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Prevalence of *Theileria annulata* infection in cattle in different regions and farming systems in Algeria

Zahra Messaoudi,[1,2] Selin Hacilarlioglu,[2] Tülin Karagenc,[2] Huseyin Bilgin Bilgic,[2] Jacinto Gomes,[3,4] Bachir Medrouh,[5] Rabah Kelanemer,[6] Fairouz Saidi,[1] William Weir[7] and Hocine Ziam[1,6]

[1]*Laboratory of Biotechnology, Environment and Health, University of Blida 1, Blida, Algeria*

[2]*Department of Parasitology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Isıklı, 09016, Türkiye*

[3]*Elvas School of Biosciences, Polytechnic Institute of Portalegre, Portugal*

[4]*Valoriza – Research Centre for Endogenous Resources Valorisation, Polytechnic Institute of Portalegre, Portugal*

[5]*Research Center for Agropastoralism, Djelfa, Algeria*

[6]*Institute of Veterinary Sciences, University of Blida 1, Blida, Algeria*

[7]*Veterinary Diagnostic Service, School of Biodiversity, One Health and Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow*

ABSTRACT: Tropical theileriosis (TT) is a tick-borne disease of cattle caused by the apicomplexan parasite *Theileria annulata*. It poses a major threat to the health of imported exotic breeds and, to a lesser extent, native breeds which have a degree of resistance to the disease. The study aimed to determine the prevalence of *T. annulata* infection in cattle in the arid and semi-arid regions of Algeria using serological and molecular methods. Bovine blood was tested for the presence of *T. annulata*-specific antibodies using the immunofluorescent antibody test (IFAT) and for parasite DNA using a polymerase chain reaction (PCR) for the gene encoding the major merozoite/piroplasm surface protein (Tams1). One hundred and sixteen individuals were screened and overall prevalences of 50.0 ± 4.6 % (IFAT) and 64.7 ± 4.4 % (PCR) were determined. There was moderate agreement between IFAT and PCR tests for the diagnosis of TT, with a sensitivity of 74.6 % and a specificity of 95.1 % for IFAT when compared against the PCR. Potential risk factors identified in this study were breed (OR = 2.25, 95% CI: 1.07–5.07), season of sampling (OR = 4.29, 95% CI: 1.97–10.9) and breeding system (OR = 8.60, 95% CI: 1.41–57.6). Sequential analysis of Tams1 PCR amplicons revealed three new haplotypes with 97.5–100% identity to each other and, overall, they were found to be similar to previously sequenced isolates from north African–south European lineage.

Keyword: Cattle; Immunofluorescent antibody test; PCR; Tams; Algeria.

Correspondence author:
H. Ziam,
Laboratory of Biotechnology, Environment and Health,
University of Blida 1, Algeria
E-mail address: veziamocine@gmail.com

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INTRODUCTION

Theileria annulata is a tick-borne apicomplexan haemoprotozoan parasite that causes tropical theileriosis in cattle and is transmitted by several species of ixodid ticks of the genus *Hyalomma* (Uilenberg, 1981). It is characterised by lymphoproliferative and lymphodestructive disease (Dobbelaere and Rottenberg, 2003) which may be fatal. The disease is endemic in tropical and subtropical regions, including the Mediterranean, the Middle East, Central Asia, India and Northern China (Purnell, 1978). It is regarded as a serious constraint for cattle breeding and results in direct losses from mortality (30–90% in introduced exotic breeds), abortion and decreased milk yield (Benchikh Elfegoun et al., 2017; Purnell, 1978). In cattle that survive the acute phase of the disease, a long-lasting carrier state develops, which is also associated with significant production and economic losses (Gharbi et al., 2011; Uilenberg, 1995).

Diagnosis of the infection in cattle is based on clinical findings paired with microscopic detection of schizont-infected leukocytes in Giemsa-stained lymph node biopsies or piroplasms in Giemsa-stained peripheral blood smears in acute cases (Purnell, 1978). However, it is difficult to discriminate *T. annulata* from non-pathogenic *Theileria* spp. microscopically, and this approach lacks the sensitivity to detect carrier animals with low piroplasm parasitaemia (d'Oliveira et al., 1995). Serological tests, such as the indirect immunofluorescence antibody test (IFAT), may also be used to detect sub-clinical infections by detecting circulating antibodies generated against schizont antigens (Burridge et al., 1974; Kiara et al., 2018). The IFAT has been shown to have higher sensitivity and specificity compared to microscopic examination (Darghouth et al., 1996a). Polymerase chain reaction (PCR), which directly detects parasite DNA, may be considered the preferred diagnostic method because of its greater sensitivity and specificity (d'Oliveira et al., 1995; Valente et al., 2023). Depending on the gene target, it has been shown to have a detection limit of approximately two to three piroplasms per microlitre of blood, which corresponds to a parasitaemia of 0.000048% (d'Oliveira et al., 1995). Several parasite-encoded genes have been developed as PCR targets for *T. annulata* including Cyclooxygenase-3 (Cox-3) and the 18S ribosomal RNA gene (18S rRNA), which are well-conserved between different *Theileria* species. The gene encoding the major merozoite/piroplasm surface protein (Tams1) has also been used as a both a diagnostic and genotyping target (Chae et al., 1998; d'Oliveira et al., 1995; Hikosaka et al., 2009; Kundave et al., 2021).

Theileria annulata is endemic in the northern part of Algeria and is mainly transmitted by *Hyalomma* ticks, particularly *H. scupense* in the humid and semi-arid areas (Bedouhene et al., 2022; Benchikh Elfegoun et al., 2017; Boulkaboul, 2003). However, from early October to mid-November many clinical cases of tropical theileriosis are observed in Algeria despite adult *H. scupense* not being active during this period (Bedouhene et al., 2022). Notably, two other species of tick considered to be vectors of *T. annulata*. However, *Hyalomma excavatum* and *Hyalomma lusitanicum,* are active at this time and they have also been implicated in disease transmission (Bedouhene et al., 2022; Benchikh Elfegoun et al., 2017). Clinical studies based on microscopic examination of blood smears have shown a prevalence of 45.5 to 95.4 % in adult cattle and the disease occurring mainly in summer, with a peak in July (Foughali et al., 2021; Kernif et al., 2024; Ziam et al., 2020). Classical molecular diagnostic techniques, PCR and real-time PCR, have revealed prevalences of 30.1 to 50.0 % in carrier cattle (Ayadi et al., 2017; Sadeddine et al., 2020; Ziam et al., 2015). The availability and use of effective diagnostic methods are critical to disease control, as outbreaks can occur when carrier cattle that have been incorrectly diagnosed with no infection are transported to non-endemic areas where the vector ticks are present (Sergent et al., 1945; Ziam, 2016).

One of the factors determining the occurrence of *T. annulata* in Algeria is the movement of cattle to livestock markets from semi-arid to humid regions and vice-versa, areas which are 500 km apart. The purchase and import of cattle can lead to an exchange of ticks between farms (Ziam, 2016), thus providing an opportunity to spread the parasite. Ayadi et al., (2017) state that the variation in the prevalence of *T. annulata* reported across the geographical range of the disease could be due to differences in animal demographics (age, sex and breed) and ecological parameters (agroecological zone, irregular application of acaricides and management practises) (Kernif et al., 2024; Ullah et al., 2021).

