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Molecular prevalence and risk factors analysis of *Theileria annulata* in cattle at Jeddah, Saudi Arabia

[Prevalência molecular e análise dos fatores de risco da Theileria annulata *em bovinos em Jeddah, Arábia Saudita]*

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ABSTRACT

Theileria annulata is a tick-borne heamoprotozoan parasite responsible for tropical theileriosis in bovine populations, which causes substantial economic losses to the livestock sector. This study aimed to determine the prevalence of *T. annulata* infection in cattle in Jeddah, Saudi Arabia, and its associated risk factors using microscopic examination of Giemsa-stained blood smears (GSBS), PCR targeting the *18S rRNA* and cytochrome b (*cyt b*) gene, and sequencing analysis. Material and Methods: A total of 200 blood samples were collected randomly from cattle between December 2022 and May 2023. Results: Results revealed that 53 samples (26.5%) were positive for *Theileria* piroplasms using GSBS and increased to 71 samples (35.5%) using PCR. The risk of *T. annulata* infection was reported to be non-significantly higher among young, female, and imported cattle. The study revealed that 53 samples (26.5%) were positive for *Theileria* piroplasms using GSBS, while the PCR technique showed 71 samples (35.5%). The level of kappa agreement between GSBS and PCR was slight (0.135). Further, GSBS showed 51% accuracy, 18.31% sensitivity, and 68.99% specificity when compared to PCR results. Analysis of the *cyt b* sequence of Saudi isolates of *T. annulata* showed 99.27-100% identity, and with *18S rRNA* sequences the identity was > 98%. Conclusion: The high infection of *T. annulata* in slaughtered cattle in Jeddah requires the implementation of adopted programs to reduce the burden of theileriosis on the Saudi economy. PCR is considered a better test for conducting periodic examinations and control programs for bovine theileriosis.

Keywords: Cattle, Polymerase chain reaction, *T. annulata*, Theileriosis

RESUMO

Theileria annulata *é um parasita hemoprotista transmitido por carrapatos responsável pela teileríase tropical em populações bovinas, causando perdas econômicas substanciais ao setor pecuário. Este estudo teve como objetivo determinar a prevalência da infecção por* T. annulata *em bovinos em Jeddah, Arábia Saudita, e seus fatores de risco associados, utilizando exame microscópico de esfregaços de sangue corados com Giemsa (GSBS), PCR direcionado ao gene* 18S rRNA *e citocromo b (*cyt b*) e análise de sequenciamento. Material e métodos: Um total de 200 amostras de sangue foram coletadas aleatoriamente de bovinos entre dezembro de 2022 e maio de 2023. Resultados: Os resultados revelaram que 53 amostras (26,5%) foram positivas para piroplasmas* Theileria *usando GSBS e aumentaram para 71 amostras (35,5%) usando PCR. O risco de infecção por* T. annulata *foi relatado como não significativamente maior entre bovinos jovens, fêmeas e importados. O estudo revelou que 53 amostras (26,5%) foram positivas para piroplasmas* Theileria *usando GSBS, enquanto a técnica de PCR mostrou 71 amostras (35,5%). O nível de concordância kappa entre GSBS e PCR foi leve (0,135). Além disso, o GSBS apresentou 51% de precisão, 18,31% de sensibilidade e 68,99% de especificidade quando comparado aos resultados da PCR. A análise da sequência cyt b de isolados sauditas de* T. annulata *mostrou 99,27-100% de identidade, e com sequências de* rRNA 18S *a identidade foi > 98%. A alta infecção por* T. annulata *em bovinos abatidos em Jeddah requer a implementação de programas*

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adotados para reduzir o impacto da teileríase na economia saudita. A PCR é considerada um teste melhor para a realização de exames periódicos e programas de controle da teileríase bovina.

Palavras-chave: bovinos, reação em cadeia da polimerase, T. annulata, *Teileríase*

INTRODUCTION

Bovine theileriosis is a tick-borne protozoal disease caused by *Theileria annulata* and transmitted by ixodid ticks of the genus *Hyalomma* (Mohammed-Ahmed *et al.,* 2018). This disease causes significant financial losses to farmers due to mortality, morbidity, reduced milk production, and high costs of acaricides, treatment, and vaccination (Perera *et al.,* 2014). Although *T. annulata* causes productivity losses in exotic cattle breeds and their crosses, naive indigenous cattle, particularly calves and adults under endemic instability, are also affected (Kasaija *et al.,* 2021). The clinical signs of theileriosis are variable and may include fever, mild nasal and ocular discharge, anorexia, salivation, enlargement of superficial lymph nodes, respiratory distress, acute anemia, jaundice, and death due to asphyxia (Osman and Al-Gaabary, 2007). The most common pathological lesions include enlargement of the lymph nodes and spleen, pulmonary emphysema, and subcutaneous and intramuscular hemorrhages.

