**Supplementary Materials**

**Table S1.** Sampling effort and ticks collected in Eswatini during June and July 2018 survey by site. The number of *Rhipicephalus* nymphs extracted and tested for tick-borne pathogens are represented in parentheses. .

| | | | | *Amblyomma hebraeum* | | | *Haemaphysalis elliptica* | *Rhipicephalus appendiculatus* | *Rhipicephalus evertsi* | *Rhipicephalus muehlensi* | *Rhipicephalus maculatus* | *Rhipicephalus simus* | *Rhipicephalus* sp. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type | Location | Site | distance sampled (m) | adult | nymph | larvae | adult | adult | adult | adult | adult | adult | nymphs (extracted nymphs) | larvae w/ festoons | larvae w/o festoons |
| wildlife conservation area | Hlane | HH1 | 1200 | 1 | 0 | 45 | 1 | 3 | 1 | 8 | 1 | 10 | 42 (20) | 586 | 11 |
| | | HH2 | 1200 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | 5 | 8 | 107 (20) | 3665 | 5 |
| | | HM1 | 1200 | 1 | 2 | 15 | 1 | 1 | 0 | 0 | 0 | 84 | 1 (1) | 120 | 120 |
| | | HH3 | 1200 | 3 | 3 | 0 | 7 | 18 | 0 | 1 | 5 | 47 | 21 (18) | 0 | 0 |
| | | LL1 | 1200 | 0 | 0 | 3992 | 0 | 2 | 0 | 1 | 0 | 42 | 69 (20) | 642 | 313 |
| | | ML1 | 1200 | 0 | 0 | 2 | 0 | 6 | 1 | 1 | 3 | 12 | 1 (1) | 74 | 4 |
| | | MM1 | 1200 | 1 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 35 | 0 (0) | 37 | 26 |
| | Mbluluzi | HL1 | 1200 | 0 | 4 | 1484 | 0 | 33 | 0 | 2 | 5 | 5 | 18 (18) | 13941 | 206 |
| | | HL2 | 1200 | 0 | 0 | 1246 | 1 | 18 | 0 | 1 | 0 | 5 | 399 (20) | 395 | 0 |
| | | HM2 | 1200 | 0 | 3 | 1418 | 0 | 0 | 0 | 0 | 0 | 1 | 159 (20) | 2051 | 41 |
| | Mlawula | LL2 | 1200 | 0 | 1 | 5 | 1 | 0 | 0 | 1 | 5 | 4 | 23 (19) | 909 | 25 |
| | | ML2 | 1200 | 0 | 1 | 4 | 45 | 1 | 0 | 1 | 1 | 18 | 69 (20) | 770 | 20 |
| | | MLA | 600 | 0 | 1 | 284 | 0 | 0 | 0 | 4 | 11 | 1 | 220 (60) | 670 | 830 |
| | Dombeya | DO | 1000 | 0 | 2 | 245 | 6 | 23 | 1 | 2 | 0 | 0 | 351 (60) | 2283 | 51 |
| | Mhlosinga | MH | 1000 | 0 | 1 | 7 | 11 | 6 | 0 | 0 | 0 | 7 | 1 (1) | 400 | 6 |
| | Nisela | NI | 1200 | 0 | 0 | 56 | 10 | 15 | 0 | 0 | 0 | 4 | 0 (0) | 52 | 264 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mixed cattle and game ranch | IYSIS | LH1 | 1200 | 0 | 0 | 973 | 0 | 0 | 0 | 0 | 0 | 0 | 6 (6) | 461 | 4 |
| | | LH2 | 1200 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 69 (20) | 1793 | 262 |
| | | LM1 | 1200 | 0 | 0 | 342 | 2 | 0 | 0 | 0 | 0 | 0 | 54 (20) | 898 | 1967 |
| | | LM2 | 1200 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 1 | 58 (20) | 609 | 470 |
| | Bushlands Ranch | BU2 | 300 | 0 | 1 | 8 | 4 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 582 | 0 |
| cattle ranch | Nkalashane Ranch | NK | 1200 | 0 | 0 | 818 | 43 | 0 | 0 | 0 | 0 | 13 | 0 (0) | 0 | 54 |
| | Ndukuyamangendla Ranch | RIC | 1200 | 0 | 1 | 508 | 9 | 0 | 0 | 0 | 0 | 18 | 1 (1) | 0 | 0 |
| communal | Lomahasha | LO1 | 300 | 0 | 1 | 528 | 2 | 1 | 0 | 0 | 0 | 0 | 0 (0) | 8 | 886 |
| | Maphiveni | MA1 | 400 | 0 | 0 | 0 | 32 | 2 | 0 | 0 | 0 | 1 | 0 (0) | 0 | 0 |
| | Ndzevane | SE | 800 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 252 | 7 |
| | Sitsatsaweni | SI | 600 | 0 | 0 | 1019 | 33 | 0 | 0 | 0 | 0 | 2 | 3 (3) | 10 | 214 |

