Molecular detection of *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae* and *Rickettsia conorii israelensis* in ticks from Israel

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Abstract

Rickettsioses are recognized as important emerging vector-borne infections of humans worldwide. Previous reports documented the presence of two spotted fever group rickettsiae in Israel, *Rickettsia conorii israelensis* and *Rickettsia felis*. The aim of this study was to characterize the diversity of rickettsiae in ticks collected from vegetation and the ground, from different parts of Israel. Non-engorged questing adult ticks were collected from 13 localities. A total of 131 tick pools, 83 of *Rhipicephalus turanicus* and 48 of *Rhipicephalus sanguineus* (each with 2–10 ticks per pool), were included in this study. In addition, 13 *Hyalomma* sp. ticks were collected. The ticks were molecularly screened for rickettsiae, targeting the citrate synthase (*gltA*) and the outer membrane protein A (*ompA*) gene loci. *Rickettsia massiliae ompA* DNA (100% sequence identity; 180 bp) was detected in 32 *Rh. turanicus* and 12 *Rh. sanguineus* tick pools. *R. conorii israelensis* was detected in three *Rh. sanguineus* pools. *Rickettsia sibirica mongolitimonae ompA* DNA (100% sequence identity; 182 bp) was found in one *Hyalomma* tick. This study reports the first detection of *R. massiliae* and *R. sibirica mongolitimonae* in ticks from Israel. This is the first report describing the presence of these human pathogens in the Middle East.

Keywords: *Hyalomma*, *Rhipicephalus*, *Rickettsia conorii israelensis*, *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae*

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Introduction

Rickettsioses are recognized as important emerging vector-borne infections of humans worldwide. They are caused by obligate intracellular Gram-negative bacteria of the genus *Rickettsia* (family *Rickettsiacea*). Spotted fever group rickettsioses are characterized by fever, headache, rash and potential eschar formation at the bite site [1,2]. Two spotted fever group rickettsiae have been detected and characterized in Israel to date, *Rickettsia conorii israelensis*, the aetiological agent of Israeli spotted fever (ISF), and *Rickettsia felis*, the aetiological agent of flea-borne spotted fever. Israel is considered to be an endemic region for ISF, and clinical cases are commonly presented. In contrast, clinical cases attributed to *R. felis* in Israeli patients have not been reported to date [3–5].

Arthropod vectors have been screened extensively for the diversity of the pathogens that they carry. The introduction of molecular techniques in the last two decades has resulted in increased detection of emerging and re-emerging vector-borne pathogens in different parts of the world [6]. The aim of this study was to characterize the diversity of rickettsiae in ticks collected from vegetation and the ground in different parts of Israel.

Materials and Methods

Tick collection

Non-engorged questing adult ticks were collected from 13 localities in the vicinity of human habitations in three different geographical regions in Israel (Caesarea, Pardes Hana, Michmoret and Alexander valley in the north; Tel Aviv, Bet

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Arif, Mazkeret Batia, Kibbutz Hulda and Kibbutz Harel in the centre; and Or Haner, Bror Hail, Reim and Tzeelim in the south). Ticks were collected from vegetation of up to 30 cm in height with the flagging technique. In addition, some ticks were collected directly from the vegetation or while moving on the ground. The ticks were identified morphologically by an experienced entomologist. A total of 1196 non-engorged adult ticks identified as *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus turanicus* (Pomerantsev, 1936) and *Hyalomma* sp. were collected during 2002–2003 and 2007–2008. Ticks of the same species collected on the same date and from the same location were pooled together in one vial. Ticks collected during the years 2002–2003 were kept in a medium containing 10% fetal bovine serum and 10% antibiotics/antimycotics (10 mg/mL streptomycin sulphate, 10 000 U/mL, penicillin G sodium, and 25 mg/L amphotericin B), and ticks collected during the years 2007–2008 were kept in 70% ethanol. All ticks were frozen at −70°C until DNA extraction.

