<table>
<thead>
<tr>
<th>Title</th>
<th>Epidemiology, risk factors, and co-infection of vector-borne pathogens in goats from Sistan and Baluchestan province, Iran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Hakimi, Hassan; Sarani, Ali; Takeda, Mika; Kaneko, Osamu; Asada, Masahito</td>
</tr>
<tr>
<td>Citation</td>
<td>PLoS ONE, 14(6), e0218609; 2019</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2019-06-20</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/39309">http://hdl.handle.net/10069/39309</a></td>
</tr>
</tbody>
</table>

© 2019 Hakimi et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

NAOSITE: Nagasaki University’s Academic Output SITE
http://naosite.lb.nagasaki-u.ac.jp
Epidemiology, risk factors, and co-infection of vector-borne pathogens in goats from Sistan and Baluchestan province, Iran

Hassan Hakimi¹, Ali Sarani², Mika Takeda¹, Osamu Kaneko¹, Masahito Asada¹*¹

¹ Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan, ² Department of Clinical Science, University of Zabol, Veterinary Faculty, Zabol, Iran

* masahitoasada@nagasaki-u.ac.jp

Abstract

Several vector-borne pathogens restrict livestock farming and have significant economic impact worldwide. In endemic areas livestock are exposed to different tick species carrying various pathogens which could result in co-infection with several tick-borne pathogens in a single host. Although the co-infection of and the interaction among pathogens are critical factors to determine the disease outcome, pathogen interactions in the vector and the host are poorly understood. In this study, we surveyed the presence of Babesia ovis, Theileria ovis, Theileria lestoquardi, Anaplasma ovis, Anaplasma phagocytophilum, and Anaplasma marginale in 200 goats from 3 different districts in Sistan and Baluchestan province, Iran. Species-specific diagnostic PCRs and sequence analysis revealed that 1.5%, 12.5%, and 80% of samples were positive for T. lestoquardi, T. ovis, and A. ovis, respectively. Co-infections of goats with up to 3 pathogens were seen in 22% of the samples. We detected a significant association between T. ovis infection and age, T. ovis infection and location (Zabol), and A. ovis infection and location (Sarbaz) by multivariate logistic regression analysis. In addition, by analyzing the data with respect to Plasmodium caprae infection in these goats, a negative correlation was found between P. caprae and A. ovis infection. This study contributes to understanding the epidemiology of vector-borne pathogens and their interplay in goats.

Introduction

Tick-borne diseases remain an economic burden for the livestock industry of tropical and subtropical regions of the world. Protozoan parasites such as Babesia spp. and Theileria spp. together with Anaplasma spp. are responsible for tick-borne diseases in small ruminants and cause great economic losses in the livestock and livestock-related industries [1, 2].

Small ruminant theileriosis is mainly caused by Theileria lestoquardi, Theileria ovis, and Theileria separata. T. lestoquardi is the most virulent species and occasionally causes death while T. ovis and T. separata are benign and cause subclinical infections in small ruminants...
Several species of Babesia have been described to cause ovine and caprine babesiosis including Babesia ovis, Babesia motasi, Babesia crassa, and Babesia foliata [4]. B. ovis is the most pathogenic and causes fever, hemoglobinuria, severe anemia, icterus and occasional death [5]. Anaplasma spp. are important for human and animal health and these pathogens are generally considered to produce mild clinical symptoms. Although several Anaplasma spp. including Anaplasma marginale, Anaplasma ovis, and Anaplasma phagocytophilum could be found in small ruminants, A. ovis is the main cause of small ruminant anaplasmosis in the world.

