Potential role of dogs as sentinels and reservoirs for piroplasms infecting equine and cattle in Riyadh City, Saudi Arabia

Running title: Dogs as reservoirs for equine/bovine piroplasms

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Summary

Canine tick-borne diseases have been considered emerging and re-emerging threats, given their increasing global prevalence. In this molecular survey, we aimed to detect and identify common tick-borne pathogens in dogs from Riyadh city in Saudi Arabia. Initially, the study included 36 dogs visiting private veterinary clinics. PCRs targeting the 18S ribosomal RNA gene (rDNA) of haemoparasites (*Babesia*, *Theileria* and *Hepatozoon*) and the 16S rDNA of Anaplasmataceae were performed. The results showed that 26 (72.2%) dogs were infected by some of the haemoparasites under investigation. The sequencing analysis of the amplicons confirmed the infections due to two parasite species *Theileria equi* and *Theileria velifera*. Further examination of guard dogs kept in the horse stables of the Riyadh Municipality revealed that the majority of the tested dogs (65.2%: 30 out of 46) were infected with either of the parasites. In addition, the genotypes of all the parasites in these dogs were identical to those of the parasites in the dogs from the veterinary clinics. Thus, it can be concluded that dogs are infected with these

haemoparasites and serve as a reservoir for both *T. equi* and *T. velifera* in the study area; however, the clinical implication of this finding is to be studied.

Keywords: Dogs; *Theileria equi*; *Theileria velifera*

Introduction

Together with the remarkable increase in the amount of DNA sequence data in open repositories, molecular diagnostic techniques have allowed the sensitive and accurate detection of tick-borne pathogens. This has evoked an ever-growing interest in evolutionary biologists to retrieve and use such data to identify orthologous sequences and depicting phylogenetic inferences in an attempt to identify species and/or genotypes more accurately. Thus, studying and controlling an infectious disease implies the need for the knowledge of all factors involved in its transmission.

Ticks (Acari: Ixodida) are haematophagous ectoparasites of terrestrial and semi-aquatic mammalian, avian, and reptilian species, which affect domestic animals and wildlife (Dantas-Torres *et al.*, 2013; Barker and Walker, 2014; Panetta *et al.*, 2017). They are important vectors for human and animal diseases, and their global distribution contributes to the increase in the incidence of emerging and re-emerging tick-borne diseases worldwide (Guglielmone *et al.*, 2013; de la Fuente *et al.*, 2017). The prevalence of vector-borne diseases in a population closely reflects the distribution and density of the vectors (Vascellari *et al.*, 2016).

Theileria spp. and *Babesia* spp. have been reported as a major constraint for the production of small ruminants and large animals in Saudi Arabia (Alanazi *et al.*, 2012,

2014; Al-Khalifa *et al*., 2009; Mostafa and Bin Dajem, 2014). However, limited information about canine vector-borne diseases in Saudi Arabia is available, with only two case reports demonstrating the presence of endemic infections with *Ehrlichia canis* and *Dirofilaria repens* (Sacchini *et al.*, 2007; Tarello, 2003).

This study was initially set up to screen for canine haemoparasites or bacteria from the family Anaplasmataceae in dogs admitted to the private veterinary clinics. The results indicated the presence of equine and bovine haemoparasites in dogs. With this in mind, we also investigated the dogs that live together with horses to understand the role of dogs as a reservoir of these parasites.

Materials and methods

Study areas

The investigation was conducted in Riyadh city, Saudi Arabia. Riyadh city is the capital of Saudi Arabia, with the following geographical positions: latitude 24°–08° north and longitude 47°–18° east. It has an area of about 1,798 km^2 and was reported to be inhabited by approximately seven million people in 2016 (General Authority for Statistic, 2016). Riyadh city has a very hot summer, with temperatures reaching up to 49°C or more, and an average temperature of 43°C. Winters are cold with windy nights. The overall climate is arid, with very little annual rainfall (22.6 mm); the relative humidity ranges from 10% to 42% throughout the year (The General Authority of Meteorology and Environmental Protection (GAMEP), Saudi Arabian Government website: http://www.pme.gov.sa).

