

Molecular detection of tick-borne pathogens in cattle from Saudi Arabia

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Abstract

Background and aim:

Babesiosis and anaplasmosis are tick-borne diseases that affect the health of cattle and may be also responsible for remarkable economic losses. This study aimed to detect the presence of the causative agents of babesiosis and anaplasmosis among cattle in different provinces in Saudi Arabia and to characterise their species genetically.

Methodology:

A total of 362 blood samples were taken from cattle in four regions (Riyadh, Al-Kharj, Al-Hasa and Al-Qassim) of Saudi Arabia and were molecularly screened by polymerase chain reaction (PCR) of partial 18S rRNA and 23S rRNA genes to detect *Babesia* and *Anaplasma* species.

Results

The overall prevalence of both *Babesia* and *Anaplasma* in cattle was 2.49% and 5.80%, respectively. *Theileria annulata* (*T. annulata*) and *Theileria ovis* (*T. ovis*) were found in seven (1.93%) and two (0.55%) of the 362 samples, respectively. *Anaplasma ovis* (*A. ovis*) was detected in 21 (5.80%) of 362 samples. Likewise, four of the cattle were found to be co-infected with more than one pathogen (1.10%). All cattle samples tested negative for other species of *Babesia*, *Theileria* and *Anaplasma*.

Conclusion

The presence of *T. ovis* and *A. ovis* has been herein reported in Saudi Arabia. The presence of two novel genotypes of *T. annulata* is also reported in Saudi Arabian cattle. Further molecular surveys on larger numbers of samples from the entire country are needed in order to address correctly the prevalence and geographical distribution of the tick-borne disease.

1. Introduction

Tick-borne diseases (TBDs) including piroplasmosis and anaplasmosis are mainly distributed in tropical and temperate regions [1, 2]. The main causative agents of bovine piroplasmosis belong to the genera *Babesia* (Piroplasmida; Babesiidae) and *Theileria* (Piroplasmida; Theileriidae). Furthermore, pathogens within the genus *Anaplasma* (Rickettsiales; Anaplasmataceae) cause bovine anaplasmosis [3]. TBDs affect the health of livestock and are responsible for heavy economic losses. They also pose a potential risk regarding transmission of disease to humans [4]. Cattle production is severely affected by various diseases, especially in the absence of adequate veterinary services [5] and many endo- and ectoparasites affect the health, productivity and performance of the animals, including ticks [6, 7].

The cattle population in Saudi Arabia numbered approximately 350,000 in 2015. The highest percentages were in the Eastern and Central regions (Saudi Arabia General Authority for StatisticsGen, 2015 at <https://www.stats.gov.sa/en>). These regions contain the most cattle imported to Saudi Arabia from Europe [8]. In Saudi Arabia, cattle are infested by several species of hard ticks (Acari, Ixodidae), mainly *Hyalomma anatolicum anatolicum*, (*H. anatolicum anatolicum*), *Amblyomma variegatum*, *Rhipicephalus praetextatus* and *Hyalomma marginatum rufipes* [9, 10, 11]. These ticks can act as vectors for a variety of pathogens of veterinary importance, and several tick-borne diseases are known to be endemic in Saudi Arabia. However, knowledge of tick-borne pathogens (TBP) in cattle of this country is very limited. A few molecular epidemiological studies have been conducted on a limited number of animals [12, 13, 14]. Therefore, the present study aimed to determine the prevalence of ixodid ticks and investigate their associated pathogens at a molecular level among cattle in Saudi Arabia.

2. Materials And Methods

Ethical approval

This study was revised and approved by the Ethical Research Committee, Deanship of Scientific Research, King Khalid University, according to the ethical principles of human and animal research (Approval no. (ECM#2019-74) - (HAPO-06-B-001).

