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Isolation of *Rickettsia helvetica* from ticks in Slovakia

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Summary. – To date, only three rickettsial species have been found in ticks in Slovakia by serological and/or molecular-biological techniques, namely *Rickettsia slovaca*, *Candidatus rickettsia* IRS, and *Rickettsia raoultii*. Recently, we succeeded in isolation of the forth species, *Rickettsia helvetica* from *Ixodes ricinus*, the most frequent tick in Slovakia. The isolation, positive for 10% of tested ticks, was performed on XTC cells by the shell-vial technique, Gimenez staining and light microscopy. The infected cell cultures contained rod-shaped particles morphologically identical to rickettsiae. The isolation was confirmed by direct detection of a fragment of the *R. helvetica* gene for citrate synthase in the positive ticks by PCR and its subsequent cloning, sequencing and comparison with the database.

Keywords: Rickettsia helvetica; isolation; Ixodes ricinus; Slovakia

Introduction

Spotted fever group (SFG) rickettsiae are Gram-negative intracellular bacteria associated with arthropods, which are maintained by transstadial and transovarial transmission (Burgdorfer and Varma, 1967). *Ixodes ricinus* and *Dermacentor marginatus* are the main sources of dispersion of SFG rickettsiae in Central and Eastern Europe (Barandika *et al.*, 2008; Bertolotti *et al.*, 2006; Psaroulaki *et al.*, 2006; Punda-Polic *et al.*, 2002). Ticks transmit rickettsial infections while feeding on humans or animals, resulting in severe diseases with characteristic clinical features such as: high fever, severe headache, and rash or eschar formation (Parola *et al.*, 2005).

R. helvetica (Burgdorfer *et al.*, 1979), *R. slovaca* (Brezina *et al.*, 1969), *R. sibirica* (Balayeva *et al.*, 1996), and *R. raoultii* (Mediannikov *et al.*, 2008) are the most prevalent rickettsial species frequently detected in Central and Eastern Europe.

R. helvetica (Beati *et al.*, 1993) was first time isolated from *I. ricinus* ticks in Switzerland (Burgdorfer *et al.*, 1979) and later on confirmed all around the old continent. It can be frequently detected in all countries from North Sweden (Nilsson *et al.*, 1997) to South France (Fournier *et al.*, 2000; Parola *et al.*, 1998) and from United Kingdom (Tijsse-Klasen *et al.*, 2011) to Ural (Nefedova *et al.*, 2008).

Twenty-nine years after the first isolation of *R. slovaca* (Brezina *et al.*, 1969) this agent was documented as a human pathogen (Raoult *et al.*, 1997). In the last decade it was repeatedly recognized as an intriguing factor of the disease called TIBOLA, in Hungary (Lakos, 2002), Germany (Pluta *et al.*, 2009), Italy (Selmi *et al.*, 2008), DEBONEL in Spain (Ibarra *et al.*, 2006), and SENLAT in France (Angelakis *et al.*, 2010).

North Asian tick typhus (Siberian tick typhus) is caused by *R. sibirica sibirica*, which belongs to the group of rickettsiae that are transmitted to humans by the ixodid ticks bite (Eremeeva *et al.*, 1993; Rehacek and Tarasevich, 1991). When it was described for the first time in the mid 1930s (Zdrodovski and Golinevitch, 1960), it was known to be spread mostly in Eastern Europe.

Clearly, the use of molecular biological methods to uncover the bacterial agents has brought new discoveries

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Abbreviations: SFG = spotted fever group; XTC = *Xenopus laevis* tissue culture; CPE = cytopathic effect

(Parola and Raoult, 2001). A new subspecies, *R. sibirica* subsp. mongolitimonae, was isolated from the blood and the skin of a patient in France (Raoult *et al.*, 1996), and later on its presence was confirmed in Portugal (de Sousa *et al.*, 2006).