The present study aimed to evaluate the detection of *T. annulata* in cattle located in arid and semi-arid regions in Algeria by IFAT and PCR. The sensitivity (Se) and specificity (Sp) of these diagnostic tests

were assessed and the risk factors associated with naturally infected cattle in Algeria determined.

MATERIALS AND METHODS

Study area

The study was carried out in two distinct bioclimatic zones: a humid site with a Mediterranean climate and a semi-arid site located in the highland region (Fig. 1). The humid area in north central Algeria has a Mediterranean climate with an average temperature of 5 °C in winter and 35 °C in summer. The average annual relative humidity is 75 % and the average annual rainfall varies between 800 and 1200 mm (Zeggane et al., 2021). The area stretches from the Djurdjura in the east to Mount Chenoua in the west and from the Mediterranean Sea in the north to the Bouira highlands in the south. It is a vast landscape interspersed with valleys and mountainous regions. The climate of the semi-arid area is cold and severe in winter with an average temperature of 3 °C. In contrast, summers are hot and dry with an average temperature of 36 °C. The average annual rainfall varies between 30 and 400 mm with the average annual relative humidity being between 25 and 28 % (Haied et al., 2017). It extends from the Parc of Jardin massif in the East to the Tiaret mountain pass in the west and from the Taguensa massif in the north to the highland of Djelfa in the south.

Study population and sampling

The study was conducted between May 2018 and September 2019 with a total of 20 farms being randomly selected from the humid (n = 10) and semi-arid (n = 10) areas. A total of 116 animals were sampled, 57 from the humid area and 59 from the semi-arid area, the vast majority (n = 111) being older than one year. The bioclimatic zone and animal husbandry system associated with each sampled animal was recorded together with its age, sex and breed. The animals were kept in semi-intensive and extensive systems and were of exotic breed (Holstein and Montbeliard) or crossbred (Atlas brown/exotic crosses). The cattle in the extensive system lived on scrubland, fallow pastures and crop residues without feed supplements. Cattle in the semi-intensive system, on the other hand, fed on fallow pastures, crop residues, hay, straw and concentrated feed. The animals are clinically healthy and have not been treated with antibiotics such as tetracycline or theilericide, particularly buparvaquone or parvaquone. Blood samples were collected in EDTA tubes from randomly selected animals in each herd. For each

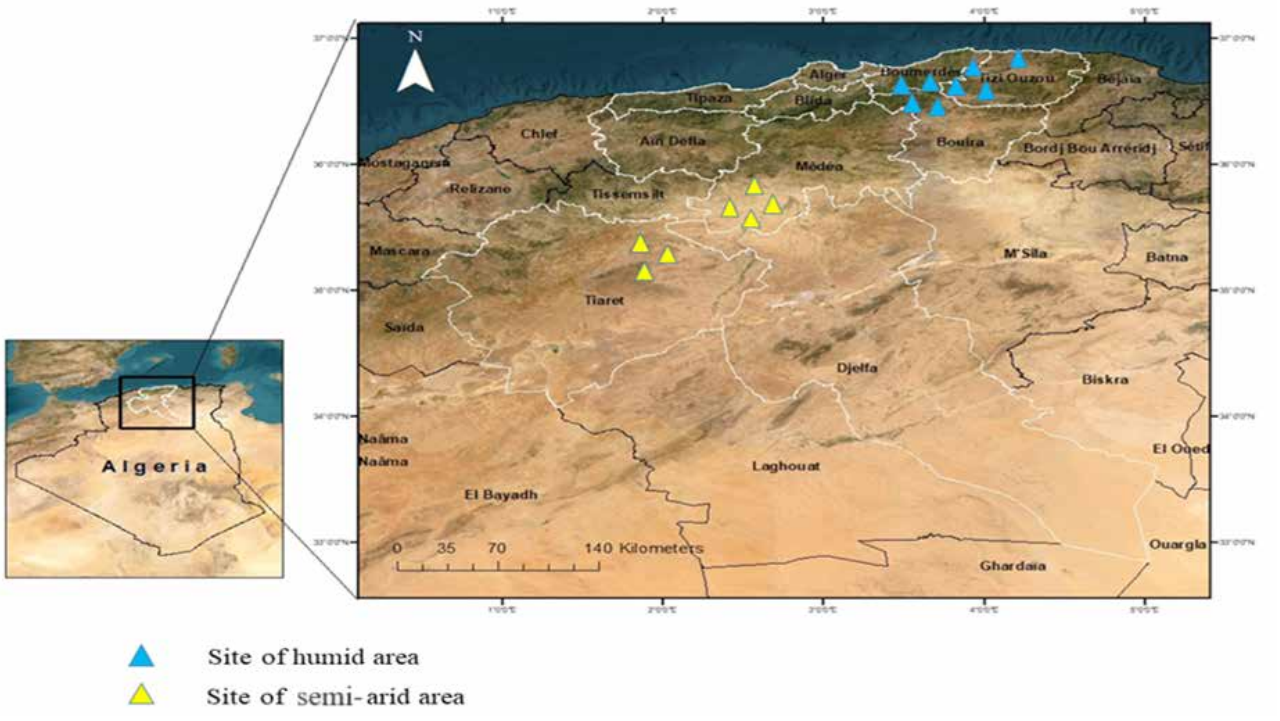

Figure 1. Geographical location of the study area, the bleu and yellow triangles indicate the localities where cattle were sampled in the humid and semi-arid areas respectively.

sample, genomic DNA was extracted from 200 µl of whole blood using the PureLink® genomic DNA extraction Kit (Invitrogen/Thermo Fisher Scientific, USA), following the manufacturer's instructions. Extracted DNA was resuspended in 100 µl elution buffer and stored at -20 °C until used in PCRs. Blood samples for serology were collected in dry Vacutainer tubes and serum was separated by centrifugation at 2,500 rpm for 10 minutes. Each serum sample was transferred to an eppendorf tube which was labelled and stored at -20 °C until further use.

Serological test

One hundred and sixteen samples were tested by IFAT using acetone-fixed *T. annulata* schizont antigen slides, which were prepared from *T. annulata*-infected cell lines at low passage levels, as described by Ilhan (2000). IFAT was performed as described by Minami et al. (1983) with minor modifications. Briefly, each serum sample was subjected to two-fold serial dilutions in phosphate-buffered saline (PBS), ranging between 1:40 and 1:1280. For each antigen slide, 10 µl of each dilution was deposited in the wells, including *T. annulata*-positive and-negative controls and PBS alone as conjugate controls. The slides were incubated at room temperature in a dark, moist chamber for 30 min and then washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO4) for 5 min each time. After drying, a dilution of fluorescein-conjugated rabbit anti-bovine IgG (1:80) with 0.1% Evans Blue (SIGMA) was added to each well and incubated for 30 min. After washing and drying, slides were mounted with 50 % glycerol in PBS and examined under a 20X or 40X objective using a blue-violet incident light fluorescence microscope (Olympus BX51, Tokyo, Japan). Fluorescence intensity for IFAT was categorised as strong (++), weak (+), or absent (−), and samples with a titre of ≥ 1:160 were considered seropositive (i.e. positive fluorescence titers ≥ 1:160) (Darghouth et al., 1996a). This relatively high threshold was chosen firstly, to fulfil the OIE recommendation of 95% specificity (World Organisation for Animal Health, 2021) and, secondly, to reduce cross-reactivity with *T. buffeli*, which is highly prevalent in the study region (Ziam et al., 2015).

