

PCR Detection of *Borrelia burgdorferi* Sensu Lato, Tick-Borne Encephalitis Virus, and the Human Granulocytic Ehrlichiosis Agent in *Ixodes persulcatus* Ticks from Western Siberia, Russia

Olga V. Morozova,^{1,2} Andrey K. Dobrotvorskyy,³ Natalya N. Livanova,³ Sergey E. Tkachev,¹ Valentina N. Bakhvalova,³ Anatoly B. Beklemishev,⁴ and Felipe C. Cabello^{2*}

Novosibirsk Institute of Bioorganic Chemistry,¹ Institute of Systematics and Ecology of Animals,³ and Institute of Biochemistry,⁴ Novosibirsk 630090, Russia, and Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595²

Received 14 February 2002/Returned for modification 9 June 2002/Accepted 25 July 2002

PCR assays were used to test adult *Ixodes persulcatus* ticks from Western Siberia, Russia, for *Borrelia burgdorferi* sensu lato, tick-borne encephalitis virus (TBEV), and the human granulocytic ehrlichiosis (HGE) agent. Of the 150 ticks that were studied, 38% were infected with *B. burgdorferi*, 46% were infected with TBEV, and 8% were infected with the HGE agent. These three pathogens were distributed in the ticks independently of one another.

Ixodid ticks transmit a great variety of pathogens to mammalian hosts, including human beings (19). Since the identification of *Borrelia burgdorferi* as the agent of Lyme disease, 11 tick-borne human bacterial pathogens in Europe, including that causing human granulocytic ehrlichiosis (HGE), have been described (19). Ticks and their animal hosts maintain a variety of pathogens in the same habitats, and as a consequence, they may be infected with two or more infectious agents (4, 7, 8, 11, 13, 15, 16, 23). Closely related *Ixodes* tick species harbor similar sets of pathogens in America and Eurasia (9). Ticks of the *Ixodes persulcatus* group are well known as major vectors of Lyme borreliosis and ehrlichiosis in North America and of tick-borne encephalitis and Lyme borreliosis in temperate Eurasia. Powassan and deer tick viruses, closely related to tick-borne encephalitis virus (TBEV), have been detected in the Eastern United States (9). However, the diversity of pathogens associated with *I. persulcatus* Schulze, the taiga tick situated in the Asian part of Russia, has not been well studied. The objectives of the present study were to estimate the infection rate of ticks in western Siberia, Russia, and to assess the prevalence of mixed infections in them.

In May of 2001, 150 unfed adult *I. persulcatus* ticks were collected by flagging vegetation along a pathway in an aspen and birch forest near Novosibirsk, where the relative densities of ticks varied from 100 to 300 per km of the route. The tick species were identified by an entomologist (10). Total nucleic acids were extracted from ticks with an IsoQuick nucleic acid extraction kit (ORCA Research, Bothell, Wash.), which was employed according to the manufacturer's instructions, followed by phenol-chloroform extraction and isopropanol precipitation (22). The presence of each pathogen was determined

by using PCR with primer sets that have previously been used to detect the pathogens concerned and that are specific for genomic regions known to be present in all the isolates (3, 21, 24, 25). The primers to detect the *bmp* genes were used to confirm the results of previous studies indicating that these genes are present in all *B. burgdorferi* sensu lato strains and appear in the following order: *bmpD*, *bmpC*, *bmpA*, and *bmpB* (12). *B. burgdorferi* DNA was detected by using five primer pairs: (i) 126 (TGCGAGTTCGCGGGAG) and 127 (TCCTAGGCATTCACCATAGACTCTT) for the *rrf* (5S)-*rrl* (23S) intergenic spacer region (21); (ii) B1 (ATGCACACTTGGTGTAACTA) and B2 (GACTTATCACCGGCAGTCTTA) for the 16S rRNA gene (17); (iii) 4 (forward) and 2 (reverse) for the *bmpD* and *bmpC* genes, respectively (12); (iv) 1 (forward) and 6 (reverse) for the *bmpC* and *bmpA* genes, respectively (12); and (v) 13 (forward) and 24 (reverse) for the *bmpA* and *bmpB* genes, respectively (12).

