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.

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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-Fornielo et al., 2003; Duscher et al., 2013; Ewing and Panciera, 2003; Karagenc et al., 2006; Maia et al., 2014; Najmi 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...
Table 1

<table>
<thead>
<tr>
<th>Tick species (number &amp; % of total ticks collected)</th>
<th>Animal host (number of ticks collected from each host species)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhipicephalus sanguineus</em> s.l. (n = 305, 59.1%)</td>
<td>Dog (n = 250)</td>
<td>Jenin 63</td>
</tr>
<tr>
<td></td>
<td>Goat (n = 13)</td>
<td>Jericho 33</td>
</tr>
<tr>
<td></td>
<td>Sheep (n = 42)</td>
<td>Nablus 20</td>
</tr>
<tr>
<td><em>Rhipicephalus bursa</em> (n = 15, 2.9%)</td>
<td>Goat (n = 1)</td>
<td>Qalqilia 89</td>
</tr>
<tr>
<td></td>
<td>Sheep (n = 14)</td>
<td>Ramallah 16</td>
</tr>
<tr>
<td><em>Rhipicephalus turanicus</em> (n = 142, 27.5%)</td>
<td>Dog (n = 6)</td>
<td>Tubas 29</td>
</tr>
<tr>
<td></td>
<td>Sheep (n = 136)</td>
<td></td>
</tr>
<tr>
<td><em>Haemaphysalis parva</em> (n = 40, 7.7%)</td>
<td>Dog (n = 35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat (n = 3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sheep (n = 2)</td>
<td></td>
</tr>
<tr>
<td><em>Haemaphysalis adleri</em> (n = 14, 2.7%)</td>
<td>Dog (n = 13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sheep (n = 1)</td>
<td></td>
</tr>
<tr>
<td>Total ticks</td>
<td></td>
<td>516</td>
</tr>
</tbody>
</table>

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 protozoan pathogens.

2.2. DNA extraction of the collected ticks

DNA was extracted from each tick using a DNA extraction kit (QIAGEN GmbH, 407244 Hilden, 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 BJI: 5’-GTC TGG TAA TTG GAA TGA TGG-3’ and BN2: 5’-TAG TTT ATG GTG AGT 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™ 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*, *H. 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 (Apol), originating from the bacteria *Xylophilus amplus* was selected because it was indicated to produce distinguishable fragment sizes for some of the species. The PCR–amplified products were digested with the Apol 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
branches was assessed by bootstrapping with 1000 pseudoreplicates. Nodes with bootstrap support less than 70% were collapsed.

2.7. Statistical analysis

Statistical analysis of the epidemiological data was carried out using the SPSS V.17.0 program. Two-tailed t-test and Pearson's correlation were performed. p-value <0.05 was considered statistically significant.

3. Results

3.1. Tick identification

A total of 516 partially fed hard ticks were collected from 189 animals (117 dogs, 66 sheep and 6 goats) in herds composed from 1–20 animals, living in six different districts. Collection included: 150 ticks from Nablus, 116 from Tubas, 105 from Qalqilia, 85 from Jenin, 36 from Jericho, and 24 from Ramallah (Fig. 1). The most abundant tick genera were Rhipicephalus (462/516, 89.5%) and Haemaphysalis (54/516, 10.5%) (Table 1). Three hundred and five (305) R. sanguineus s.l. ticks were collected from all the six districts. 142 Rhipicephalus turanicus from five districts, and 40 Haemaphysalis parva were collected from five districts. Only 15 Rhipicephalus bursa were collected from sheep sampled in the Nablus and Tubas districts. Fourteen Haemaphysalis adleri were collected from dogs and sheep. Ha. adleri was identified according to Feldman-Muhsam (1951, 1954). The majority of collected ticks were in the adult stage (females: 247/516, 47.9% and males: 237/516, 45.9%) and only a small part (32/516, 6.2%) were nymphs.

3.2. Pathogen detection and identification

Pathogens were detected in 25 ticks from dogs which comprised 8.2% of the total number of ticks collected from dogs, and in 29 ticks collected from sheep which comprised 14.9% of the ticks from sheep (Table 2). No pathogens were detected in ticks from goats. The animals carrying the infected ticks were apparently healthy and did not show any overt disease manifestations.

PCR and sequencing of DNA of all positive PCR products (n = 54) from the study identified the presence of one Theileria spp. (T. ovis), two Babesia spp. (B. ovis and B. vogelii) and one Hepatozoon spp. (He. canis). Overall, a total of 54 ticks (10.5%) were positive for pathogens which included: T. ovis (28/516, 5.4%), He. canis (21/516, 4.1%).