Molecular detection of *Theileria annulata* using species-specific primers

Theileria annulata DNA was specifically amplified by PCR using previously described forward (5′-CAA ATT CGA GAC CTA CTA CGA TG-3′) and reverse (5′-CCA CTT RTC GTC CTT AAG CTC G-3′) primers from the Tams1 gene (Santos et al., 2013) yielding an amplicon of approximately 319 bp."

The PCR reaction was performed in a final volume of 25 µl containing 12.5 µl of NZYTaq II 2x Green Master Mix (NZY Tech, Portugal), 5.5 µl of ultrapure water, 2 µl of 0.4 mM of each primer (Eurofins Genomics, Germany) and 5 µl of template DNA solution. The reactions were performed in an automated thermal cycler (MJ Mini, Bio-Rad) and comprised an initial denaturation step at 94 ºC for 30 seconds, followed by 35 cycles of denaturation (94 ºC for 30 s), primer annealing (55 ºC for 30 s) and extension (72 ºC for 30 s). A final extension at 72 ºC for 10 min was performed and the samples held at 4 ºC thereafter. The amplified products were visualised under UV light after electrophoresis on a 1.5 % (w/v) agarose gel (in 1× TBE buffer) stained with GreenSafe Premium (NZY Tech, Portugal), and the size of the amplified products was estimated using a 100 base pair DNA ladder (GeneDireX®, GeneDireX Inc., USA).

Phylogenetic analysis

From the PCR-positive samples, four were selected, each representing a distinct geographical region, and subsequently purified and sequenced using the Sanger sequencer (Eurofins Genomics, Germany). Each sequence regenerated was then individually compared with previously published sequences from the GenBank™ NCBI database using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin/). Haplotype diversity (Hd) and nucleotide diversity (π) were calculated using DNA Sequence Polymorphism (DNAsp 6) (Rozas et al., 2017). The resulting sequences are available in GenBank under accession numbers PP537629–PP537632. Phylogenetic analysis was conducted using 29 reference *T. annulata* sequences from Europe, Africa, and Asia, sourced from the NCBI database. The phylogenetic tree was reconstructed using the Maximum Likelihood (ML) method implemented in MEGA12 (Kumar et al., 2024), with branch support evaluated through 1000 bootstrap replications. The optimal nucleotide substitution model identified was the Tamura 3-parameter model with a discrete Gamma distribution (+G) (Tamura, 1992). The resulting tree was rooted using *Theileria lestoquardi* (LC430946) as the outgroup. To assess the potential novelty of haplo-

types identified within *T. annulata,* an analysis was conducted on all *Tams1* sequences corresponding to *T. annulata* present in GenBank. ClustalW alignment was performed on sequences using MEGA12. Representative sequences for each haplotype were identified using DNAsp 6. To examine the global genetic relationships among the distinct haplotypes of *T. annulata*, a haplotype network analysis was conducted using the Median Joining method within the Network12 software (Bandelt et al., 1995).

Statistical analysis

Descriptive and analytic statistics were performed using the R statistical language version 4.3.1 (R Core Team., 2021). The overall prevalence and infection rates according to the different parameters studied were calculated, as were the confidence intervals or the standard errors of the different techniques. The results of the serological method were compared with that of the PCR by calculating sensitivity, specificity and kappa index (McHugh, 2012; Viera and Garrett, 2005). A kappa (κ) measure of agreement test was performed to compare the performance of the two tests; $\kappa < 0$ indicates no agreement beyond chance, while a κ-value between 0.81 and 0.99 indicates almost perfect agreement. A κ-value between 0.41 and 0.60 indicates a moderate level of agreement. In a second phase of analysis, univariate comparisons were performed using the Chi-squared test for the different variables. Finally, the parameters with statistically significant differences ($P < 0.05$) were analysed using a multivariate binary logistic model to interpret the risk factors associated with *T. annulata* infection.

RESULTS

Seroprevalence

The results of the IFAT study are summarised in Table 1, including a univariate analysis, which highlighted differences in seroprevalence in several of the analytical groups. For sera diluted at 1/160, 58 of the 116 samples were found to be positive for *T. annulata* antibodies, corresponding to an overall seroprevalence of 50.0 ± 4.6 %. The rate of infection with *T. annulata* was 67.3 ± 6.3 % in crossbred, 52.9 ± 12.1 % in Holstein and 27.3 ± 6.7% in Montbeliard breeds, with evidence of differing seroprevalence among breeds ($P < 0.001$). Overall, adults had a seroprevalence (52.3 ± 4.7 %) while young cattle did not show any evidence of exposure, a result which only approached statistical significance ($P = 0.0571$). There was no significant difference between males and females nor between cattle located in the humid and the semi-arid region. The seasonality of sampling was associated with a difference in *T. annulata* seroprevalence ($P < 0.01$), the highest being in autumn (70.3 ± 7.5 %), with the lowest in spring (35.5 ± 7.6%) and an intermediate level in summer. Finally, the infection rate was found to be higher in extensively farmed cattle with rates of 69. 2 ± 7.3% compared to those kept semi-intensively (40.3 ± 5.5%) and this was statistically significant ($P < 0.01$).

PCR prevalence

Of the 116 DNA cattle tested, 75 were found to be positive for *T. annulata* by Tams PCR, corresponding to an overall prevalence of 64.7 ± 4.4 %, slightly higher than that of the serological study. The highest prevalence was recorded in crossbreds with a rate of 89.19 ± 4.2 % compared to Holstein and Montbeliard, where rates of 70.6 ± 11.0% and 31.8 ± 8.70 % were recorded, respectively (Table 2). These differences were statistically significant ($P < 0.001$) and mirrored the serological results. Similarly, the highest infection rate was recorded in autumn (89.2 ± 4.4 %), with lowest in the spring (51.3 ± 8.0 %) with seasonality influencing prevalence ($P < 0.001$). While, again, cattle sex did not exert an influence, the cattle in the humid region were found to have a higher prevalence (77.2 ± 5.5 %) compared to those in the semi-arid region, with a rate of 52.5±7.2% ($P < 0.01$). Again, echoing the serological results, in cattle reared in semi-intensive systems, *T. annulata* infection was detected at lower level than in the extensive systems, with respective rates of 51.9 ± 5.6 % and 89.7 ± 4.7 ($P < 0.001$) (Table 2).

Comparison of diagnostic techniques

Of the 116 blood samples collected, only two cattle were found to be serologically positive and PCR negative, whereas 19 samples were PCR positive but IFAT negative (Table 3). The κ-coefficient indicated a moderate agreement ($\kappa = 0.62$) between IFAT and PCR (Table 3). The sensitivity and specificity of the IFAT technique for the detection of *T. annulata* were 74.6% and 95.1%, respectively, compared with the PCR test (Table 3).

Risk factors associated with *T. annulata* in the cattle population

Univariate analysis of demographic factors and husbandry parameters revealed that the statistically significant risk factors ($P < 0.05$) for *T. annulata*

Table 1. Characteristics of cattle infected with *T. annulata* using serology technique IFAT.