Accurate and conclusive laboratory testing is essential for confirming *Theileria* infection. Although theileriosis is commonly diagnosed by microscopic examination of Giemsa-stained peripheral blood smears or by detecting macro schizonts in the lymph node biopsy smears (Perera *et al.,* 2014; Hayati *et al.,* 2020), this method is not sufficiently sensitive to detect infection in carrier animals (Pienaar *et al.,* 2020)**.** Furthermore, serological diagnosis using IFA and ELISA may yield false positives due to cross reactivity or inaccurate specific immune response (Billiouw *et al.,* 2005). Blood and biochemical profiles, such as bilirubin, cholesterol, albumin, also total proteins, can be useful within diagnosing several diseases (Alnahari *et al.,* 2025). However, since its introduction, PCR has demonstrated greater sensitivity, specificity, and accuracy for detecting *T. annulata* in infected animal hosts, even in cases of low parasitemia (Bilgic *et al.,* 2010).

To date, several studies have been carried out in different regions of Saudi Arabia to identify and diagnose theileriosis in cattle. In Taif, Riyadh, and Al-Ahsa, studies (Ghafar and Amer, 2019; Alanazi *et al.,* 2019) reported that more than 5% of cattle were infected with *T. annulata* by PCR. In contrast, studies conducted in Qassim and Jizan (Omer *et al.,* 2002; Al-Khalifa *et al.,* 2009) reported a prevalence of 15.4-25% based on the examination of blood smears. However, there is currently no available data on theileriosis in cattle in Jeddah, except for one study that reported *T. ovis* in sheep and goats from Jeddah using PCR targeting the *18S rRNA* gene (Metwally *et al.,* 2021). Therefore, the objectives of the present study were to estimate the prevalence and associated risk factors of theileriosis in cattle in Jeddah, to evaluate the diagnostic performance of Giemsa-stained blood smears compared to PCR, and to genetically characterize *T. annulata* isolates by targeting the *18S rRNA* and cytochrome b (*cyt b*) genes.

ETHICAL ASPECTS

The research was submitted to the *Ethics Committee on Animal Use* of the University of Jeddah, Saudi Arabia, and approved under the number (UJ-232430040).

MATERIALS AND METHODS

From December 2022 to May 2023, a total of 200 blood samples were randomly collected from cattle from the central slaughterhouse in Jeddah city, located in the western region of Saudi Arabia (21° 32′ 36″ N, 39° 10′ 22″ E). Data including sex, breed, and age (categorized into three groups according to reference 4) were recorded. Blood samples (5 mL) were aseptically collected from the jugular vein using a vacuum tube with ethylenediamine tetra acetic acid (EDTA) and transported in an ice box to the Department of the College of Science at the University of Jeddah Laboratory for preparation of thin blood smears and preserved at −20°C for molecular analysis (Selim *et al.,* 2022).

Briefly, a small drop of fresh blood was placed on the first third of a clean glass microscopic slide, then spread with another slide and left to air dry. The blood film was fixed in methanol alcohol for 2 min and then stained with Giemsa stain for 30 min and examined under an oil immersion lens at x100 (Lempereur *et al.,* 2017)**.**

DNA extraction was performed using the Qiagen DNA Purification Kit (Promega, USA), as per the manufacturer's instructions, and stored at -20°C until future use. For the amplification of targeted genes of *Theileria* spp., both on genus and species-specific levels. The PCR was performed as per previously described PCR assays**.** The amplification of the *18S rRNA* gene (778 bp) was performed using previously described primers: F: 5'-GAAACGGCTACCACATCT-'3, R: 5'-AGTTTCCCCGTGTTGAGT-'3 (Cao *et al.,*2013). The cycling conditions were a cycle at 95°C for 5 min and 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s, and a final extension for 10 min at 72°C. The amplification of the *cyt b* gene (312 bp) was performed using previously described primers; F: 5′-ACT TTG GCC GTA ATG TTA AAC–3′ and R: 5′-CTC TGG ACC AAC TGT TTG G–3′ (Bilgiç *et al.,* 2013). The cycling conditions of a cycle at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s and a final extension at 72°C for 10 min. Positive and negative (PCR-grade water) controls were included for each run. PCR product (10 µl) was electrophoresed on a 2% agarose gel containing 5 µl/ml ethidium bromide in Tris-acetate-EDTA buffer at 110 V and visualized under UV light**.**

The PCR products of the *Cyt b* (312 bp) and *18S rRNA* (778 bp) genes were purified by the QIAquick PCR purification kit (QIAGEN) and subjected to sequencing by using an automated DNA sequencer (ABI 3730XL, Solgent Co. Ltd., South Korea). DNA BaserV3 software was used to read the sequences. Sequences identified were aligned with the previously published *T. annulata* sequences in GenBank by the Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST), and analyzed by the MegAlign software (DNASTAR, Madison, WI, USA) using the Clustal W method. Divergence estimation within the *Cyt b* and *18S rRNA* genes sequenced in this study was calculated in MEGA X by comparing with the other available *Cyt b* and *18S rRNA* sequences of *T. annulata* from the GenBank. Divergence is calculated by MEGA X.