**Table S2.** Sampling effort and ticks collected in Eswatini during December 2018 and June and July 2019 surveys by site in wildlife conservation areas. The number of *Rhipicephalus* nymphs extracted and tested for tick-borne pathogens are represented in parentheses. .

| | | | | *Amblyomma hebraeum* | | | *Haemaphysalis elliptica* | *Rhipicephalus appendiculatus* | *Rhipicephalus evertsi* | *Rhipicephalus muehlensi* | *Rhipicephalus maculatus* | *Rhipicephalus simus* | *Rhipicephalus* sp. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Session | Location | Site | distance sampled (m) | adult | nymph | larvae | adult | adult | adult | adult | adult | adult | nymphs (extracted nymphs) | larvae w/ festoons | larvae w/o festoons |
| December 2018 | Hlane | HH1 | 800 | 0 | 2 | 0 | 0 | 2 | 0 | 1 | 17 | 22 | 3 (3) | 0 | 179 |
| | | HM1 | 800 | 0 | 1 | 137 | 3 | 1 | 0 | 1 | 0 | 35 | 5 (5) | 8 | 1076 |
| | | HH3 | 800 | 4 | 0 | 63 | 0 | 0 | 0 | 0 | 4 | 15 | 11 (11) | 87 | 1853 |
| | | LL1 | 800 | 0 | 0 | 914 | 0 | 1 | 0 | 0 | 0 | 22 | 19 (19) | 172 | 2278 |
| | Mbluluzi | HL1 | 800 | 0 | 4 | 4575 | 0 | 2 | 0 | 4 | 9 | 0 | 99 (20) | 0 | 226 |
| | | HL2 | 800 | 0 | 1 | 1610 | 0 | 0 | 0 | 1 | 3 | 1 | 136 (20) | 226 | 575 |
| | | HM2 | 800 | 0 | 0 | 349 | 0 | 0 | 0 | 0 | 6 | 1 | 145 (20) | 20 | 101 |
| | IYSIS | LH2 | 800 | 0 | 0 | 350 | 0 | 0 | 1 | 0 | 1 | 0 | 3 (3) | 42 | 511 |
| | | LM2 | 800 | 0 | 0 | 18 | 0 | 0 | 0 | 0 | 6 | 0 | 10 (10) | 306 | 400 |
| | Mlawula | LL2 | 800 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 7 | 3 | 102 (20) | 97 | 570 |
| | | ML2 | 800 | 1 | 2 | 8 | 0 | 0 | 0 | 3 | 1 | 26 | 165 (20) | 895 | 588 |
| June and July 2019 | Hlane | HH1 | 400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 9 (9) | 704 | 57 |
| | | HM1 | 400 | 0 | 0 | 27 | 0 | 1 | 0 | 0 | 0 | 21 | 0 (0) | 28 | 1 |
| | | HH3 | 400 | 0 | 0 | 4 | 1 | 0 | 1 | 1 | 0 | 3 | 6 (6) | 350 | 72 |
| | | LL1 | 400 | 0 | 0 | 649 | 0 | 0 | 0 | 1 | 0 | 12 | 24 (20) | 555 | 98 |
| | Mbluluzi | HL1 | 400 | 0 | 0 | 196 | 2 | 1 | 1 | 0 | 0 | 2 | 30 (20) | 1864 | 432 |
| | | HL2 | 400 | 0 | 0 | 168 | 0 | 1 | 0 | 0 | 0 | 0 | 36 (20) | 1009 | 68 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HM2 | 400 | 1 | 0 | 59 | 0 | 2 | 0 | 0 | 0 | 4 | 114 (20) | 2758 | 39 |
| | IYSIS | LH2 | 400 | 0 | 0 | 32 | 0 | 0 | 0 | 0 | 0 | 0 | 17 (17) | 202 | 464 |
| | | LM2 | 400 | 0 | 0 | 23 | 0 | 1 | 0 | 0 | 0 | 0 | 33 (20) | 316 | 553 |
| | Mlawula | LL2 | 400 | 0 | 0 | 54 | 0 | 0 | 0 | 0 | 1 | 2 | 3 (3) | 1671 | 77 |
| | | ML2 | 400 | 0 | 0 | 129 | 0 | 0 | 0 | 0 | 0 | 6 | 15 (15) | 483 | 73 |