Dogs

Initial investigation included 36 dogs (19 male and 17 female) who visited two private veterinary clinics in Riyadh City. Clinical symptoms, including fever (cut-off value ≥ 39.5°C), diarrhoea, weakness, emaciation, reddish eyes and haematouria, were recorded by the veterinarians. Second part of this study was conducted on 46 guard dogs (21 male and 25 female) who were kept at 37 horse stables in Riyadh municipality. These dogs were apparently healthy and did not show any clinical signs.

Blood and DNA extraction

A volume of 2-5 ml blood of the cephalic vein were drawn from each dog into EDTA vacuum tubes (BD Vacutainer® Tube, Gribbles Pathology, VIC, Australia) and subsequently dispatched to the Laboratory of Parasitology, Shaqra University, for DNA extraction. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), eluted in 50 µl of elution buffer as per manufacturer's instruction, and stored at −20°C prior to use.

PCR and sequencing

PCR detecting *Babesia*, *Theileria,* and *Hepatozoon* parasites (BTH-PCR1) was carried out to amplify the parasite's 18S ribosomal RNA gene (rDNA) using BTH 18S 1st F: 5'-GTGAAACTGCGAATGGCTCATTAC-3' and BTH 18S 1st R: 5'-AAGTGATAAGGTTCACAAAACTTCCC-3' for primary amplification. This was followed by the nested BTH-PCR2 using BTH 18S 2nd F: 5'-GGCTCATTACAACAGTTATAGTTTATTTG-3' and BTH 18S 2nd R: 5'-CGGTCCGAATAATTCACCGGAT-3' for secondary amplification as described previously (Masatani *et al.*, 2017). PCR detecting members from the Anaplasmataceae family was conducted to amplify bacterial 16S rDNA using EHR16SD: 5'-GGTACCYACAGAAGAAGTCC-3' and EHR16SR: 5'-

TAGCACTCATCGTTTACAGC-3' (Parola *et al.,* 2000). PCR reactions were performed in a 25 µl-reaction mixture containing 12.5 µl of 2 × Gflex PCR Buffer (Mg2+, dNTP plus) (TaKaRa Bio Inc., Shiga, Japan), 0.5 µl of Tks Gflex DNA Polymerase (1.25 units/µl) (TaKaRa Bio Inc.), 200 nM of each primer, 1.0 µl of template DNA or 5-fold diluted first PCR product, and water. The reaction conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, annealing temperature of 55°C for 30 s and extension at 68°C for 90 s, followed by a final extension at 68°C for 5 min. The PCR products were subjected to electrophoresis in a 1.2% agarose gel stained with Gel-Red™ (Biotium, Hayward, CA). The PCR products were purified by using the NucleoSpin Gel and PCR Clean Up Kit (Takara Bio Inc.). Cycle sequencing reactions were performed using the nested primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI Prism 3130 x genetic analyser (Applied Biosystems) according to the manufacturers' instructions.

Sequence data analysis

Sequences obtained were manually edited using the ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan). The obtained sequences were compared with those available in public databases using nucleotide BLASTn at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted by using MEGA version 7.0 (Kumar et al., 2016). Sequences were aligned with closely related sequences retrieved from the GenBank using MUSCLE algorisms implemented in MEGA (Kumar et al., 2016). A neighbour-joining method was used to construct rooted phylogenetic tree with 1,000 bootstrap replicates. The sequences obtained in the present study were submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) under

accession numbers LC431545–LC431547 for *T. equi* and LC431548–LC431551 for *T. velifera*.

Statistical analysis

To understand the association of parasite infections with clinical symptoms mentioned above, age and sex of dogs, we performed multivariate logistic regression analysis using statistical software R version 3.1.2. Possible multicollinearity between the variables was assessed by calculating variance inflation factor (VIF). Since multicollinearity was observed among "weakness", "emaciation" and "reddish eyes" (VIF: >10), we excluded the variable "weakness" from the model. We then calculated the odds ratio and its 95% confidence interval for each association. We also conducted a likelihood-ratio test to evaluate the significance of each variable in the model.