For the phylogenetic tree, the analysis was performed by using the Maximum Likelihood method (ML) based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree for the heuristic search was obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. In the bootstrap analysis, 1000 replications were used. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGAX (Tamura *et al.,* 2021).

The SPSS software (SPSS for Windows Version 22, SPSS Inc., Chicago, USA) was used to analyze the data. The category variables were examined using Chi-square test. Statistical significance was defined as a *P*-value < 0.05.

RESULTS

Out of 200 samples, 53 (26.5%) samples were found positive in GSBS, showing characteristic shaped piroplasm. Microscopic examination of stained thin blood smears revealed *T. annulata* (Fig. 1). *Theileria* piroplasms were observed mostly as signet rings or dots (Fig. 1A); some were detected in pyriform shape inside the red blood cells (Fig. 1B). As seen in Tables 1 and 2, the prevalence rate of *T. annulata* piroplasms in imported breed of cattle was higher (26.8%, 95% CI: 0.19-0.35) than in local ones (25.97%, 95% CI: 0.18-0.26). Regarding the age factor, a high prevalence rate was recorded among cattle ≤ 1 year old (27.7%, 95% CI: 0.18-0.39) compared to cattle >1 year old. Furthermore, 33.3% (95% CI: 0.06-0.79) prevalence was reported among females compared to 26.4% (95% CI: 0.21-0.33) prevalence in males. All differences were statistically non-significant (Tables 1 and 2).

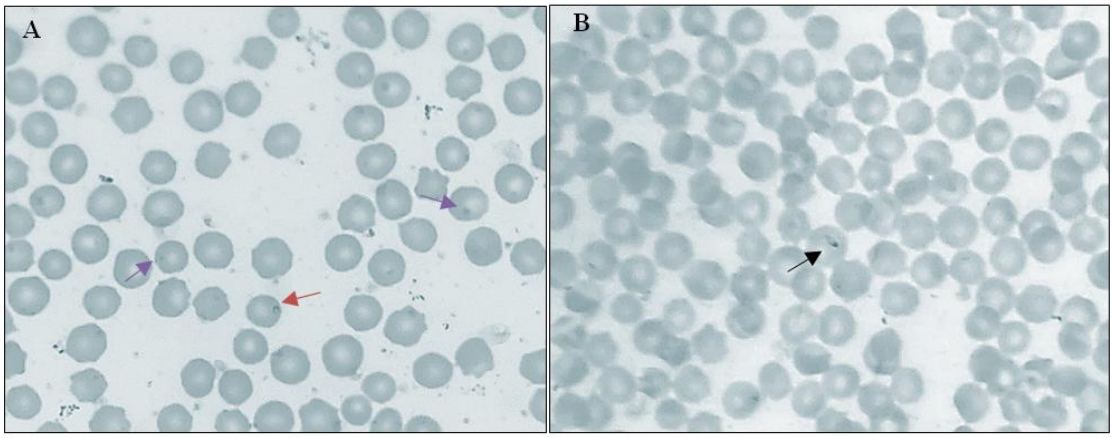

Figure 1. Blood smears demonstrating intracellular piroplasm of T. annulata (X 100). The presence of (A) signet ring (red arrow) and dot (blue arrow), (B) pyriform shaped (black arrow)

Table 1. Prevalence of *T. annulata* among cattle and associated risk factors using microscopic and PCR assays

Variables		Blood smear				PCR			
		Pos. *n*	Prev. %	95% CI	*P*-value	Pos. *n*	Prev. %	95% CI	*p*-value
Age	≤1y (*n*=65)	18	27.7	0.18-0.39	0.8 ^{NS}	19	29.2	0.19 - 0.41	0.2 ^{NS}
	>1y (*n*=135)	35	25.9	0.19- 0.34		51	37.8	0.30 - 0.46	
Sex	Females (*n*=3)	1	33.3	0.06-0.79	0.8 ^{NS}	2	66.7	0.20 - 0.94	0.3 ^{NS}
	Males (*n*=197)	52	26.4	0.21-0.33		69	35.03	0.29 - 0.42	
Origin	Local (*n*=77)	20	25.97	0.18-0.26	0.9 ^{NS}	22	28.6	0.19 - 0.39	0.11^{NS}
	Imported (*n*=123)	33	26.8	0.19-0.35		49	39.8	0.32 - 0.49	
Total (*n* = 200) *		53	26.5	0.21-0.33		71	35.5	0.29-0.42	

