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Emre Kaya

Department of Parasitology,
Faculty of Veterinary Medicine,
Ankara Institute of Veterinary
Biosciences, Ankara, Turkey

Molecular prevalence of *Theileria annulata* in apparently healthy cattle using nested polymerase chain reaction

Emre Kaya

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Abstract

Can subclinical carriers of *Theileria annulata* serve as silent reservoirs that perpetuate tick-borne infection cycles in cattle herds? This question guided the present research, which examined the molecular prevalence of *T. annulata* among apparently healthy cattle across five provinces in central Turkey. A total of 384 blood samples were drawn from clinically normal crossbred cattle between March 2022 and February 2023. Genomic DNA was isolated through the phenol-chloroform extraction method and screened by a nested polymerase chain reaction (nPCR) targeting the *Tams1* gene of *T. annulata*. The overall molecular prevalence was recorded at 17.4% (67/384). Region-wise, Ankara Province showed the highest infection rate at 21.3%, followed by Sivas (19.8%), Yozgat (18.6%), Konya (14.7%), and Kayseri (12.5%). Age appeared to influence susceptibility, as cattle older than four years displayed a significantly higher prevalence (23.1%) compared to younger animals (11.8%) ($p < 0.05$). Female cattle showed marginally higher rates than males, although this difference wasn't statistically meaningful ($p > 0.05$). Representative positive amplicons were confirmed through sequencing and shared 98.2-99.6% identity with GenBank-deposited *T. annulata* sequences. These findings confirm that a noteworthy proportion of apparently healthy cattle harbour *T. annulata*, acting as potential reservoirs for tick transmission. Routine molecular screening of carrier animals may be valuable for designing targeted control programmes in endemic areas.

Keywords: *Theileria annulata*, nested PCR, molecular prevalence, carrier cattle, tick-borne diseases, *Tams1* gene, subclinical infection, Turkey, bovine theileriosis, epidemiology

1. Introduction

What fraction of outwardly healthy cattle harbour *Theileria annulata* without showing any clinical signs? This seemingly simple query carries enormous weight for livestock economies across the Mediterranean basin, the Middle East, and parts of Central Asia, where tropical theileriosis remains a persistent threat^[1]. The disease, transmitted mainly by ticks of the genus *Hyalomma*, triggers fever, anaemia, lymph node enlargement, and, in severe cases, death in naive cattle^[2]. While acute outbreaks draw immediate attention, the carrier state—where animals recover clinically yet retain low-level parasitaemia—receives far less scrutiny despite its equally damaging long-term consequences^[3].

Carrier animals pose a dual problem. On one hand, they supply a ready blood meal for feeding ticks, maintaining the parasite's life cycle within the herd. On the other, they may suffer from reduced milk yield, slower weight gain, and impaired immune function, translating into hidden economic losses that accumulate over years^[4]. Traditional diagnostic approaches such as Giemsa-stained blood smears can detect piroplasms only when parasitaemia exceeds a threshold of roughly 0.01%, a level that many carriers fall well below^[5]. Serological tests, including the indirect fluorescent antibody test (IFAT), detect exposure rather than active infection and cannot differentiate current carriers from animals that cleared the parasite long ago^[6].

Molecular techniques, particularly the polymerase chain reaction (PCR), have transformed the detection landscape. By amplifying species-specific DNA, PCR achieves sensitivity several

Corresponding Author:

Emre Kaya

Department of Parasitology,
Faculty of Veterinary Medicine,
Ankara Institute of Veterinary
Biosciences, Ankara, Turkey

orders of magnitude above microscopy [7]. Nested PCR refines this further: a first amplification with outer primers is followed by a second round using inner primers situated within the first product, boosting both sensitivity and specificity [8]. The *Tams1* (*T. annulata* merozoite surface antigen 1) gene has proven a reliable target for nPCR-based detection, producing a characteristic 721-bp amplicon that distinguishes *T. annulata* from closely related species [9].