Sampling procedures

The cross-sectional study was conducted from May to October 2018 in four regions of Saudi Arabia: Riyadh (24°0'N, 45°30'E); Al-Kharj (24°08'N, 47°18E); Al-Hasa (25°23'N, 49°36E) and Al-Qassim (25°48'N, 42°52E) (**Figure 1**). All cows were apparently healthy at the time of sampling and was screened for tick infestation. Ticks found within 15 min were collected (2–5 ticks/infested animal) and placed in labelled tubes containing 70% ethanol that were individualised per cow. Ticks were identified to the species level by examination of morphological features and use of morphological keys [15, 16, 17]. Each cow was categorised according to age and gender.

Blood samples were collected from each animal (2–5 ml) from the jugular vein into vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer® Tube, Gibbles Pathology, VIC, Australia) and transported in ice boxes to the molecular laboratory, Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, for DNA extraction.

Molecular procedures

Total genomic DNA (gDNA) was isolated from the blood samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following manufacturer's instruction. Aliquots of between 50µl and 100µl of gDNA from each sample were stored at –20°C prior to molecular analysis.

The primers were designed to target a conserved region that encoded for ribosomal 18S rRNA (969 bp) and 238S rRNA(485 bp) to detect the DNA of *Babesia* [18] and *Anaplasma* [19] respectively (**Table 1**). Polymerase chain reaction (PCR) assays were performed in an automated BIO-RAD Thermal Cycler (BIO-RAD, Singapore) using one PCR master mix (GeneDireX, Taiwan). The PCR conditions for the *Babesia* amplification were: one incubation step at 95°C for 15 min; 35 cycles of 1 min at 95 °C; 30 s of annealing at 58 °C and 1 min at 72 °C; followed by a final extension for 5 min at 72 °C. Positive and negative controls were included. PCR conditions of *Anaplasma* amplification were: an initial denaturation step at 95 °C for 15 min; then 40 cycles that consisted of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, a 1 min extension at 72 °C, a final extension cycle at 72 °C for 7 min and cooling of the reactions at 15 °C. Distilled water samples were used as negative controls. Positive genomic controls of *Theileria annulata* (*T. annulata*) and *Anaplasma marginale* were included. The amplifications were confirmed by electrophoresis on a 1.5% agarose gel, stained with Red Safe and examined by ultraviolet transillumination. A DNA molecular weight marker (100bp DNA Ladder H3 RTU, GeneDireX, Taiwan) was used to estimate the size of the products. The PCR products of the positive samples were purified by use of a PCR Clean-Up & Gel Extraction Kit (GeneDireX, Taiwan) according to the manufacturer's instructions.

Sequencing and phylogenetic analyses

The purified PCR products were sequenced at Macrogen Lab Technology, Korea. The obtained sequences were assembled using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). The obtained sequences of *Babesia* and *Anaplasma* were submitted to GenBank and then compared with those available in the GenBank database by the US National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Moreover, the sequences obtained from positive samples and to sequences of validated species already available in GenBank were aligned using Bioedit software version 7.0.5.3 (Clustal W multiple alignment) [20]. For taxonomic analyses, the maximum-likelihood phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software

version X [21] with 500 bootstrap replications.

Statistical analysis

The significant differences regarding the prevalence of infections with *Babesia* and *Anaplasma* and their risk factors, such as gender and age of the cattle and their levels of tick infestation, were calculated by χ^2 test using a Statistical Package for the Social Sciences (SPSS) program, version 20.0, at $P < 0.05$.

Results

Of the 362 cattle examined, nine (2.49%) were found to be infected with *Babesia*, 21 (5.80%) with *Anaplasma* and four (1.10%) were found co-infected with more than one pathogen (**Table 2**). *Anaplasma ovis* (*A. ovis*) was the most prevalent pathogen (5.80%) followed by *T. annulata* (1.93%) and *T. ovis* (0.55%) (**Table 2**). All the samples tested negative for other species of *Babesia*, *Theileria* and *Anaplasma*. Among the four provinces, the highest prevalence of infection observed in Riyadh (12.14%) and the lowest recorded in Al-Qassim (3.03%). In the region of Al-Kharj, there was no infection by TBDS (**Table 2**).