NS: The result is not significant at p < .05. *, P value between BS and PCR is 0.052 NS

Table 2. Risk for T. annulata infection according to animal origin, age and sex

Variables	Comparisons		
	Age	Sex	Origin
Risk	0.37 (> 1 y)	0.66 (Females)	0.39 (Imported)
	0.29 (≤ 1 y)	0.35 (Males)	0.28 (Local)
Risk ratio	1.3 (CI $_{95\%}$ = 0.8 - 1.99)	1.9 (CI $_{95\%}$ = 0.84 – 4.3)	1.7 (CI $_{95\%}$ = 0.9 – 3.1)
z-value	1.2	1.5	1.6
p-value	0.3	0.13	0.11
Odds ratio	1.5 (CI $_{95\%}$ = 0.8 - 2.8)	3.7 (CI $_{95\%}$ = 0.3 – 41.7)	1.4 (CI $_{95\%}$ = 0.75 - 2.5)

The PCR assay detected 71 samples (35.5%) positive for *T. annulata*. As shown in Table 1 and Fig. 2, prevalence increased non-significantly among cattle > 1 year of age (37.8%, 95% CI: 0.30 - 0.46, *P*=0.2), females (66.7%, 95% CI: 0.20 - 0.94, *P*=0.3), and imported cattle (39.8%, 95% CI: 0.32 - 0.49, *P*=0.11). The PCR included 57 samples previously tested negative by Giemsa staining (Table 3).

A comparison of the diagnostic characteristics between GSBS and PCR in the examination of 200 blood samples collected showed that the PCR detected most of the samples and the GSBS detected the least. All percentages of positivity for each test are seen in Table 1. The PCR's ability to identify more blood samples increased the number of *T. annulata*-positive blood samples to 71, compared to 53 positive samples using GSBS. Not all samples positive with GSBS were also positive with PCR (Table 3).

Therefore, 57 GSBS-negative blood samples were PCR positive. According to the negative and positive results, the Kappa agreement between the GSBS and PCR revealed slight agreement (0.135; 95% CI: 0.261 -0.008; Table 3). In addition to the results of kappa, GSBS showed 51.00% accuracy, 18.31% sensitivity, and 68.99% specificity in the detection of *T. annulata* in cattle when compared to PCR results as a gold standard test (Table 4).

Figure 2. Detection of *T. annulata* DNA in native cattle. Lane M (50 bp DNA ladder). Lane 1 (positive control of *T. annulata*). Lane 2 to 18 (positive samples)

Table 3. Estimated K-value, SD and 95% CI for the test agreement (Blood smear vs. PCR) for *T. annulata* infection in two hundred cattle

Variable		PCR		Total	*K*- vale	SD	0.95% CI
Blood smear		Pos.	Neg.		0.135	0.065	0.261 -0.008
	Pos.	14	39	53	Slight		
	Neg.	57	90	147	agreement		
Total		71	129	200			

SD: standard error, CI: Confidence Interval

Table 4. Estimation of blood smear sensitivity and specificity compared to the PCR results

Statistic	Value %	95% CI
True positive	14	
False positive	39	
True negative	90	
False negative	57	
Sensitivity	18.31	10.13 - 29.27
Specificity	68.99	60.25 - 76.84
Positive Likelihood Ratio	0.59	0.34 - 1.03
Negative Likelihood Ratio	1.18	1.01 - 1.39
Disease prevalence (*)	35.50	28.88 - 42.56
Positive Predictive Value (*)	24.53	15.73 - 36.14
Negative Predictive Value (*)	60.54	56.67 - 64.29
Accuracy (*)	51.00	43.85 - 58.12

*, these values based on the prevalence of *T. annulata*

The partial *Cyt b* gene sequence (312 bp) was sequenced. The sequences of the four Saudi isolates were aligned with the related *T. annulata Cyt b* gene sequences in the GenBank database, and the alignment of the identified sequences with retrieved *Cyt b* sequences showed 99.27-100% identity. The BLASTn search of the identified nucleotide sequences with the reference *T. annulata Cyt b* gene (MN492496) showed 100% identity among the three sequences (PP470770-2) and with the four-sequence (PP470773) showed 99.9% identity with a substitution at one nucleotide (Fig. 3). Pairwise genetic distance of the four sequences with the reference sequence revealed one nucleotide substitution, indicating a very little genetic distance of 0.01. These results suggest a little genetic diversity between Saudi isolates based on the *Cyt b* gene.

