Rickettsia hoogstraalii sp. nov., isolated from hard- and soft-bodied ticks

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A novel spotted fever group *Rickettsia* was found in *Haemaphysalis sulcata* ticks collected from sheep and goats in Croatia in 2006. At the same time, a genetically identical organism was co-isolated with the embryonic cell line CCE3 obtained from the soft tick *Carios capensis* in Georgia, USA. In this study, further phenotypic and genotypic characteristics of the novel rickettsial strain present in *H. sulcata* ticks were investigated. Based on the cultivation of bacteria in mosquito and Vero cell cultures, the presence of rickettsiae in tick tissues and cell cultures [confirmed by transmission electron microscopy (TEM)] and the amplification and sequencing of five rickettsial genes, it was demonstrated that the novel *Rickettsia* strain fulfils the criteria to be classified as a novel species. The name *Rickettsia hoogstraalii* sp. nov. is proposed for the new strain. *Rickettsia hoogstraalii* sp. nov., an obligately intracellular bacterium, was grown in Vero cells and arthropod CCE3, ISE6 and C6/36 cell lines. The morphology of the cells of the novel species was typical of SFG rickettsiae. The small coccobacillary appearance of the bacteria was apparent with light microscopy. A Gram-negative bacterial cell wall and a cytoplasmic membrane separated by a narrow periplasmic space were visible by TEM. To date, *Rickettsia hoogstraalii* sp. nov. has been isolated from two species of ticks, *H. sulcata* and *C. capensis*. The novel species appears to be geographically widely distributed, having been detected in Croatia, Spain and Georgia, USA. Although no information is available regarding the possible pathogenicity of the novel species for vertebrate hosts, *R. hoogstraalii* sp. nov. has a cytopathic effect in Vero, CCE3 and ISE6 cells. Sequence analyses of the 16S rRNA, 17 kDa, *gltA*, *ompA* and *ompB* genes indicated that even though *R. hoogstraalii* sp. nov. was closely related to *Rickettsia felis*, it represents a separate species within the spotted fever group. The type strain of *R. hoogstraalii* sp. nov. is strain Croatia^T (=DSM 22243^T=UTMB 00003^T).

Croatia is an endemic region for three distinct rickettsial diseases: Mediterranean spotted fever, murine typhus and rickettsial pox. Mediterranean spotted fever is caused by tick-transmitted *Rickettsia conorii* in the coastal area from

Zadar to Dubrovnik (Punda *et al.*, 1984; Tartaglia, 1939). Murine typhus is caused by flea-transmitted *Rickettsia typhi* in southern Croatia (Punda-Polic *et al.*, 2008) and rickettsial pox is transmitted by the mite-borne *Rickettsia akari* in the northern part of the country (Radulovic *et al.*, 1996). Two further human rickettsial pathogens have been identified recently in southern Croatia. DNA sequences from *Rickettsia slovaca* and *Rickettsia aeschlimannii* were detected in *Dermacentor marginatus* and *Hyalomma marginatum* ticks, respectively, but as yet, no human infections caused by these rickettsiae have been reported in Croatia (Duh *et al.*, 2003; Punda-Polic *et al.*, 2002).

Abbreviations: CPE, cytopathic effect; D-MEM-GlutaMAX, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; L-15-Gmax, Leibovitz 15 Glutamax medium; SFG, spotted fever group; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the *gltA*, 16S rRNA, *ompA*, *ompB* genes and the 17 kDa protein of the novel *Rickettsia* isolate are FJ767737, FJ767735, EU816565, FJ767738 and FJ767736, respectively.

