Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus* Midichloria mitochondrii and *Babesia canis vogeli* in ticks from Israel

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Abstract

Ticks are vectors of important pathogens of human and animals. Therefore, their microbial carriage capacity is constantly being investigated. The aim of this study was to characterize the diversity of domestic animal pathogens 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 1196 ticks in 131 pools—83 pools of *Rhipicephalus turanicus* and 48 of *Rhipicephalus sanguineus* (with two to ten ticks per pool)—were included in this study. In addition, 13 single free-roaming *Hyalomma* spp. ticks were collected. Screening by molecular techniques revealed the presence of *Ehrlichia canis*, *Anaplasma platys*, *Anaplasma bovis* and *Babesia canis vogeli* DNA in *R. turanicus* ticks. *E. canis*, *A. bovis*, *B. canis vogeli* and *Candidatus* Midichloria mitochondrii DNA sequences were detected in *R. sanguineus* ticks. *Candidatus* Midichloria mitochondrii DNA was also detected in *Hyalomma* spp. ticks. Neither *Hepatozoon* spp. nor *Bartonella* spp. DNA was detected in any of the ticks examined. This study describes the first detection of *E. canis* in the tick *R. turanicus*, which may serve as a vector of this canine pathogen; *E. canis* was the most common pathogen detected in the collected questing ticks. It also describes the first detection of *A. bovis* and *Candidatus* Midichloria mitochondrii in Israel. To the best of the author's knowledge, this is the first report describing the detection of DNA of the latter two pathogens in *R. sanguineus*, and of *A. bovis* in *R. turanicus*.

Keywords: *Anaplasma bovis*, *Anaplasma platys*, *Babesia canis vogeli*, *Candidatus* Midichloria mitochondrii, *Ehrlichia canis*, Israel, ticks
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Introduction

Ticks are vectors of important pathogens of humans and animals. The geographical distribution and habitats of ticks have expanded in recent years. Major drivers of this phenomenon include environmental changes, globalization and global warming [1,2]. Because of their increased distribution, ticks have been extensively screened for the diversity of pathogens that they carry [3]. 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 [2].

Several tick-borne pathogens have been reported to infect dogs in Israel, including *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli* and *Hepatozoon canis* [4–6]. Of these pathogens, dogs and wild canids in Israel are most frequently exposed to *E. canis* [7–10]. However, the extent of tick infection with these pathogens has not been investigated in Israel to date.

In a previous study carried out in Israel by the authors, several tick species, including *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus turanicus* (Pomerantsev, 1936) and *Hyalomma* spp., were collected from vegetation and the ground, identified and screened for rickettsial organisms. Several spotted fever group rickettsiae, pathogenic to humans, including *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae* and *Rickettsia conorii israelensis*, were detected, some for the first time in Israel and the Mediterranean region [3]. The aim of this study was to characterize the diversity of pathogens of veterinary importance in the same ticks and to investigate whether ticks in Israel are infected with *Bartonella* species.

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. Ticks were collected from the following locations: Caesarea, Pardes Hana, Michmoret and Alexander valley in the north; Tel Aviv, Bet Arif, Mazkeret Batia, Kibbutz Hulda and Kibbutz Harel in the centre; and Or Haner, Bror Hail, Reim and Tzeelim in the south (Fig. 1). The ticks were collected from vegetation of up to 30 cm in height with the flagging technique. In addition, some ticks were manually collected from the vegetation or while moving on the ground. The ticks were identified morphologically with standard taxonomic keys [11,12]. A total of 1196 non-engorged adult ticks identified as *R. sanguineus*, *R. turanicus* and *Hyalomma* spp. 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 (two to ten ticks per vial). Ticks collected during the years 2002–2003 were initially 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 then frozen at −70°C until DNA extraction.