TBEV RNA was detected by reverse transcription-PCR (RT-PCR) with two primer sets: (i) E1 and E2, corresponding to the TBEV envelope gene *E*, and (ii) NS1 and NS2, corresponding to the TBEV nonstructural gene NS1 (3). These primers correspond to regions that are highly homologous and present in all virus isolates (3). The HGE agent was detected by nested amplification of the heat shock *groESL* operon with primers HS1 (TGGGCTGGTANTGAAAT) and HS6 (CCCGGACAYACCTTC) in the first PCR and with 5- μ l aliquots of the first reaction mixtures with primers HS43 (ATWGCWAARGAAGCATAGTC) and HS45 (ACTTCACGYTCA TAGAC) in the second nested PCRs (20, 24). Primers for nested PCR were kindly provided by D. Liveris (New York Medical College, Valhalla). Sequencing of PCR products with primer 126 was performed at the Cancer Center at Columbia University, New York, N.Y. Nucleotide sequences were compared using the interactive program CLUSTALW.

The results of PCR detection of different pathogens in *I. persulcatus* ticks are shown in Fig. 1. *B. burgdorferi* DNA was

* Corresponding author. Mailing address: Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595. Phone: (914) 594-4182. Fax: (914) 594-4176. E-mail: cabello@nymc.edu.