In Iran small ruminant farming is widely practiced with 52 and 26 million heads of sheep and goats, respectively, being raised mainly by small-scale farmers [6]. In regions with harsh and severe environments, such as central and southeast Iran, goat raising dominates. The great diversity of the environment in Iran affects the distribution of ticks, and thereby the pathogens transmitted. Several epidemiological studies are available regarding tick-borne pathogens in small ruminants in Northern and Western regions in Iran [7–10]. However, there is a scarcity of data regarding the prevalence of Babesia, Theileria, and Anaplasma spp. infecting small ruminants in southeastern Iran. This study investigated the prevalence of tick-borne pathogens in Sistan and Baluchestan province, in the southeastern part of Iran where it borders with Afghanistan and Pakistan; and where the frequent border-crossing animal passage facilitates the circulation of tick-borne pathogens between countries [11].

In endemic areas livestock are bitten by vectors carrying multiple pathogens or different vectors transmitting various pathogens, which result in co-infections in the host. The interaction among different pathogens within a host are complex and may result in protection against virulent pathogens or exacerbate the clinical symptoms [12]. In a study done on the indigenous African cattle in Kenya, it was shown that co-infection with less pathogenic Theileria spp. in calves results in a decreased mortality associated with virulent T. parva which is likely the result of cross protection [13]. A recent study also showed a negative interaction between B. ovis and T. ovis in sheep, indicating infection with the less pathogenic T. ovis produces protection against highly pathogenic B. ovis [14]. In contrast, co-infection of B. ovata and T. orientalis, two parasites which are transmitted via the same tick species, exacerbate the symptoms and produce clinical anemia in cattle [15]. Recently we identified the goat malaria parasite, Plasmodium caprae, in goat samples originating from Sistan and Baluchestan province, Iran [16]. However, nothing is known for this pathogen except some DNA sequence and morphology. In order to gain insights into pathogenicity and interaction of this parasite with other vector-borne pathogens in the host, we examined the prevalence of tick-borne piroplasms and Anaplasma spp. in these goat samples and evaluated the interplay among the identified pathogens.

Materials and methods

Sampling sites and blood collection

Blood samples were collected from 200 goats (95 males and 105 females) from 3 districts in Sistan and Baluchestan province, including Zabol (n = 51), Sarbaz (n = 125), and Chabahar (n = 24) as shown in S1 Fig. These samples were utilized for screening of Plasmodium caprae in this region [16]. Age of the goats were recorded by 0.5-year interval based on the owner’s report. Average ages were 1.9 years old (range: 1–5 years old) for male and 3.0 years old (range: 1–6 years old) for female goats. In each district, blood samples were collected from different farms. Sampling was done in January, June, and November of 2016 and July of 2017. Blood sampling and DNA extraction was performed as described [16].
**Ethical statement**

Sampling of goats was performed with the informed consent of the farm owners. All procedures were carried out in compliance to the ethical guidelines for the usage of animal samples of University of Zabol. This study was approved by the Ethics Committee of University of Zabol (permission number: IRUOZ.ECRA.2016.01).

**Detection of Babesia spp. Theileria spp. and Anaplasma spp. by species-specific PCR**

Each DNA sample was screened for *B. ovis*, *T. lestoquardi*, *T. ovis*, *A. phagocytophilum*, *A. ovis*, and *A. marginale* by species-specific PCR as described [17–21]. Small subunit ribosomal RNA (SSUrRNA) was the target gene for detection of *B. ovis* and *T. ovis* while specific primers targeting merozoite surface antigen gene (*ms1*) were used for detection of *T. lestoquardi*. *A. ovis*, and *A. marginale* were screened using primers targeting major surface protein 4 gene (*msp-4*); and for specific detection of *A. phagocytophilum*, primers targeting *epank1* gene were used (Table 1).