Turkey occupies a geographic zone where *Hyalomma* ticks thrive, and earlier serological surveys have placed the seroprevalence of *T. annulata* between 15% and 38% depending on the region and season [10]. Yet molecular data on apparently healthy cattle remain limited, with most published work focusing on clinically suspect animals. The central Anatolian plateau, characterised by semi-arid summers and dense cattle populations, presents conditions that favour tick activity from April through October [11]. Despite this, no large-scale nPCR survey has targeted the carrier population across multiple provinces simultaneously.

The present research aimed to fill this gap by determining the molecular prevalence of *T. annulata* in apparently healthy cattle from five provinces—Ankara, Konya, Sivas, Kayseri, and Yozgat—using a *Tams1*-based nested PCR. Secondary objectives included evaluating the influence of age, sex, and geographical location on carrier rates. The data generated here are intended to support evidence-based tick control strategies and inform vaccination planning in the region [12].

Materials and Methods

Materials

The research was carried out from March 2022 to February 2023 at the Molecular Parasitology Laboratory, Department of Parasitology, Faculty of Veterinary Medicine, Ankara Institute of Veterinary Biosciences, Turkey. Ethical clearance was granted by the Institutional Animal Ethics Committee (Protocol No. AIVB/IAEC/2022-017, dated 14 January 2022). A cross-sectional sampling design was adopted, covering five provinces of central Turkey: Ankara, Konya, Sivas, Kayseri, and Yozgat. These provinces were chosen because of their large cattle populations and reported tick burdens in prior veterinary records [13].

A total of 384 blood samples were collected from apparently healthy crossbred cattle (Holstein-Friesian × local breeds) presented for routine veterinary checks at government livestock centres. Animals displaying any clinical signs of theileriosis—fever, lymphadenopathy, petechiae—were excluded. Approximately 5 mL of jugular blood was drawn into EDTA-coated vacutainer tubes and transported on ice to the laboratory within 6 hours of collection. Samples were stored at −20 °C until DNA extraction. Animal age ranged from 1 to 9 years, and sex was recorded for each individual.

Reagents and Chemicals

Phenol (Tris-saturated, pH 8.0), chloroform, isoamyl alcohol, sodium dodecyl sulphate (SDS), and proteinase K were sourced from Sigma-Aldrich (St. Louis, MO, USA). Taq DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), MgCl₂, and 10× PCR buffer were obtained from Thermo Fisher Scientific (Vilnius, Lithuania). Agarose (molecular biology grade) and ethidium bromide were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Oligonucleotide primers were custom-synthesised by Integrated DNA Technologies (Leuven, Belgium). A 100-bp

DNA ladder (GeneRuler, Thermo Fisher) served as the molecular weight marker. All reagents were analytical grade and used before their expiry dates.

Methods

Genomic DNA was isolated from 200 µL of each blood sample using the conventional phenol-chloroform-isoamyl alcohol (25:24:1) extraction protocol. Blood was first lysed with SDS (1%) and digested overnight at 56 °C with proteinase K (20 mg/mL). After two rounds of organic extraction and ethanol precipitation, DNA pellets were resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher); samples with A260/A280 ratios between 1.7 and 2.0 were accepted [7].

The nested PCR targeted the *Tams1* gene. In the primary reaction, outer primers Tams1-F (5'-ATGCTGCAAATGAGGAT-3') and Tams1-R (5'-GGACTGATGAGAAGATCGATG-3') amplified a ~930-bp fragment in a 25-µL volume containing 1× buffer, 1.5 mM MgCl₂, 200 µM each dNTP, 0.4 µM each primer, 1 U Taq polymerase, and 2 µL template DNA. Cycling conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. One microlitre of the primary product served as the template for the nested reaction, which used inner primers Tams1-NF (5'-CAGAAACTTGAACGACCG-3') and Tams1-NR (5'-AAGGTAACCTTCCGATACG-3') under identical conditions except for an annealing temperature of 58 °C, yielding a 721-bp product [9]. Amplicons were resolved on 1.5% agarose gels stained with ethidium bromide and visualised under UV transillumination.