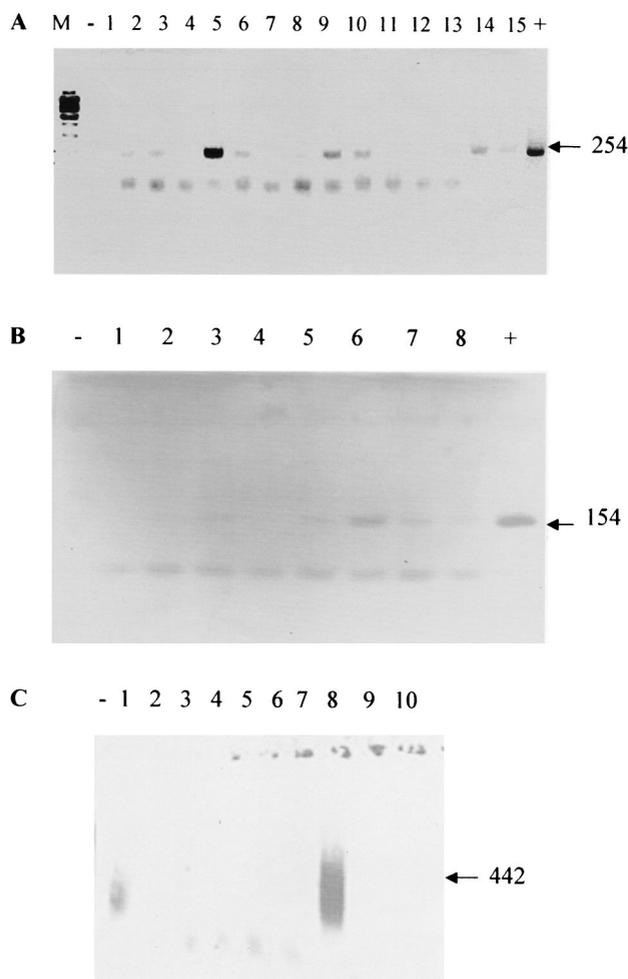


FIG. 1. PCR detection of tick-borne pathogens. (A) Products of PCR with rRNA primers 126 and 127, specific to *B. burgdorferi* (strain B31) DNA, after electrophoresis in 1% agarose gel. Lane M, DNA markers (Minnesota Molecular Hi-Lo); lane -, negative control; lanes 1 to 15, PCR products with DNAs isolated from individual ticks; lane +, positive control with *B. burgdorferi* (strain B31) DNA. (B) Products of RT-PCR with envelope gene E1 and E2 primers corresponding to TBEV (Sofyin strain) genomic RNA (3) after electrophoresis in 2% agarose gel. Lane -, negative control; lane +, positive control with TBEV (Sofyin strain) RNA; lanes 1 to 8, products of the RT-PCR with RNA isolated from ticks. (C) Products of nested PCR with the *groESL* primer pairs specific for the HGE agent (20) after electrophoresis in 1.5% agarose gel. Lane -, negative control; lanes 1 to 10, products of the nested PCR. Arrows on the right indicate the position of the expected amplicon and its number of base pairs.

readily detected by direct PCR (Fig. 1A), while TBEV RNA was revealed by RT-PCR (Fig. 1B). However, the results of direct PCR with HGE-specific primers were negative, and DNA of this pathogen was detected only after nested PCR (Fig. 1C). Thus, the numbers of *Borrelia* organisms, TBEVs, and HGE agents in ticks were different.

Primers 126 and 127, corresponding to the *rfl* (5S)-*rml* (23S) intergenic spacer region (21) of *B. burgdorferi*, and primers B1 and B2, derived from the 16S rRNA gene (17), demonstrated similar levels of tick infection when used in PCR on parallel aliquots of total DNA from 50 ticks ($\chi^2 = 32.52$; $df = 1$; $P < 0.001$). Moreover, PCR with three primer pairs specific to *bmp*

TABLE 1. Prevalence of human pathogens in *I. persulcatus* ticks from western Siberia, Russia, detected by PCR, RT-PCR, and nested PCR

Pathogens	No. of ticks examined	Prevalence (% \pm SD [sampling error]) ^a
<i>B. burgdorferi</i> sensu lato	150	38.0 \pm 4.0
TBEV	100	46.0 \pm 5.0
HGE	50	8.0 \pm 3.8
<i>B. burgdorferi</i> -TBEV coinfection	100	18.0 \pm 3.8
<i>B. burgdorferi</i> -HGE coinfection	50	6.0 \pm 3.4

^a Calculated as $\sqrt{\frac{p \times (100 - p)}{n}}$, where p is the percentage of positive samples and n is the sample size.

paralogous gene family 36 revealed the same relative order of the *bmpD*, *C*, *A*, and *B* genes among the American (12) and Russian (data not shown) *B. burgdorferi* sensu lato isolates. Comparative analysis of nucleotide sequences of products of PCR of DNA from Siberian ticks with primer 126 showed a 95 to 98% homology with *Borrelia*. The use of TBEV-specific E1 and E2 as well as NS1 and NS2 primers revealed the same infection rate (46.0% \pm 7.2%) in two different pools of ticks ($n = 50$). Nucleotide sequences of the *E* gene fragment from TBEV Siberian strains have been previously published (3).

TBEV and *B. burgdorferi* were the most frequently found pathogens in these 150 ticks (Table 1). The 38% rate of infection with *B. burgdorferi* is consistent with data from the microscopic examination of *I. persulcatus* ticks from western Russia (1, 14, 15). A high prevalence of these spirochetes has also been detected by PCR in taiga ticks from the Pre-Ural region (18). The frequency of *B. burgdorferi*-positive ixodid ticks in Siberia was close to those previously published for ticks in New York (54%) (6) and New Jersey (43%) (25). However, infection of *I. scapularis* with TBEV was never observed in North America, while approximately half of the *I. persulcatus* ticks examined in Siberia harbored TBEV (Table 1).

Ehrlichiae of the HGE genogroup have previously been reported only for *I. persulcatus* ticks from the Baltic region of Russia (8) and from northeastern China (5). Our finding of the HGE agent in this species in the Asian region of Russia is, therefore, the first report of such infection from this area. The infection rate of ticks with the HGE agent (Table 1) was significantly lower than that of ticks with TBEV or *B. burgdorferi* ($P < 0.001$) but was similar to the 9% infection rate for ticks found in Westchester County, N.Y. (6).