**Cloning and sequencing**

PCR products of all positive samples for *T. ovis* or *T. lestoquardi* and three positive samples for *A. ovis* randomly selected from each sampling village (21 samples in total) were sequenced. The amplified PCR products were recovered from agarose gels and cloned into the Zero Blunt TOPO vector (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer’s protocol. Following transformation three *E. coli* colonies were selected, the plasmids were extracted and purified, and the gene sequences were analyzed using BigDye Terminator v1.1 and an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA). The single nucleotide polymorphisms (SNPs) found in the obtained sequence were confirmed by repeating the amplification, cloning, and sequencing process. *T. lestoquardi ms1* (*Tlms1*), *T. ovis SSUrRNA* (*ToSSUrRNA*), and *A. ovis msp-4* (*Aomsp-4*) sequences from this study were deposited in GenBank (*T. ovis*: LC430938 and LC430939, *A. ovis*: LC430940, LC430941 and LC430942, *T. lestoquardi*: LC430943, LC430944, LC430945, LC430946, LC430947 and LC430948).

**Statistical analysis**

The associations of pathogens with sex, age, and sampling location were analyzed by two-tailed Fisher’s exact test and logistic regression analysis. Factors with at least borderline significance

### Table 1. List of primers used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequences</th>
<th>Fragment (bp)</th>
<th>Anelling temp (˚C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong></td>
<td><strong>Reverse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. ovis</em> SSUrRNA</td>
<td>TGGCAGGACCTTGGTCTTCT</td>
<td>549</td>
<td>62</td>
<td>Aktas et al. (2005) [17]</td>
</tr>
<tr>
<td><em>T. ovis</em> SSUrRNA</td>
<td>TCGAGACCTTCGGGGT</td>
<td>520</td>
<td>60</td>
<td>Aktas et al. (2006) [19]</td>
</tr>
<tr>
<td><em>T. lestoquardi</em> ms1-2</td>
<td>GTGCCGCAAGTGAGTGAGTGAGTCAG</td>
<td>730</td>
<td>55</td>
<td>Taha et al. (2011) [18]</td>
</tr>
<tr>
<td><em>A. ovis</em> msp-4</td>
<td>TGAAGGAGGGGTATGAGG</td>
<td>347</td>
<td>62</td>
<td>Torina et al. (2012) [21]</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em> epank1</td>
<td>GAGATGCTAGGAGAGCAGGAGGACTCTT</td>
<td>444</td>
<td>54–62 (Touch-down PCR)</td>
<td>Walls et al. (2000) [20]</td>
</tr>
<tr>
<td><em>A. marginale</em> msp-4</td>
<td>CTGAAGGGGAGTAATGGG</td>
<td>344</td>
<td>60</td>
<td>Torina et al. (2012) [21]</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0218609.t001
(p < 0.15) according to univariate analysis were included in the multivariate analysis. Backward-stepwise elimination was used to generate a minimum adequate model and excluded variables (p > 0.05) were retested in the minimum model. Two-tailed Fisher’s exact test and logistic regression analysis were also used to evaluate the significance of association between co-infected pathogens. A correlation coefficient (Rij) between the different pairs of pathogens was measured as described [22]. Twenty-seven negative goats were excluded from the calculation of correlation coefficient. Statistical analysis was performed using EZR version 1.27 [23].

Results and discussion

Prevalence of T. lestoquardi, T. ovis, B. ovis and Anaplasma spp. in goat blood samples from Sistan and Baluchestan province, Iran

All 200 samples analyzed were negative for B. ovis, A. marginale, and A. phagocytophilum; while 3 (1.5%), 25 (12.5%), and 160 (80%) were positive for T. lestoquardi, T. ovis, and A. ovis, respectively (Table 2). In Zabol, 19/51 (37.3%) and 50/51 (98%) samples were positive for T. ovis and A. ovis, respectively, while T. lestoquardi was not detected. In Sarbaz, 3/125 (2.4%), 6/125 (4.8%) and 87/125 (69.6%) samples were positive for T. lestoquardi, T. ovis and A. ovis, respectively. None of the samples from Chabahar were positive for T. lestoquardi and T. ovis while 23/24 (95.8%) were positive for A. ovis. Sequence analysis of obtained PCR products confirmed that the species identities judged by PCR diagnosis were correct. In spite of low infection rate of T. lestoquardi, 6 different nucleotide sequences of ms1 was obtained which were relatively diversified and showed 97.9–99.7% identity values. Two nucleotide sequences for ToSSUrRNA and 3 nucleotide sequences for 3 Aomsp-4 identified in this study were relatively conserved with 99.8% and 99.3–99.7% sequence identity, respectively.