In addition to these recognized rickettsial pathogens, the existence of a novel spotted fever group (SFG) *Rickettsia* was demonstrated recently in *Haemaphysalis sulcata* ticks collected from sheep and goats in different locations in the southern part of Croatia (Duh *et al.*, 2006). A high infection rate for these rickettsiae in *H. sulcata* ticks, up to 26%, was measured at different tick collection sites. Bacteria were partially characterized by using molecular techniques. Phylogenetic analysis of the complete citrate synthase *gltA* gene and portions of genes for 17 kDa and OmpB proteins showed that the novel rickettsial strain had the highest similarity to the human pathogen *Rickettsia felis* (Duh *et al.*, 2006). At the same time in the USA, a rickettsial endosymbiont 'Candidatus *Rickettsia hoogstraalii*' was co-isolated with the arthropod embryonic cell line CCE3. The cell line was obtained from a soft tick, *Carios capensis*, collected from the nests of brown pelicans in coastal Georgia, USA (Mattila *et al.*, 2007). The authors stressed the cytopathic nature of the endosymbiont for CCE3 and ISE6 cells. Interestingly, the *gltA* and 17 kDa gene sequences of the endosymbiont showed 100% similarity to the gene sequences of the novel *Rickettsia* strain detected in *H. sulcata* ticks in Croatia.

Given the wide geographical distribution of this novel rickettsial strain, its presence in different species of ticks collected from different hosts and its molecular similarity to human pathogens such as *R. felis* and *Rickettsia australis*, we decided to further characterize the novel rickettsial strain from *H. sulcata* ticks and describe its phenotypic and genotypic characteristics. Based on the cultivation of bacteria in cell cultures, the presence of rickettsiae in tick tissues and in cell cultures, as confirmed by transmission electron microscopy, and the amplification and sequencing of five rickettsial genes, we evaluated whether this new *Rickettsia* strain warranted classification as a novel species.

Three adult *H. sulcata* ticks, collected in 2000 from the same sheep in southern Croatia, were selected for the cultivation attempts. The ticks were repeatedly immersed in 5% bleach and 70% ethanol and washed with sterile water. After manual homogenization in 800 µl Leibovitz 15 Glutamax medium (L-15-Gmax) with 5% fetal calf serum (FCS), the tick homogenate was used for the inoculation of C6/36 mosquito cells. Two shell-vial cultures, containing a monolayer of approximately 40 000 cells, were inoculated with 300 µl tick homogenate and centrifuged at 700 g for 1 h. A 1 ml aliquot of L-15-Gmax medium with 5% FCS and with or without antibiotics was added to the shell-vial cultures. The vials were incubated at 26 °C. The rest of the tick homogenate was used for DNA extraction. On the third day of incubation, the medium was changed to antibiotic-free medium in both vials. Every third day, fresh medium was added and the infection of cells was assessed by Diff-Quik staining and by PCR and sequencing of a portion of the 17 kDa gene. Rickettsial DNA was detected after 12 days of shell-vial cultivation. The 17 kDa sequence of the rickettsial isolate was identical to that of the novel *Rickettsia* strain amplified directly from *H. sulcata* ticks and

to 'Candidatus *R. hoogstraalii*' isolated from *C. capensis* ticks (GenBank accession nos DQ081185 and EF629538, respectively). Small coccobacilli were seen on a smear after 24 days of cultivation (Fig. 1a). After 33 days, 90% of the C6/36 cells in the vial were infected. Infected cells were harvested by using glass beads and further inoculated into 25 cm² flasks containing a monolayer of fresh C6/36 cells. A 6 ml sample of L-15-Gmax medium with 5% FCS was added to the flasks, which were then incubated at 26 °C. The subsequent five passages required 13–17 days for 90%

Fig. 1. Cultivation of *Rickettsia hoogstraalii* sp. nov. in C6/36 and Vero E6 cells. (a) Small coccobacillary shape of the novel strain cultivated in Vero E6 cells viewed with light microscopy; (b) immunofluorescence of *R. hoogstraalii* sp. nov. antigen with *R. conorii* antibody-containing human sera, titre 1 : 80; (c) immunofluorescence negative control.

of the cells to become infected. The fifth passage of the novel *Rickettsia* was used for the inoculation of Vero E6 cells. A 1 ml aliquot of inoculum was added to 25 cm² flasks containing a monolayer of fresh Vero E6 cells without medium and the flasks were then shaken for 2 h at 300 r.p.m. Flasks were incubated at 28 °C and after 2 h, 6 ml Dulbecco's modified Eagle's medium (D-MEM-GlutaMAX) with 5% FCS was added. The novel *Rickettsia* strain infected up to 90% of the Vero E6 cells within 15 days. In contrast to the cultivation of the novel strain in C6/36 cells, in which no cytopathic effect (CPE) was observed, the novel *Rickettsia* produced a severe CPE in Vero E6 cells. Infected cells were harvested and used either for the production of *Rickettsia* antigen or divided into 1 ml aliquots to be stored at -80 °C.