Coinfection with *B. burgdorferi* and TBEV was observed in 18% of *I. persulcatus* ticks from western Siberia, while coinfection with *B. burgdorferi* and the HGE agent was found in 6% of these ticks. Contingency analysis indicated that *B. burgdorferi* and TBEV as well as *B. burgdorferi* and the HGE agent were independently distributed in the tick population ($\chi^2 = 1.00$, $df = 1$, $P = 0.32$, and $\chi^2 = 1.15$, $df = 1$, $P = 0.28$, respectively). The epidemiological significance of different pathogens that coexist and are associated with the same tick species is evident. Residents of areas of endemicity are exposed to the risk of two or more different tick-borne infections after single tick bites, and coinfection may alter the clinical manifestations and response to the treatment of Lyme borreliosis, tick-borne encephalitis, or HGE.

It has been postulated that antagonism between *Borrelia* and

TBEV takes place in the same vector because of possible reciprocal inhibition of different pathogens during reproduction in ticks or animals (2). The statistically similar expected and observed values of the coinfection of ticks with *Borrelia* and TBEV in this study (Table 1) are consistent with the independent distribution of these pathogens in the ticks and do not support the postulated mutual inhibition. Thus, these two pathogens do not seem to interfere with each other in ticks and are apparently not involved in any antagonistic relationships in the tick hosts (15, 16).

These studies were supported in part by grant 02-01-113 from the Russian program "Vaccines of New Generation," grant N19 of the Program of the Integration in Basic Sciences of the Siberian Branch of the Russian Academy of Sciences, and grant R01 AI43063 from the National Institute of Allergy and Infectious Diseases to F.C.C.

We thank Henry P. Godfrey and Harriett Harrison (New York Medical College, Valhalla) for their help in the preparation of the manuscript and Dionysios Liveris and Ira Schwartz (New York Medical College) for the HGE agent-specific primers.