Figure 3. Nucleotide sequence alignment of Saudi *T. annulata cyt b* gene variants (this study, GenBank Accession No. PP70770-3) with reference *T. annulata* isolate from Iran (GenBank Accession No. MN432496). Identical bases (dots) and sites of variation (yellow box)

The 778 bp nucleotide sequences corresponding to the partial *18S rRNA* gene sequence were amplified and sequenced. The sequences of the six Saudi isolates were compared with the other related *18S rRNA* gene sequences in the GenBank database and the alignment of the identified sequences with reference *T. annulata 18S rRNA* sequences showed > 98 % identity. Six sequences were deposited in the GenBank database under the accession numbers PP462079, PP462080, PP462081, PP462082, PP462083, and PP462084. Alignment of the six sequenced *T. annulata* isolates with the reference *T. annulata* 18SrRNA gene (KU554731, KM288519, OR364144, and MK838106) showed that three sequences (PP462079, PP462082, and PP462084) were 100% identical. But lower identity (97.95–99.99%) was estimated when aligned with the other three sequences, PP462080, PP462081, and PP462083, which showed 1, 3, and 8 nucleotide substitutions, respectively, with a genetic distance of 0.01 - 0.02 (Fig. 4).

A phylogenetic tree based on the partial *Cyt b* sequences (312 bp) of Saudi isolates and sequences from Egypt, Sudan, Iran, Turkey, Iraq, China, Tunisia, and India was generated using the Maximum Likelihood method. The tree is divided into two clades. The studied isolates grouped into a single clade and were closely related to the Iranian *T. annulata* isolate with 99% nodal support but distinct from other known *T. annulata* from Egypt, Sudan, Turkey, Iraq, China, Tunisia, and India, and the estimated pairwise distance ranged between 0.48 and 0.51 (Fig. 5). Further, phylogenetic tree based on *18S rRNA* gene sequences classified the obtained isolates into three subtypes (ST). ST1 included one sequence (PP462083) with eight nucleotide substitutions. ST2 included one sequence (PP462081) with three nucleotide substitutions. ST3 included four sequences (PP462079, PP462082, and PP462084) with one nucleotide substitution. These isolates were closely related to *T. annulata* from Egypt, China, Tajikistan, and Pakistan but distinct from other known *T. annulata* from other countries (Fig. 6).

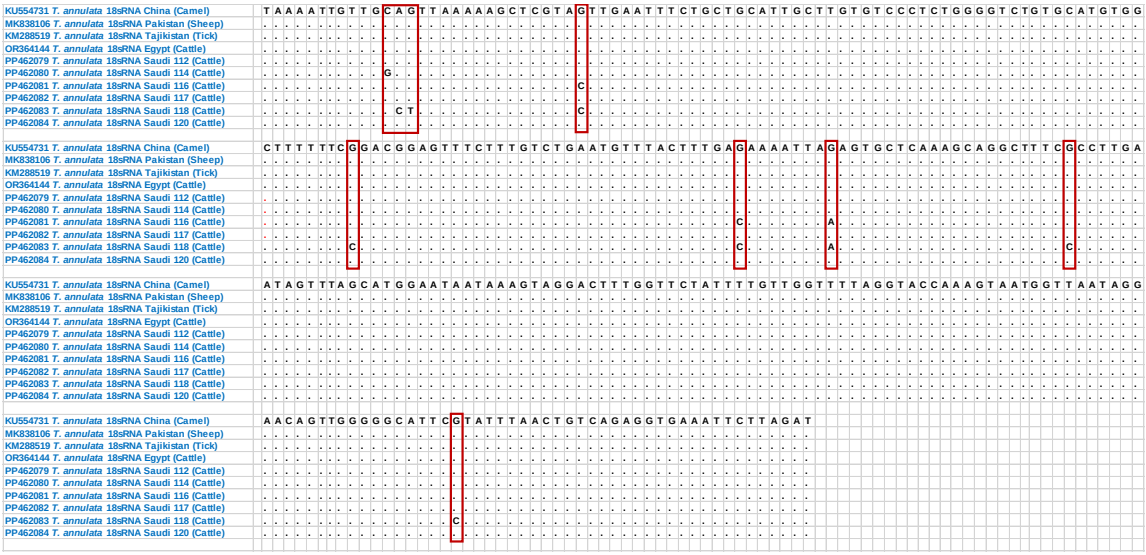

Figure 4. Nucleotide sequence alignment of Saudi *T. annulata 18S rRNA* gene variants (this study, GenBank Accession No. PP462079-83) with reference *T. annulata* isolate from China (GenBank Accession No. KU554731), Pakistan (MK838106), Tajikistan (KM288519), and Egypt (OR364144). Identical bases (dots) and sites of variation (red boxes)