		Cut-off												
Factors	**Category**	**1/40**	**%±SE**	**1/80**	**%±SE**	**1/160**	**%±SE**	**p-value**	**1/320**	**%±SE**	**1/640**	**%±SE**	**1/1280**	**%±SE**
Breed	MB	21/44	47.7±7.5	20/44	45.4±7.5	12/44	27.3±6.7	0.0003***	7/44	15.9±5.5	6/44	13.6±5.1	6/44	13.6±5.1
	HLT	16/17	94.1±5.7	14/17	87.5±8.0	9/17	52.9±12.1		4/17	23.5±10.2	4/17	23.5±10.2	1/17	5.9±5.7
	Crossbred	46/55	83,6±4.9	43/55	78.2±5.5	37/55	67.3±6.3		13/55	23.6±5.7	5/55	9.1±3.8	4/55	7.3±5.3
Age	Younger's	3/5	60.0±21.9	3/5	60.0±21.9	0/5	0.0	0.0571^{NS}	0/5	0,0	0/5	0.0	0/5	0.0
	Adults	80/111	72.1±4.2	74/111	66.7±4.4	58/111	52.3±4.7		24/111	21.6±3.9	15/111	13.5±3.2	11/111	9.9±4.0
Sex	Females	74/102	72.5±4.4	69/102	67.6±4.6	53/102	52.0±4.9	0.3934^{NS}	22/102	30.3±4.5	13/102	12.7±3.2	10/102	9.8±2.9
	Males	9/14	64.3±12.8	8/14	57.1±13.2	5/14	35.7±12.6		2/14	14.3±9.3	2/14	14.3±9.3	1/14	7.1±6.8
Season	Spring	20/39	51.3±8.0	20/39	51.3±8.0	14/39	35.9±7.6	0.0082**	5/39	12.8±5.3	1/39	2.6±2.5	0/39	0.0
	Summer	26/40	65.0±7.5	23/40	57.5±7.8	18/40	45.0±7.8		8/40	20.0±6.3	8/40	20.0±6.3	6/40	15.0±5.6
	Autumn	37/37	100.0	34/37	91.9±4.4	26/37	70.3±7.5		11/37	29.7±7.5	6/37	16.2±6.0	5/37	13.5±5.6
Region	Humid	41/57	71.9±5.9	37/57	64.9±6.3	33/57	57.9±6.5	0.0946^{NS}	8/57	14.0±4.5	3/57	5.3±2.9	0/57	0.0
	semi-arid	42/59	71.2±5.8	40/59	67.8±6.0	25/59	42.4±6.4		16/59	27.1±5.7	12/59	20.3±5.2	11/59	18.6±5.0
Husbandry	Semi-intensive	50/77	64.9±5.4	47/77	61.0±5.5	31/77	40.3±5.5	0.0031**	16/77	20.8±4.6	12/77	15.6±4.1	11/77	14.3±3.9
	Extensive	33/39	84.6±5.7	30/39	76.9±6.7	27/39	69.2±7.3		8/39	20.5±6.4	3/39	7.7±4.2	0/39	0.0
	Total	83/116	71.6±4.1	77/116	66.4±4.3	58/116	50.0±4.6		24/116	20.7±3.7	15/116	12.9±3.1	11/116	9.5±2.7

SE: Standard Error, ^{NS}: Not significant, *: P < 0.05, **: P < 0.01, ***: P < 0.001

Table 2. Characteristics of cattle infected with *T. annulata* using PCR technique.

Factors	Categories	Tams	%±SE	P-value
Breed	MB	14/44	31.8±7.0	0.000***
	HLT	12/17	70.6±11.0	
	Crossbred	49/55	89.1±4.2	
Age	Youngers	1/5	20.0±17.8	0.032*
	Adults	74/111	66.7±4.4	
Sex	Females	68/102	66.7±4.7	0.530^{NS}
	Males	7/14	50.0±13.3	
Season	Spring	20/39	51.3±8.0	0.000***
	Summer	22/40	55.0±7.8	
	Autumn	33/37	89.2±4.4	
Region	Humid	44/57	77.2±5.5	0.003**
	semi-arid	31/59	52.5±6.5	
Husbandry	Semi-intensive	40/77	51.9±5.6	0.000***
	Extensive	35/39	89.7±4.8	
	Total	75/116	64.7±4.4	

SE: Standard Error, NS: Not significant, *: P < 0.05, **: P < 0.01, ***: P < 0.001
Tams: major merozoïte/piroplasm surface protein

Table 3. Comparative of serology IFAT and PCR assay results of *Theileria annulata* detection.

Diagnostic technique	PCR		Sensibility=74.6%
	Positives	Negatives	Specificity=95.1%
IFAT serology			
Positive	56	2	K value=0.6274
Negative	19	39	

infection were breed, season and husbandry system, on the basis of the results of the IFAT test (Table 1). PCR testing revealed the same factors along with sex and region of origin (Table 2).

In order to determine the relative importance of the factors highlighted in the univariate analyses, multivariate regression modelling was undertaken, the results of which are illustrated in Table 4. Modelling the IFAT results in this way revealed that only 'season' and 'husbandry system' were factors associated with *T. annulata* exposure. Autumn was the season with the highest risk of being seropositive and was associated with an odds ratio (OR) of 2.81 which had a 95 % confidence interval (CI) of between 1.53 and 5.48. Cattle in semi-extensive systems had a four-fold higher risk of being infected with *T. annulata*, with an OR of 4.06 (95 % CI: 1.19 - 15.35). In contrast, PCR results showed that breed, season and husbandry system were potential risk factors for *T. annulata* infection in cattle (P < 0.05) with ORs of 2.25 (95 % CI: 1.07 - 5.07), 4.29 (95 % CI: 1.97 - 10.9) and 8.60 (95 % CI: 1.41 - 57.6), respectively (Table 4). Age and region were found to not be statistically significant (Table 4).

Genetic diversity and haplotype analysis of *T. annulata* isolates

A multiple sequence alignment of the four sequences revealed three different haplotypes (Fig. 2). The sequence PP537629 showed a similarity of 98.53 % with the sequence of *T. annulata*, which is documented in GenBank under the accession numbers PV750534, AF214879 and PV750545. The sequence PP537630 shared 98.90 % similarity with the sequence of *T. annulata*, which is stored in GenBank under accession number KF765519. In contrast, se-

Table 4. Multivariate logistic regression analysis of risk factors associated with *T. annulata* infection

		PCR				IFAT 1/160			
Risk factor	**Parameters**	**P/N**	**OR**	**95% CI**	***p* value**	**P/N**	**OR**	**95% CI**	***p* value**
Age	Young <1year (reference)	1/5	1.7799	0.197-39.8	0.6416^{NS}	0/5	--	--	--
	Adult >1 year	74/111				58/111			
Breed	Crossbred (reference)	14/44				37/55			
	Holstein	12/17	2.2536	1.07-5.07	0.0388*	9/17	1.4601	0.81-2.62	0.19930^{NS}
	Montbeliard	49/55				12/44			
Season	Autumn (reference)	19/39				26/37			
	Summer	23/40	4.2990	1.97-10.9	0.0007***	18/40	2.8100	1.53-5.48	0.00136 **
	Spring	33/37				14/39			
Region	Semi-arid (reference)	44/57	0.8265	0.203-3.16	0.7819^{NS}	25/59	--	--	--
	Humid	31/59				33/57			
Husbandry	Semi-intensive (reference)	40/77	8.6093	1.41-57.6	0.0208*	31/77	4.0692	1.19-15.35	0.02950 *
	Extensive	35/39				27/39			