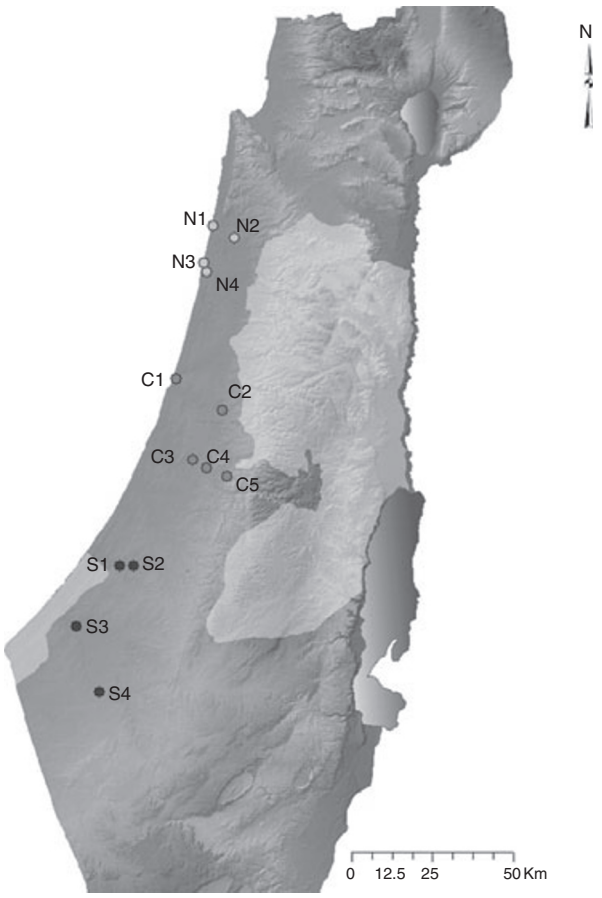

FIG. 1. Tick collection localities in three geographical regions in Israel. N, northern Israel; C, ventral Israel; S, southern Israel. N1, Caesarea; N2, Pardes Hana; N3, Michmoret; N4, Alexander Valley; C1, Tel Aviv; C2, Bet Arif; C3, Mazkeret Batia; C4, Kibbutz Hulda; C5, Kibbutz Harel; S1, Or Haner; S2, Bror Hail; S3, Reim; S4, Tzeelim.

DNA extraction, PCR amplification and sequencing

Fifty millilitres of phosphate-buffered saline were added to each vial containing ticks after elimination of the remaining ethanol and medium. 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 by a DNA extraction kit (Illustra Tissue Mini Spin kit; GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions.

Extracted DNA was molecularly screened for *Bartonella* spp. by high-resolution melt real-time PCR, with the primers described in Table 1, according to a previously described protocol [13,14]), and for *Ehrlichia* spp., *Babesia* spp., *Hepatozoon* spp. and *A. platys* by conventional PCR, with the primers described in Table 1, according to protocols previously described [15–19]. PCR products were purified with a PCR purification kit (ExoSAP- IT; USB, Cleveland, OH, USA) and sequenced. DNA sequencing was carried out with the BigDye Terminator cycle sequencing chemistry from Applied Biosystems (Foster City, CA, USA), an ABI 3700 DNA Analyzer and the ABI's 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 *R. turanicus* and 48 of *R. sanguineus*, each with two to ten adult ticks per pool, were included in this study. One hundred and three of the 131 pools (78%) contained ten ticks each. In addition, 13 adult ticks of *Hyalomma* spp. were placed in single tubes and analysed separately.

Microbial DNA in *R. turanicus*

E. canis DNA was detected in eight pools (9.6%), *A. platys* DNA was detected in one pool (1.2%), *Anaplasma bovis* DNA was detected in four pools (4.8%) and *B. canis vogeli* DNA was detected in one pool (1.2%; Table 2).

TABLE 1. Targeted genes and list of primers used in this study

Pathogen	Gene	Primer	Sequence (5′ → 3′)	Size (bp)	Reference
Ehrlichia spp.	16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	345	15
		EHR16SR	TAGCACTCATCGTTTACAGC		
Anaplasma platys	16S rRNA	Platys	GATTTTTGTCGTAGCTTGCTATG	679	16
		EHR16SR	TAGCACTCATCGTTTACAGC		
Babesia spp.	18S subunit	PIROA	AATACCCAATCCTGACACAGGG	400	17
		PIROB	TTAAATACGAATGCCCCCAAC		
		RLB R3 + BIO	CTTTAACAAATCTAAGAATTTCACCTCTGACAGT	450	18
		RLB F2	GACACAGGGAGGTAGTGACAAG		
Bartonella spp.	*rpoB*	600f	CGTGCAACAGAAGATTTAGATC)	202	13
		800r	CGA TTC GCA TCA TCA TTT TC		
	gltA	Bhcs.781p	GGGGACCAGCTCATGGTGG	313	14
		Bhcs.1137n	AATGCAAAAAGAACAGTAAACA		
Hepatozoon spp.	18S subunit	HEP F	ATACATGAGCAAAATCTCAAC	626–666	19
		HEP R	CTTATTATTCCATGCTGCAG		