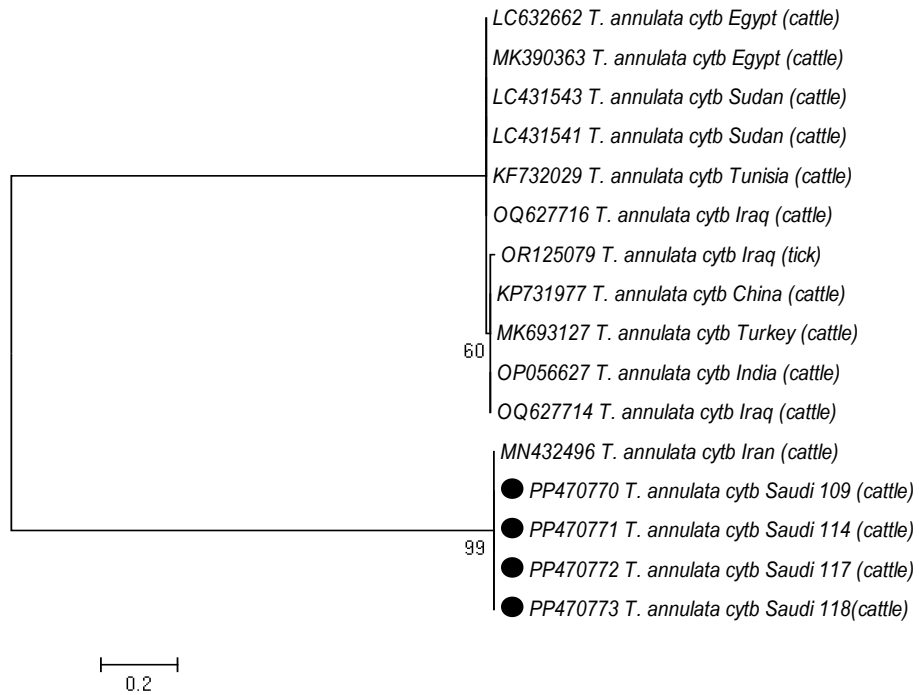

Figure 5. Molecular phylogenetic analysis of the partial *cyt b* gene sequence of *T. annulata* by the maximum likelihood method

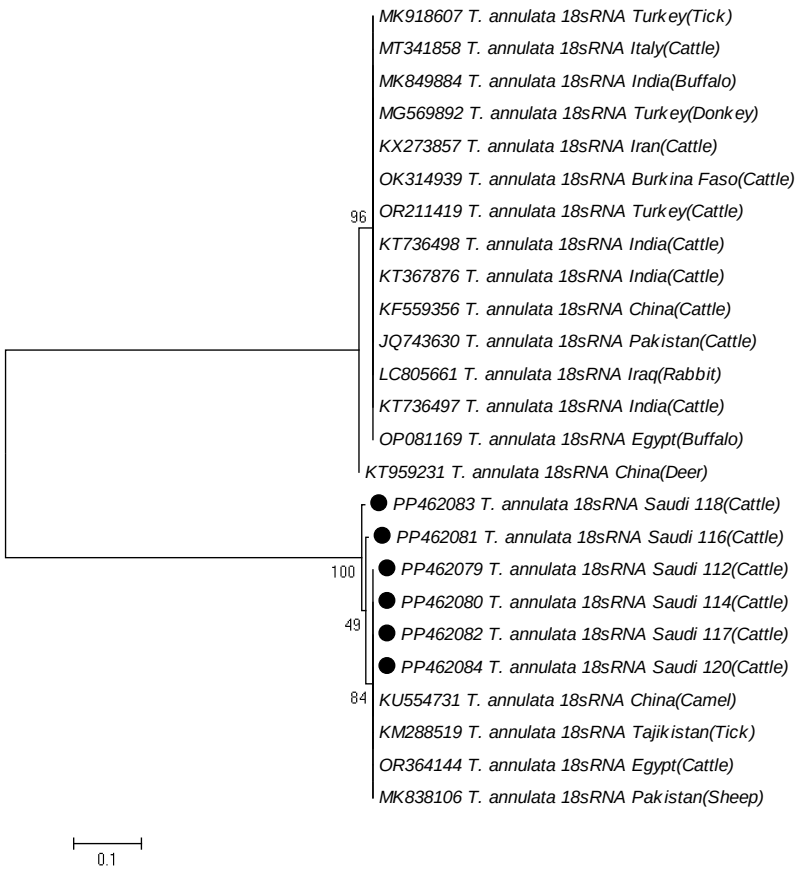

Figure 6. Molecular phylogenetic analysis of the partial *18S rRNA* gene sequence of *T. annulata* by the maximum likelihood method

DISCUSSION

Animal husbandry plays an important role in the economy. However, several haemoprotozoan diseases constrain livestock production in many developing countries and are responsible for high rates of morbidity and mortality, leading to reduced production of meat, milk, and other livestock by-products (Khan *et al.,* 2004)**.** Bovine tropical theileriosis (*T. annulata* infection) is of great economic importance to many countries where millions of cattle are at risk of exposure to this disease. The present study aimed to estimate the prevalence of *T. annulata* infection in cattle at the main slaughterhouse in Jeddah city. The findings confirmed that theileriosis is endemic among cattle in Jeddah, western Saudi Arabia, as the infection was detected in most slaughtered cattle. Furthermore, the infection showed no significant association with sex, origin, or age, suggesting that all cattle were equally exposed to the pathogen.