^{NS}: Not significant, *: P < 0.05, **: P < 0.01, ***: P < 0.001

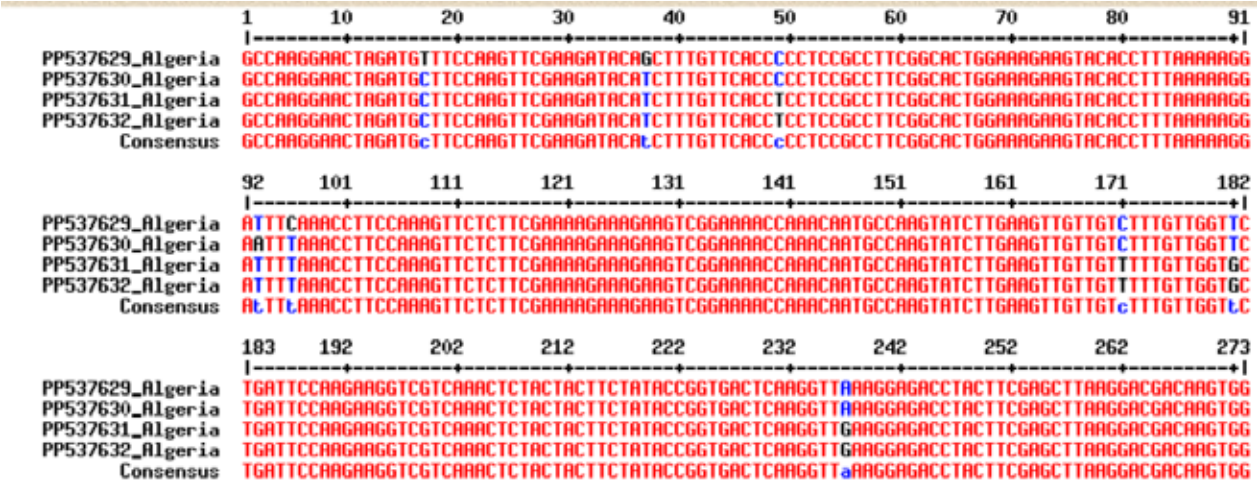

Figure 2. A multiple sequence alignment of the four *T. annulata* Tams1 gene sequences (273 bp) obtained in this study, revealing eight polymorphic sites, was performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin/).

quences PP537631 and PP537632 shared the same haplotype, and each had 98.53% similarity to the same reference KF765519. This alignment resulted in eight polymorphic sites and a higher genetic diversity (Hd=0.833) and nucleotide diversity (π = 0.01709).

Phylogeny of *T. annulata*

A maximum likelihood tree was constructed to infer the evolutionary relationships between the *T. annulata* isolates using the partial Tams1 gene sequences. A total of 34 sequences were analysed, including four isolates from the present study and reference sequences from GenBank. The phylogenetic tree was constructed with *Theileria lestoquardi* (LC430946) as outgroup (Fig. 3). The analysis revealed two main clades: The first included isolates from India, Iran, China and several Middle Eastern countries. This indicates a genetically diverse and geographically extensive group that had relatively high pairwise divergence values, e.g. 6.53%–10.64% between Indian and Chinese or Iranian isolates (Table 5). In contrast, the second large group was more geographically restricted and comprised mainly isolates from North Africa and Southern Europe, including Algeria, Egypt, Tunisia and Spain. In particular, the four Algerian isolates from the present study (PP537629, PP537630, PP537631 and PP537632), shown by green circles, belonged to this second group and formed a strongly supported subgroup with the Egyptian isolates KF765519 and AB917283 (bootstrap = 94). Within this subgroup, PP537631 and PP537632 showed particularly close genetic relationships with a pairwise distance of 1.86%, indicating the presence of shared haplotypes (Table 5). PP537630 was in the same cluster and showed a divergence of 1.86% to PP537631 and PP537632 and only 1.48% to the Egyptian isolate KF765519, suggesting common ancestry or recent gene flow between the Algerian and Egyptian strains. In contrast, PP537629, although belonging to the same North African group, showed greater divergence with genetic distances between 1.48% and 2.62% to the other Algerian sequences, forming a distinct branch that may represent a genetically differentiated local variant (Table 5). In addition, the genetic distances between the Algerian isolates and those of the first main group (e.g. from China, Iran or India) ranged from 3.76 % to over 9.3 %, emphasising the deep evolutionary separation between the two main lineages (Table 5). This phylogenetic structure, which is supported by both tree topology and pairwise genetic distances, emphasises the existence of regionally clustered but genetically heterogeneous populations of *T. annulata* in Algeria and North Africa.

Haplotype Network

A haplotype network was created using 414 partial Tams1 gene sequences from GenBank, comprising data from 19 countries (Table 5), including the sequences generated in this study (Fig. 4). To ensure the accuracy of the sequence analysis, short sequences or those with degenerate nucleotides were excluded. The analysis identified 181 haplotypes, indicating

