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Molecular detection of *Theileria*, *Babesia*, and *Hepatozoon* spp. in ixodid ticks from Palestine

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ABSTRACT

Ixodid ticks transmit various infectious agents that cause disease in humans and livestock worldwide. A cross-sectional survey on the presence of protozoan pathogens in ticks was carried out to assess the impact of tick-borne protozoa on domestic animals in Palestine. Ticks were collected from herds with sheep, goats and dogs in different geographic districts and their species were determined using morphological keys. The presence of piroplasms and *Hepatozoon* spp. was determined by PCR amplification of a 460–540 bp fragment of the 18S rRNA gene followed by RFLP or DNA sequencing. A PCR-RFLP method based on the 18S rRNA was used in order to detect and to identify *Hepatozoon*, *Babesia* and *Theileria* spp. A total of 516 ticks were collected from animals in six Palestinian localities. Five tick species were found: *Rhipicephalus sanguineus* sensu lato, *Rhipicephalus turanicus*, *Rhipicephalus bursa*, *Haemaphysalis parva* and *Haemaphysalis adleri*. PCR-based analyses of the ticks revealed *Theileria ovis* (5.4%), *Hepatozoon canis* (4.3%), *Babesia ovis* (0.6%), and *Babesia vogeli* (0.4%). *Theileria ovis* was significantly associated with ticks from sheep and with *R. turanicus* ticks ($p < 0.01$). *H. canis* was detected only in *R. sanguineus* s.l. and was significantly associated with ticks from dogs ($p < 0.01$).

To our knowledge, this is the first report describing the presence of these pathogens in ticks collected from Palestine. Communicating these findings with health and veterinary professionals will increase their awareness, and contribute to improved diagnosis and treatment of tick-borne diseases.

1. Introduction

Ixodid (hard) ticks are vectors of numerous diseases among animals and humans throughout the world (Harrus and Waner, 2011; Inokuma et al., 2003). *Hepatozoon* spp. and the piroplasms which include *Babesia* and *Theileria* spp. are tick-borne intracellular parasites that infect vertebrates. Some piroplasm species cause major economic losses to the livestock industry in Asia, resulting in weight loss, decreased meat and milk production, abortions and death (Zintl et al., 2003). Infections vary in severity from sub-clinical to acute with fever, anemia, severe lethargy, and circulatory shock (Homer et al., 2000; Zintl et al., 2003). Several species of piroplasms are transmitted to sheep and goats by ixodid ticks. Ovine theileriosis is caused by several species of *Theileria* and transmitted by ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* (Bishop et al., 2004). While bovine theileriosis has been extensively studied, ovine theileriosis has received less scientific attention (Gao et al., 2002). *Hepatozoon canis* is a protozoan transmitted by the *Rhipicephalus sanguineus* sensu lato group and present in Europe, Asia, America and Africa (Aydin et al., 2015; Criado-Fornelio et al., 2003; Duscher et al., 2013; Ewing and Panciera, 2003; Karagenc et al., 2006; Maia et al., 2014; Najm et al., 2014; Tolnai et al., 2015). This parasite is transmitted to dogs by the ingestion of ticks containing mature oocysts. *H. canis* infections range from being sub-clinical to severe.

To date, there are no data on the presence and geographic distribution of hard ticks, and the extent of tick infection with piroplasm pathogens in Palestine. We therefore aimed in this study to collect ixodid ticks found on small ruminants and dogs in different

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Table 1
Different tick species collected from studied animals in different areas of Palestine.

Tick species (number & % of total ticks collected)	Animal host (number of ticks collected from each host species)	Area					
		Jenin	Jericho	Nablus	Qalqilia	Ramallah	Tubas
Rhipicephalus sanguineus s.l. (*n* = 305, 59.1%)	Dog (*n* = 250)	63	33	20	89	16	29
	Goat (*n* = 13)	0	0	0	0	0	13
	Sheep (*n* = 42)	0	0	11	0	0	31
Rhipicephalus bursa (*n* = 15, 2.9%)	Goat (*n* = 1)	0	0	0	0	0	1
	Sheep (*n* = 14)	0	0	3	0	0	11
Rhipicephalus turanicus (*n* = 142, 27.5%)	Dog (*n* = 6)	2	3	0	1	0	0
	Sheep (*n* = 136)	0	0	111	0	0	25
Haemaphysalis parva (*n* = 40, 7.7%)	Dog (*n* = 35)	15	0	3	9	7	1
	Goat (*n* = 3)	0	0	0	0	0	3
	Sheep (*n* = 2)	0	0	0	0	0	2
Haemaphysalis adleri (*n* = 14, 2.7%)	Dog (*n* = 13)	5	0	1	6	1	0
	Sheep (*n* = 1)	0	0	1	0	0	0
Total ticks	516	85	36	150	105	24	116

districts of Palestine to study the protozoan pathogens found in them by molecular techniques.