Immunofluorescence assay slides with antigen from the novel rickettsial strain were prepared as described by Avsic-Zupanc *et al.* (1999) and tested with *R. conorii* and *R. typhi* antibody-containing human sera. In a preliminary experiment, only three human serum samples were tested. For two sera, the IgG antibody titre against the *R. conorii* antigen was previously determined as 1:160 and 1:320 (data not shown). No cross-reactivity was observed when the novel *Rickettsia* antigen was tested with *R. typhi*-positive serum; however, immunofluorescence was clearly visible with *R. conorii*-positive sera at the same titre as determined for the *R. conorii* antigen (Fig. 1b, c).

For transmission electron microscopy (TEM), 18 adult *H. sulcata* ticks were collected from sheep in southern Croatia in 2007. Ticks were washed in 70% ethanol, rinsed in sterile water and processed for TEM. Ten of the 18 live ticks were dissected and the remainder were stored at -80 °C. Salivary glands, midgut and ovaries were removed from the dissected ticks, placed into separate tubes containing PFGPA fixative and stored at 4 °C (Ito & Rikihisa, 1981). The PFGPA fixative consisted of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% picric acid, 0.03% CaCl₂ and 0.05 M cacodylate buffer. The additional parts of the ticks were used for DNA extraction and PCR. Tissues from two ticks, in which the presence of rickettsiae was confirmed by PCR, were processed further. The ticks were washed three times in 0.1 M cacodylate buffer and shipped to the University of Texas Medical Branch (UTMB, Galveston, TX, USA) where ultrathin sections for TEM were prepared as described previously (Popov *et al.*, 2007). In addition, one 25 cm² flask with C6/36 cells infected with the novel *Rickettsia* from *H. sulcata* ticks was also processed for TEM.

In tick salivary glands, TEM revealed multiple rickettsiae located free in the cytosol of epithelial cells. Clearance of the cytoplasmic contents was observed around the rickettsiae. The ultrastructure of the novel *Rickettsia* cells demonstrated the morphology typical of SFG rickettsiae with the cell wall and cytoplasmic membrane separated by a narrow periplasmic space (Fig. 2a). Rickettsiae were also seen in midgut cells (Fig. 2b), although they were less

numerous than in the salivary glands. No rickettsiae were observed in ovaries. TEM also demonstrated multiple rickettsiae in infected C6/36 cells. Binary fission of rickettsiae was observed in infected C6/36 cells as well as in tick midgut samples (Fig. 2c, d). The morphology seen in Fig. 2e is suggestive of rickettsial movements; rickettsiae lying flat on the cell surface are entering the cell whereas those in the cytoplasmic protrusions (filopodia) are exiting the cell (Fig. 2f). It is typical for SFG rickettsiae to enter or exit cells at different speeds using tails formed by actin polymerization.

The molecular and phylogenetic characteristics of the new isolate were determined. DNA was extracted with a BioSprint 15 DNA Blood kit (Qiagen) using the third passage of the novel *Rickettsia* strain isolated in C6/36 cells from *H. sulcata* ticks. The complete *gltA* gene, a large part of the 16S rRNA and *ompA* genes (without the repetitive region) and partial *ompB* and 17 kDa genes were amplified and sequenced as described previously (Duh *et al.*, 2006; Gracner *et al.*, 2009; Roux & Raoult, 1995). The nucleotide sequences of five genes from the unidentified *Rickettsia* strain amplified directly from *H. sulcata* and of '*Candidatus R. hoogstraalii*' from *C. capensis* ticks have been published previously (Duh *et al.*, 2006; Gracner *et al.*, 2009; Mattila *et al.*, 2007). Based on sequence pair distances and alignments, performed with the CLUSTAL W algorithm (Lasergene), identical nucleotide sequences for the *gltA*, 16S rRNA, 17 kDa and *ompB* genes were demonstrated from three separate sources of the novel *Rickettsia* strain: the rickettsial isolate cultivated in C6/36 cells, the unidentified *Rickettsia* sp. amplified directly from *H. sulcata* and '*Candidatus R. hoogstraalii*' from *C. capensis* ticks. Analysis of the *ompA* gene revealed 99.8% sequence similarity between the novel *Rickettsia* strain isolated in C6/36 cells and the unidentified *Rickettsia* sp. amplified directly from *H. sulcata* ticks (Gracner *et al.*, 2009). When 1200 bp of the *ompA* gene of the *Rickettsia* isolate cultivated in C6/36 cells was compared with '*Candidatus R. hoogstraalii*' from *C. capensis* ticks, it was shown that the gene sequences were 99.3% similar. Comparison of the novel *Rickettsia* isolate to other SFG or typhus group (TG) rickettsiae sequences available in GenBank revealed different similarities as shown in Table 1. The sequence similarity between the novel *Rickettsia* isolate and other rickettsiae ranged from 98.3% to 99.7% for the 16S rRNA gene, 86.5% to 97.2% for the *gltA* gene, 84.5% to 97.1% for the 17 kDa gene and 80.2% to 87.5% for the *ompA* gene (Table 1).