**Table S3.** Density of questing ticks in protected versus unprotected sites during June and July 2018. Bold represents statistical significance.

| Tick Species and Life Stage | Protected area sample mean density (per 100m) | Unprotected area sample mean density (per 100m) | t-test; df = 25 |
|---|---|---|---|
| *Amblyomma hebraeum* larvae | 41.6 | 76.0 | t = -1.1; p = 0.3 |
| *Rhipicephalus* (*Boophilus*) larvae | 21.7 | 56.0 | t = -0.2; p = 0.8 |
| *Rhipicephalus* larvae | 134.5 | 6.0 | **t = 4.0; p < 0.001** |
| *Rhipicephalus* nymphs | 7.7 | 0.1 | **t = 2.6; p = 0.014** |
| *Rhipicephalus appendiculatus* adult | 0.89 | 0.12 | t = 1.2; p = -0.25 |
| *Rhipicephalus evertsi* adult | 0.02 | 0 | NA |
| *Rhipicephalus maculatus* adult | 0.22 | 0 | NA |
| *Rhipicephalus muehlensi* adult | 0.17 | 0 | NA |
| *Rhipicephalus simus* adult | 1.14 | 0.53 | t = 0.7; p = 0.5 |
| *Haemaphysalis elliptica* adult | 0.43 | 3.1 | **t = -3.6; p = 0.001** |

**Table S4.** Mean density of questing ticks at resampled sites. Paired t-tests with 95% confidence levels. Bold represents statistical significance.

| Tick Species and Life Stage | Repeated sites: 2018 (per 100m) | Repeated sites: 2019 (per 100m) | t-test; df = 10 | Repeated sites: Winter 2018 (per 100m) | Repeated sites: Summer 2018 (per 100m) | t-test; df = 10 |
|---|---|---|---|---|---|---|
| *Amblyomma hebraeum* larvae | 62.2 | 30.5 | t = -0.9; p = 0.4 | 62.2 | 91.2 | t = -1.5; p = 0.2 |
| *Rhipicephalus* (*Boophilus*) larvae | 11.1 | 44.0 | **t = -3.1; p = 0.01** | 11.1 | 95.0 | **t = -4.9; p < 0.001** |
| *Rhipicephalus* larvae | 165.3 | 225.9 | t = -2.1; p = 0.07 | 165.3 | 21.1 | **t = 2.3; p = 0.05** |
| *Rhipicephalus* nymphs | 6.98 | 6.55 | t = 0.1, p = 0.9 | 6.98 | 8.09 | t = -0.06, p = 0.9 |
| *Rhipicephalus appendiculatus* adult | 0.57 | 0.14 | t = 1.49; p = 0.2 | 0.57 | 0.07 | t = 2.0; p = 0.07 |
| *Rhipicephalus evertsi* adult | 0.02 | 0.05 | t = -0.8; p = 0.4 | 0.02 | 0.01 | t = 0.2; p = 0.8 |
| *Rhipicephalus maculatus* adult | 0.14 | 0.02 | **t = 2.6; p = 0.03** | 0.14 | 0.61 | **t = -3.1; p = 0.01** |
| *Rhipicephalus muehlensi* adult | 0.11 | 0.05 | t = 1.1; p = 0.3 | 0.11 | 0.15 | t = -0.53; p = 0.6 |
| *Rhipicephalus simus* adult | 1.64 | 1.16 | t = 1.1; p = 0.3 | 1.64 | 1.42 | t = 0.37; p = 0.7 |
| *Haemaphysalis elliptica* adult | 0.44 | 0.07 | t = 1.2; p = 0.3 | 0.44 | 0.03 | t = 1.4; p = 0.2 |