TABLE 2. Microbial DNA fragments detected in *Rhipicephalus sanguineus*, *Rhipicephalus turanicus* and *Hyalomma* spp. ticks from Israel, and sequence identity with GenBank-deposited sequences

Pathogen	Sample	Tick	Accession number	Percentage identity
Ehrlichia canis 16S rRNA	40/1	*R. sanguineus*	EU123923.1	100
	40/7	*R. sanguineus*	AY621071.1	99
	40/8	*R. sanguineus*	EU439944.1	96
	40/12	*R. sanguineus*	AY621071.1	99
	42/1	*R. sanguineus*	EU439944.1	99
	42/2	*R. turanicus*	EU439944.1	100
	44/5	*R. turanicus*	AY621071.1	99
	46/5	*R. turanicus*	EU439944.1	99
	46/9	*R. turanicus*	EU439944.1	99
	RE 01	*R. turanicus*	AY621071.1	99
	RE 05	*R. turanicus*	AY621071.1	100
	RE 06	*R. turanicus*	EU439944.1	99
	RE 16	*R. turanicus*	EU439944.1	99
Anaplasma platys 16S rRNA	42/4	*R. turanicus*	EF139459.1	99
Anaplasma bovis 16S rRNA	40/19	*R. sanguineus*	AF470698.1	99
	44/3	*R. turanicus*	AF470698.1	99
	RE 08	*R. turanicus*	AF470698.1	97
	RE 13	*R. turanicus*	AF470698.1	98
	RE 18	*R. turanicus*	EU181143.1	100
Candidatus Midichloria mitochondrii 16S rRNA	40/1	*R. sanguineus*	EU315771.1	99
	00701-1a	*R. sanguineus*	EU315771.1	99
	43/4	*Hyalomma* sp.	AM411594.1	98
	44/1	*Hyalomma* sp.	AM411594.1	99
	44/6	*Hyalomma* sp.	AM411594.1	99
	RE 11	*Hyalomma* sp.	AM411594.1	97
	ZE 07	*Hyalomma* sp.	AM411594.1	98
Babesia canis vogeli 18S subunit	40/1	*R. sanguineus*	AB248733.1	100
	47/2	*R. sanguineus*	AB248733.1	100
	008021	*R. turanicus*	AB248733.1	100

Microbial DNA in *R. sanguineus*

E. canis DNA was detected in five pools (10.4%), *A. bovis* DNA was detected in one pool (2.1%), *Candidatus* Midichloria mitochondrii DNA was detected in two pools and *B. canis vogeli* DNA was detected in two pools (4.2%; Table 2).

Microbial DNA in *Hyalomma* spp.

Candidatus Midichloria mitochondrii DNA was detected in five single ticks (38.5%; Table 2).

A. bovis and *C.* Midichloria mitochondrii

A. bovis and *C.* Midichloria mitochondrii DNA was amplified by the primers used for the amplification of *E. canis* 16S rRNA (EHR16SD and EHR16SR; Table 1), and identified only after DNA sequences were obtained (Table 2). Neither *Hepatozoon* spp. nor *Bartonella* spp. DNA could be detected in any of the tick samples.

Geographical distribution

E. canis DNA was detected in *R. turanicus* and *R. sanguineus* ticks from all three geographical regions in Israel. Four of the five *A. bovis*-positive pools were of *R. turanicus* ticks from southern Israel. Six of the seven *C.* Midichloria mitochondrii-positive ticks and pools were from southern Israel; five of these were single *Hyalomma* spp. ticks. Two of the three *B. canis vogeli*-positive pools were from southern Israel. The only *A. platys*-positive pool detected was from central Israel.

