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Hantavirus RNA not detected in *Ixodes ricinus* ticks

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Abstract

A total of 174 unfed *Ixodes ricinus* ticks (104 nymphs, 36 males, and 34 females) collected in the Roztocze National Park in eastern Poland were examined by the PCR and nested PCR methods for the presence of hantavirus RNA. None of the examined *Ixodes ricinus* specimens showed the presence of the hantavirus-specific RNA, in spite of using 2 genetic markers and the clearly positive results obtained with the positive control. Thus, a hypothesis about the possible participation of ticks in the transmission of hantaviruses, has so far not been confirmed.

Key words

hantaviruses, epidemiology, *Ixodes ricinus*, PCR, Poland

INTRODUCTION

Hantaviruses (HDV) are enveloped single-stranded RNA viruses belonging to the family Bunyaviridae. Infections caused by hantaviruses pose an increasing global problem and are regarded as 'emerging infectious diseases' [1-4]. Hantaviruses are distributed worldwide with over 150,000 HDV cases being registered annually [2]. In Eurasia, 2 forms of the disease are distinguished:

1) severe Haemorrhagic Fever with Renal Syndrome (HFRS) caused by Hantaan virus (HTNV), Dobrava virus (DOBV) and Seoul virus (SEOV), with the mortality rate amounting up to 20%;
2) the considerably milder Nephropathia Epidemica (NE) caused mainly by Puumala virus (PUUV) and Saaremaa virus (SAAV) [3-6]. In America, there occurs the very severe Hantavirus Cardio-Pulmonary Syndrome (HCPS), caused mainly by the Sin Nombre virus (SNV) and Andes virus (ANDV).

Small rodents are both reservoir and vectors of the disease. PUUV is mainly transmitted by bank voles (*Myodes glareolus*), DOBV by yellow-necked mice (*Apodemus flavicollis*), and SAAV by striped field mice (*Apodemus agrarius*) [1, 6]. Human infection occurs mainly by inhalation of dust polluted with rodent excrements. Nevertheless, some other modes of infection are considered, including transmission by blood-sucking arthropods [7-9]. Houck *et al.* [7] detected RNA of the HCPS-causing Bayou hantavirus in trombiculid mites (chiggers) and an ixodid tick collected in Texas. Gamasid and chigger mites have been PCR positive for HTNV in the People's Republic of China [8]. Thus, the role of mites in the transmission of the Old and New World hantaviruses is documented. Valiente Moro *et al.* [9] expressed an opinion that mites belonging to the super-family Dermanyssoidea should be considered as potential vectors of various pathogens, including hantaviruses.

Recently, the first focus of hantavirus disease (HDV) has been established in the Carpathian mountains in southeast Poland, close to foci described earlier in Slovakia [3, 5, 10]. A total of 13 serologically-confirmed clinical cases of HDV were described, of which 10 were HFRS cases caused by DOBV and 3 were NE cases caused by PUUV [3, 5]. In seroepidemiological studies of forestry workers in eastern Poland, Knap *et al.* [11] and Grygorczuk *et al.* [12] found a positive response to hantaviruses in 2.5% and 8.7%, respectively, which indicates the possibility of occupational exposure.

As forestry workers in eastern Poland are often exposed to tick bite, we undertook an attempt to screen *Ixodes ricinus* ticks collected in that region, specifically in the work area of anti-HFRS seropositive forestry workers in the Roztocze National Park [11], for the presence of hantavirus RNA.

MATERIALS AND METHODS

Collection of ticks. Unfed ticks were collected during summer/autumn season in 2009 and 2010 from Roztocze National Park, situated on the area of Roztocze Highlands (southeastern part of the Lublin province, eastern Poland). Ticks were collected by dragging a woolen flag over lower vegetation and litter along the paths and edges of coniferous and mixed forests. After collection, the ticks were placed in plastic vials and stored at -80°C for further investigation. A total of 174 *Ixodes ricinus* ticks (104 nymphs, 36 males, and 34 females) were collected.