Four phylogenetic trees were drawn from an alignment of 239 bp of the 17 kDa gene sequence, 766 bp of the 16S rRNA gene, 1217 bp of the *gltA* gene and 3178 bp of the *ompA* gene sequence. The phylogenetic trees were inferred with TREECON software using the Kimura 80 model and the neighbour-joining tree algorithm. The support for the tree nodes was calculated with 1000 bootstrap replicates (Fig. 3). Efforts were made to include the nucleotide sequences of the respective genes from all of the recognized

Fig. 2. The ultrastructure of *R. hoogstraalii* sp. nov. as demonstrated by TEM. (a) *R. hoogstraalii* sp. nov. in salivary glands of a *H. sulcata* tick; (b) *R. hoogstraalii* sp. nov. in the midgut of a *H. sulcata* tick; (c) binary fission of cells of *R. hoogstraalii* sp. nov. in the midgut of a *H. sulcata* tick; (d) binary fission of *R. hoogstraalii* sp. nov. cells in C6/36 mosquito cell; (e) cells of *R. hoogstraalii* sp. nov. entering and/or exiting a C6/36 mosquito cell; (f) cell of *R. hoogstraalii* sp. nov. exiting a C6/36 mosquito cell, filopodia. Bars, 0.5 µm (a, c), 1 µm (b), 0.2 µm (d, f) and 2 µm (e).

Rickettsia species with a few exceptions due to the non-availability of gene sequences in GenBank or because the existing sequences were too short to be included in the analysis. Based on all four phylogenetic trees, the novel *Rickettsia* isolate was consistently positioned between *R. felis* and other rickettsiae within the SFG (Fig. 3).

Discussion

Historically, the description of novel species of rickettsiae has been justified by differences in their epidemiology, serology and intracellular growth characteristics (Walker, 2007). Recently, gene sequence-based guidelines for taxonomic classification have been proposed and used for the classification and identification of novel rickettsial species (Fournier *et al.*, 2003). Although the authors of this proposal stated that these criteria were objective and sufficient for the description of novel species of the genus *Rickettsia*, gene sequence-based criteria alone are inadequate for defining prokaryotic species and are incapable of keeping pace with the levels of diversity that are being

revealed in nature (Fournier *et al.*, 2003; Gevers *et al.*, 2005).

In this study, we describe phenotypic as well as genotypic features of the new *Rickettsia* isolated in C6/36 and Vero E6 cells from *H. sulcata* ticks and propose the name *Rickettsia hoogstraalii* sp. nov. for this novel species. The status of the bacterium was unclear on the basis of the molecular and epidemiological characteristics found for the novel rickettsiae detected in *H. sulcata* ticks from Croatia and reported by Duh *et al.* (2006) and Gracner *et al.* (2009). Gene sequence analysis conducted by Pérez-Osorio *et al.* (2008) revealed the close resemblance of the novel rickettsial strain to the human pathogen *R. felis*, although the analysis of different genes gave inconsistent results. The aim of this study was to isolate and cultivate the novel *Rickettsia* species. Because of its genetic similarity to *R. felis*, a mosquito cell line was chosen and a low temperature, 26 °C, was used for cultivation. The isolation of the *Rickettsia* strain from *H. sulcata* ticks was successful and we were able to propagate it in subsequent passages. In addition, we were able to infect Vero E6 cells with the new