**Table S5.** The percent sequence identity of identified bacterial and protozoan species in this study to reference sequences obtained from Genbank. The percent sequence identity describes how similar a sequence from this study is to a reference sequence and was calculated for each gene from pathogen species by dividing the number of matching nucleotides between the sequence obtained in this study and the reference sequence by the total number of nucleotides.

| **Pathogen Species/Genotypes** | **Gene** | **Reference Genbank Accession Number** | **Sequence Identity** |
|---|---|---|---|
| *Rickettsia africae* | *ompA* | CP001612 | 99.3-100% |
| | *ompB* | CP001612 | 100% |
| | *gltA* | CP001612 | 100% |
| Candidatus *Rickettsia barbariae* | *ompA* | EU272186 | 99.3% |
| | *ompB* | EU272187 | 100% |
| | *gltA* | EU272185 | 99.3% |
| *Rickettsia conorii* | *ompA* | AE006914 | 99.6-100% |
| | *ompB* | AE006914 | 100% |
| | *gltA* | AE006914 | 100% |
| *Rickettsia massiliae* | *ompA* | CP000683 | 100% |
| | *ompB* | CP000683 | 100% |
| | *gltA* | CP000683 | 99.5-100% |
| *Rickettsia* sp. A | *ompA* | CP019435, *R. raoulti* | 97.6% |
| | *ompB* | CP019435, *R. raoulti* | 98.5% |
| | *gltA* | CP019435, *R. raoulti* | 99.6% |
| *Rickettsia* sp. B | *ompA* | KT835137, *Rickettsia* sp. SSSE | 100% |
| | *ompB* | CP013133, *Rickettsia rhipicephali* | 99.2% |
| | *gltA* | CP013133, *R. rhipicephali* | 99.8% |
| *Rickettsia* sp. C | *ompA* | NC_017065, *R. slovaca* | 92.2% |
| | *ompB* | NC_017065, *R. slovaca* | 94.5-96.8% |
| | *gltA* | NC_017065, *R. slovaca* | 99.4% |
| *Ehrlichia ruminantium* | *groEL* | NC_005295 | 98.3% |
| *Ehrlichia minasensis* | *16S* | MH500005 | 99.7-100% |
| | *groEL* | JX629806 | 98.6% |
| *Babesia caballi* from *R. simus* | *18S* | EU642514 | 98.6% |
| | | KX375825 | 98.8% |
| *Babesia caballi* from *R. appendiculatus* | *18S* | EU642514 | 97.1% |
| | | KX375825 | 97.3% |
| *Babesia* sp. | *18S* | HQ437690, *Babesia* sp. | 99.0% |
| *Hepatozoon* sp. | *18S* | KF939627, *Hepatozoon* sp. | 98.8% |
| | | MN723845, *Hepatozoon ophisauri* | 98.8% |
| | | MN791089, *Hepatozoon canis* | 92.2% |
| *Theileria equi* | *18S* | EU888902 | 99.0% |
| *Theileria ovis* | *18S* | AY260172 | 99.2% |
| *Theileria taurotragi* | *18S* | MH393312 and MK131255 | 100% |