Enrichment of the Rickettsial family by new members, e.g. „Cadiz agent" in Spain (Marquez *et al.*, 1998), „*Candidatus rickettsia IRS*" in Slovakia (Sekeyova *et al.*, 2000), *R. monacensis* in Germany (Simser *et al.*, 2002), or „*Candidatus R. barbariae*" in Sardinia (Mura *et al.*, 2008), brought new data about the viability and variability of these exciting microorganisms. A list of possible new pathogens which can be found in Europe has been widely extended.

The aim of this study was identification of rickettsial species circulating in ticks in Slovakia with the emphasis on so far undetected ones.

Materials and Methods

Collection of ticks. In the spring of 2010, 30 *I. ricinus* adult ticks (assigned as Ir1-Ir30) were collected from vegetation in the Podunajske Biskupice district of the south-eastern area of Slovakia. The ticks were identified according to standard taxonomic key (Nosek and Sixl, 1982) and frozen at -80°C.

Isolation of rickettsiae from ticks in XTC-2 cells. Ticks were thawed, sterilized by immersion in iodinated alcohol for 10 min, rinsed with distilled water for 10 min, and dried on sterile filter paper under a laminar flow hood. Each tick was longitudinally cut into two pieces. One half of each tick was triturated in 1 ml Leibowitz medium (L medium) (Gibco, UK), and the mixture was placed into a shell-vial containing monolayers of XTC cells. The shell-vials were centrifuged at 700 x g for 1 hr, and the supernatant was then replaced with 1 ml of L medium containing 4% fetal calf serum (FCS) and 2 mmol/l L-glutamine (PAA Laboratories, Austria). After 6 days of incubation at 34°C in a CO_2 incubator, scraped XTC cells were applied to a microscope slide and stained by the method of Gimenez (Gimenez, 1964), and/or Giemsa (Giemsa, 1904) to detect rickettsiae. Supernatant with de-touched cells from infected shell-vials were transferred into 25 cm^2 flasks. Fresh L medium was added and the flasks were incubated at 34°C. The medium was changed every 4 days. The isolated strains were subcultured in XTC cells by subsequent trypsinizations.

Reference rickettsiae. R. conorii (Moroccan strain VR-141) was obtained from the American type culture collection. *R. helvetica* type strain C9P9 was kindly supplied by Dr. W. Burgdorfer (Rocky Mountain Laboratories, Hamilton, Montana, USA). *R. raoultii* Khabarovsk VR-1596 (T) was obtained from the laboratories of Reference Center for Rickettsioses (Faculty of Medicine, Marseille, France).

PCR. PCR was used for the detection of SFG rickettsiae in tick suspensions as described previously (Beati *et al.*, 1992; Regnery *et al.*, 1991). The DNA of the remaining half-ticks was extracted, purified, and recovered into 80 µl of sterile water, according to the QIAamp tissue kit procedure (Qiagen, Germany). The amplification of a 382 base pair (bp) fragment of the gene encoding for the citrate synthase was performed using previously described oligonucleotide primer pairs Rp CS.877p and Rp CS.1258n (Bioprobe Systems, France). A negative (distilled water) and a positive *R. raoultii* strain Khabarovsk VR-1596 (T), *R. helvetica* type strain C9P9, and *R. conorii* (Moroccan strain VR-141) controls were included in each test. PCR conditions were applied as described previously (Beati *et al.*, 1992; Roux *et al.*, 1997). Reactions were performed in automated DNA thermal cyclers (GeneAmp PCR system 2400 and 9700; Applied Biosystems, France). The amplification products were visualized on a 1% agarose gel stained with ethidium bromide and examined by using ultraviolet (UV) transillumination. A DNA size marker (Boehringer-Mannheim V, Germany) was used to estimate the size of DNA fragments.

Sequencing and sequence alignment. PCR products were purified by using a QIAquick spin PCR purification kit (Qiagen) and sequenced by using a DNA sequencing kit (dRhodamine Terminator cycle sequencing ready reaction; Applied Biosystems), according to the manufacturer´s instructions. All sequences were assembled and edited with Auto Assembler software (version 1.4; Perkin-Elmer, France) and compared with those of the rickettsiae present in the GenBank database using the BLAST search tool. Obtained sequences were aligned and analyzed by using the ClustalW which is a part of the Bisance software package (Dessen *et al.*, 1990), and phylogenetic tree was constructed by using Mega v.4 software. Similarity levels between pairs of sequences were determined with DNASIS software (Hitachi Software Engineering America, USA).