Fig. 1. Map describing the districts of Palestine studied and the number of ticks collected in each district. The overall infection rates with tick-borne pathogens identified are in brackets.
Table 2  
Pathogens found in the different tick hosts according to the animal host of ticks.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Tick host</th>
<th>Animal host of infected ticks</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dog</td>
<td>Sheep</td>
<td>Goat</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Theileria ovis</td>
<td>B. bursa s.l.</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bursa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. parva s.l.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. parva</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. turanicus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total Theileria</td>
<td>1</td>
<td>27</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>B. ovis</td>
<td>B. bursa s.l.</td>
<td>1 (1%)</td>
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<td>0</td>
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</tr>
<tr>
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<td>1</td>
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<tr>
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<td>H. parva s.l.</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total B. ovis</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>H. canis</td>
<td>B. bursa s.l.</td>
<td>20 (11%)</td>
<td>1</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bursa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. parva s.l.</td>
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<td>0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>H. parva</td>
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<td>0</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. turanicus</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
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<td>Total H. canis</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>B. vogeli</td>
<td>B. bursa s.l.</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bursa</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. parva s.l.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. parva</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. turanicus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total B. vogeli</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total infected ticks</td>
<td></td>
<td>54</td>
<td>25</td>
<td>29</td>
<td>0</td>
<td>54</td>
</tr>
</tbody>
</table>

§ nymphal tick.

p < 0.01, prevalence compared to presence of the specified pathogen in other tick species.

The rate of He. canis DNA detection was significantly higher in male ticks (15/21, 71.4%) compared to female (5/21, 23.8%) and nymphal ticks (4.7%, p < 0.01). The presence of Babesia/Theileria DNA was not significantly different in female (16/28, 57%) than in male (12/28, 43%) and nymphal ticks (3.5%). Overall, 12.0% of the ticks tested were pathogen-positive: 11.1% of the R. sanguineus s.l. and 12.7% of the R. turanicus were positive for pathogens. All T. ovis infections were detected in R. bursa, R. sanguineus s.l. and R. turanicus from sheep. T. ovis was significantly associated with ticks from sheep and also with R. turanicus ticks (p < 0.01). The DNA of He. canis was detected only in R. sanguineus s.l. and He. canis was significantly associated with ticks from dogs and also with R. sanguineus ticks (p < 0.01). The DNA of B. ovis was detected in R. bursa Ha. parva and R. sanguineus s.l. while the DNA of B. vogeli was detected in R. sanguineus s.l. None of the studied pathogens were found in Ha. adleri (Table 1).

3.3. DNA Sequencing and RFLP

The 18S rRNA PCR yielded a product of approximately 465–542 bp (Fig. 2a). A 542 bp band was amplified from He. canis positive samples. It was clearly distinguished from bands amplified for the other pathogen species with T. ovis and B. vogeli producing a 489 bp band and B. ovis producing a 465 bp band. PCR products digested with Apol showed three fragments of 394, 122 and 26 bp for He. canis, four fragments of 244, 121, 115 (both latter bands observed as one heavy band) and 26 bp for T. ovis, but only two fragments of 367 and 122 bp for B. vogeli and two fragments of 343 and 122 bp for B. ovis (Fig. 2b). The lower band of 26 bp was not visualized on agarose gel for all species. Specifically, RFLP was able to distinguish between piroplasms and He. canis, since both species T. ovis and B. vogeli showed the same PCR size product. RFLP of PCR positive samples of He. canis, B. vogeli, B. ovis and T. ovis, were run in parallel and used as a positive control in each digestion reaction.

Twenty of the 54 (37%) sequenced PCR products were analyzed blindly, validated and confirmed the PCR RFLP profiles described above. Representative confirmed partial sequences of the 18S rRNA gene of two He. canis samples from ticks collected from dogs, two B. vogeli from dog ticks, two B. ovis from ticks found on a sheep and a dog, and one T. ovis from a sheep tick identified in the present study were deposited in GenBank under accession nos. KT587799, KT587790, KT587791, KT587792, KT587793, KT587794 and KT587795, respectively.

3.4. Phylogenetic analysis

Three phylogenetic trees of Hepatozoon, Babesia and Theileria, were constructed from the 18S rRNA gene sequences generated in this study with comparison to selected sequences available in GenBank.

The first tree (Fig. 3A) represents all the sequences of He. canis generated in this study (n = 21). Three clades were formed with most of the study sequences falling into two clades. One clade included sequences from other countries such as Israel, Sudan, Austria and Spain, while two clades included only sequences from Palestine.

Concerning Babesia sequences, the second phylogenetic tree analysis (Fig. 3B) found two major clades for Babesia species and showed evidence of two monophyletic clades, one consisting of B. vogeli with B. canis and the other including B. ovis with well-supported separation among them. The comparison of the 18S rRNA B. ovis sequences revealed 99% homology between Palestinian sequences and those from Spain (AY150058.1), Turkey (JF923656.1) and Albania (KF681514.1).
Fig. 3. Neighbor joining phylogenogram trees. Phylogenetic analysis was constructed by the UPGMA method with bootstrap of 1000 replications using Mega 6 program. The Embank accession numbers, species of infected animals and country of origin from which the sequences were derived are included for each sequence. New GenBank accessions for sequences derived from the present study are highlighted in bold letters. The number of identical sequences is in brackets. Selected reference piroplasm and He. canis sequences from GenBank are also shown. *Isospora belli* and *Theileria cervi* were been used as out-groups. (A) Phylogenetic tree based on partial sequences of the 18S rRNA gene of *Hepatozoon canis* comparing sequences generated in this study to sequences from other countries. (B) Phylogenetic tree based on partial sequences of the 18S rRNA gene of *Babesia* species comparing sequences generated in this study to sequences from other countries. (C) Phylogenetic tree based on partial sequences of the 18S rRNA gene of *Theileria* comparing sequences generated in this study to sequences from other countries.
The third phylogenetic tree consisted of all T. ovis sequences generated in this study (Fig. 3C). Here, the 18S rRNA gene sequences of T. ovis (n = 26) described herein formed well supported clades of sequences that were identical to each other and all revealed 100% homology to the T. ovis reference sequence (accession no. KP019206.1) deposited in GenBank with the exception of two sequences (21.16D and 21.38A).