Discussion

This study evaluated questing adult ticks collected in field locations by flagging, whereas, in some other studies, ticks taken off animals were evaluated. The presence of pathogens in non-engorged questing ticks is suggestive of the ticks' ability to serve as carriers of the respective pathogens and possibly to transmit them. Obviously, either the pathogens were transmitted from the parent generation, or the ticks acquired the infection while feeding in the larval or nymphal stage and transmitted it trans-stadially to the adult stage. The identity of the animal hosts on which the ticks fed in their earlier life stages was not known. It can be presumed that the ticks collected fed on several domestic and wild animal species. *R. sanguineus* and *R. turanicus* are known to parasitize a variety of host animals [12]. Five different organisms were detected in the ticks in this study, including *E. canis*, *A. platys*, *A. bovis*, *B. canis vogeli* and *C.* Midichloria mitochondrii. The first four are known animal pathogens. In a previous study performed on DNA from the same tick, *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae* and *Rickettsia conorii israelensis*, three spotted fever group rickettsial pathogens, were detected [3]. These findings indicate the ability of ticks to carry a wide range of pathogens, and highlight the importance of ticks as vectors for human and animal pathogens.

A. bovis, the aetiological agent of monocytotropic anaplasmosis, is suspected to cause a clinical disease in ruminants [20,21]. In the current literature there is a lack of comprehensive data on the epidemiology and clinical importance of this organism. This article reports the first molecular detection of *A. bovis* in Israel, both in *R. sanguineus* and in *R. turanicus*. To the best of our knowledge, this is the first report of the detection of *A. bovis* DNA in these latter tick species. Detection of *A. bovis* was previously reported in *Hyalomma* spp. from Iran [22], *Hyalomma* spp., *Rhipicephalus appendiculatus* and *Amblyomma variegatum* ticks from Africa [23], *Haemaphysalis megaspinosa*, *Ixodes persulcatus* and *Ixodes ovatus* from Japan [21,24–26] and *Haemaphysalis longicornis* ticks from Korea [27,28]. *A. bovis* DNA was also detected in several domestic and wild ruminants, including cattle and deer [21], and in cottontail rabbits from North America [29].

This article reports the first molecular detection of *C.* Midichloria mitochondrii in ticks from Israel and the Middle East. *C.* Midichloria mitochondrii is an intracellular alphaproteobacterial symbiont that was previously detected in several hard tick (Ixodidae) species [30,31]. The bacterium was found to be localized both in the cytoplasm and in the intermembrane space of the mitochondria of tick ovarian cells. It is the first bacterium shown to reside within the mitochondria [30]. The possible role of this endosymbiont in the ticks is yet to be determined. In this study, *C.* Midichloria mitochondrii was detected in five *Hyalomma* spp. ticks and in two *R. sanguineus* pools. To the best of our knowledge, this is the first report of the detection of this bacterium in *R. sanguineus* ticks.

E. canis was detected in about 10% of the pools, and was the most commonly detected pathogen in this study. As most pools (78.6%) contained ten ticks each, the prevalence of *E. canis* in single ticks could be expected to be lower. Reports on the seroprevalence of *E. canis* antibodies in dogs, jackals and foxes in Israel have given frequencies ranging from 30% to 36%, suggesting a high level of exposure to this pathogen [4,7,8].

E. canis DNA was detected in both *R. sanguineus* and *R. turanicus*. To the best of the authors' knowledge, this is the first report of *E. canis* DNA detection in *R. turanicus*. In contrast to what was found for *E. canis*, low rates of *B. canis vogeli* and *A. platys* infection were recorded in this study, and no *H. canis* infection was found (Table 2). These findings are in agreement with clinical observations; *E. canis* is a commonly encountered canine disease in Israel, whereas the other pathogens are less frequently encountered [9].

Several *Bartonella* spp. have been identified in humans, animals and their flea vectors in Israel [32–34]. *Bartonella* DNA was not detected in the ticks included in this study. Although *Bartonella* DNA was previously detected in ticks, there is no definitive evidence for the ability of ticks to transmit *Bartonella* spp. [35,36].

In conclusion, *E. canis* was the most commonly detected pathogen in this study, and was detected for the first time in *R. turanicus*. This article describes for the first time the detection of *A. bovis* and *C.* Midichloria mitochondrii in Israel. This is the first report describing the molecular detection of these two pathogens in *R. sanguineus*, and of *A. bovis* in *R. turanicus*. Clinicians should be aware of the presence of these pathogens in this region. The role of *C.* Midichloria mitochondrii in ticks has yet to be explored.

Transparency Declaration

The authors declare no dual or conflicting interests.

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