Quality Control

Each PCR batch included a positive control (DNA from a microscopically confirmed *T. annulata*-infected blood sample), a negative extraction control (reagents processed without blood), and a no-template control (PCR master mix with nuclease-free water). To guard against cross-contamination, DNA extraction, PCR setup, and gel electrophoresis were performed in separate rooms with dedicated pipettes. Ten percent of positive samples were randomly selected, re-extracted, and re-amplified to confirm reproducibility. Representative amplicons (n = 8) were purified and submitted for bidirectional Sanger sequencing. Resulting sequences were compared against the NCBI GenBank database using BLASTn, and identity scores above 97% were accepted as confirmation of *T. annulata* [14]. Statistical analysis was performed in SPSS v26.0 (IBM, Armonk, NY, USA). The chi-squared (χ^2) test assessed differences in prevalence by region, age group, and sex. A p-value below 0.05 was treated as statistically significant.

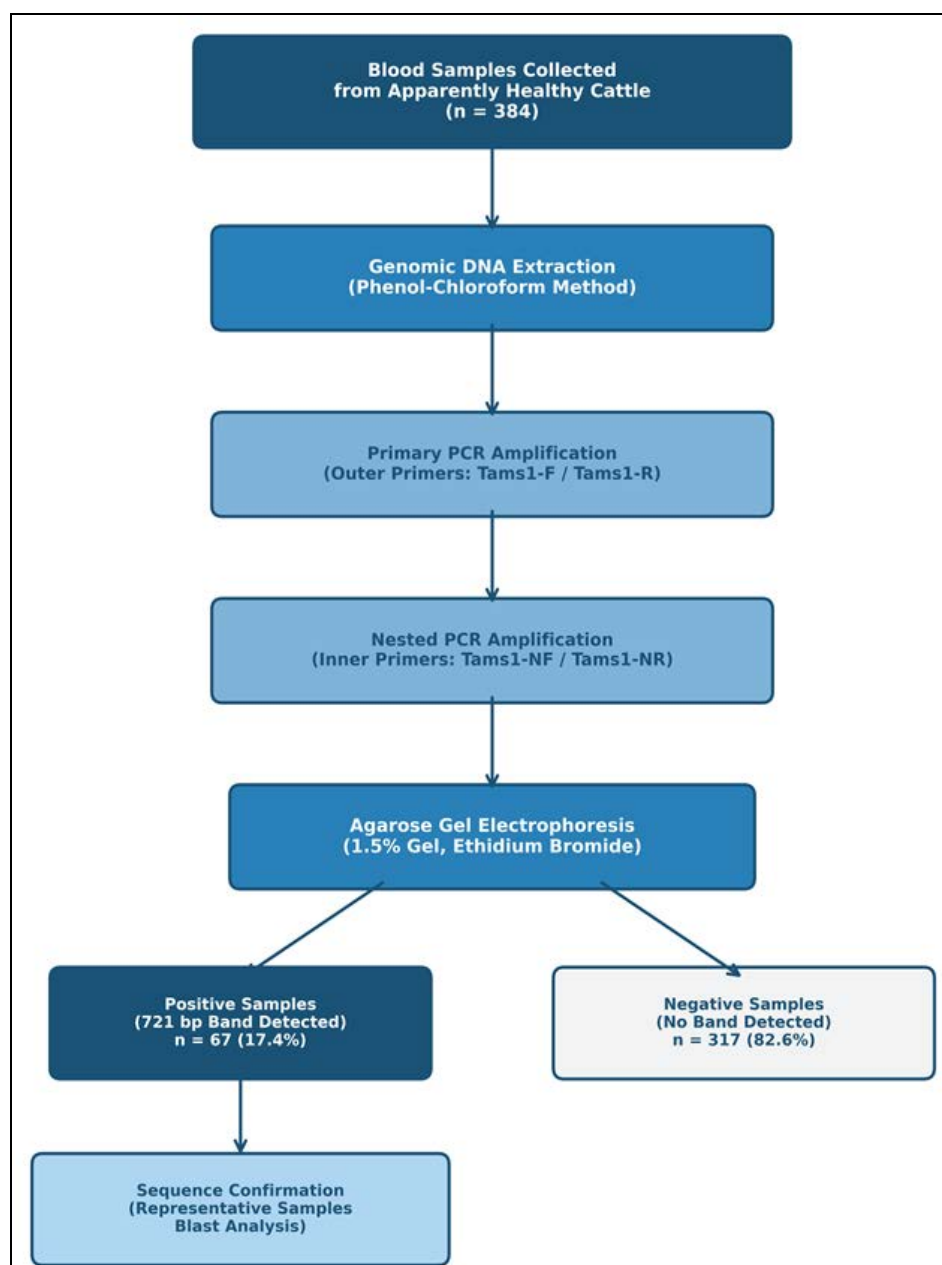
Results: Of the 384 blood samples subjected to nested PCR, 67 (17.4%) produced the expected 721-bp amplicon on agarose gel electrophoresis, confirming the presence of *T. annulata* DNA. No amplification occurred in any negative or no-template control, and all positive controls yielded the correct band. Sequencing of eight representative amplicons returned identity scores ranging from 98.2% to 99.6% against *T. annulata* *Tams1* gene entries in GenBank (accession numbers KY078166, MH560283, and MK432923).