| | | | |
|---|---|---|---|
| *Theileria velifera* | *18S* | MN595045 | 99.7% |
| *Theileria* sp. (sable) | *18S* | GU733378 | 99.2% |
| *Theileria* sp. (giraffe) | *18S* | FJ213582 | 100% |
| *Theileria* sp. (waterbuck) | *18S* | KF597072 | 100% |
| *Theileria* sp. Tragelaphini B | *18S* | MK131247 | 98.7% |
| *Theileria* sp. Tragelaphini D | *18S* | MK131250 | 98.7-100% |
| *Theileria* sp. Tragelaphini E | *18S* | MK131251 | 99.3-99.7% |
| *Theileria* sp. Aepycerotini | *18S* | MK131245 | 100% |
| *Theileria* sp. (impala) | *18S* | MK131253 | 100% |
| *Theileria* sp. (rodent) | *18S* | MT269267 | 99.5% |
| | | MK484070 | 98.8% |
| *Theileria* sp. A | *18S* | EU053199, *Theileria* sp. (dog) | 96.9% |
| | | GU733378, *Theileria* sp. (sable) | 95.6% |
| *Theileria* sp. B | *18S* | MK131255, *Theileria taurotragi* | 96.9% |

**Table S6.** Tick infection prevalence (no. infected/no. tested), with 95% confidence intervals for prevalence estimates in brackets, considering all adult ticks and the subset of nymphal ticks tested for tick-borne pathogens by site and sampling session.

| | | Tick Infection Prevalence | | |
|---|---|---|---|---|
| Location | Site | Winter 2018 | Summer 2018 | Winter 2019 |
| Hlane | HH1 | 4/45; 8.9% [2.5-21.2%] | 4/47; 8.5% [2.4-20.4%] | 1/10; 10%[0.3-44.5%] |
| | HH2 | 11/38; 28.9% [15.4-45.9%] | - | - |
| | HM1 | 23/90; 25.6% [16.9-35.8%] | 6/45; 13.3% [5.1-26.8%] | 3/22; 13.6%[2.9-34.9%] |
| | HH3 | 25/103; 24.3% [16.4-33.7%] | 6/34; 17.6% [6.8-34.5%] | 3/12; 25%[5.5-57.2%] |
| | LL1 | 15/65; 23.1% [13.5-35.2%] | 5/42; 11.9% [4-25.6%] | 7/33; 21.2%[9-38.9%] |
| | ML1 | 6/24; 25% [9.8-46.7%] | - | - |
| | MM1 | 9/40; 22.5% [10.8-38.5%] | - | - |
| Mbluluzi | HL1 | 21/67; 31.3% [20.6-43.8%] | 9/39; 23.1% [11.1-39.3%] | 4/26; 15.4%[4.4-34.9%] |
| | HL2 | 10/45; 22.2% [11.2-37.1%] | 4/26; 15.4% [4.4-34.9%] | 6/21; 28.6%[11.3-52.2%] |
| | HM2 | 5/24; 20.8% [7.1-42.2%] | 0/28; 0% [0-12.3%] | 5/27; 18.5%[6.3-38.1%] |
| Mlawula | LL2 | 7/31; 22.6% [9.6-41.1%] | 6/33; 18.2% [7-35.5%] | 1/6; 16.7%[0.4-64.1%] |
| | ML2 | 16/87; 18.4% [10.9-28.1%] | 5/53; 9.4% [3.1-20.7%] | 2/21; 9.5%[1.2-30.4%] |
| | MLA | 16/77; 20.8% [12.4-31.5%] | - | - |
| Dombeya Nature Estate | DO | 24/94; 25.5% [17.1-35.6%] | - | - |
| Mhlosinga Nature Reserve | MH | 5/26; 19.2% [6.6-39.4%] | - | - |
| Nisela Game Reserve | NI | 4/29; 13.8% [3.9-31.7%] | - | - |
| IYSIS | LH1 | 0/6; 0% [0-45.9%] | - | - |
| | LH2 | 5/25; 20% [6.8-40.7%] | 0/5; 0% [0-52.2%] | 3/17; 17.6% [3.8-43.4%] |
| | LM1 | 2/22; 9.1% [1.1-29.2%] | - | - |
| | LM2 | 3/27; 11.1% [2.4-29.2%] | 0/16; 0% [0-20.6%] | 3/21; 14.3% [3-36.3%] |
| Bushlands Ranch | BU2 | 3/5; 60% [14.7-94.7%] | - | - |
| Nkalashane Ranch | NK | 23/56; 41.1% [28.1-55%] | - | - |

| | | | | |
|---|---|---|---|---|
| Ndukuyamangendla Ranch | RIC | 6/29; 20.7% [8-39.7%] | - | - |
| Lomahasha | LO1 | 0/4; 0% [0-60.2%] | - | - |
| Maphiveni | MA1 | 17/35; 48.6% [31.4-66%] | - | - |
| Ndzevane | SE | 0/1; 0% [0-97.5%] | - | - |
| Sitsatsaweni | SI | 5/38; 13.2% [4.4-28.1%] | - | - |