Results and Discussion

Three rickettsia-positive ticks Ir6, Ir9, and Ir16 were detected by the PCR with the primers Rp CS.877p and Rp CS.1258n. The result of the amplification was visualized on an ethidium bromide stained, 1% agarose gel with an approximate size of 382 bp. This profile was identical to that of all three rickettsiae used as a positive control (Fig. 1).

Three positive isolates of rickettsiae were recovered from *I. ricinus* ticks Ir6, Ir9, and Ir16, respectively, using the shell-vial technique. The isolates were subcultured on XTC cells. No cytopathic effect was observed. Staining by the method of Gimenez (Gimenez, 1964), and Giemsa (Giemsa, 1904) revealed small, intracellular, rod-shaped bacteria (Fig. 2).

A 382 bp sequence of PCR-derived fragments of the citrate synthase gene was generated from Ir6, Ir9, and Ir16. Comparison of these sequences with the corresponding sequences of the SFG rickettsiae revealed 100% similarity between *R. helvetica* sp. Podunajske Biskupice, Ir6, Ir9, and Ir16 isolates, and *R. helvetica* type strain C9P9 (U59723) (Roux *et al.*, 1997) (Fig. 3).

We have isolated and characterized *R. helvetica* from 10% of the *I. ricinus* ticks collected in the district of Podunajske Biskupice in South-Eastern Slovakia. This district is unique in the region, for its moderate climate, which is suitable for the prevalence of various tick species. *I. ricinus* is a most frequently human biting exophilic tick, widely distributed in spring months, active in circulation of rickettsiae both as vector and host (Boldis *et al.*, 2008; Chmielewski *et al.*, 2009; Siuda *et al.*, 2009; Špitalská *et al.*, 2008).

Our isolate of *R. helvetica* sp. Podunajske Biskupice, is genetically similar to the type strain C9P9 (Reference Center for Rickettsioses, Marseille, France), one of the first strains of this species isolated at the Rocky Mountain Laboratories from *I. ricinus* ticks collected in the Staatswald, Bern, Switzerland (Beati *et al.*, 1994; Burgdorfer *et al.*, 1979). In Slovakia, seroprevalence of antibodies to rickettsiae in human sera (Kovacova *et al.*, 2006), or positivity of DNA extracted from blood samples of domestic and wild hosts, have been deeply studied before (Špitalská *et al.*, 2008).

Molecular evidence and serological screening, namely immunofluorescence testing of several intriguing rickettsial agents, including *R. helvetica*, in humans was recently provided in a study of a cohort of sera obtained from patients who reported to overcome tick or insect bite, and prevailed over the disease with "unknown etiology" (Sekeyova *et al.*, 2012). However, even if we confirmed that several of these cases were linked to rickettsia, no isolation was provided. To our knowledge, this is the first report of the isolation of *R. helvetica*, sp. Podunajske Biskupice, in the studied territories. After discovery of *R. slovaca* (Brezina *et al.*, 1969), "*Candida-*

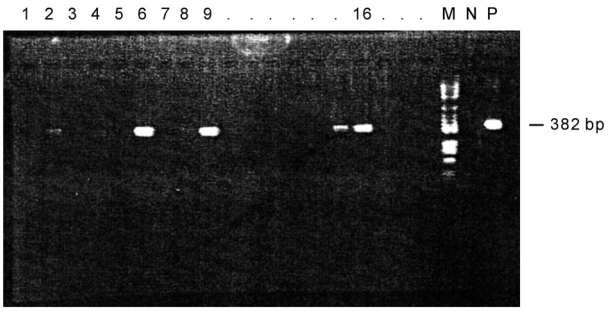

Fig. 1

Identification of *R. helvetica* by PCR

Agarose gel electrophoresis of PCR products stained with ethidium bromide, (tick number = 1–19, positive control = P, negative control = N, and DNA size marker = M). 382 bp band in positive samples Ir6, Ir9, and Ir16 is clearly visible.