2. Materials and methods

2.1. Tick collection and identification

Ticks were collected from small ruminant herds composed of 1–20 animals in six rural districts of Palestine between January and September of 2014. Ticks were collected from all sheep and goats present in the same herd and all dogs accompanying the herd. The ticks were taken off from the skin of animals and collections were carried out once in each sampled herd. All ticks found on each animal were collected and immediately introduced into 70% ethanol and kept at −20 °C until taxonomic identification based on morphological criteria was applied (Feldman-Muhsam, 1951, 1954; Pegram et al., 1987). Ticks were then used for DNA extraction for the detection of tick-borne protozoal pathogens.

2.2. DNA extraction of the collected ticks

DNA was extracted from each tick using a DNA extraction kit (QIAGEN GmbH, 40724Hilden, Germany) following the manufacturer's instructions. Each tick was crushed individually with a disposable sterile scalpel in a micro tube. After digestion with proteinase K (20 μg/ml), samples were applied to columns for absorption and washing of DNA. DNA was eluted in 100 μl of buffer and stored at 4 °C until used as template for PCR amplification.

2.3. Polymerase chain reaction

The PCR reactions for detection of piroplasmid and *Hepatozoon* spp. in ticks were performed using primers BJ1: 5′-GTC TTG TAA TTG GAA TGA TGG-3′ and BN2: 5′-TAG TTT ATG GTT AGG ACT ACG-3′ which amplify a fragment of 460–540 bp of the 18S rRNA gene of the genus *Babesia* as described previously (Casati et al., 2006) with the following modification: the PCR reactions were performed in a total volume of 25 μl using PCR-Ready Supreme^{TM} mix (Syntezza Bioscience, Jerusalem) including the primers at 1 μM final concentration. The PCR amplification program performed by a thermocycler (Mastercycler Personal, Eppendorf) included an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s and final extension 72 °C for 5 minutes. Five microliters of the PCR products were analyzed on 2% Tris-acetate-EDTA buffer (TAE 1X) agarose gels and visualized under UV transillumination.

2.4. DNA sequencing

PCR products were sequenced using an ABI 3730xl DNA Analyzer (Hylab Co., sequencing service). The products were sequenced in both directions with the same primers as for PCR. The chromatograms were checked and the sequences were assembled by the Bio-edit software. The 18S rRNA sequences were aligned using the Multalin Multiple sequence alignment tool (http://multalin.toulouse.inra.fr/multalin/). In order to verify whether the size of the amplified fragment of the detected microbial species in ticks is sufficient to discriminate the different species, the DNA sequences were compared with the GenBank database by the nucleotide sequence homology search facilitated by the National Centre for Biotechnology Information (NCBI) using the BLAST analysis database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The species were identified based on being the first match by BLAST and having >97% identity with this match.

2.5. PCR-RFLP

For species identification, the partial 18S rRNA DNA sequences of *T. ovis, He. canis, B. ovis and B. vogeli* obtained in the present study and reference strains from GenBank were mapped for restriction site polymorphisms using the NEBcutter V2.0 program available at http://tools.neb.com/NEBcutter2/index.php. The restriction enzyme, XapI (ApoI), originating from the bacteria *Xylophilus ampelinus* was selected because it was indicated to produce distinguishable fragment sizes for some of the species. The PCR-amplified products were digested with the ApoI restriction enzyme (Thermo, Germany) according to the manufacturer's recommendations. Each digestion reaction was set up in 15 μl volume containing 1.5 μl of the 10X reaction buffer and 1 μl of restriction enzyme. Digested PCR products were analyzed on 2% TAE agarose gels and visualized under UV transillumination.

2.6. Phylogenetic analysis

Phylogenetic analyses of the 18S rRNA sequences were performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) applying the neighbor joining and maximum likelihood algorithms. Phylogenetic tree analysis was conducted by the MEGA 6 program using UPGMA program. The reliability of internal