**Table S7.** The number and species identity of ticks with coinfections of pathogen species belonging to *Anaplasma, Ehrlichia, Rickettsia,* and *Theileria* observed among 1716 ticks.

| | Anaplasma sp. | Ehrlichia sp. | Theileria velifera | Theileria sp. waterbuck | Theileria ovis | Theileria sp. Tragelaphini B | Theileria sp. Tragelaphini D | Theileria taurotragi | Theileria sp. Aepycerotini | Theileria sp. Tragelaphini E |
|---|---|---|---|---|---|---|---|---|---|---|
| Rickettsia barbariae | 1 R. simus | - | - | - | - | - | - | - | - | - |
| Rickettsia conorii | 5 H. elliptica | - | - | - | - | - | - | - | - | - |
| Rickettsia sp. B | 1 R. simus | - | - | - | - | - | - | - | - | - |
| Rickettsia massiliae | - | 1 R. simus | - | - | - | - | - | - | - | - |
| Rickettsia africae | - | - | 3 A. hebraeum | 2 A. hebraeum | - | - | - | - | - | - |
| Anaplasma sp. | - | - | - | - | 1 R. appendiculatus | - | - | - | - | - |
| Ehrlichia sp. | - | - | - | - | - | 1 R. muehlensi | 2 R. appendiculatus | 1 R. appendiculatus | 2 R. appendiculatus | - |
| Ehrlichia minasensis | - | - | - | - | - | - | - | - | - | 1 R. simus |

**Table S8.** Conventional PCR primers and reaction conditions used in this study. All conventional PCR assays were performed in 25 μL reactions, using 5 μL of 5x GoTaq® Reaction Buffer and 200 μM of each dNTP. Cycling conditions were as follows: 95°C for 3 min; specified number cycles of 95°C for 30 seconds (except for Tick ITS2 for 60 seconds and Tick 12S for 15 seconds), specified annealing conditions, and specific elongation; followed by 72 °C for 7 min and hold at 4 °C.