Several species of Theileria can infect small ruminants and T. ovis and T. lestoquardi were reported previously from Iran [8, 24, 25]. While there is no report on T. ovis prevalence in the goat population in Iran, the prevalence of T. lestoquardi is 6.25% (by semi-nested PCR diagnosis targeting SSUrRNA) and 19% (by microscopic diagnosis with Giemsa-stained smear) in West Azerbaijan and Kurdistan provinces, respectively, in western Iran [26, 27]. The prevalence of T. lestoquardi in sheep ranges from 6.6% in Razavi Khorasan province in northeast Iran to 33% in Fars province in central Iran, which is one of the most important endemic regions for ovine theileriosis in Iran [8, 28]. T. ovis is more prevalent in sheep and ranges from 13.2% in western Iran to 73% in central Iran [8, 28]. The overall infection rate of T. lestoquardi in this study was 1.5%, which is relatively low compared to the previous reports from Iran [26, 27]. This difference may originate from the difference in sampling time, as all the positive samples in this study were collected in summer. The climate diversity which affects the distribution and infestation of the tick vector in various regions in Iran [29] may also contribute to a difference in infection rates. Detection method could be another possible contributing factor to this difference. T. ovis was present in 12.5% of samples and this is the first molecular report of this parasite in goats in Iran. B. ovis is one of the most important and highly pathogenic parasites

Table 2. Pathogens identified in different districts in Sistan and Baluchestan province.

<table>
<thead>
<tr>
<th>District</th>
<th>T. ovis</th>
<th>T. lestoquardi</th>
<th>A. ovis</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zabol</td>
<td>19 (37.3%)</td>
<td>0</td>
<td>50 (98%)</td>
<td>51</td>
</tr>
<tr>
<td>Sarbaz</td>
<td>6 (4.8%)</td>
<td>3 (2.4%)</td>
<td>87 (69.6%)</td>
<td>125</td>
</tr>
<tr>
<td>Chabahar</td>
<td>0</td>
<td>0</td>
<td>23 (95.8%)</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>25 (12.5%)</td>
<td>3 (1.5%)</td>
<td>160 (80%)</td>
<td>200</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0218609.t002
that infects small ruminants and prevalent in different regions in Iran [9]. Although *Rhipicephalus bursa*, the tick vector of *B. ovis*, exists in Sistan and Baluchestan [29], we could not detect this pathogen in this study suggesting that *B. ovis* may not be common in the surveyed region.

Goat anaplasmosis in Iran is mainly caused by *A. ovis* and *A. marginale* [10]. The infection rate of *A. ovis* is from 34.7% (by nested PCR diagnosis targeting *msp4*) in western Iran to 63.7% (by PCR-RFLP analysis targeting *msp4*) in northern and northeastern Iran [10, 30]. The overall infection rate of *A. ovis* in goats was 80% in this study which was higher than other reported areas in Iran. The difference in sampling time, diagnosis method, geographical, and climate variation may contribute to the differential prevalence of this pathogen.