Results

Dogs admitted to the private veterinary clinics

BTH PCR showed positive results for haemoparasite infections in 26 dogs (72.2%), while PCR for detecting members of the Anaplasmataceae family yielded negative results in case of all dogs (Table 1). All positive samples were further subjected to direct Sanger sequencing. Sequence alignment and BLAST analysis revealed that 7 and 19 samples were *T. equi* and *T. velifera*, respectively. Almost entire 18S rDNA sequence was obtained from 13 samples, resulting in 3 and 4 different genotypes for *T. equi* and *T. velifera*, respectively (Table 2). Three genotypes of *T. equi* were divided into two clusters in a phylogenetic tree (Figure 1). *T. equi* genotype 1 was found in 2 samples with 100% similarity to the sequences of *T. equi* reported from Saudi Arabia (KJ801922-KJ801937), Turkey (MG569904-5), Israel (KX227620- KX227630), Brazil (KJ573370), and USA

(JX177673). *T. equi* genotypes 2 and 3 (1 sample each) had 98% identity with *T. equi* available in the database. Likewise, *T. velifera* further resulted into 4 genotypes, all of which were clustered into one single clade in a phylogenetic tree (Figure 1). Genotype 1 was the most prevalent and detected in 4 samples, followed by genotype 2 (n = 3) and genotypes 3 and 4 (1 sample each) (Table 2). The alignment of the sequences obtained from this study is provided in supplementary Figure S1. There were one or two nucleotide differences observed between *T. velifera* genotypes.

Table 3 indicates the number of dogs positive for infection by each parasite and the results of the clinical observations. Clinical signs obtained from the private clinics showed that a majority of the dogs (n = 31) had pyrexia (body temperature above 39.5°C). Diarrhoea was also common in the tested dogs. All the dogs were administered with 120 mg/mL of imidocarb dipropionate (Imizol, Schering Plough Animal Health), and the dogs with severe haematological disorder were administered with Phenamidine Isethionate B. Vet. C 5% m/v by subcutaneous injection (0.3 ml per kg body mass). A statistical analysis did not find any association between the parasite infection status and clinical symptoms, age, and sex of the dogs (Wald test, $P > 0.05$), except that *T. equi* infection was found to be associated with age (Wald test, $P < 0.05$) (Supplementary Table S1).

Dogs kept in horse stables of Riyadh Municipality

BTH PCR showed positive results for haemoparasite infections in 30 dogs (65.2%) (Table 1). None of the samples yielded positive results for members of the Anaplasmataceae family by PCR. Sequencing analysis of the amplified products identified that 8 and 22 were infected with *T. equi* and *T. velifera*, respectively. A total of 21 samples yielded almost entire 18S rDNA sequences, which resulted in 2 different genotypes for both *T.*

equi (genotypes 1 and 2) and *T. velifera* (genotypes 1 and 2) (Table 3). All four genotypes were identical to those found in the dogs obtained from the veterinary clinics.

Discussion

It is generally acknowledged that dogs play an important role in transmitting tick-borne diseases by: (i) carrying ticks with a broad host range, (ii) acting as a domestic reservoir for certain nidicolous ticks, and (iii) possibly carrying ticks at all life stages that are not attached to the host or that may have been interrupted during feeding (Otranto et al., 2015; Dantas-Torres and Otranto D, 2016).

The results of the current study provide molecular evidence for the presence of *T. equi* and *T. velifera*, which are known to be equine and bovine parasites belonging to the genus *Theileria*. In the recent past, *T. equi* was detected in clinically ill dogs in Croatia (Beck *et al.*, 2009) and South Africa (Rosa *et al*., 2014); although these studies reported only one and two cases of *T. equi* infections, respectively, the present study indicated a high prevalence of *T. equi* in the tested dogs. Moreover, we detected *T. velifera* in a total of 41 dogs (Table 1). To the best of our knowledge, a direct detection of this parasite in canine blood has not yet been reported. This parasite was recently detected in ticks (*Dermacentor marginatus*, *Haemaphysalis parva*, *Haemaphysalis sulcate*, and *Rhipicephalus sanguineus*) collected from sheep and dogs in Greece by the reverse line blot (RLB) assay (Chaligiannis *et al.*, 2018). The presence of *T. equi* and *T. velifera* in dogs is not surprising, since these dogs share the same habitat with other domestic animals. Collectively, our study provides evidence for not excluding the dogs from the epidemiology of the infections caused by these parasites.

