotypes as well as on the tolerance to heavy metal ions.

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