RNA isolation. After thawing, the ticks were investigated individually. Each specimen was crushed separately in liquid nitrogen and then homogenized with a syringe needle and suspended in buffer containing guanidine isothiocyanate (GITC), inhibiting RNA-ase enzyme. Total RNA was extracted from homogenized ticks using the RNeasy Mini Kit (Qiagen, USA) according to the producer's instructions. The amounts of extracted RNA measured with NanoDrop ND1000 spectrophotometer (USA) were in the range of 10–60 ng (for nymphs and adults respectively).

Reverse Transcription Reverse transcription was carried out using the QuantiTect Reverse Transcription kit (Qiagen, USA) according to the producer's instructions.

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PCR and nested PCR reaction. The reactions were performed according to Arai *et al.* [13].

To amplify a hantavirus S- and L-segment sequences, the polymerase chain reactions (PCR) were carried out with the following primers: 1. for the region of the S-segment: outer OSM55: 5'-TAGTAGTAGACTCC-3' and HTN-S6: 5'-GCTCIGGATCCATITCATC-3'; inner Cro2R: 5'-AIGAYTGRTARAAIGAIGAYTTYTT-3' and PHS-5F: 5'-TAGTAGTAGACTCCTTRAARAGC-3'

2. for the region of the L-segment: outer HAN-L-F1: 5'-ATGTAYGTBAGTGCWGATGC-3' and HAN-L-R1: 5'-AACCADTCWGTYCCRTCATC-3'; inner HAN-L-F2: 5'-TGCWGATGCHACIAARTGGTC-3' and HAN-L-R2: 5'-GCRTCRTCWGARTGRTGDGCA-3'.

The first PCR reaction was conducted in a 50 µl reaction volume containing the following mix of reagents: 2.5 U *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer containing 15 mM $MgCl_2$, additional 1.25 mM $MgCl_2$ (all from Qiagen, USA), 250 mM each of dNTP (Fermentas, Lithuania), 0.25 µM of each of primer (Eurogentec, Seraing, Belgium), nuclease-free water (Applied Biosystems, USA) and 2 µl of cDNA (10 × diluted in nuclease-free water). The reaction was performed in C1000 Thermal Cycler (BioRad, USA) under the following conditions: initial denaturation at 94°C for 30 sec, 2-degree step-down annealing from 46°C-38°C for 40 sec, and elongation at 72°C for 1 min; then 30 cycles (denaturation 94°C for 30 sec, annealing at 42°C for 40 sec, elongation at 94°C for 30 sec); and final extension step at 72°C for 7 min.

Nested PCR reaction was carried out under the same conditions with 2 µl of the first amplification product. For detection of the specific reaction products (1,083 bp for the S-segment and 347 bp for the L-segment), electrophoresis was performed in 2% agarose gel under standard conditions. After ethidium bromide staining, the strips were read under UV light.

As a positive control, the antigens of 6 hantaviruses (Hantaan, Sin Nombre, Puumala, Dobrava, Seoul, Saremaa) were retrieved from slides of the commercial kit for detection of anti-hantavirus IgG antibodies by the immunofluorescence method (Euroimmune, Germany). RNA was isolated using Qiamp Viral RNA Mini Kit (Qiagen, USA) according to manufacturer's procedure. RNase-free water was used as a negative control.

Precautions to exclude contamination were as follows: One-use tips with filters (PCR-clean/dualfilter/sterile) and one-use Eppendorf test tubes (PCR-clean, free of detectable human DNA, DNase, RNase, PCR inhibitor) were used. The glass and plastic surfaces were treated with RNase Zap (Ambion, RNA Company, USA) which completely removes contamination with RNase.

RESULTS AND DISCUSSION

None of the examined *Ixodes ricinus* specimens showed the presence of the hantavirus-specific RNA in spite of using 2 genetic markers and the clearly positive results obtained with the positive control. Thus, a hypothesis about the possible participation of ticks in the transmission of hantaviruses, raised by one of our team (J. D.) [5, 11] has not been confirmed. Nevertheless, the role of ticks as potential vectors of hantaviruses cannot be excluded, and for a definitive solution to this problem, an examination of a greater number of tick specimens and species is required.

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