REFERENCES

- Alekseev, A. N., E. A. Arumova, L. A. Burenkova, and S. P. Chunikhin. 1993. Some peculiarities of the Lyme disease distribution and of the behavior of *Ixodes* ticks infected with it. *Parazitologiya* 27:389–397. (In Russian.)
- Alekseev, A. N., L. A. Burenkova, I. S. Vasil'eva, E. V. Dubinina, and S. P. Chunikhin. 1996. The functioning of foci of mixed tick-borne infections on Russian territory. *Med. Parazitol.* 4:9–16. (In Russian.)
- Bakhvalova, V. N., V. A. Rar, S. E. Tkachev, V. A. Matveeva, L. E. Matveev, A. S. Karavanov, A. K. Dobrotvorsky, and O. V. Morozova. 2000. Tick-borne encephalitis virus strains of Western Siberia. *Virus Res.* 70:1–12.
- Baumgarten, B. U., M. Rölinghoff, and C. Bogdan. 1999. Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. *J. Clin. Microbiol.* 37:3448–3451.
- Cao, W.-C., Q.-M. Zhao, P.-H. Zhang, J. S. Dumler, X.-T. Zhang, L.-Q. Fang, and H. Yang. 2000. Granulocytic ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. *J. Clin. Microbiol.* 38:4208–4210.
- Chang, Y. F., V. Novosel, C. F. Chang, J. B. Kim, S. J. Shin, and D. H. Lein. 1998. Detection of human granulocytic ehrlichiosis agent and *Borrelia burgdorferi* in ticks by polymerase chain reaction. *J. Vet. Diagn. Investig.* 10:56–59.
- Christova, I., L. Schouls, I. van de Pol, J. Park, S. Panayotov, V. Lefterova, T. Kantardjiev, and J. S. Dumler. 2001. High prevalence of granulocytic ehrlichiae and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks from Bulgaria. *J. Clin. Microbiol.* 39:4172–4174.
- Dubinina, E. V., and A. N. Alekseev. 1999. The biodiversity dynamics of the causative agents of diseases transmitted by ticks in the genus *Ixodes*: an analysis of multiyear data. *Med. Parazitol.* 2:13–19. (In Russian.)
- Ebel, G. D., A. Spielman, and S. R. Telford. 2001. Phylogeny of North American Powassan virus. *J. Gen. Virol.* 82:1657–1665.
- Filippova, N. A. 1977. Ixodid ticks of the subfamily Ixodinae. *Fauna USSR* 4:393. (In Russian.)
- Fingerle, V., U. G. Munderloh, G. Liegl, and B. Wilske. 1999. Coexistence of ehrlichiae of the phagocytophila group with *Borrelia burgdorferi* in *Ixodes ricinus* from Southern Germany. *Med. Microbiol. Immunol.* 188:145–149.
- Gorbacheva, V. Y., H. P. Godfrey, and F. C. Cabello. 2000. Analysis of the *bmp* gene family in *Borrelia burgdorferi* sensu lato. *J. Bacteriol.* 182:2037–2042.
- Jenkins, A., B.-E. Kristiansen, A.-G. Allum, R. K. Aakre, L. Strand, E. J. Kleveland, I. van de Pol, and L. Schouls. 2001. *Borrelia burgdorferi* sensu lato and *Ehrlichia* spp. in *Ixodes* ticks from southern Norway. *J. Clin. Microbiol.* 39:3666–3671.
- Kolchanova, L. P. 1997. Spontaneous tick infection with *Borrelia* and the degree of their individual infectiousness in different landscape subzones of Tyumen province. *Med. Parazitol.* 1:49–50. (In Russian.)
- Korenberg, E. I., Y. V. Kovalevskii, A. S. Karavanov, and G. G. Moskvitina. 1999. Mixed infection by tick-borne encephalitis virus and *Borrelia* in ticks. *Med. Vet. Entomol.* 13:204–208.
- Korenberg, E. I., L. Y. Gorban, Y. V. Kovalevskii, V. I. Frizen, and A. S. Karavanov. 2001. *Emerg. Infect. Dis.* 7:459–462.
- Marconi, R. T., and C. F. Garon. 1992. Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis. *J. Clin. Microbiol.* 30:2830–2834.
- Nefedova, V. V., E. I. Korenberg, L. N. Nesterenko, A. L. Gintsburg, Y. V. Kovalevskii, and N. B. Gorelova. 2001. Comparative evaluation of effectiveness of *Borrelia* indication in ixodid ticks (Ixodidae) by methods of dark-field microscopy and polymerase chain reaction (PCR). *Parazitologiya* 35:3–8. (In Russian.)
- Parola, P., and D. Raoult. 2001. Tick-borne bacterial diseases emerging in Europe. *Clin. Microbiol. Infect.* 7:80–83.
- Petrovec, M., J. W. Sumner, W. L. Nicholson, J. E. Childs, F. Strle, J. Barlić, S. Lotrič-Furlan, and T. Avšič-Zupanc. 1999. Identity of ehrlichial DNA sequences derived from *Ixodes ricinus* ticks with those obtained from patients with human granulocytic ehrlichiosis in Slovenia. *J. Clin. Microbiol.* 37:209–210.
- Postic, D., M. V. Assous, P. A. D. Grimont, and G. Baranton. 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rfl* (5S)-*rfl* (23S) intergenic spacer amplicons. *Int. J. Syst. Bacteriol.* 44:743–752.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., p. 127. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schouls, L. M., I. Van de Pol, S. G. T. Rijpkema, and C. S. Schot. 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *J. Clin. Microbiol.* 37:2215–2222.
- Sumner, J. W., W. L. Nicholson, and R. F. Massung. 1997. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. *J. Clin. Microbiol.* 35:2087–2092.
- Varde, S., J. Beckley, and I. Schwartz. 1998. Prevalence of tick-borne pathogens in *Ixodes scapularis* in a rural New Jersey County. *Emerg. Infect. Dis.* 4:97–99.