Of the 362 examined cattle, 245 (67.7%) were infested by ticks (n=541). of which, 328 (60.6%) were males and 213 (39.4%) were females. The most representative tick species were *H. anaticum anaticum* (207, 38.3%), *H. dromedarii* (116, 21.4%), *H. impeltatum* (97, 18%), *Rhipicephalus (Boophilus) annulatus* (71, 13.1%), *Rhipicephalus turanicus* (47, 8.7%) and *H. excavatum* (three, 0.5%). Data regarding sex and age of the sampled cattle along with the number and percentage positivity for TBPs are reported in **Table 3**.

The PCR products showed the expected band size for each pathogen (**Figures 2 and 3**). By BLAST analysis, *T. annulata* and *T. ovis* were identified. Two novel genotypes of *T. annulata* were identified in this study (GenBank: MN826137 and MN826138) that were 99.8% identical to *T. annulata* in *H. anaticum anaticum* from Hofuf, Saudi Arabia (GenBank: MK300062) and 99.2% identical to *T. annulata* in cattle from Egypt (GenBank: MN625888 and MN625889). Moreover, a new genotype of *T. ovis* (GenBank: MN826139) was detected in cattle with 98% similarity to those of sheep from Egypt (GenBank: MN625886). In addition, in the present study, a novel *A. ovis* genotype was detected in cattle (GenBank: MN880224) with 99% similarity to those of *A. ovis* detected in cattle from Egypt (GenBank: MN625933). The phylogenetic tree of these genotypes are illustrated in **Figures 4 and 5**.

Discussion

The high prevalence of tick infestation (67.7%) and of TBPs (8.3%) among cattle in Saudi Arabia represents a potential threat to the health and welfare of these animals as well as to livestock production. The most prevalent tick species was *Hyalomma anaticum anaticum*, which is considered to be the main species that parasitises cattle [10, 22, 23]. Camels are the main host for the species *Hyalomma dromedarii*, although it can also infest cattle, sheep, goats, horses and donkeys [24]. The species *Hyalomma impeltatum* infests a wide range of mammalian hosts including cattle [25]. These tick species are the main vectors of *Babesia* spp., *Theileria* spp. and *Anaplasma* spp. of domestic and wild ungulates in Saudi Arabia [12, 13, 26, 27, 28, 29, 30]. *Rhipicephalus (Boophilus) annulatus* has been reported from cattle in Jazan in the south-west and from animals in central parts of Saudi Arabia [31, 32]. This tick species is an efficient vector of *Babesia bovis* globally [33]. The other two species herein identified in cattle, *Rhipicephalus turanicus* and *Hyalomma excavatum*, usually infest sheep, goats and horses [10, 11, 31] begin found herein on cattle might be due to husbandry practices in desert areas, where livestock share the same environment.

The overall TBP infection prevalence in cattle reported in this study was 8.3% which is in agreement with previous findings [13, 26]. Also findings of this study indicate that *T. annulata* is present in the central and eastern parts of Saudi Arabia, which overlap with the distribution of *H. anaticum anaticum*, the putative vector of *T. annulata* [8, 31].

T. ovis is a non-pathogenic parasite that is widespread in Saudi Arabia, particularly in sheep and goats [34]. Moreover, according to experimental transmission studies [35], *H. anaticum anaticum* could be the vector of this parasite in Saudi Arabia. This tick has been found to infest sheep and goats in Saudi Arabia [10, 31].