DNA extraction, real-time PCR amplification and sequencing

After elimination of the ethanol and medium remains from each vial containing ticks, 50 mL of phosphate-buffered saline was added. Each sample was manually homogenized with plastic microtube pestles for 1 min, and then centrifuged for 10 s at 2000 ***g***. The upper fraction was collected from each vial, and DNA was extracted with a DNA extraction kit (Illustra Tissue Mini Spin kit; GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Initial detection of *Rickettsia* species was performed by screening all DNA samples by a real-time PCR assay targeting a 133-bp fragment of the citrate synthase gene (*gltA*), using primers rico173F (CGACCCGGGTTTTATGTCTA) and rico173R (ACTGCTCGCCACTTGGTAGT), designed for this study. Real-time PCR was carried out with an initial hold for 15 min at 95°C, followed by 50 cycles of 5 s at 95°C, 30 s at 57°C (fluorescence acquisition on the HRM channel) and 1 s at 72°C. The melting phase started at 60°C, each step rising by 1°C (fluorescence acquisition on the HRM channel), and finished at 90°C with a hold for 90 s at the first step and 5 s at the subsequent steps. Hybridization started at 90°C and fell to 50°C, by 1°C at each step. The positive samples were further analysed by targeting a 178–189-bp fragment of the outer membrane protein A gene (*ompA*), using primers 107F (GCTTTATTCACCACCTCAAC) and 299R (TRATCACCACCGTAAGTAAAT) [7], with an initial hold for 15 min at 95°C, followed by 50 cycles of 10 s at 95°C, 30 s at 56°C (fluorescence acquisition on the HRM channel) and 6 s at 72°C. The melting phase started at 60°C, each step rising by 1°C (fluorescence acquisition on the HRM channel), and finished at 95°C with a hold for 90 s at the first step and 1 s at the subsequent steps. Hybridization started at 90°C and fell to 50°C, by 1°C at each step. Both real-time PCR reactions were carried out using the Rotor Gene 6000 cycler (Corbett Research, Sydney, Australia). PCR was performed in 20-μL reaction volumes containing 4 μL of DNA, 1.5 μL of each primer, 0.6 μL of cyto9, 2.4 μL of double-distilled water, and 10 μL of Thermo-Start PCR Master Mix (Thermo Scientific, Loughborough, UK). DNA extracted from cultured *R. conorii israelensis* was used as a positive control, and two negative control samples containing all the ingredients of the reaction except DNA were used for all trials.

PCR products were purified using a PCR purification kit (ExoSAP-IT; USB, Cleveland, OH, USA) and sequenced. DNA sequencing was carried out by utilizing the BigDye terminator cycle sequencing chemistry from an Applied Biosystems ABI 3700 DNA Analyzer (Foster City, CA, USA), and the ABIs data collection and sequence analysis software. Further analysis was performed with Sequencher software, version 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA).

Results

Ticks

A total of 131 pools, 83 of *Rh. turanicus* and 48 of *Rh. sanguineus*, each with 2–10 adult ticks per pool, were included in this study. In addition, 13 adult ticks of *Hyalomma* spp. were placed in single tubes and analysed separately.

Rickettsiae

The rickettsial *gltA* gene fragment was detected and sequenced in 23 of the 48 (48%) *Rh. sanguineus* tick pools, in 51 of the 83 (61%) *Rh. turanicus* tick pools, and in five of the 13 (38%) *Hyalomma* sp. ticks. Sequences of the *gltA* fragment were identical in most of the tested samples (99–100% sequence identity to a large number of rickettsial species and strains), and could not assist in species identification. Therefore, the *ompA* gene was further targeted. Table 1 shows the *ompA* gene sequence similarities observed with respect to those deposited in GenBank.

Rickettsial *ompA* DNA was found in 16 of the 23 *gltA*-positive *Rh. sanguineus* pools (Table 1), 12 of which were positive for *Rickettsia massiliae*. All of these sequences were identical and were deposited in GenBank (new accession number GU212859); *ompA* DNA from three *Rh. sanguineus* pools was identical to that from *R. conorii israelensis*. The three sequences were also identical to each other and were deposited in GenBank (accession number GU212863). One

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