LETTER TO THE EDITOR

SPECIFIC DETECTION OF *RICKETTSIA SLOVACA* BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF *sca*4 GENE

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For many years, occurence of rickettsiae belonging to the spotted fever group (SFG) infesting *Ixodes ricinus, Haemaphysalis inermis, H. punctata, Dermacentor marginatus, D. reticulatus* (the order *Acari,* the family *Ixodidae*) ticks has been continuously monitored in different localities of Slovakia (*1, 2, 3*). It was believed that *Dermacentor* ticks were infested with *Rickettsia slovaca* in a high percentage. This hypothesis was not confirmed or proved false, because the methods used for the assessment of rickettsia presence were a hemocyte test and PCR with primers targeting 16S rDNA, *gltA, ompA, ompB* genes. These methods were not able to distinguish *R. slovaca* from other rickettsiae. The correct identification of the rickettsiae species could be done only by sequence analysis.

Therefore, we tried to devise a new test suitable for an easy differentiation of *R. slovaca* from other rickettsiae. This study reports a development of a quick, cheap, and easy differentiation method based on the restriction fragment length polymorphism (RFLP) of the *sca*4 gene.

R. slovaca belonging to the family *Rickettsiaceae*, the genus *Rickettsia* is a rod shaped obligate intracellular parasite isolated from *D. marginatus* ticks collected in central Slovakia in 1968. Initially, the isolated rickettsial strains indicated as "B" or "D" were classified as the members of SFG rickettsiae closely related to *R. sibirica* (*4*). Using complement fixation and microagglutination tests, the strains were definitively identified as the new rickettsial species and shortly the designation *R. slovaca* was proposed *(5).* In 1998, Sekeyová *et al*. suggested that *R. slovaca* should be considered as a separate taxonomic species on the basis of the distinctive clinical, epidemiological, phenotypic, and genotypic features (*6*).

R. slovaca is transmitted to the hosts largely by salivary glands and faeces of *D. marginatus* ticks. Other rickettsiae are found in all organs of the infested ticks and can be transmitted transovarially and transstadially (*7*). Occurrence of *D. marginatus* is generally common throughout the Central Asia and Europe except for northern Europe. The ticks are active during early spring, autumn, and winter. Adult ticks inhabit forests and pastures and frequently bite people entering these biotopes. Since its discovery, *R. slovaca* has been detected in many European countries, e.g. in Portugal, France, Croatia, Spain (*8, 9, 10, 11*). The role of wild animals in circulation of *R. slovaca* is not well-defined. However, study on wild boars in northern Spain suggests that wild boars are exposed to *R. slovaca* infection and in this way the pathogen is well established also in the wild cycle of *D. marginatus (11)*.

R. slovaca had been considered as a non-pathogenic microorganism for many years. However, the first case of human infection caused by *R. slovaca* was reported in France in 1997 *(9).* Following the bite of *D. marginatus,* the afflicted woman developed fever, arthralgia, fatigue, eschar of the scalp, and enlarged cervical lymph nodes. This case was

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Abbreviations: RFLP = restriction fragment length polymorphism; SFG = spotted fever group

well-documented by seroconversion, molecular detection of *R. slovaca*, and by the isolation of the etiologic agent from biting ticks. Clinically similar cases were detected also in Hungary and Spain (*12*, *13*).

For differentiation of *R. slovaca* from other rickettsiae we decided to use the enzymatic digestion of the *sca*4 gene. The restriction enzymes *Hae*III and *Pho*I were selected as optimal using software NEB Cutter and Enzyme Finder of the New