Comparison of parasite genotypes in terms of location showed that some genotypes were shared between parasites from dogs brought to the clinics and those from dogs in the horse stables. This fact suggests that dogs might transmit parasites to horses in *Theileria*-free regions, which results in the expansion of *Theileria*-endemic areas. This may also warrant the testing of dogs travelling to disease-controlled areas or countries not only for traditionally recognized dog parasites, but also for other horse and cattle piroplasms. In the present study, genotyping was conducted on the sequences of 18S rDNA, where only a small number of nucleotide differences were observed between genotypes. Moreover, PCR amplicons were sequenced directly without cloning, which might have masked the presence of multiple genotypes in a single animal. To better understand the transmission of these parasites between animals, further studies employing highly polymorphic markers are required.

The brown dog tick (*R. sanguineus*), one of the most widely distributed ticks worldwide and a vector of many pathogens affecting dogs, is also a vector for equine piroplasmosis (Scoles and Ueti, 2015). The same tick species also infests cattle and horses (Schoeman, 2009). In the study areas, there are several cattle farms near the horse stables. Although a direct physical contact between cattle and dogs was not confirmed, it is possible that the dogs entered the farms and acquired the ticks, since the dogs roam freely. Though several tick species including *R. sanguineus* have been recorded in Riyadh (Al-Khalifa *et al.*, 1986; Alanazi *et al.,* 2018), no information regarding the vector of the *Theileria* spp. in dogs in Saudi Arabia is available. Further studies should include the surveys on ticks to understand the lifecycle of the parasites in the tested areas.

Dogs appeared to be susceptible to *T. equi* and *Theileria* spp. infection, but systematic investigations on the clinical impacts of these infections, for which pale mucous

membranes, bleeding, lethargy, thrombocytopenia, anaemia, and myelofibrosis are the main clinical manifestations, are still relatively rare (Criado *et al*., 2006; and Rosa *et al*., 2014). Infection of dogs with cattle piroplasms is not uncommon. For instance, *T. annulata* has been reported from an asymptomatic dog in Spain and Iran (Bigdeli *et al*., 2012; Criado *et al*., 2006). These findings led us to agree with the assumption that some piroplasm species may lack host specificity or that distinct yet undiscovered piroplasm species closely related to those already recognised may exist, as these parasites were merely classified based on phenetic relationships rather than at deep molecular characterization levels.

The current study showed no clear association between *Theileria* infection and clinical outcomes in dogs, suggesting that healthy dogs might carry *Theileria* spp. In fact, guard dogs kept at the horse stables were apparently healthy and did not show any clinical signs of infections. This unclear association may also be related to the study design whereby all the dogs tested in the statistical analysis visited clinics. Limitations of the current statistical analysis includes a lack of data on possible variables such as breed and weight of dogs. Further studies are essential for understanding the association between *Theileria* infection and clinical outcomes in dogs. In addition, a diagnostic protocol to detect asymptomatic infection of *Theileria* in dogs is yet to be established.

Conclusion

The current study has confirmed that *T. equi* and *T. velifera* are highly prevalent in dogs in Riyadh city, and we presume that dogs can be potential reservoirs for these parasites, which primarily infect equines and cattle. Further investigation is required to determine the potential biological or ecological vectors of these pathogens both under experimental

and field conditions. Moreover, further experimentation is also needed to confirm the clinical sings of infection in dogs, although some literatures have already provided circumstantial evidences supporting this claim.

Ethics approval and consent to participate

Sampling procedure was reviewed and approved by the Ethical Committee of the Department of Biological Science at Faculty of Science and Humanities, Shaqra University, Kingdom of Saudi Arabia (Approval no. SH 03-2018). Informed consent was sought from animal owners.

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Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure Legend

Figure 1. Phylogenetic tree of 18S rDNA of haemoparasites detected in dogs in the private veterinary clinics and the horse stables using a maximum likelihood. All bootstrap values from 1,000 replications are shown on the interior branch nodes. The sequences obtained in the present study are shown in bold. GenBank/EMBL/DDBJ accession numbers are given after the species name.