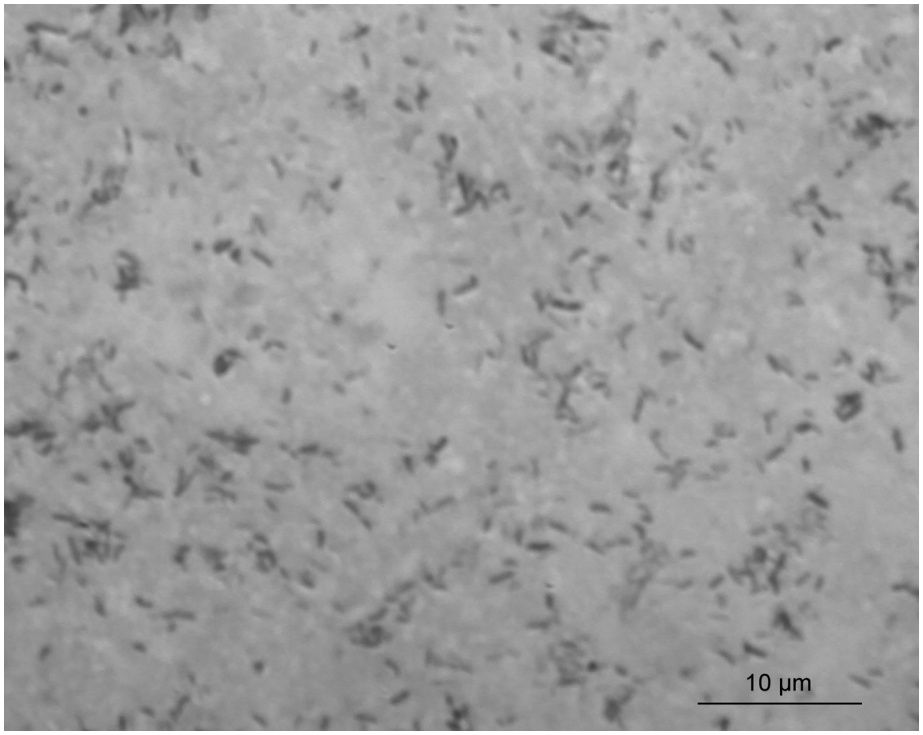

Fig. 2

***R. helvetica* grown in XTC-2 cells**

Light microscopy of cells stained by Gimenez.