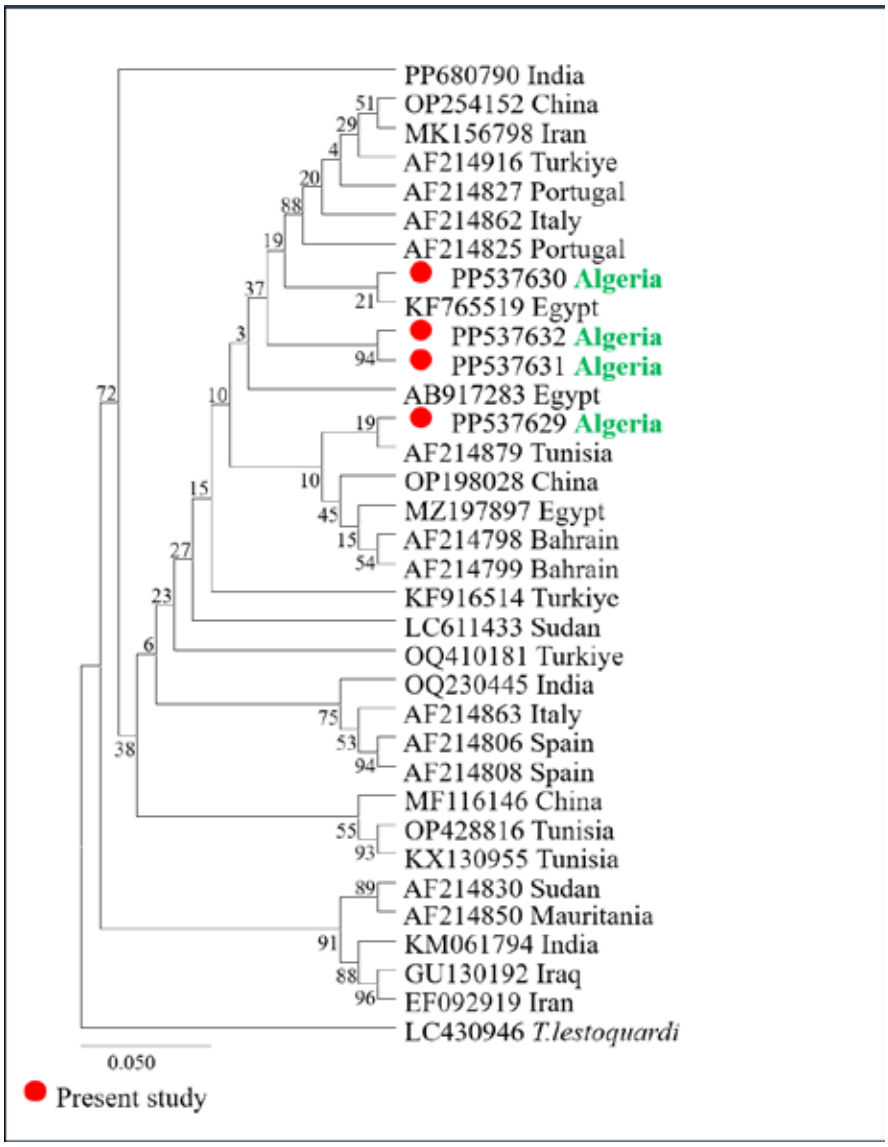

Figure 3. Phylogenetic relationships among *T.annulata* Tams1 sequences are shown based on GenBank accession numbers and countries of origin. The analysis used 34 sequences in total. A maximum likelihood phylogenetic tree of the Tams1 gene (273 bp) was constructed using the Tamura 3-parameter model in MEGA12, with 1,000 bootstrap replicates.

a very high genetic diversity (Hd = 0.9853). The calculated nucleotide diversity ($\pi = 0.06061$) reflects a considerable degree of heterogeneity in the population (Fig. 4). The haplotypes labelled in green (H1, H2 and H3) represent the three new Algerian haplotypes identified in this study. These haplotypes formed a separate but linked cluster. H1 and H2 were separated by a single mutation, indicating close evolutionary relatedness, while H3 diverged slightly more but remained clearly linked to the same local lineage (Fig. 4). The Algerian haplotypes were most closely related to H30, H31 and H32, which correspond to southern European sequences from Portugal, and to H82 and H83, which represent north African sequences from Egypt, suggesting that they are integrated into a broader north African–south European lineage (Fig. 4).

DISCUSSION

The present study was conducted in a large geographic area in northern Algeria, considered to be endemic for tropical theileriosis, where tick vectors are abundant and numerous factors favour transmission of *T. annulata* (Boulkaboul, 2003; Kernif et al., 2024; Ziam et al., 2015). In this study, 50.0 % of cattle were found to be positive for *T. annulata* antibodies when tested by IFAT, while 64.7 % were positive for *T. annulata* DNA by PCR. Overall, the high prevalence of the parasite observed in this study is in accordance with previous studies in North Africa (Abaker et al., 2017; Ayadi et al., 2017; Gharbi et al., 2020; Jacquiet et al., 1994; Rahali et al., 2014; Ziam et al., 2015). Climatic conditions in the area favour tick activity and extensive livestock farming allows direct contact between livestock and ticks (Bedouhene et al., 2022; Boulkaboul, 2003).

Table 5. Estimation of evolutionary divergence between 34 nucleotide sequences with 273 aligned positions. Evolutionary distances were calculated as percentages using the Tamura 3-parameter model.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1 Hl PP537632 Algeria																																		
2 Hl PP537631 Algeria	**0.00**																																	
3 H2 PP537630 Algeria	**1.86**	**1.86**																																
4 H3 PP537629 Algeria	**2.62**	**2.62**	**1.48**																															
5 AF214827 Portugal	2.99	2.99	3.38	4.94																														
6 AF214862 Italy	3.37	3.37	3.77	5.34	0.37																													
7 AF214916 Turkiye	3.76	3.76	4.16	5.73	0.74	1.11																												
8 AF214825 Portugal	3.76	3.76	3.38	4.93	0.74	1.11	1.48																											
9 OP254152 China	4.14	4.14	3.76	4.54	1.11	1.48	1.11	1.85																										
10 MK156798 Iran	4.53	4.53	3.37	4.15	1.48	1.86	1.48	1.48	0.37																									
11 KF765519 Egypt	1.48	1.48	1.11	1.86	2.99	3.38	3.76	2.99	3.38	2.99																								
12 AF214879 Twusia	4.16	4.16	2.99	1.48	4.17	4.56	4.95	4.16	3.77	3.38	3.38																							
13 AB917283 Egypt	3.76	3.76	3.38	1.86	4.54	4.94	5.33	4.54	4.15	3.76	3.00	1.86																						
14 LC611433 Sudan	2.62	2.62	2.99	2.99	4.93	5.32	5.71	4.92	5.31	4.92	3.37	3.76	3.37																					
15 AF214798 Bahrain	4.55	4.55	4.16	3.39	6.12	6.53	6.52	6.11	5.32	4.92	3.77	4.16	3.76	4.16																				
16 OP198028 China	4.55	4.55	3.38	1.86	6.12	6.53	6.92	6.11	5.72	5.32	3.77	2.61	2.24	4.14	2.24																			
17 MZ1n897 Egypt	6.13	6.13	4.93	3.38	7.74	8.15	8.56	7.73	7.32	6.91	5.34	4.14	2.99	4.93	3.00	1.48																		
18 OQ410181 Turkiye	3.00	3.00	4.15	2.61	5.32	5.71	6.11	5.31	5.71	5.32	2.99	3.37	2.99	2.61	3.00	2.99	3.00																	
19 MF116146 China	6.53	6.53	7.74	6.12	8.96	9.38	9.79	8.95	9.38	8.96	6.52	6.92	5.72	4.54	6.53	5.72	5.74	3.38																
20 OP428816 Twusia	6.12	6.12	5.72	4.15	7.73	8.14	8.55	7.72	7.32	6.91	5.33	4.92	2.99	4.14	4.54	3.76	3.00	2.99	2.62															
21 KX130955 Twusia	6.12	6.12	5.72	4.15	7.73	8.14	8.55	7.72	7.32	6.91	5.33	4.92	2.99	4.14	4.54	3.76	3.00	2.99	2.62	0.00														
22 PP680790 India	4.54	4.54	5.72	4.94	6.91	7.32	7.72	6.90	7.32	6.91	4.54	5.72	5.32	4.16	3.77	4.54	4.56	2.24	2.61	5.33	5.33													
23 AF214806 Spain	5.74	5.74	5.72	4.15	8.98	9.40	9.82	8.97	8.56	8.14	5.73	4.92	5.72	4.56	5.74	5.32	5.34	3.39	3.76	5.73	5.73	2.62												
24 AF214808 Spain	6.13	6.13	6.11	4.54	8.97	9.39	9.80	8.96	8.55	8.13	6.12	4.92	5.71	4.55	5.73	5.32	5.33	3.38	3.76	5.72	5.72	2.61	0.37											
25 AF214863 Italy	7.76	7.76	8.57	6.93	10.23	10.66	11.08	10.22	10.66	10.23	7.75	6.92	7.74	6.55	7.76	7.32	6.53	4.55	4.93	7.75	7.75	3.76	2.62	2.61										
26 OQ230445 India	5.34	5.34	6.12	5.34	7.73	8.14	8.55	7.72	8.14	7.73	5.33	5.32	6.12	4.96	5.34	5.72	4.94	3.00	3.38	6.13	6.13	1.48	1.87	1.86	2.24									
27 AF214830 Sudan	10.26	10.26	9.80	9.80	10.22	10.65	10.23	10.21	9.79	9.37	11.08	10.63	10.63	8.17	8.14	8.12	8.14	8.57	7.72	9.79	9.79	6.12	7.74	7.73	8.15	7.32								
28 KM061794 India	10.23	10.23	9.80	10.66	9.37	9.79	8.54	9.36	8.96	8.55	9.39	11.50	11.50	9.83	9.80	9.78	10.63	9.39	9.37	12.36	12.36	7.72	9.39	9.37	9.81	8.13	6.10							
29 AF214799 Bahrain	5.74	5.74	4.94	3.38	7.34	7.75	7.74	7.32	6.52	6.11	4.95	3.37	4.15	4.54	1.86	2.23	2.23	3.38	6.92	4.93	4.93	4.95	4.55	4.54	5.73	4.16	8.96	10.64						
30 GU130192 Iraq	9.80	9.80	9.38	10.23	8.95	9.37	8.95	8.94	8.55	8.13	8.96	11.06	11.06	9.40	8.56	8.54	7.72	8.15	8.13	11.08	11.08	5.72	8.15	8.14	7.73	6.11	4.14	2.61	8.55					
31 EF092919 Iran	9.80	9.80	9.38	10.23	8.95	9.37	8.95	8.94	8.55	8.13	8.96	11.06	11.06	9.40	8.56	8.54	7.72	8.15	8.13	11.08	11.08	5.72	8.15	8.14	7.73	6.11	4.14	2.61	8.55	0.00				
32 AF214850 Mauritania	10.23	10.23	9.80	10.63	9.37	9.79	9.38	9.36	9.79	9.37	10.22	11.48	11.48	8.14	9.80	8.94	8.95	8.55	7.71	10.62	10.62	6.91	8.55	8.54	8.97	8.13	2.24	5.31	9.79	3.37	3.37			
33 KF916514 Turkiye	4.16	4.16	3.76	2.24	5.72	6.12	5.73	5.71	4.54	4.15	3.38	2.99	2.61	2.99	3.00	3.38	4.16	2.61	5.32	4.15	4.15	4.15	4.54	4.54	6.53	4.94	8.98	9.40	3.38	8.98	8.98	9.80		
34 LC430946 *T.lestoquardi*	16.32	16.32	15.42	16.80	16.79	17.26	16.80	16.78	15.89	15.43	15.43	18.20	17.72	14.52	14.53	15.87	15.90	15.43	15.89	15.89	15.89	14.54	16.35	16.37	18.25	16.35	15.44	17.27	15.88	15.47	15.47	15.44	16.37	