A higher prevalence of *T. ovis* infection in goats with >1 year of age was observed (one positive out of 39 goats ≤1 years old, 24 positive out of 161 goats >1 years old were positive, *p* < 0.05 by two-tailed Fisher’s exact test), however no statistical differences were observed between the prevalence of other pathogens and the age of goats (Table 3). In a study from goats in Saudi Arabia, *T. ovis* was significantly less prevalent in animals <1 year of age [31]. Similarly, *T. ovis* infection was more prevalent in the goats >1 year old in Turkey [32]. The higher prevalence of *T. ovis* in adults (>1 year) may be due to a more frequent contact of adult goats to the tick vector. Additionally, male goats were significantly more frequently infected with *T. ovis* (17 out of 95 male goats and 8 out of 105 female goats were positive) and *A. ovis* (87 out of 95 male goats and 73 out of 105 female goats were positive) (*p* < 0.05 by two-tailed Fisher’s exact test; Table 3), which is consistent to the previous report for *T. ovis* [31] and *A. ovis* [32]. Differences in the management of male and female animals during pregnancy, labor, and lactation may affect the degree of the exposure to tick vectors, which in turn may contribute to higher prevalence of these pathogens in male animals [33]. The prevalence of pathogen among the sampling locations were different for *T. ovis* and *A. ovis* (*p* < 0.05 by two-tailed Fisher’s exact test). These correlations were further analyzed by multivariate logistic regression analysis. Significant association between *T. ovis* infection and age, *T. ovis* infection and Zabol, and *A. ovis* infection and Sarbaz was detected by this analysis, whereas significant correlation between pathogen and sex was not detected (Table 4). Zabol is located around 500 km north of Sarbaz and Chabahar with a drier climate which may affect the distribution of ticks and subsequently *T. ovis*. However, the data showed that *T. ovis* and *A. ovis* infections correlate with different locations suggesting other factor such as difference in farm management and tick control situation may also contribute to the prevalence of these pathogens.

There is no epidemiological report on tick-borne pathogens in small ruminants in Afghanistan, and similar reports are limited from Pakistan and not from the western region [34, 35]. Thus, the result of this study serves as a useful reference to estimate the prevalence of tick-borne piroplasms and *Anaplasma* spp. in the Afghanistan and the Pakistan regions neighboring the Sistan and Baluchestan province of Iran.

Table 3. Factors associating with the infection evaluated by Fisher’s exact test.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Age</th>
<th>Odds ratio</th>
<th>95% Confidence interval</th>
<th>p value</th>
<th>Sex</th>
<th>Odds ratio</th>
<th>95% Confidence interval</th>
<th>p value</th>
<th>Location</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1</td>
<td>≤1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. ovis</em></td>
<td>Positive</td>
<td>1</td>
<td>24</td>
<td>0.15</td>
<td>0.0036–0.99</td>
<td><strong>0.033</strong></td>
<td>17</td>
<td>8</td>
<td>2.63</td>
<td>1.01–7.43</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>38</td>
<td>137</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. lestoquardi</em></td>
<td>Positive</td>
<td>1</td>
<td>2</td>
<td>2.08</td>
<td>0.035–40.98</td>
<td>0.48</td>
<td>2</td>
<td>1</td>
<td>2.23</td>
<td>0.11–133.1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>38</td>
<td>159</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. ovis</em></td>
<td>Positive</td>
<td>33</td>
<td>127</td>
<td>1.47</td>
<td>0.53–4.65</td>
<td>0.51</td>
<td>87</td>
<td>73</td>
<td>4.73</td>
<td>1.98–12.65</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0218609.t003
Interplay between *P. caprae* and other pathogens co-infected in the goat

Because the goat malaria parasite, *P. caprae*, was detected in 28 samples among these 200 samples in the previous study [16], we evaluated the possible effect of a specific pathogen infection against the other pathogens identified in this study. A correlation coefficient value (R$_{ij}$) was calculated for each two-pathogen interaction [22]. Co-infections are summarized in Table 5. A strong negative correlation between *P. caprae* and *A. ovis* infections with R$_{ij}$ value of –0.593 was observed (p < 0.01 by two-tailed Fisher’s exact test, Table 6) and their double infection was only 8%. Infection of *P. caprae* and *T. ovis* showed a relatively weak negative correlation, yet significant (R$_{ij}$ value: –0.182, p < 0.05 by two-tailed Fisher’s exact test). However, all *T. ovis* positive samples were also positive for *A. ovis* and a relatively weak, though significant, positive correlation was observed (R$_{ij}$ value: 0.118, p < 0.01 by two-tailed Fisher’s exact test). The co-infections were further analyzed by multivariate logistic regression analysis including age, sex, and location information and a significant correlation was detected between *P. caprae* and *A. ovis* infections (p < 0.05, odds ratio: 0.26, 95% confidence interval: 0.11–0.61).