This study reports two novel *T. annulata* genotypes (GenBank: MN826137 and MN826138) that are 99.8% identical to *T. annulata* in *H. anaticum anaticum* from the Hofuf region of Saudi Arabia (GenBank: MK300062) and 99.2% to *T. annulata* in cattle from Egypt (GenBank: MN625888 and MN625889). The results are in agreement with already published data of occurrence of *T. annulata* in cattle in Saudi Arabia [8, 13, 14]. Moreover, a new genotype of *T. ovis* (GenBank: MN826139) has been detected in cattle blood with 98% similarity to those of *T. ovis* detected in sheep from Egypt (GenBank: MN625886). A novel *A. ovis* genotype has been identified in Saudi cattle (GenBank: MN880224) with 99% similarity to those of *A. ovis* detected in cattle from Egypt (GenBank: MN625933). However, this study do not cover all Saudi Arabian regions and dose give a clear picture of TBPs distributions and prevalence in Saudi cattle, therefore further epidomolgical and molecular studies are required in order to elaborate better strategic control plans and assess the risk to cattle health and productivity.

Conclusion

This is the first study that reported *T. ovis* and *A. ovis* in cattle from Saudi Arabia, Futhermore, two new genotypes of *T. annulata* in cattle were recorded and therefore, cattle may be considered as carriers for *Babesia* and *Anaplasma* spp. of other ruminants in Saudi Arabia. Further extensive molecular surveys on a large numbers of of animals from the whole country are needed in order to address correctly the prevalence and geographical distribution of TBPs.. This in turn will help in the design and implementation of effective preventive and control measures.

Declarations

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Declarations

Ethics approval and consent to participate

The performance of blood sampling for this study was approved by the Ethical Research Committee, Deanship of Scientific Research, King Khalid University, according to the ethical principles of human and animal research (Approval no. (ECM#2019-74) (HAPO-06-B-001).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors shared in the design of the plan of this study. ADA, MSA, ASA, MYA collected blood samples from cattle and extracted DNA from the blood samples. ADA, HHAMA, AMA, BSME and SA participated in the conduction of PCR protocols, purification of PCR products, sequence analyses, construction of the phylogenetic tree and analyses of the data. ADA, SA, HHAMA and AMA co-wrote the manuscript. ADA,MSA and SA reviewed and wrote the final draft of the manuscript. All authors revised and approved the final version of the manuscript.

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Tables

Table 1: Primers and target genes of pathogens investigated.

pathogens	Target gene	Primer Name	Primers (5'-3')
Piroplasma	18S rRNA	piro18S-F1	GCGAATGGCTCATTAAACA
		piro18S-F4	CACATCTAAGGAAGGCAGCA
		piro18S-F3	GTAGGGTATTGGCCTACCG ^a
		piro18S-R3	AGGACTACGACGGTATCTGA ^a
Anaplasmataceae	23S rRNA	Ana23S-212f	ATAAGCTGCGGGGAGTTGTC
		Ana23S-753r	TGCAAAAAGGTACGCTGTCAC

Table 2: Prevalence of infection among cattle with tick-borne pathogens according to regions of Saudi Arabia.

Region	Examined animals	<i>Theileria annulata</i>		<i>Theileria ovis</i>		<i>Anaplasn</i>
		Positive	%	Positive	%	Positive
Riyadh	173	6	3.5	1	0.6	16
Al-Kharj	101	0	0.00	0	0.00	0
Al-Hasa	55	1	1.8	1	1.8	4
Al-Qassim	33	0	0.00	0.00	0.00	1
Total	362	7	1.93	2	0.55	21

ns= not significant

3: Risk factors associated with Piroplasma and Anaplasma infections detected by PCR in cattle at different provinces of Saudi Arabia.