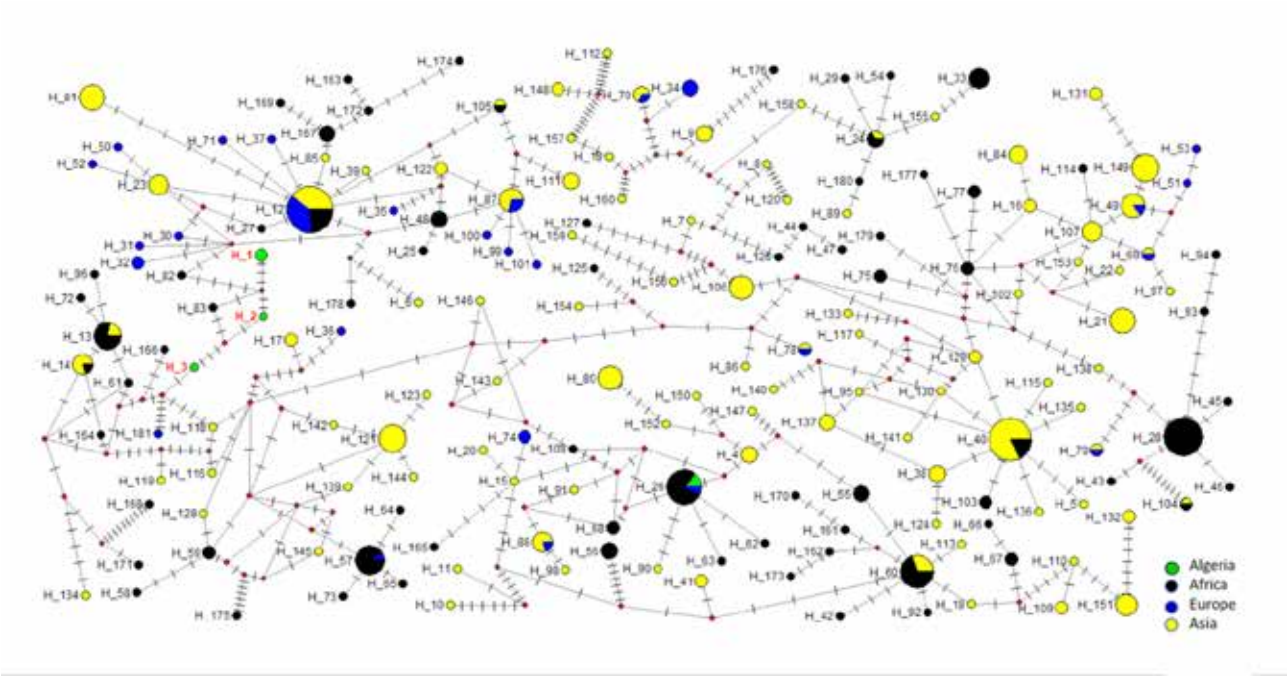

Figure 4. Haplotype network of *T. annulata* sequences. Each circle represents a unique haplotype, with colours indicating geographic origin. The circle size indicates the haplotype frequency. The lines show the evolutionary relationships, with hatch marks representing nucleotide substitutions. Ancestral haplotypes are represented by small red circles.

Our results showed that the sensitivity and specificity of IFAT were significantly lower than those of PCR, with a kappa (κ) coefficient of 0.62. We attribute the low sensitivity of the IFAT to a weak specific immune response against the parasite and the presence of a low level of antibodies during the long-term carrier period (Burridge et al., 1974). Although specificity appears relatively high, there exists the possibility of cross-reactivity in antibody response with other *Theileria* species (Burridge et al., 1974; Leemans et al., 1999), such as *T. buffeli* which is known to be widespread in Algeria (Ziam et al., 2015; Ziam et al., 2016). The two animals that were positive for IFAT and negative for PCR provide circumstantial evidence supporting the hypothesis of cross-reactivity. At the same time, this result implies a relatively low prevalence of *T. buffeli* in the sampled population, although the frequency of putative co-infection with both parasites cannot be determined from these results. Nineteen cattle were PCR positive and IFAT negative, suggesting that antibodies to *T. annulata* in some cattle may be of shorter duration than the infection itself. Similar observations were also made by Darghouth et al., (1996a) using a schizont antigen based IFAT. In addition, it is also possible that in spring, some cases of latent infection were not detected by serology (Darghouth et al., 1996a).