Table 1. Gene sequence similarity (%) of four genes of *R. hoogstraalii* sp. nov. strain Croatica^T, compared with sequences of recognized species of the genus *Rickettsia* available in GenBank

Alignment was performed based on sequence pair distances with the CLUSTAL W algorithm (Lasergene software package). GenBank accession numbers are reported in Fig. 3. —, Nucleotide sequences were either not available or too short to be included in the analysis; NE, do not exist in TG rickettsiae; NA, not amplifiable.

Species	<i>R. hoogstraalii</i> sp. nov. strain Croatica ^T			
	16S rRNA gene	<i>gltA</i>	17 kDa	<i>ompA</i>
<i>R. rickettsii</i> strain Bitterroot	99.5	93.5	96.2	86.9
<i>R. peacockii</i> strain Skalkaho ^T	99.3	—	96.2	87.0
<i>R. conorii</i> strain NIAID Malish 7 ^T	99.7	93.6	96.2	86.5
<i>R. sibirica</i> strain 246 ^T	99.6	93.8	96.2	86.4
<i>R. parkeri</i> strain NIAID maculatum 20 ^T	99.6	93.7	96.2	86.6
<i>R. slovaca</i> strain 13-B	99.6	93.7	—	86.9
<i>R. africae</i> strain ESF-5	99.6	93.5	—	86.8
<i>R. helvetica</i> strain C9P9	99.6	93.5	—	—
<i>R. massiliae</i> strain Mtu1 ^T	99.6	93.8	—	87.3
<i>R. montanensis</i> strain M/5-6	99.2	94.1	96.7	86.4
<i>R. rhipicephali</i> strain Burkdorfer 3-7-female 6 ^T	99.6	93.3	96.7	87.5
<i>R. aeschlimannii</i> strain MC16 ^T	99.5	94.0	—	87.3
<i>R. honei</i> strain TT-118	99.5	93.6	96.2	86.7
<i>R. asiatica</i> strain IO-1 ^T	99.0	—	95.0	NA
<i>R. tamurae</i> strain AT-1 ^T	99.5	94.2	95.8	—
<i>R. japonica</i> strain YM	99.5	93.3	96.2	86.5
<i>R. akari</i> strain MK Kaplan ^T	99.1	93.8	95.0	—
<i>R. australis</i> strain NIAID Phillips 32 ^T	99.3	94.4	97.1	80.2
<i>R. felis</i> strain URRWXC2	99.7	97.2	93.7	80.2
<i>R. typhi</i> strain Wilmington ^T	98.7	89.9	88.7	NE
<i>R. prowazekii</i> strain Brein1 ^T	98.8	90.2	87.9	NE
<i>R. canadensis</i> strain 2678 ^T	98.3	90.0	84.5	—
<i>R. bellii</i> strain 369442-1	99.1	86.5	94.1	—

isolate and establish continuous growth of the strain in this cell line. The only difference noted during the cultivation of the novel *Rickettsia* strain in different cell lines was that a CPE was observed in Vero cells but not in mosquito cells. Similarly, ‘*Candidatus R. hoogstraalii*’, which was co-isolated with the *C. capensis* embryonic cell line CCE3 and is genetically identical to the novel *Rickettsia* strain isolated from *H. sulcata* ticks, was also shown to be cytopathic for different tick cells (Mattila *et al.*, 2007). The presence and morphology of the novel *Rickettsia* strain isolated from *H. sulcata* ticks was further demonstrated by TEM. Multiple rickettsial cells with typical morphology were seen in infected mosquito cells and in tick salivary glands and midgut epithelial cells. Given the capability of the novel rickettsial strain to cause CPE in different cell lines and the absence of rickettsial cells in tick ovaries, we assumed that the novel *Rickettsia* strain was not an endosymbiont of ticks. In addition, rickettsial antigen obtained from the novel *Rickettsia* isolate was used for the production of immunofluorescence assay slides and it showed a high affinity to antibodies in the serum of a human patient infected with *R. conorii*. However, the true pathogenic potential of the novel *Rickettsia* isolate remains to be proven. Five gene sequences, *gltA*, 16S rRNA, *ompA*, *ompB*

