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# Isolation of a *Rickettsia slovaca*-Like Agent from *Dermacentor variabilis* Ticks in Vero Cell Culture

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## Abstract

*Rickettsia slovaca* is transmitted by *Dermacentor marginatus* ticks, and is the causative agent of tick-borne lymphadenopathy and *Dermacentor*-borne necrosis erythema lymphadenopathy throughout Europe. It has not been found in New World ticks, nor have tick-borne lymphadenopathy or *Dermacentor*-borne necrosis erythema lymphadenopathy been reported in humans in the Americas. Here we describe the isolation of a *R. slovaca*-like agent from *D. variabilis* nymphs from a colony of ticks derived from field collected adults.

### Keywords

*Rickettsia slovaca*; *Dermacentor variabilis*; *Rickettsia* isolation

## Introduction

*Rickettsia slovaca* is the causative agent of tick-borne lymphadenopathy and *Dermacentor*-borne necrosis erythema lymphadenopathy. It was first isolated from *Dermacentor marginatus* ticks in 1968 (Brezina et al. 1969), but wasn't recognized as a human pathogen until 1997 (Raoult et al. 1997). Since then, it has been observed throughout Europe, with *D. marginatus* as the recognized tick vector (Marquez et al. 2006). *Dermacentor spp.* in the United States are known vectors of rickettsial agents, including *Rickettsia rickettsii*, *R.montanensis*, and *R. phillipi* (364D) (Ammerman et al. 2004). *R. slovaca* is, historically, a strictly European pathogen and as such has not been found in New World ticks nor been implicated as a cause of human disease in the United States.

Nymphs used in this study originated from a colony of *D. variabilis* established from adult ticks collected in North Carolina and Virginia. These ticks were obtained for the purpose of introducing fresh genetic material into our currently maintained laboratory colony. Upon receipt, the ticks were tested and found positive for rickettsial DNA. The origin of the infection in ticks is unknown, but sequence analysis showed 100% identity at six gene targets to the 1968 isolate of *R. slovaca* from *D. marginatus* in Slovakia (Gen- Bank

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accession no. CP003375.1, Zemtsova et al. 2016). Here we describe the first cell culture isolation of this rickettsial agent from *Dermacentor variabilis* ticks.

## Materials and Methods

The infected *D. variabilis* were from a colony derived from wild ticks collected in southeastern Virginia and northeastern North Carolina and maintained in a laboratory for several generations before being sent to our lab. Ten infected nymphs were surface sterilized by immersion for 10 minutes each in 70% ethanol and 10% Clorox solution. They were then rinsed in sterile water and air dried on sterile filter paper. Ticks were triturated in 2mL modified Eagle's medium and added to a T25 flask of confluent Vero E6 cells. An additional 3mL of media was added to the flask, consisting of modified Eagle's medium supplemented with 10% fetal bovine serum, 4mM l-glutamine, nonessential amino acids, HEPES, sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 μg/mL amphotericin B. Cells were maintained at 34°C in an incubator with 5% $CO_2$. After 24 hours, the inoculum was removed and 5mL of medium, without antibiotics and antimycotics, was added. Thereafter, media was refreshed once weekly. The culture was monitored for signs of infection by examining cells fixed to glass slides with absolute methanol and stained with acridine orange (Becton, Dickinson and Company, Sparks, MD). Slides were then viewed under a microscope fitted for fluorescent microscopy for identification of live, rod-shaped, Rickettsia-like organisms. For cryopreservation, cells from a T25 flask (~25% infected) were detached with glass beads, spun at 300 × *g*, resuspended in 3mL of sucrose phosphate glutamate solution (Bovarnick et al. 1950), supplemented with 0.005M $MgCl_2 \cdot 6H_2O$ and Renografin (13.6 mL/liter), aliquoted, and stored in liquid nitrogen.

DNA from the cell culture was extracted using the Qiagen DNEasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocols and eluted in 200 μL (final volume). Multiple locus sequence analysis of the cultured rickettsial agent was done for the 17 kDa *glt*A, *omp*B, *omp*A, *rpo*B, and *sca*4 genes. Sequences were performed according to the same protocols as, and compared with, those of *Rickettsia* detected in *D. variabilis* and characterized in our laboratory previously (Zemtsova et al. 2016). A BLAST search was used to identify homologous sequences. Tick species identification was carried out by sequencing 12S mitochondrial DNA (Beati and Keirans 2001).

## Results and Discussion

Small rods consistent with *Rickettsia*-like organisms were observed in culture by acridine orange staining 10 days post inoculation. Primary cultures of the *R. slovaca*-like agent did not initially show signs of cytopathic effects, and the monolayer remained intact for 14 days post-inoculation. Only after the first passage did the cells show cytopathic effects, beginning on day 4 with complete destruction of the monolayer by day 9. Additionally, live rickettsia were successfully recovered from cultures that had been stored for one year in liquid nitrogen.

Sequences of the cell culture PCR products targeting the *omp*A, 17 kDa, *glt*A, *omp*B, *sca*4, and *rpo*B genes showed 100% identity to those obtained from colony *D. variabili*s (GenBank

acc. nos. KR559552, KR559550, KR559551, KR559553, KR559554, and KR559549), as well as to a 1968 isolate of *R. slovaca* from *Dermacentor marginatus* ticks collected in Slovakia (GenBank acc. no. CP003375.1). Sequencing of the 12S mitochondrial ribosomalDNAconfirmed that the ticks used for isolation were in fact *D. variabilis* (100% identity to GenBank acc. no. S83088.1).

Here we report the first isolation of a *Rickettsia sp*. identical, at six gene targets, to the European pathogen, *R. slovaca* from North American *D. variabilis* ticks. As far as we know, these ticks were field collected in North Carolina and Virginia and subsequently used to supplement our uninfected colony for the prevention of inbreeding. The infection was detected during routine testing procedures of both tick colonies and animal hosts. The primary suspects were *R. montanensis* or *R. rickettsii*, both of which are known to occur in *D. variabilis* in that region. The identification and isolation of *R. slovaca* from these ticks was unexpected. The original source and the history of *R. slovaca* introduction into *D. variabilis* ticks remain unknown (despite our sincere and diligent efforts to elucidate these). We can only state, unequivocally, that neither our laboratory nor the external facility from which ticks were acquired ever worked with any *R. slovaca* isolates or known European vectors of this pathogen. Based on the available data, it would be premature to claim that *R. slovaca* is actually present in natural populations of *D. variabilis*. Yet, isolation of this agent in cell culture from nymphal *D. variabilis*, which were fed as larvae on laboratory guinea pigs, confirms that the American dog tick is capable of maintaining the live pathogen and transmitting it both transovarially and transstadially. Therefore, it is likely that in case of *R. slovaca* importation (whether past or future) to North America this pathogen may be maintained locally by *D. variabilis*. Vector and reservoir competency studies as well as increased testing of wild *D. variabilis* will help determine whether this is a pathogen of consequence to public health in the United States

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