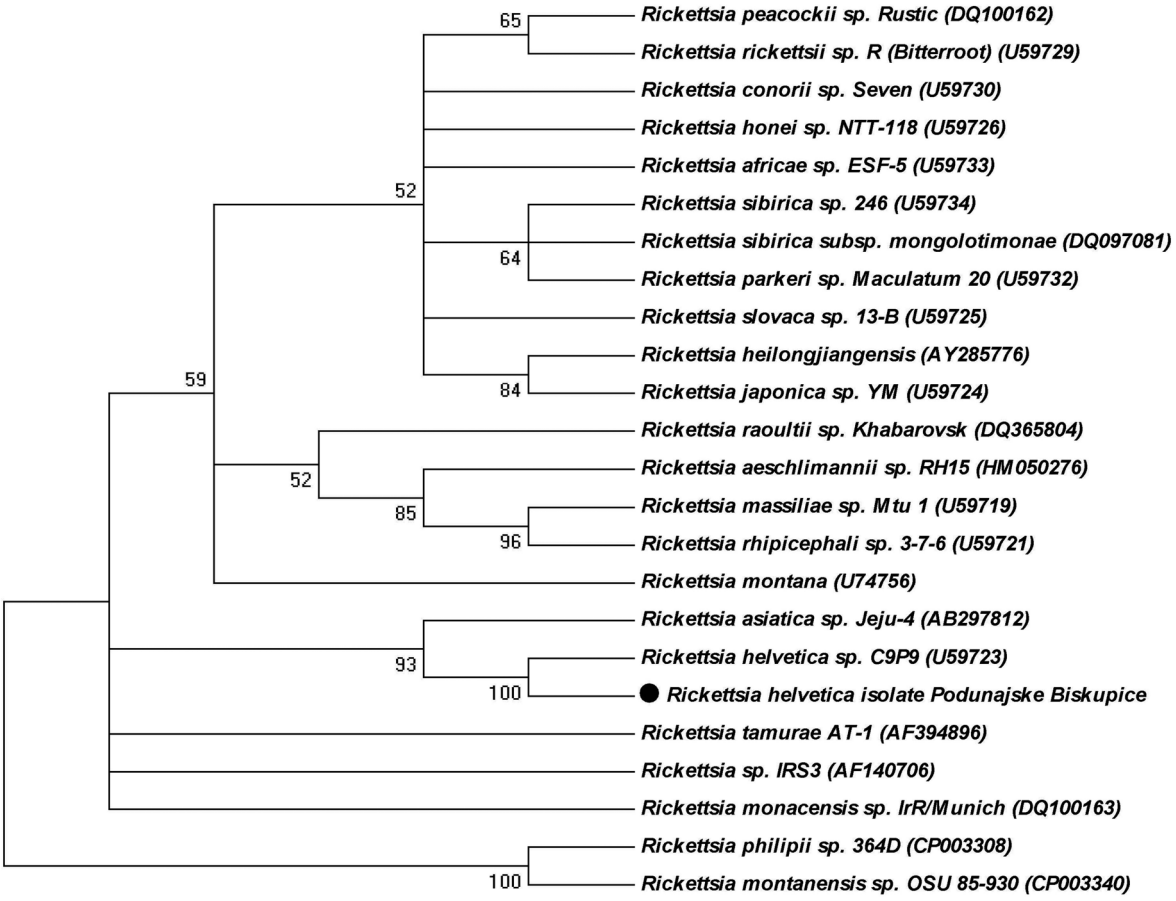

Fig. 3

Phylogenetic tree of *Rickettsiae* genus based on citrate synthase gene

tus rickettsia IRS" (Sekeyova *et al.*, 2000), and recent isolation of *R. raoultii* (Spitalska *et al.*, 2012), this is the fourth species of the Rickettsia genera that was successfully isolated from ticks. We have obtained the isolate *R. helvetica* sp. Podunajske Biskupice using complex of established methods such as the shell-vial technique and Gimenez staining, in combination with modern and sensitive detection methods, PCR and gene sequencing.

Since the citrate synthase gene of SFG rickettsiae has already been sequenced before (Roux *et al.*, 1997), we were able to compare the obtained sequences of a new isolate with other corresponding sequences available in the rickettsial database.

A confirmation of *R. helvetica* in *I. ricinus* by isolation is epidemiologically important. Prevalence of this agent was long-established in several European countries, e.g. in Switzerland (Peter *et al.*, 1981), France (Parola *et al.*, 1998) (Fournier *et al.*, 2000), Denmark (Nielsen *et al.*, 2004), Slovakia (Kovacova *et al.*, 2006), and there are no borderlines for this species, as it was found also in Japan (Matsumoto and Inokuma, 2009) or even in Kamchatka Penninsula (Pukhovskaia *et al.*, 2010).

Its pathogenicity to humans is variably described despite long lasting evidence in nature. The organism was first considered to be nonpathogenic, then after providing an array of serosurveys was linked to the disease with flu-like symptoms, associated with fever, headache, and myalgia. It may cause many health problems, from mild to serious complications.

In order to clarify and assign a causative agent in the future, we would like to investigate a larger group of ticks and a larger cohort of territorially corresponding human sera. Furthermore, we intend to focus our attention to the isolation trials, being aware of the fact that a yield of endoparasite germ is a final evidence of the causative agent, a source of disease.

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