### Table 5. Co-infection of pathogens in goat samples from Sistan and Baluchestan province.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Positive numbers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single infection</strong></td>
<td></td>
</tr>
<tr>
<td><em>T. lestoquardi</em></td>
<td>1 (0.5)</td>
</tr>
<tr>
<td><em>A. ovis</em></td>
<td>118 (59)</td>
</tr>
<tr>
<td><em>P. caprae</em></td>
<td>12 (6)</td>
</tr>
<tr>
<td><strong>Double infection</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. ovis</em> &amp; <em>T. ovis</em></td>
<td>24 (12)</td>
</tr>
<tr>
<td><em>A. ovis</em> &amp; <em>T. lestoquardi</em></td>
<td>1 (0.5)</td>
</tr>
<tr>
<td><em>A. ovis</em> &amp; <em>P. caprae</em></td>
<td>16 (8)</td>
</tr>
<tr>
<td><strong>Triple infection</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. ovis</em>, <em>T. ovis</em> &amp; <em>T. lestoquardi</em></td>
<td>1 (0.5)</td>
</tr>
</tbody>
</table>

*From Kaewthamasorn et al, 2018 [16]*

https://doi.org/10.1371/journal.pone.0218609.t005

### Table 6. Fisher’s exact test for the co-infection of two pathogens.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th><em>A. ovis</em></th>
<th><em>T. ovis</em></th>
<th><em>T. lestoquardi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. caprae</em></td>
<td>–0.593 (0.0036)</td>
<td>–0.182 (0.029)</td>
<td>–0.059 (1)</td>
</tr>
<tr>
<td><em>A. ovis</em></td>
<td>–</td>
<td>0.118 (0.0055)</td>
<td>–0.131 (0.49)</td>
</tr>
<tr>
<td><em>T. ovis</em></td>
<td>–</td>
<td>–</td>
<td>–0.072 (0.33)</td>
</tr>
</tbody>
</table>

Correlation coefficient between two pathogens (R$_{ij}$) is presented. The p-value is shown in the bracket. p-values were calculated by Fisher’s exact test.

https://doi.org/10.1371/journal.pone.0218609.t006
The negative correlation between two vector-borne pathogens could also happen in the host or the vector by competing for resources such as space like available erythrocytes, nutrients, or impact on the immune response. We found a negative correlation between *P. caprae* and *A. ovis*. Mosquitoes are the likely vector for *P. caprae*, while *A. ovis* and *T. ovis* are transmitted by ticks; thus excluding the possibility of negative interference in the vector and highlighting likely competition in the goat [36, 37]. A negative association was reported between *Theileria annulata*, a protozoan parasite responsible for tropical theileriosis, and *A. marginale* [22]. In a study that was done using blood samples from sick sheep, the authors showed that the presence of *T. ovis* was negatively correlated with *B. ovis*, indicating that infection with low pathogenic *T. ovis* protects sheep from infection with highly pathogenic *B. ovis* [14]. An absolute exclusion was shown to exist between *T. annulata* and *B. bovis*, since the authors did not find any co-infection in cattle samples in Algeria [22]. The negative correlation between two pathogens could happen through modification of host immune response such as development of cross-protection immunity. Alternatively, this may be due to a mechanical interference between pathogens since all these pathogens infect host erythrocytes. However, there is no data on the erythrocyte type preference and receptors for these pathogens. Studies on the molecular mechanism of erythrocyte invasion and modification mediated by these pathogens would provide important insights behind these observations; however, such information are scarce, if any. Given the fact that *P. caprae* observed in the goats had very low parasitemia, below the microscopy detection limit [16], we consider that the interference by *P. caprae* against *A. ovis* and *T. ovis* is quite unlikely and exclusion may take place through modulating the host immune system or mechanical interference by *A. ovis* and *T. ovis*.