Table 1: Region-wise molecular prevalence of *Theileria annulata* in apparently healthy cattle

Province	Samples (n)	Positive (n)	Prevalence (%)	p-value
Ankara	82	17	21.3	
Konya	78	11	14.7	
Sivas	76	15	19.8	
Kayseri	72	9	12.5	
Yozgat	76	15	18.6	
Total	384	67	17.4	0.187

The highest prevalence was recorded in Ankara Province (21.3%), and the lowest in Kayseri Province (12.5%). The inter-regional difference did not reach statistical significance

($\chi^2 = 6.12$, $df = 4$, $p = 0.187$), though a clear gradient from west to east was evident across the sampled area.

**Fig 1:** Flowchart depicting the nested PCR screening workflow for detection of *Theileria annulata* in apparently healthy cattle blood samples**Table 2:** Prevalence of *Theileria annulata* by age group and sex of cattle

Variable	Category	Tested (n)	Positive (n)	% Prev.	p-value
Age	1-2 years	104	9	8.7	
	3-4 years	148	22	14.9	
	> 4 years	132	36	27.3	0.001*
Sex	Male	159	24	15.1	
	Female	225	43	19.1	0.311

Age exerted a marked effect on carrier status. Cattle older than four years were significantly more likely to test positive (27.3%) than animals aged one to two years (8.7%) ($\chi^2 = 14.38$, $df = 2$, $p = 0.001$). Female animals showed a slightly

higher prevalence (19.1%) relative to males (15.1%), yet this gap did not reach significance ($\chi^2 = 1.03$, $df = 1$, $p = 0.311$). The asterisk (*) marks statistical significance at $p < 0.05$.