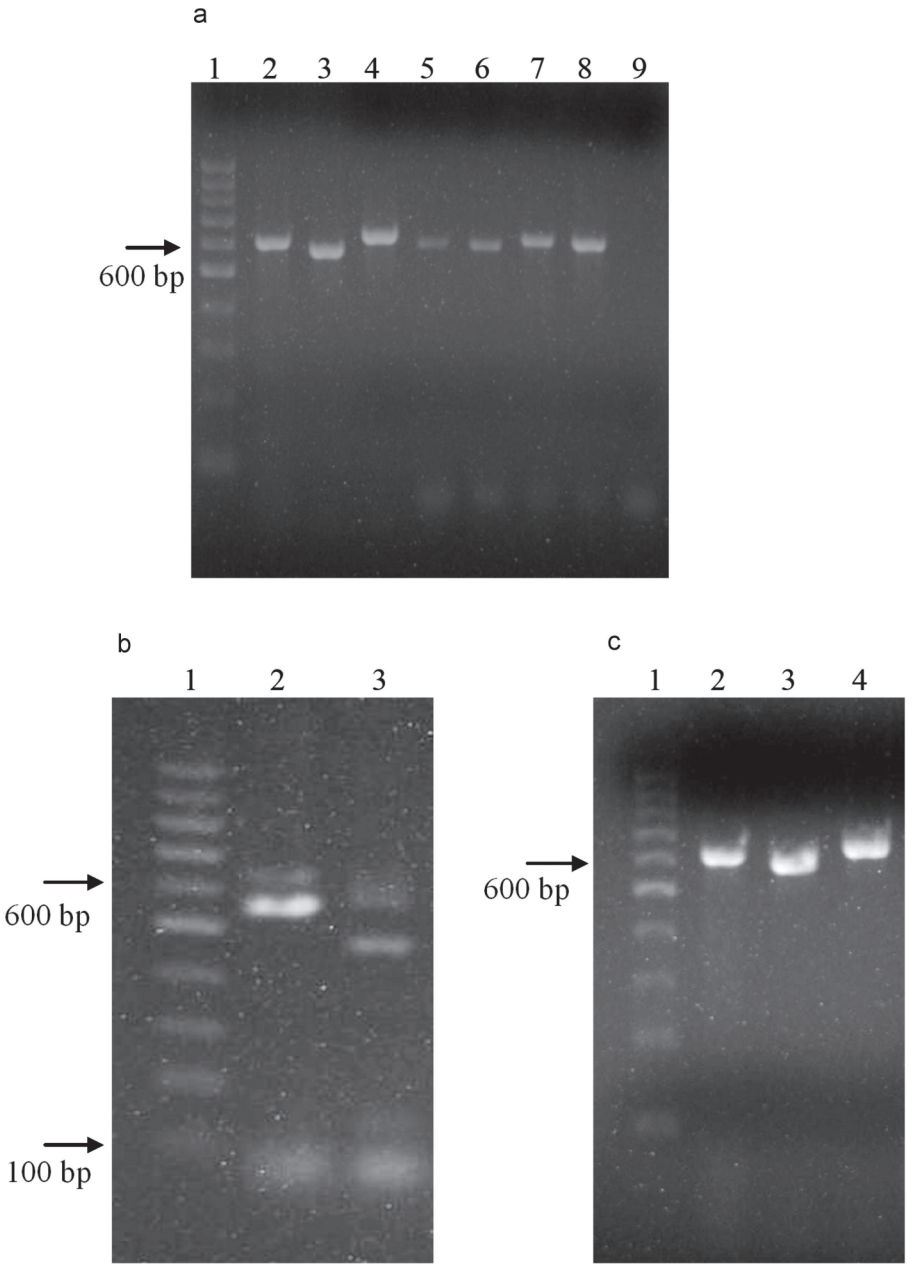

Fig. 1

RFLP analysis of *Hae*III digested PCR products of *sca 4* gene for various rickettsiae

(a) 100 bp molecular marker (lane 1), PCR products of *sca 4* gene for *R. heilongjiangensis* (lane 2), *Rickettsia* sp. IRS4 (lane 3), *R. helvetica* (lane 4), *R. raoulti* (lane 5), *R. slovaca* (lane 6), *R. typhi* (lanes 7, 8). Negative control (lane 9). (b) 100 bp molecular marker (lane 1), restriction profiles of *R. typhi* (lane 2), and *R. slovaca* (lane 3). (c) 100 bp molecular marker (lane 1), restriction profiles of *R. heilongjiangensis* (lane 2), *Rickettsia* sp. IRS4 (lane 3), and *R. helvetica* (lane 4).

England BioLabs Inc. (http://www.neb.com). The PCR products of *sca*4 gene of *R. slovaca* and *R. typhi* using primers D767Forward and D1390Reverse identified from sequence data of *R. conorii* (*14*) can be digested with these enzymes. We took advantage of the fact that the PCR products of *sca*4 gene of other *Rickettsiae* spp. available in GeneBank are not digested by *Hae*III and *Pho*I enzymes.