Tables

Table 1. Results of PCR and sequencing for haemoparasite and Anaplasmataceae infections in dogs in the private veterinary clinics and the horse stables.

Location	No. tested	No. positive for *T. equi*	No. positive for *T. velifera*	No. positive for Anaplasmataceae
Private veterinary clinics	36 (19/17)†	7 (4/3)	19 (11/8)	0
Horse stables	46 (21/25)	8 (4/4)	22 (10/12)	0
Total	82 (40/42)	15 (8/7)	41 (21/20)	0

†(male/female)

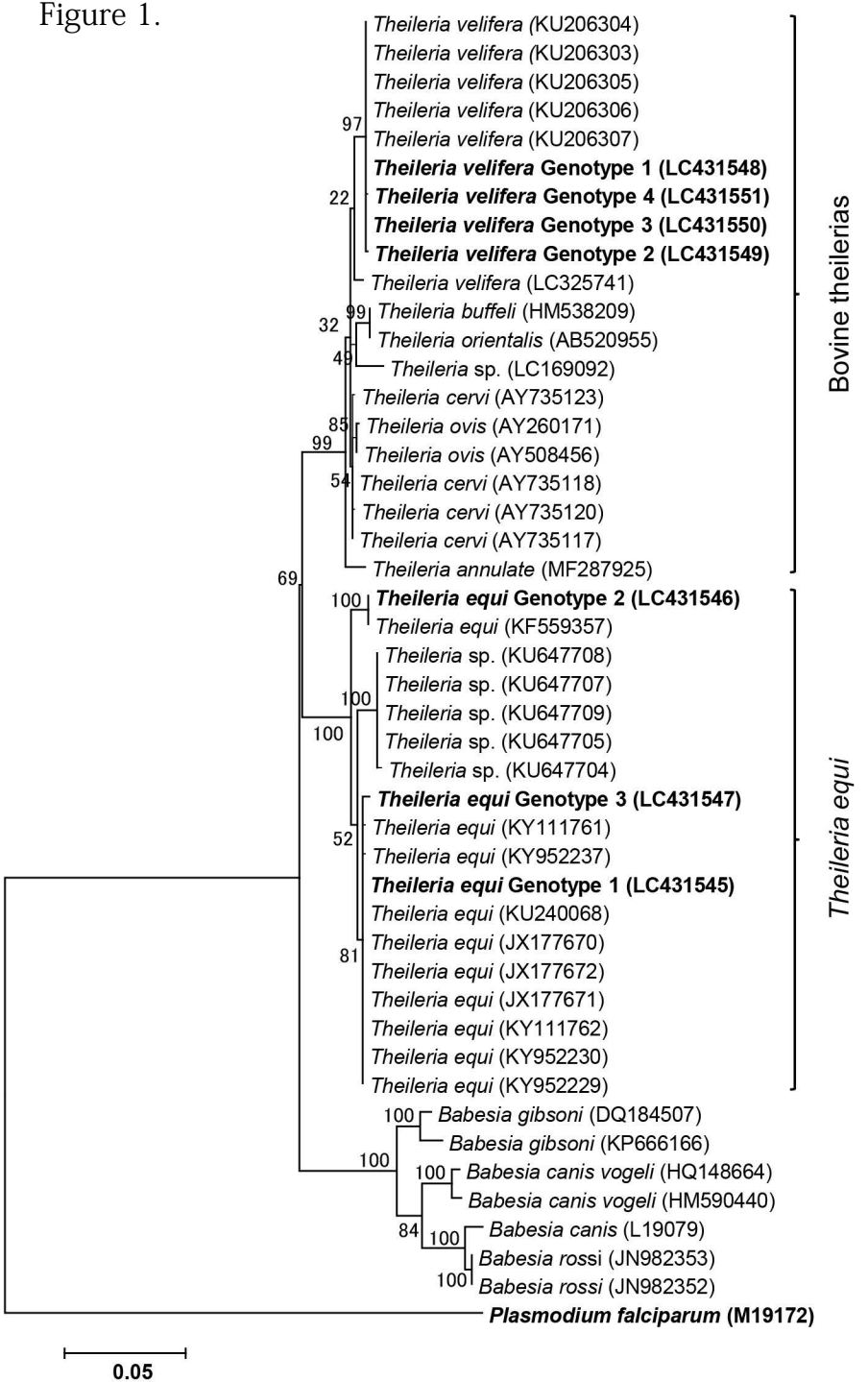
Table 2. Parasite genotypes detected in dogs in the private veterinary clinics and the horse stables.