The best example of positive correlation among two vector-borne pathogens is between *Borrelia burgdorferi*, the causative agent of Lyme disease, and *Babesia microti*, the primary agent of human babesiosis, both of which are transmitted by the tick *Ixodes ricinus* [38]. Co-infection of these two pathogens are common and enhance the transmission and emergence of *B. microti* in human population in USA, possibly by lowering the ecological threshold for establishment of *B. microti* [12, 39]. Immunosuppression by one pathogen may predispose the host to the second pathogen. This phenomenon could be seen in *B. microti* with the parasites *Trypanosoma musculi* and *Trichuris muris* in mice [40, 41]. Moreover, the possibility of co-infection increases if the pathogens are transmitted by the same vector [40]. Both *T. ovis* and *A. ovis* are transmitted by the same tick, *R. sanguineus*, in the region thus positive correlation may be a result of the simultaneous inoculation of these pathogens to the goat by ticks or by enhancing pathogen fitness and transmission by tick vector [37, 42]. In addition, positive correlation of *T. ovis* and *A. ovis* infection may suggests the absence of cross protection between these pathogens; one eukaryotic protozoan parasite and the other prokaryotic bacteria. One infection appears to increase the susceptibility to the other pathogen. Co-infection is often associated with exacerbation of symptoms, thus, competition among pathogens could be beneficial to the host [15]. It is worth investigating the mechanism for competitive interaction among these pathogens.

**Conclusions**

The distribution of *T. lestoquardi*, *T. ovis*, and *A. ovis* in different regions in Iran is well reported. However, in this study we focused in Southeast of Iran, Sistan and Baluchestan province, where no reports exist, and showed the co-infection of these pathogens. Co-infection of several pathogens might influence the pathogenesis in the host and may jeopardize correct diagnoses. We showed a negative correlation between *A. ovis* and *P. caprae*, suggesting possible interference via immunity or against erythrocyte invasion by the other pathogen. The results...
of this study may contribute to understand these pathogen interactions in the host, and aid in designing preventive measures of tick-borne pathogens in the region. However, limited sample size is a constraint factor of this study and our findings needs to be extended by studies with large-scale samples from different geographical regions as well as experimental infection studies.

Supporting information

S1 Fig. Map of Iran showing sampling sites in Sistan and Baluchestan province, Iran. (TIF)

Acknowledgments

The authors are grateful to Paul Frank Adjou Moumouni and Xuenan Xuan from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine for providing positive control DNA for performing PCR. We thank Thomas J. Templeton from our institute for his critical reading of the manuscript. This work was conducted at the Joint Usage/Research Center on Tropical Disease, Institute of Tropical Medicine, Nagasaki University. HH is a recipient of the JSPS Postdoctoral Fellowship for foreign researchers from the Japan Society for the Promotion of Science.

Author Contributions

Conceptualization: Hassan Hakimi, Masahito Asada.
Data curation: Hassan Hakimi, Ali Sarani, Mika Takeda, Osamu Kaneko.
Formal analysis: Hassan Hakimi, Masahito Asada.
Funding acquisition: Osamu Kaneko, Masahito Asada.
Investigation: Hassan Hakimi, Ali Sarani, Mika Takeda, Masahito Asada.
Methodology: Hassan Hakimi, Mika Takeda.
Resources: Ali Sarani.
Supervision: Osamu Kaneko, Masahito Asada.
Validation: Masahito Asada.
Writing – original draft: Hassan Hakimi.
Writing – review & editing: Ali Sarani, Mika Takeda, Osamu Kaneko, Masahito Asada.

References