Factor	Total animals	Bebesia		Anapl	
		Positive	%	Positive	
Age (year)	≤1	225	5	2.22	17
	>1	137	4	2.92	4
	x ²		0.111		8.0
	Sig.		ns		0.0
Gender	Male	201	7	3.48	16
	Female	161	2	1.24	5
	x ²		2.778		5.7
	Sig		ns		0.0
Ticks	Yes	245	8	3.27	19
	No	117	1	0.85	2
	x ²		5.444		13.7
	Sig.		0.020		<0.0

Sig= significant , ns= not significant

Figures





Figure 1

Map showing the location of the study areas in Saudi Arabia. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

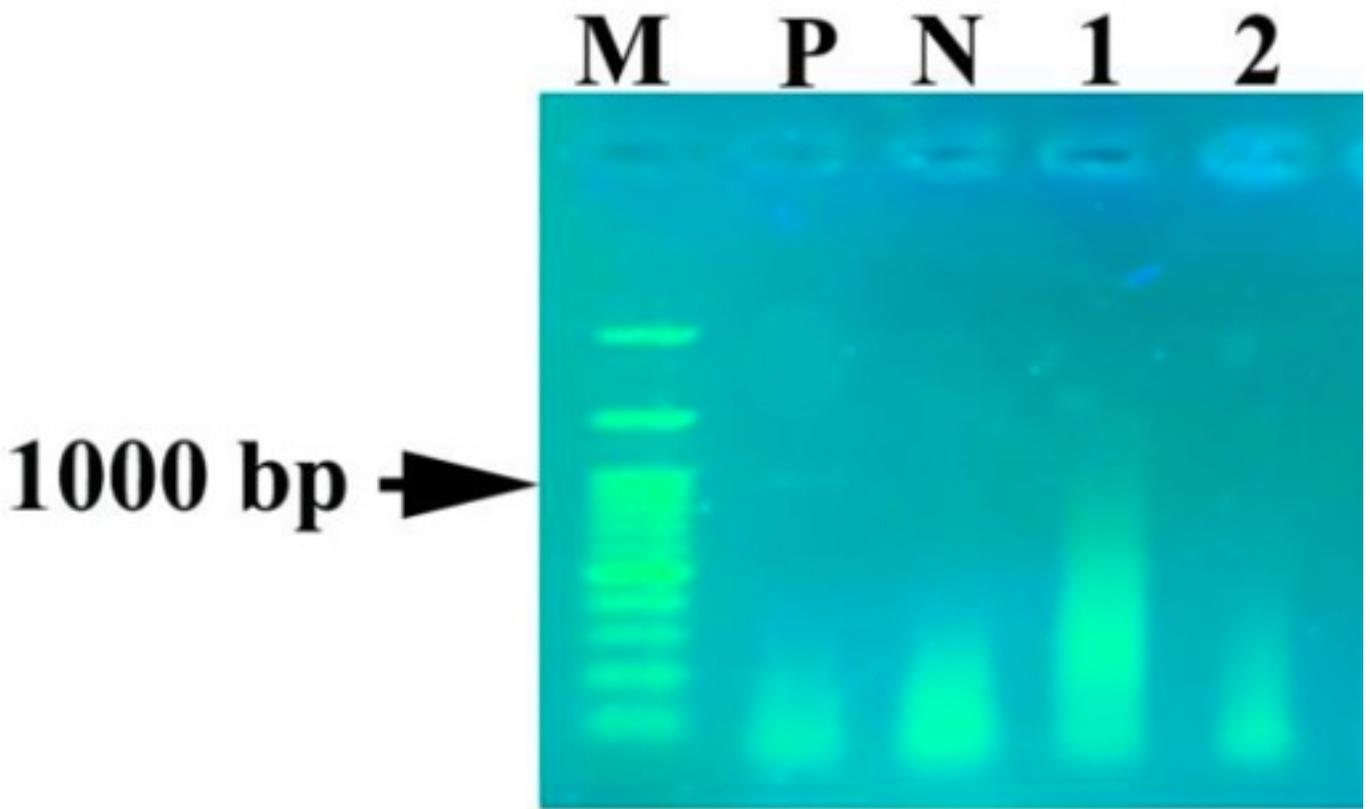


Figure 2

Image of agarose gel electrophoresis (1.5%) showing 1100 pb band for the amplification of 18S rRNA gene of Piroplasma. Lane M: DL 2000 marker; Lane P: positive control; Lane N: negative control; lanes 1-11: negative samples; lane 12: positive sample.