	T. equi			*T. velifera*			
Location	Genotype 1	Genotype 2	Genotype 3	Genotype 1	Genotype 2	Genotype 3	Genotype 4
Private veterinary clinics	2	1	1	4	3	1	1
Horse stables	5	1	0	10	5	0	0
Total	7	2	1	14	8	1	1

Table 3. Clinical information and parasite species detected in dogs admitted to the private veterinary clinics.

	Symptom					
Parasite	Fever	Diarrhoea	Weakness	Emaciation	Reddish eyes	Haematouria
T. equi (n = 7)	7	5	2	1	1	0
T. velifera (n = 19)	17	10	4	2	1	1
Negative (n = 10)	7	4	1	1	0	0
Total	31	19	7	4	2	1

Figure 1.


Theileria velifera (KU206304)
Theileria velifera (KU206303)
Theileria velifera (KU206305)
Theileria velifera (KU206306)
Theileria velifera (KU206307)
Theileria velifera Genotype 1 (LC431548)
Theileria velifera Genotype 4 (LC431551)
Theileria velifera Genotype 3 (LC431550)
Theileria velifera Genotype 2 (LC431549)
Theileria velifera (LC325741)
Theileria buffeli (HM538209)
Theileria orientalis (AB520955)
Theileria sp. (LC169092)
Theileria cervi (AY735123)
Theileria ovis (AY260171)
Theileria ovis (AY508456)
Theileria cervi (AY735118)
Theileria cervi (AY735120)
Theileria cervi (AY735117)
Theileria annulate (MF287925)
Theileria equi Genotype 2 (LC431546)
Theileria equi (KF559357)
Theileria sp. (KU647708)
Theileria sp. (KU647707)
Theileria sp. (KU647709)
Theileria sp. (KU647705)
Theileria sp. (KU647704)
Theileria equi Genotype 3 (LC431547)
Theileria equi (KY111761)
Theileria equi (KY952237)
Theileria equi Genotype 1 (LC431545)
Theileria equi (KU240068)
Theileria equi (JX177670)
Theileria equi (JX177672)
Theileria equi (JX177671)
Theileria equi (KY111762)
Theileria equi (KY952230)
Theileria equi (KY952229)
Babesia gibsoni (DQ184507)
Babesia gibsoni (KP666166)
Babesia canis vogeli (HQ148664)
Babesia canis vogeli (HM590440)
Babesia canis (L19079)
Babesia rossi (JN982353)
Babesia rossi (JN982352)
Plasmodium falciparum (M19172)
Bovine theilerias
Theileria equi
0.05

Supplementary Data

Supplementary Table S1. Odds ratios for parasite infections with age, sex and clinical symptoms.

	T. velifera (95% CI)	*T. equi* (95% CI)
Age	1.41 (0.85, 2.34)	0.24† (0.08, 0.76)
Sex	0.76 (0.18, 3.18)	0.80 (0.07, 8.60)
Fever	1.67 (0.22,12.45)	40821294.65 (0.00, ∞)
Diarrhoea	1.88 (0.34, 10.32)	2.86 (0.15, 55.61)
Emaciation	0.68 (0.07, 6.90)	1.46 (0.04, 47.61)
Reddish eyes	1.53 (0.07, 35.94)	10.43 (0.04, 2512.73)
Haematouria	17643787.38 (0.00, ∞)	0.00 (0.00, ∞)

† denotes p-value < 0.05 with likelihood ratio test.

CI, confidence interval.

Supplementary Figure S1. Alignment of 18S rRNA sequences of *T. velifera* and *T. equi.*