The overall prevalence of *T. annulata* infection was 26.5% by GSBS and 35.5% by PCR. Previous studies in Saudi Arabia have reported varying prevalence rates, ranging from 1-5% in Taif, Riyadh, and Al-Ahsa by PCR testing (Ghafar and Amer, 2019; Alanazi *et al.,* 2019), and 15.4 - 25% in Qassim and Jazan by blood smear testing (Omer *et al.,* 2002; Al-Khalifa *et al.,* 2009). To the best of our knowledge, this is the first report confirming the presence of *T. annulata* in cattle in Jeddah, KSA. In contrast, the prevalence obtained in this study was higher than the 28.5% reported in cattle from Pakistan (Parveen *et al.,* 2021) and the 18.2% reported in China (Guo *et al.,* 2018). Such variation in prevalence rates may be attributed to differences in sample size, animal species, diagnostic techniques, cattle population density, and the level of farmers' awareness (Elhaig and Wahdan, 2023).

Although PCR enhanced the detection of *Theileria* in GSBS-negative blood samples, we found a slight kappa agreement (0.135, 95% CI = 0.261 - 0.008) between GSBS and PCR results. Furthermore, GSBS demonstrated 51% accuracy, 18.31% sensitivity, and 68.99% specificity when compared to PCR results. The results agree with a previous study conducted in Pakistan, which reported that PCR was more sensitive than microscopic examination for detecting *T. annulata* in cattle with moderate kappa agreement ($\kappa = 0.6$) between the microscopic examination and PCR (Ullah *et al.,* 2021). Furthermore, globally, PCR is now used to diagnose *Theileria* infection, especially to identify carrier animals (Bilgic *et al.,* 2010; Shayan and Rahbari, 2005) and microscopic examination was reported to have lower sensitivity (Azizi *et al.,* 2008; Noaman, 2014) due to the destruction of the piroplasmic form due to RBC hemolysis, thick smear development, poor staining, lack of microscopic skill, and low parasitemia (Hoghooghi *et al.,* 2011; Nayel *et al.,* 2012). Our results and previous ones indicate that PCR is better for the diagnosis of theileriosis**.**

In the current survey, the risk of *T. annulata* infection non-significantly increased by 1.3 times in cattle > 1 year compared to cattle ≤ 1 year. A previous report found that *T. annulata* infection was higher in adult cattle (1-3 years) than in young cattle aged < 1 year (Abaker *et al.,* 2017). Another study has found an increased infection rate of *T. annulata* in adult cattle (≥ 6 years) compared to young cattle (< 2 years), with a significant association between the infection and cattle age (Sallemi *et al.,* 2018). Although there was no significant difference between age groups and *T. annulata* infection, this emphasized the influence of increasing age factors in this study, possibly due to older cattle being frequently re-infected, ticks being less attractive to calves, or the persistence of colostral antibodies (Gharbi *et al.,* 2014).

Although the sex difference was not statistically significant, females had a higher prevalence of *T. annulata* (risk ratio = 1.9) than males, which could be attributed to sampling bias. This is endorsed by findings from a comparable study in Tunisia (Sallemi *et al.,* 2018). The tendency of females to examine more positively compared to males is consistent with a previous study in Pakistan (Ullah *et al.,* 2021). This could be due to female cattle having more hormonal fluctuations, a weak or disturbed immune response during pregnancy or lactation, and the presence of more ticks on their bodies, all of which make females more susceptible to *T. annulata* infection (Tuli *et al.,* 2015; Parveen *et al.,* 2021).

Regarding the origin of cattle, the prevalence of *T. annulata* was high in imported cattle, with a risk ratio about 1.9 times higher ($P > 0.05$) than that in local cattle. A similar result was documented in Pakistan (Ullah *et al.,* 2021), which may reflect reduced tick infestation in local breeds because of an elevated level of tick resistance developed during generations of long-term exposure to vector ticks (Tuli *et al.,* 2015; Glass, 2001). Additionally, the native breed would become more resistant to stresses that could make them more susceptible to infection after becoming acclimated to the local environment (Durrani *et al.,* 2010; Parveen *et al.,* 2021). In contrast to our results, a report from Sudan found a higher prevalence of *T. annulata* in local breeds compared to crossbred cattle using GSBS, IFAT, and PCR. This may be due to the exotic breeds being intensively managed including the use of insecticides to control ticks, good nutrition, hygiene, and careful veterinary care (Abaker *et al.,* 2017).