4. Discussion

Ticks are vectors of important pathogens of human and animals which cause theileriosis (Jalali et al., 2014), hepatoparous (Pirata et al., 2015) and babesiosis (Theodoropoulos et al., 2006). Several tick species, including R. sanguineus s.l., R. turanicus and additional tick species have been identified in neighboring countries (Harrus et al., 2011a; Keysary et al., 2011; Salant et al., 2014). In this study, we found that R. sanguineus s.l. and R. turanicus are frequently found on dogs, sheep and goats often without obvious host specificity. Among five tick species examined for Theileria, Babesia and Hepatozoon spp., four (R. bursa, R. turanicus, R. sanguineus s.l. and Ha. parva) were found to be infected by one or more pathogen species. The rate of H. canis detection was significantly higher in male ticks, this could be due to the locomotion capacity of males seeking a new host (Solano-Gallego et al., 2012), while the presence of Babesia/Theileria was not significantly different in between the sexes.

Our finding that ticks from sheep have a higher infection rate of Theileria sp. compared to ticks from goats is in agreement with results reported directly from the blood of these animal species in a study from Ethiopia (Gebrekidan et al., 2014). This may suggest that different tick species are involved in the transmission of Theileria sp. in sheep from Palestine.

About two thirds (57%) of the positive ticks which were infected with T. ovis were of the species R. turanicus. However, the results of this study and the detection of T. ovis in R. turanicus could suggest that it is a possible vector responsible for transmission of T. ovis as proposed previously (Razmi et al., 2013). Neither B. ovis nor B. vogeli or H. canis infection was detected in this tick species.

Ovine babesiosis caused by B. ovis is an important tick-borne hemoparasitic disease of small ruminants (Schnittger et al., 2003) often leading to loss in productivity and in some cases mortality (Bai et al., 2002; Erster et al., 2016; Esmaeilnejad et al., 2014a; Rjeibi et al., 2014). The two 18S rRNA gene B. ovis sequences in the present study (from ticks feeding on a sheep and a dog, respectively) had high similarity to a B. ovis sequence deposited in GenBank (AY150058.1), from an infected goat from Spain. The results presented in this study have also demonstrated the existence of B. ovis in R. bursa collected from sheep. Our results agree with other studies that reported B. ovis in Rhipicephalus spp. including R. bursa, R. sanguineus, and R. turanicus (Erster et al., 2016; Rjeibi et al., 2014). R. bursa plays an important role as a natural vector of B. ovis and has been reported as the only vector for B. ovis that can transovarially transmit this Babesia species to sheep and goat (Altay et al., 2008).

Ovine theileriosis has been reported in various countries such as Ethiopia, Iran and China (Gebrekidan et al., 2014; Iqbal et al., 2013; Liu et al., 2008). In our study, the only species of Theileria found in ticks from Palestinian sheep was T. ovis with a high infection rate. Contrary to a report from Tunisia (M’Ghirbi et al., 2008), which detected T. ovis in sheep and goats, this species was only present in ticks from sheep in our study. This could be because both the number of goats and the number of ticks obtained from them was very low. However, this species of Theileria reportedly causes subclinical infections in small ruminants (Jalali et al., 2014).

Theileria ovis from sheep in this study showed low genetic diversity. The reported sequences for the 18S ssu rRNA gene (489 bp length) from five countries (Tunisia, Turkey, Iraq, Iran and China) had 100% homology with our sequences, except for two, and clustered together in a clade clearly distinct from T. cervi.

Canine babesiosis is a clinically important disease affecting dogs. The most common piroplasm in dogs in the Mediterranean region

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5. Conclusions

This is the first report describing the presence of several tick-borne pathogens of veterinary importance in Palestine. The RFLP method based on digestion of the 18S RNA amplicon with the restriction enzyme Apol proved useful for discriminating not only between the piroplasms *T. ovis* and *B. vogeli*, but also to confirm *He. canis* infection. It was possible to rely on the sizes of the upper fragments (394 bp for *He. canis*, 244 bp for *T. ovis*, 367 bp for *B. vogeli* and the 343 bp for *B. ovis*) to distinguish between these pathogens. The results from this study suggest that vector-borne infections of dogs and small ruminants are common, widespread and in need of further investigation in Palestine in order to establish effective measures to control their vector ticks.

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