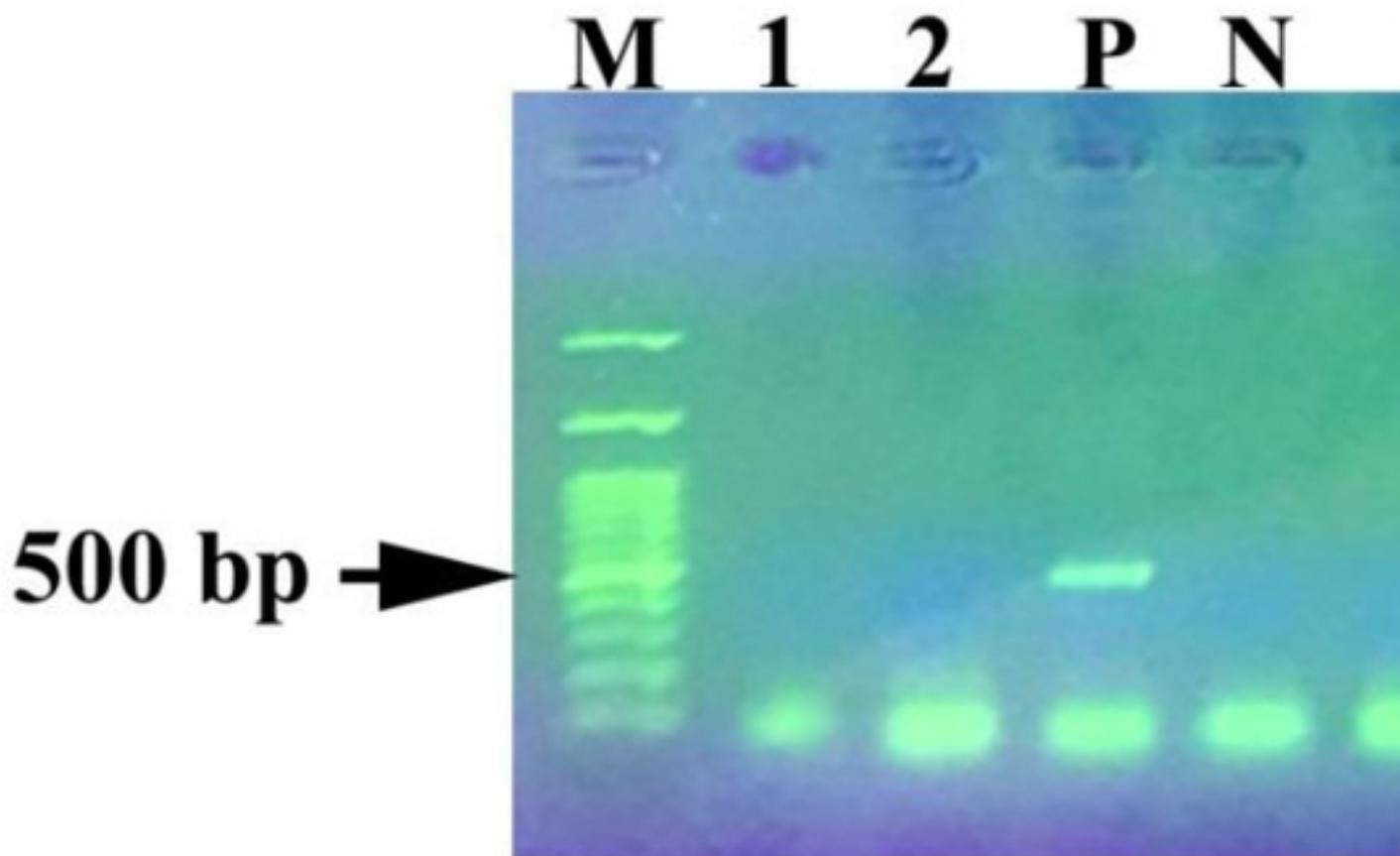
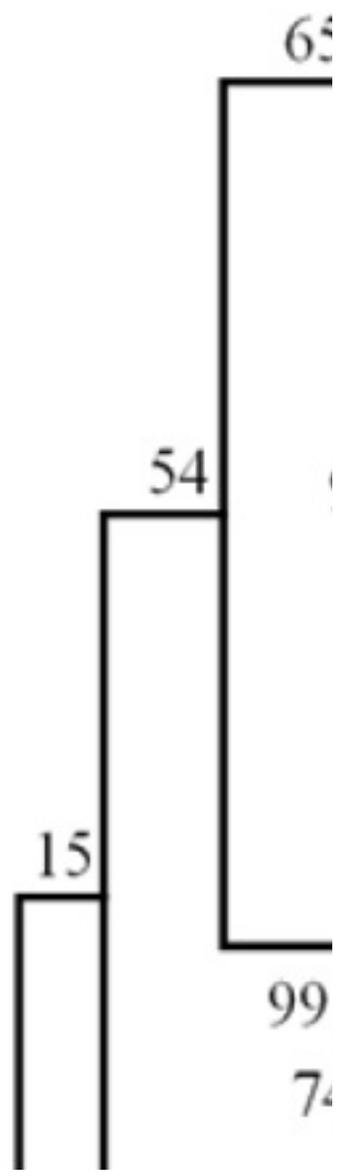