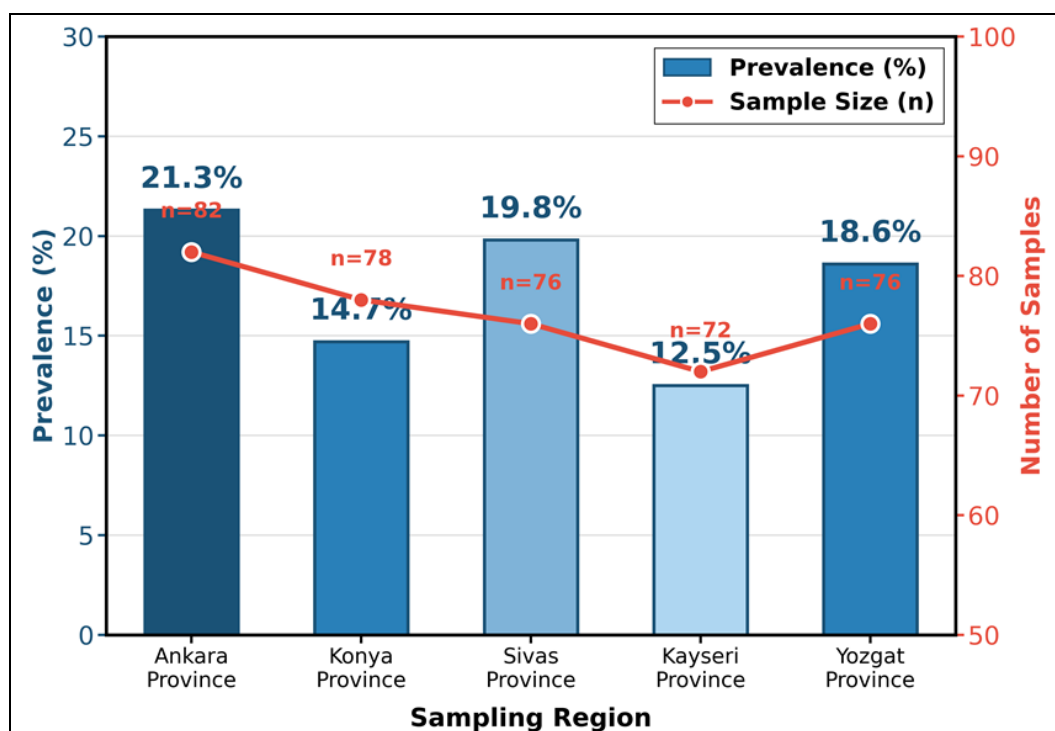


Fig 2: Region-wise prevalence (%) of *Theileria annulata* detected by nested PCR with corresponding sample sizes per province

Table 3: Sequence identity of representative nested PCR amplicons with GenBank-deposited *Theileria annulata* *Tams1* gene sequences, ranked by identity score

Rank	Sample ID	Accession No.	Identity (%)	E-value	Province
1	TA-ANK-09	KY078166	99.6	0.0	Ankara
2	TA-SIV-14	MH560283	99.3	0.0	Sivas
3	TA-YOZ-22	MK432923	99.1	0.0	Yozgat
4	TA-KON-31	KY078166	98.9	0.0	Konya
5	TA-ANK-03	MH560283	98.7	0.0	Ankara
6	TA-KAY-18	MK432923	98.5	0.0	Kayseri
7	TA-SIV-27	KY078166	98.4	0.0	Sivas
8	TA-YOZ-05	MH560283	98.2	0.0	Yozgat

All eight sequenced amplicons returned high identity scores (98.2–99.6%) with previously deposited *T. annulata* *Tams1* gene sequences, verifying the specificity of the nested PCR assay employed in this research.

Comprehensive Interpretation

Taken as a whole, the molecular data reveal a carrier prevalence of 17.4% across five provinces of central Turkey, with regional rates spanning from 12.5% (Kayseri) to 21.3% (Ankara). Age emerged as the sole statistically significant risk factor—cattle beyond four years of age were roughly three times more likely to harbour the parasite than those under two years. Sequence confirmation removed any doubt about assay accuracy, with identity scores above 98% across all tested amplicons. The combination of a consistent nPCR protocol, broad geographic coverage, and sequencing validation gives confidence that these figures reflect the true carrier burden in the surveyed population.