The *sca*4 genes of various rickettsiae, as *R. helvetica, Rickettsia* sp. IRS4*, R. heilongjiangensis, R. raoultii* obtained from ticks, *R. slovaca* from L929 cells, and *R. typhi* obtained from an antigen purified from chicken yolk sacks by gradient centrifugation were PCR amplified using a primer pair D767Forward and D1390Reverse. PCR products of 623, 590, 605, 623, 653, and 653 bp in size were obtained for *R. slovaca, Rickettsia* sp. IRS4, *R. heilongjiangensis, R. raoultii, R. typhi,* and *R. helvetica,* respectively (Fig. 1a). Negative control (nuclease-free water) was included in the assay. DNA amplifications were performed in PTC-200 Peltier thermal cycler (MJ-Research). Enzymatic digestion for identification of *R. slovaca* was performed by incubation of 17 µl of the PCR amplified products with 2 µl of enzyme buffer and 1 µl (10 units) of *Hae*III restriction endonuclease (Takara Bio) for 3 hrs at 37°C. PCR products and digested products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Their size was determined by 100 bp DNA Ladder (SBS Genetech). The PCR products of *R. slovaca* and *R. typhi* were digested to the expected profiles, *R. slovaca* to 2 fragments 477 and 146 bp long and *R. typhi* to 2 fragments 575 and 78 bp long (Fig. 1b). Other rickettsiae as *Rickettsia* sp. IRS4, *R. heilongjiangensis, R. raoultii,* and *R. helvetica* were not digested under these conditions (Fig. 1c).

In the present study, a specific profile was found after the enzymatic digestion of *sca*4 gene with *Hae*III enzyme to distinguish *R. slovaca* from other rickettsiae. *R. slovaca* could be differentiated from other rickettsiae by comparison of the DNA patterns of amplified parts from the *ompA, ompB,* and *sca*4 genes (*15, 16*). However, the digestion profiles of *sca*4 gene allow the easiest and prompt approach for differentiation of *R. slovaca* from other rickettsiae that could be useful for evaluation of infections with *R. slovaca* in vectors and hosts.

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References

(1) Řeháček J, Kováčová E, Kováč P, *Folia Parasitol.* **23**, 69–73, 1976.
(2) Řeháček J, Kováčová E, Lisák V, Rumin W, *Folia Parasitol*. **37**, 285–286, 1990.
(3) Špitalská E, Kocianová E, Výrosteková V, *Biologia*, **57,** 585–591, 2002.
(4) Brezina R, Řeháček J, Áč P, Majerská M, *Acta Virol.* **13**, 142–145, 1968.
(5) Úrvölgyi J, Brezina R, *Proc. 2nd Intern. Symp. on Rickettsiae and Rickettsial Diseases.* Smolenice, pp. 299–305, 1978.
(6) Sekeyová Z, Roux V, Xu W, Řeháček J, Raoult D, *Int. J. System. Bacteriol.* **48**, 1455–1462, 1998.
(7) Řeháček J, *Acta Sc. Nat.* **18**, 1–50, 1984.
(8) Bacellar F, Nuncio MS, Alves MJ, *Enferm. Infec. Microbiol. Clin.* **13**, 218–223, 1995.
(9) Raoult D, Berbis Ph, Roux V, Xu W, Maurin M, *Lancet* **350**, 112–113, 1997.
(10) Punda-Polic V, Petrovec M, Trilar T, Duh D, Bradaric N, Klismanic Z, Avsic-Zupanc T, *Exp. Appl. Acarol.* **28**, 169–176, 2002.
(11) Ortuno A, Quesada M, Lopez-Claessens S, Castella J, Sanfeliu I, Anton E, Segura-Porta F, *Vector-Borne Zoonotic Dis.* **7**, 59–64, 2007.
(12) Lakos A, *Wien. Klin. Wochenschr*. **114**, 648-654, 2002.
(13) Oteo JA, Ibarra V, Blanco JR, Martínez de Artola V, Márquez FJ, Portillo A, Raoult D, Anda P, *Clin. Microbiol. Infect*. **10**, 327–331, 2004.
(14) Sekeyová Z, Roux V, Raolut D, *Int. J. Sys. Evol. Microbiol.* **51**, 1353–1360, 2001.
(15) Eremeeva M, Yu X, Raoult D, *J. Clin. Microbiol.* **32**, 803–810, 1994.
(16) Roux V, Fournier PE, Raoult D, *J. Clin. Microbiol.* **34**, 2058–2065, 1996.