Four and six sequences herein this study were partially sequenced using the *Cyt b* and *18S rRNA* gene, respectively, and recognized as *T. annulate*. The phylogenetic tree based on the *18S rRNA* was useful to infer the association between the collected Saudi isolates with a high bootstrap value. The Saudi sequences were classified into three subtypes, and all subtypes clustered with *T. annulata* strains from Egypt, China, Tajikistan, and Pakistan. Further, forming a separate clade on the evolutionary tree (Kundave *et al.,* 2014; George *et al.,* 2015). The analysis of *18S rRNA* of *T. annulata* showed genetic diversity within seven hosts, at least 5 groups from different regions, and different hosts (cattle, sheep, ticks, rabbits, donkeys, and deer). The pattern in Saudi isolates (3 subtypes with 1, 3, and 8 nucleotide substitutions) reflects that different *T. annulata* isolates have distributed in cattle and globally reflects that different genotypes have a distinctive geographic distribution with different hosts. Previously, genetic diversity among *T.*

annulata strains based on the *18S rRNA* gene has been reported in many studies (Chansiri *et al.,* 1999; Manuja *et al.,* 2006; Aktas *et al.,* 2007; Sivakumar *et al.,* 2014; George *et al.,* 2015). A study conducted in India (George *et al.,* 2015) showed that phylogenetic analysis of *18S rRNA* sequences divided *T. annulata* isolates into two groups with 0.1-8.6% divergence, and the Indian isolates clustered with the Chinese strain, possibly due to change in evolution and host adaptation. Furthermore, it is possible that both hosts and tick vectors had an impact on how the changing forms of *Theileria* parasites evolved (Sivakumar *et al.,* 2014). To fully understand the origin and pathogenesis of the *T. annulata* circulating in Saudi cattle, more research is necessary.

Further, we also performed phylogenetic analysis and alignment of the obtained *T. annulata* isolates using the *Cyt b* gene. Multiple alignment revealed four isolates clustering with the Iranian strain (GenBank access. No. MN432496). Among the four isolates, three sequences (PP470770-2) were 100% identical, and the fourth sequence (PP470773) showed a single nucleotide substitution with a genetic distance of 0.01, which may be due to change in evolution and may lead to adaptation (George *et al.,* 2015)**.** In addition, the formation of a distinct clade on the phylogenetic tree is a finding supported by previous studies in Tunisia (Mhadhbi *et al.,* 2015) and Pakistan (Ahmad Atif *et al.,* 2023). These studies showed that sequencing and evolutionary analysis based on *Cyt b* showed the presence of genetic diversity among *T. annulata* strains, which led to the substructure of the *T. annulata* population in the phylogenetic tree and this due to the difference in agricultural climate zones. However, a study performed in Pakistan (Parveen *et al.,* 2021) showed that the nucleotide homology was 97-99% between Pakistani isolates and *T. annulata* isolates recovered from GenBank. Furthermore, the phylogenetic tree grouped the sequences into a stable monophyletic cluster with the *Cyt b* gene of *T. annulata* from China, Iran, India, Turkey, and Spain.

Low heterogeneity was detected at the *Cyt b* and *18S rRNA* genes, despite >97.9% identity being reported, and all grouped in a single clade, suggesting a common ancestor. A study from Pakistan evaluated the genetic property of the *Cyt b* gene of *T. annulata* isolates, and phylogenetic analysis showed that there were 9 and 21 unique haplotypes in the isolates derived from buffalo and cattle, and all of them clustered into a single lineage, indicating a single origin (Ali *et al.,* 2022)**.** Interestingly, the nucleotide substitutions found in this study at the *Cyt b* and *18S rRNA* genes, notably at the *18S rRNA* gene, in cattle indicate a need for additional research. The increasing number of detected substitutions suggests the existence of multiple subtypes, which enhances the possibility of identifying novel strains (Mahapatra *et al.,* 2017).

CONCLUSION

This work reports the parasitological and molecular prevalence of bovine theileriosis and characterization of *T. annulata* in cattle from Jeddah, Saudi Arabia by targeting *Cyt b* and *18S rRNA* genes. All sequences of *T. annulata* that are obtained here are located close to each other, indicating their geographic proximity, with a low nucleotide diversity. These findings provide baseline data for future studies on the *T. annulata* genetic diversity and can inform efforts to prevent and control the spread of the disease in Saudi Arabia.

CONTRIBUTORS

M.N. Alsulami, T.H. Alwuthaynani, H.Y. Alnahari: study conception, objective, discussion, manuscript writing, and approval of the version to be published. H.H. Albohiri, N.M. Batawee: formal analysis and data curation.

DATA AVAILABILITY STATEMENT

The research data are available upon request.

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