According to the World Organisation for Animal Health (2021), IFAT remains the recommended reference test for most parasites, particularly for epidemiological studies of diseases such as tropical theileriosis (Darghouth et al., 1996a; Rahali et al., 2014). Our results of 50.0 % seroprevalence at a serum dilution of 1/160 corroborates one reported in Turkey (Inci et al., 2007). In contrast, the positivity rate obtained by IFAT in the present study is higher than the documented rates of 19.8 % - 25.7 % in Tunisia (Darghouth et al., 1996a), 35.4 % in Morocco (Rahali et al., 2014), 17.6 % in Mauritania (Jacquiet et al., 1994), 31.0 % in Sudan (Abaker et al., 2017) and 26.0 % in Italy (Gargano et al., 2021). The difference in prevalence rates in these countries reflects the diversity of geo-climatic conditions that influence the distribution and activity of tick vectors (Bedouhene et al., 2022; Estrada-Peña et al., 2004).

Unlike IFAT, PCR is a sensitive and specific test that can detect *T. annulata* DNA, even at low levels of parasitaemia, and has the advantage of detecting the parasite in the blood during the first days of infection (d'Oliveira et al., 1995; Mans et al.,

2015). Our results agree with previous reports showing that PCR is more sensitive and specific than IFAT (Dumanli et al., 2005; El Damaty et al., 2021; Martín-Sánchez et al., 1999) as noted by the two cattle that were IFAT-positive and PCR-negative, and nineteen IFAT-negative and PCR-positive. At the time of sampling, all animals were clinically healthy. These cattle probably had low parasitaemia and a low level humoral immune response (Mans et al., 2015).

Cattle breed was a significant risk factor determining *T. annulata* prevalence (P = 0.0388, OR = 2.25, CI = 1.07 - 5.07). The low rates of *T. annulata* in improved cattle compared to crossbred cattle are probably due to the semi-intensive system in which they are kept and where acaricides are systematically applied, as previously reported (Ayadi et al., 2017; Foughali et al., 2021; Kernif et al., 2024).

The prevalence of *T. annulata* was lower in the semi-intensive rearing system than in the extensive rearing system and this relationship is supported by the multivariate analysis (OR: 8.60, 95 % CI: 1.41 - 57.6, P = 0.0208). This may be explained by extensively reared cattle being more exposed to ticks than their semi-intensively reared counterparts (Calleja-Bueno et al., 2017; Flach and Ouhelli, 1992). In addition, the semi-intensive system allows for better monitoring of animals entering barns as owners have the opportunity to pay more attention to their animals, as indicated previously (Ayadi et al., 2017).

The results obtained in the present study demonstrate that season is a potential risk factor for *T. annulata* infection (multivariate: P = 0.0007, OR: 4.29, 95 % CI: 1.97 - 10.9). *Theileria annulata* DNA and antibodies were detected more frequently in summer and autumn than in spring. These results are consistent with clinical cases of tropical theileriosis in Algeria occurring from May to early September, with a peak in July (Benchikh Elfegoun et al., 2017; Ziam et al., 2020) and from October to November (Bedouhene et al., 2022). Our findings are consistent with the activity of *Hyalomma scupense*, which extends from the end of May, with a peak in July, to the end of August (Bedouhene et al., 2022; Boulkaboul, 2003). Our findings also support the involvement of *H. lusitanicum* and/or *H. excavatum*, which are active from October to November, corresponding with the second annual period of tropical theileriosis in the country (Bedouhene et al., 2022; Boulkaboul, 2003).

Our results show a high seroprevalence of *T. annulata* in cattle over twelve months of age, which is logical as these animals will have already undergone a tropical theileriosis season. The sampled cattle originate from two regions where tropical theileriosis is enzootic and it is likely that there is endemic stability in adult animals in the cattle populations, as reported in Tunisia (Darghouth et al., 1996b). Consequently, any new cattle which are introduced into these regions will likely become infected with *T. annulata* and develop disease. Clinical cases of TT are noted to occur sporadically in these two regions when there is an imbalance in the host-parasite relationship. In addition to the influx of naïve cattle to an endemic zone, disease may be triggered by other factors. For example, sirocco, stress due to milk production, pregnancy and calving may precipitate clinical disease in dairy cattle (Darghouth et al., 2003; Sergent et al., 1945).

To confirm that IFAT-positive cattle were infected with *T. annulata*, we amplified and sequenced the *Tams1* gene, which encodes a merozoite surface protein expressed during both the schizont and erythrocytic stages of the parasite (Glascodine et al., 1990). This gene is present as a single copy in the haploid genome and is known for its high antigenic variability (Gubbels *et al*., 2000). Due to these properties, *Tams1* has been widely used in studies investigating the genetic diversity and population structure of *T. annulata* (Gubbels et al., 2000; Katzer et al., 2006). Consistent with previous studies reporting the high polymorphism of the *Tams1* gene (Gubbels et al., 2000; Katzer et al., 2006), we observed considerable sequence variation among the 181 *Tams1* sequences from 19 countries retrieved from GenBank. Genetic analyses revealed high haplotype diversity (Hd = 0.9853), nucleotide diversity (π = 0.06061) and genetic distance (Table 5), reflecting a considerable degree of heterogeneity within and between the *T. annulata* population. The four Algerian sequences identified in this study exhibited notable diversity, with three distinct haplotypes (H1, H2, H3) detected among them. These sequences displayed high haplotype diversity (Hd = 0.833), nucleotide diversity (π = 0.01709), and were associated with eight polymorphic sites (Fig. 2). BLAST analysis indicated that these haplotypes are novel. Phylogenetic analysis revealed that the Algerian isolates clustered with *T. annulata* sequences from North Africa and Southern Europe, particularly those from Egypt, Tunisia, and Spain (Fig. 3). These findings were further supported by haplotype network analysis, which demonstrat-

ed that the Algerian haplotypes are closely related to haplotypes H30–H32 (Portugal) and H82–H83 (Egypt), suggesting that they form part of a broader North African–Southern European lineage. This genetic relatedness may reflect historical and ongoing livestock trade among Mediterranean countries.

CONCLUSIONS

The present study documents the prevalence of *T. annulata* in the humid and semi-arid regions of Algeria using PCR and IFAT over the course of a year. Both methods showed that season and rearing systems are potential risk factors for *T. annulata* infection, with parasite DNA and antibodies being detected more frequently in cattle kept in extensive systems during the autumn. These findings agree with knowledge of local tick activity and animal husbandry and illustrate that a large proportion of adult cattle are in the carrier state of infection. This information, allied with the relatively low level of reported incidence of clinical disease, supports the contention that tropical theileriosis in Algeria may be generally considered endemically stable, as in some neighbouring countries.

AUTHOR CONTRIBUTIONS

ZM: conceptualisation, data curation, methodology and writing- original draft. **SH**: conceptualisation and investigation. **TK and HBB**: conceptualisation, data curation, formal analysis, methodology, funding acquisition, project administration, software, supervision and writing-review & editing. **JG**: conceptualisation, data curation, formal analysis, methodology, funding acquisition, project administration, software, supervision and writing-review & editing. **BM**: conceptualisation, data curation, formal analysis, methodology and statistical analysis. **RK and FS**: validation and visualisation. **WW**: funding acquisition, project administration and writing-review & editing. **HZ**: conceptualisation, data curation, formal analysis, methodology, funding acquisition, project administration, software, supervision and writing-review & editing. All authors analysed, reviewed and edited the manuscript's final version and approved it for publication.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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