and 17 kDa, were amplified from the novel *Rickettsia* strain isolated in C6/36 cells from *H. sulcata* ticks. Four of the gene sequences were identical to those of the *Rickettsia* sp. amplified directly from *H. sulcata* ticks and ‘*Candidatus R. hoogstraalii*’ from *C. capensis* ticks whereas the *ompA* gene sequences were not identical but were highly similar (99.8 % and 99.3 %, respectively). When phylogenetic analysis was performed based on the alignments of *gltA*, 16S rRNA, *ompA*, and 17 kDa nucleotide sequences, the novel *Rickettsia* isolate either clustered together with *R. felis* or was found in a separate branch between *R. felis* and other SFG rickettsiae.

The first informal description of the proposed novel species dates back to 2006, when these bacteria were detected in *H. sulcata* ticks collected from sheep and goats in Southern Croatia (Duh *et al.*, 2006). Although the bacteria were characterized only by molecular methods, the *H. sulcata* ticks were collected over three consecutive years in different locations in Croatia and were always found to be infected with rickettsiae at an overall prevalence rate of 22.8 %. A name, ‘*Candidatus R. hoogstraalii*’, was proposed by a second group of scientists who co-isolated the same rickettsial strain with the embryonic cell line CCE3 and published the results in 2007 (Mattila *et al.*, 2007).

Fig. 3. Phylogenetic relationship between *R. hoogstraalii* sp. nov. isolated in mosquito cell lines from *H. sulcata* ticks and recognized rickettsial species available in GenBank. The analysis was performed with the Kimura 80 model and neighbour-joining tree evaluation supported by 1000 bootstraps (TREECON software). (a) Phylogenetic tree based on the alignment of 3178 bp of the *ompA* gene; (b) tree based on the alignment of 766 bp of the 16S rRNA gene; (c) tree based on the alignment of 239 bp of the 17 kDa gene; (d) tree based on the alignment of 1217 bp of the *gltA* gene.

Interestingly, cell line CCE3 was obtained from a soft tick *C. capensis* collected from nests of brown pelicans in coastal Georgia, USA. In this paper, we have described additional and sufficient information to formally describe the novel rickettsial species. In order to avoid further confusion among recognized rickettsial species, both groups of authors have agreed to name the novel species *Rickettsia hoogstraalii* sp. nov.

Description of *Rickettsia hoogstraalii* sp. nov.

Rickettsia hoogstraalii (hoog.straal'i.i. N.L. gen. masc. n. *hoogstraalii* of Hoogstraal, named in honour of Dr Harry Hoogstraal who contributed significantly to the general knowledge on ticks).

An obligately intracellular bacterium which can be grown in Vero E6 cells at 28 °C with D-MEM-GlutaMAX supplemented with 5 % FCS. Also grows in tick CCE3 and ISE6 cell lines and the mosquito C6/36 cell line (Mattila *et al.*, 2007). The morphology is typical of SFG rickettsiae. The Gram-negative cell wall and cytoplasmic membrane are separated by a narrow periplasmic space as determined by TEM. Rickettsiae-infected cells can be stained with Diff-Quik, which reveals the small coccobacillary cells. To date, has been isolated only from two species of ticks, *H. sulcata* and *C. capensis* (Mattila *et al.*, 2007). Shows a cytopathic effect in Vero, CCE3 and ISE6 cells, but no information is available on the possible pathogenicity of these bacteria for vertebrate hosts. Widely distributed and has been detected in Croatia, Spain and Georgia, USA (Duh *et al.*, 2006; Mattila *et al.*, 2007; Portillo *et al.*, 2008). The 16S rRNA, 17 kDa, *gltA*, *ompA* and *ompB* gene sequence analyses indicate that *R. hoogstraalii* sp. nov. is a separate species from other SFG rickettsiae. Most closely related to *R. felis* (Duh *et al.*, 2006; Gracner *et al.*, 2009).

The type strain, strain Croatia^T (=DSM 22243^T=UTMB 00003^T), was isolated from *H. sulcata* ticks collected in Croatia in 2000.

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