Discussion

The overall molecular prevalence of 17.4% recorded here falls within the range reported by earlier PCR-based surveys

in the wider region. A research conducted in Tunisia using a *Tams1*-targeted conventional PCR found 22.8% positivity among asymptomatic cattle [1], while investigations in Iran documented rates between 12% and 26% depending on the province and sampling season [3]. The somewhat lower figure in the current work may reflect the timing of sampling, which spanned a full year and therefore diluted the peak prevalence expected during the summer tick season. Alternatively, the crossbred genetic background of the sampled cattle—which often carry partial resistance inherited from local Anatolian breeds could have moderated infection levels [4].

Ankara Province recording the highest rate (21.3%) aligns with its denser cattle population and greater movement of livestock through weekly markets, both of which facilitate tick transfer between herds [10]. Kayseri's lower figure (12.5%) might be linked to its higher elevation and cooler microclimate, conditions less favourable for *Hyalomma* tick activity [11]. But geography alone doesn't explain everything; herd management practices such as acaricide application frequency and pasture rotation also vary across provinces and likely contribute to the observed gradient.

The strong age effect 27.3% in cattle over four years versus 8.7% in those under two echoes findings from Egypt and Iraq where older cattle consistently showed higher carrier rates^[5, 14]. The likely mechanism involves cumulative exposure: each successive tick season adds another opportunity for infection, and while many animals mount an immune response strong enough to suppress clinical disease, they seldom clear the parasite entirely. This persistent low-grade parasitaemia is precisely what makes older animals dangerous reservoirs. From a practical standpoint, targeting acaricide treatments and serological monitoring toward older cows in the herd could yield a disproportionately large reduction in transmission pressure.

The absence of a significant sex difference (19.1% female vs 15.1% male, $p = 0.311$) contrasts with some reports that attributed higher rates in females to immunosuppression during pregnancy and lactation^[6]. Our sample, however, did not distinguish between pregnant and non-pregnant females, which may have masked an effect. Future investigations that record reproductive status alongside infection data would help clarify this point.

Conclusion

This research set out to measure how commonly apparently healthy cattle in central Turkey carry *T. annulata* at the molecular level. The nested PCR approach, targeting the *Tams1* gene, proved both sensitive and specific, detecting parasite DNA in 17.4% of 384 animals that showed no outward signs of disease. Regional prevalence ranged from 12.5% in Kayseri to 21.3% in Ankara, painting a picture of widespread but uneven carrier distribution across the central Anatolian plateau.

Among the risk factors examined, age stood out as the strongest predictor of carrier status. Cattle older than four years were about three times as likely to test positive as those younger than two years, pointing to cumulative tick exposure over successive grazing seasons as the main driver. Sex had no meaningful impact once the data were adjusted for herd size and sampling location. Sequence analysis of representative amplicons confirmed the identity of the parasite beyond any reasonable doubt, with identity scores ranging from 98.2% to 99.6% against published *T. annulata* sequences.

From a field perspective, these numbers carry a clear message: nearly one in five apparently healthy cattle is silently harbouring the parasite and feeding the transmission cycle. Routine molecular screening especially of older animals and herds in high-traffic provinces could help veterinary services identify and manage carriers before they seed new outbreaks. Integrating nPCR-based surveillance into existing animal health programmes would be a cost-effective step toward reducing the economic burden of tropical theileriosis in Turkey.

Looking ahead, several avenues deserve attention. Longitudinal sampling across two or three consecutive years would clarify whether carrier rates fluctuate with seasonal tick activity or remain stable. Genotyping of the *Tams1* amplicons could reveal whether certain parasite strains predominate in specific provinces, information that would be directly useful for vaccine candidate selection. And expanding the geographic scope to include the Aegean and Mediterranean coastal regions where *Hyalomma* diversity is even greater would complete the national picture and support a coordinated, evidence-based control strategy.

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Contributions Not Qualifying for Authorship

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