Figure 3

Image of agarose gel electrophoresis (1.5%) showing 520 pb band for the amplification of 23S rRNA gene of Anaplasma. Lane M: DL 2000 marker; Lane P: positive control; Lane N: negative control; lanes 1-8, 11, 12: negative samples; lanes 9, 10: positive samples.



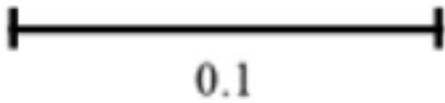
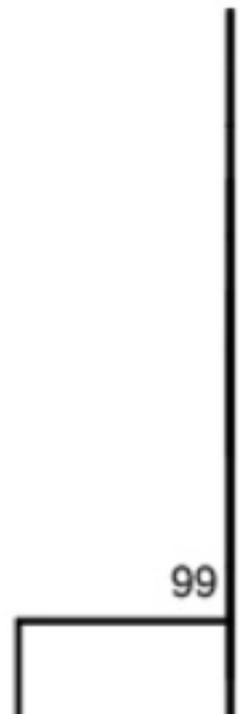
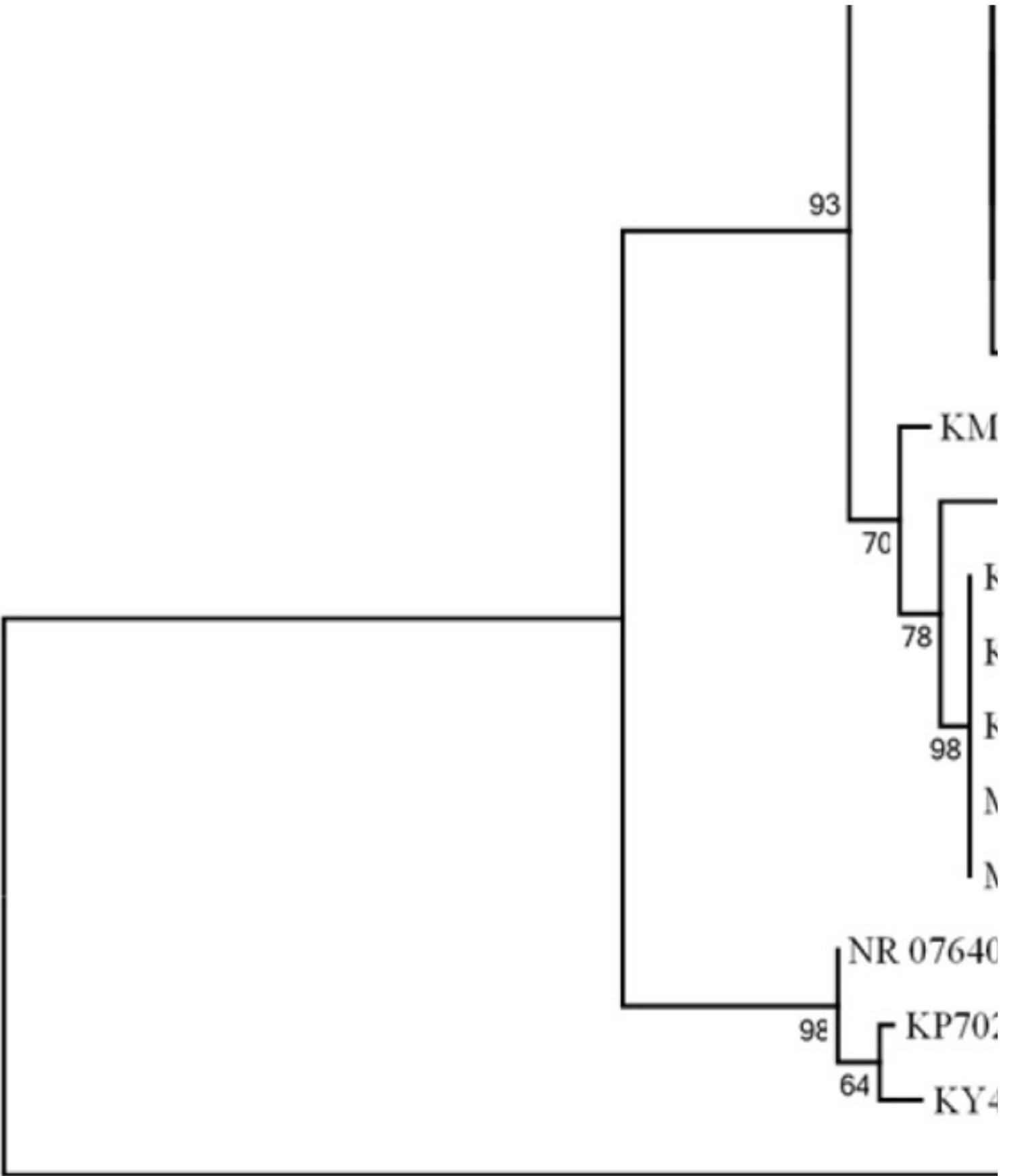
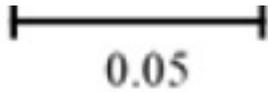


Figure 4

18S rRNA-based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Theileria* sp. in the present study (**bold**) related to other *Theileria* sp. available in GenBank. The sequences of 18S rRNA were aligned using CLUSTAL W and phylogenetic inferences were constructed in MEGA X using Maximum Likelihood based on Tamura-Nei Model for nucleotide sequences with 500 bootstrap replicates. There was a total of 491 positions in the final dataset. The scale bar represents a 5% nucleotide sequence divergence.







0.05

Figure 5

23S rRNA-based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Anaplasma* sp. in the present study (bold) related to other *Anaplasma* sp. available in GenBank. The sequences of 23S rRNA were aligned using CLUSTAL W and phylogenetic inferences were constructed in MEGA X using Maximum Likelihood based on Tamura-Nei Model for nucleotide sequences with 500 bootstrap replicates. There was a total of 435 positions in the final dataset. The scale bar represents a 5% nucleotide sequence divergence.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.pdf](#)