| Target Organism | Target Gene | Primer Name | Primer Orientation | 5′-3′ sequence | Ref | Primer Conc (nM) | $MgCl_2$ Conc (mM) | GoTaq® DNA polymerase (U) | Cycles | Annealing (temp [°C], time [s]) | Elongation (temp [°C], time [s]) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tick | ITS2 | 3SAF | Forward | CTAAGCGGTGGATCACTCGG | [1] | 500 | 2.25 | 0.2 | 35 | 55, 60 | 72, 120 |
| | | ITS2R | Reverse | ATATGCTTAAATTCAGCGGG | [2] | 500 | | | | | |
| Tick | 12S | T1B | Forward | AAACTAGGATTAGATACCC | [3] | 100 | 3.25 | 0.5 | 5 | 51, 30 | 68, 30 |
| | | T2A | Reverse | AATGAGAGCGACGGGCGATGT | | 100 | | | 25 | 53, 30 | 70, 30 |
| Tick | CO1 | LEP-F1 | Forward | ATTCAACCAATCATAAAGATATTGG | [4] | 500 | 2.5 | 1.0 | 35 | 54, 30 | 72, 60 |
| | | LEP-R1 | Reverse | TAAACTTCTGGATGTCCAAAAAATCA | | 500 | | | | | |
| Tick | CO1 | Chel-CO1-F1 | Forward | TACTCTACTAATCATAAAGACATTGG | [5] | 500 | 2.5 | 1.0 | 5 | 45, 45 | 72, 60 |
| | | Chel-CO1-R1 | Reverse | CCTCCTCCTGAAGGGTCAAAAAATGA | | 500 | | | 35 | 50, 45 | 72, 60 |
| *Rickettsia.* | ompA | Rr190.70p | Forward | ATGGCGAATATTTCTCCAAAA | [6] | 600 | 2.25 | 0.625 | 40 | 54.8, 45 | 72, 30 |
| | | 90.701 | Reverse | GTTCCGTTAATGGCAGCATCT | [7] | 600 | | | | | |
| *Rickettsia* | ompB | 120-M59 | Forward | CCGCAGGGTTGGTAACTGC | [8] | 300 | 2.0 | 1.25 | 40 | 50, 30 | 72, 60 |
| | | 120-807 | Reverse | CCTTTTAGATTACCGCCTAA | | 300 | | | | | |
| *Rickettsia.* | gltA | CS409d | Forward | CCTATGGCTATTATGCTTGC | [9] | 200 | 2.25 | 1.25 | 40 | 53, 30 | 72, 55 |
| | | Rp1258n | Reverse | ATTGCAAAAAGTACAGTGAACA | | 200 | | | | | |
| Anaplasmataceae | 16S | Ehr-16S-D | Forward | GGTACCYACAGAAGAAGTCC | [10] | 200 | 2.25 | 1.25 | 40 | 54, 60 | 72, 60 |
| | | Ehr-16S-R | Reverse | TAGCACTCATCGTTTACAGC | | 200 | | | | | |
| *Anaplasma.* | rpoB | Ana-rpoBF | Forward | GCTGTTCCTAGGCTYTCTTACGCGA | [11] | 500 | 2.25 | 1.25 | 40 | 55, 60 | 72, 60 |
| | | Ana-rpoBR | Reverse | AATCRAGCCAVGAGCCCCTRTAWGG | | 500 | | | | | |
| *Ehrlichia* | groEL | Ehr-groEL-F | Forward | GTTGAAAARACTGATGGTATGCA | [11] | 500 | 2.25 | 1.25 | 40 | 50, 60 | 72, 60 |
| | | Ehr-groEL-R | Reverse | ACACGRTCTTTACGYTCYTTAAC | | 500 | | | | | |
| | 18S | ILO-9029 | Forward 1 | CGGTAATTCCAGCTCCAATAGCGT | [12] | 200 | 1.5 | 1.0 | 30 | 53, 30 | 72, 60 |
| | | ILO-9030 | Reverse 1 | TTTCTCTCAAGGTGCTGAAGGAGT | | 200 | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Apicomplexa (*Babesia, Theileria, Hepatozoon*) | (nested cPCR) | MWG4-70 | Forward 2 | AGCTCGTAGTTGAATTTCTGCTGC | | 200 | 1.5 | 1.0 | 30 | 55,30 | 72, 60 |
| | | ILO-7782 | Reverse 2 | AACTGACGACCTCCAATCTCTAGTC | | 200 | | | | | |

**Table S9.** Real-time PCR primers, probes, and reaction conditions used in this study.

| Target Organism | Target Gene | Primer Name | Primer Orientation | 5′-3′ sequence | Ref | Primer/Probe Concentration (nM) | Initial Denature | Denaturation, annealing, and extension |
|---|---|---|---|---|---|---|---|---|
| Anaplasmataceae | 23S | Tt-Ana-F | Forward | TGACAGCGTACCTTTTGCAT | [13] | 200 | 95 °C for 10 min | 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min |
| | | Tt-Ana-R | Reverse | GTAACAGGTTCGGTCCTCCA | | 200 | | |
| | | Tt-Ana-S | Probe | 6-FAM™-GGATTAGAC/ZEN/CCGAAACCAAG-Iowa Black® | | 250 | | |
| *Rickettsia* | 17-kd | R17K135F | Forward | ATGAATAAACAAGGKACNGGHACAC | [14] | 800 | 95 °C for 15 min | 40 cycles of 95 °C for 10 seconds, 60°C for 10 sec, and 72 °C for 30 sec |
| | | R17K249R | Reverse | AAGTAATGCRCCTACACCTACTC | | 800 | | |
| | | R17KbC | Probe | 6-FAM-TTGGTTCTCAATTCGGTAAGGGTAAAGG – Black Hole Quencher® | | 100 | | |

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