

Field evaluation of an in-house schizont antigen-based ELISA and PCR for detection of *Theileria annulata* antibodies and active infection in vaccinated and non-vaccinated cattle in the Miankaleh Peninsula, Iran

Gholamreza Habibi¹, Mohammad Hossein Fallah Mehrabadi², Asghar Afshari¹, Amin Shahedi¹, Biyouk Agha Tanha¹, Qader Khezrian³, Saeid Fathi^{1*}

¹Department of Parasite Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

² Department of Epidemiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

³Large Animal Veterinarian, Private section, Qazvin, Iran

Correspond author: Saeid Fathi: Saeidfathi2012@gmail.com. Parasite Vaccine Research and Production

Department of Razi Institute, Hesarak, Karaj, IR Iran,

P.O. Box: 31975/148,

Postal Code: 31987

Tel.: 026 3405 0400 (3586),

Fax: 026 3455 2194,

Gholamreza Habibi: DVM, PhD. Dept. of Parasite Vaccine Research and Production of Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran.

<https://orcid.org/0000.0003.0052.4608>

Asghar Afshari: PhD in Parasitology. Dept. of Parasite Vaccine Research and Production of Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran.

<https://orcid.org/0000-0002-7161-5681>

Amin Shahedi: MS in Genetic. Dept. of Parasite Vaccine Research and Production of Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran

Biyouk Agha Tanha: Department of Parasite Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
Qader Khezrian: Large Animal Veterinarian, Private section, Qazvin, Iran.

**Saeid Fathi: PhD in Parasitology. Dept. of Parasite Vaccine Research and Production of Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran.*

<https://orcid.org/0000.0002.9794.2356>

ABSTRACT

Introduction: Tropical theileriosis caused by *Theileria annulata* is a major cattle disease in hyperendemic regions such as the Miankaleh Peninsula. This study evaluated molecular and serological diagnostic methods in vaccinated and non-vaccinated cattle.

Materials and Methods: Blood samples from 28 vaccinated and 29 non-vaccinated cattle were analyzed using PCR targeting the *Tams-1* gene and schizont antigen-based indirect ELISA (iELISA). Diagnostic performance and agreement between methods were assessed.

Results: The iELISA showed high sensitivity but moderate specificity, likely due to cross-reactivity. PCR detected active *T. annulata* infection in 18/28 vaccinated cattle (64.3%) and 14/29 non-vaccinated cattle (48.3%), with no significant difference between groups (RR = 1.33; 95% CI: 0.82–2.16; P = 0.23). Antibodies were detected by iELISA in 23 vaccinated cattle (82.1%) and 21 non-vaccinated cattle (72.4%), with no significant difference between groups (RR = 1.13; 95% CI: 0.84–1.54; P = 0.40). Agreement between PCR and iELISA was moderate (κ = 0.55), with most discordant results being PCR-negative/iELISA-positive, consistent with previous exposure, vaccination, or antibody persistence. Dot-blot ELISA showed diagnostic performance comparable to iELISA while being simpler and more cost-effective.

Conclusion: The in-house schizont-based iELISA, combined with PCR, is a useful approach for surveillance of *T. annulata* in endemic areas. Frequent PCR positivity in clinically healthy, including vaccinated, cattle indicates continued parasite circulation with subclinical infection, supporting the integration of vaccination with molecular surveillance.

Keywords: *Theileria annulata*; Indirect Enzyme-linked immunosorbent assays (iELISAs); Cattle; Miankaleh Peninsula; Iran

1. Introduction

Bovine theileriosis, caused by the protozoan parasite *Theileria annulata*, is a significant disease affecting cattle worldwide [1]. Tropical theileriosis is a tick-borne disease transmitted primarily by *Hyalomma* and *Rhipicephalus* species [2]. It poses a substantial risk, particularly to imported purebred cattle [3]. Globally, approximately 250 million cattle are at risk of exposure to this disease [4]. In Iran, Theileriosis represents a considerable threat to livestock health and productivity [5].

Native cattle (*Bos indicus*) exhibit lower susceptibility to the disease, whereas exotic and cross-bred cattle (*Bos taurus*) are more vulnerable to tropical theileriosis. Transplacental transmission in neonatal calves has also been reported, often leading to severe clinical outcomes [6,7]. Several factors influence the occurrence and control of the disease, including cattle breed, age, tick population density, presence of active ticks on the animals, and management practices [8].

Diagnosis of *T. annulata* infection primarily relies on microscopy, molecular methods, and serological assays, each presenting distinct advantages and limitations. Historically, clinical signs and microscopic examination of stained blood smears have been used to diagnose acute Theileriosis. However, detection of long-standing carrier cattle with low parasitemia is challenging, complicating efforts to control transmission, especially when such animals are relocated to non-endemic regions [9]. Although blood smear microscopy is straightforward and effective for acute infections, it has limited sensitivity in detecting carrier animals due to their low parasitemia levels [10]. Notably, carrier animals can be identified by elevated antibody titers [11].

Molecular diagnostics, particularly polymerase chain reaction (PCR), provide highly sensitive and specific detection of *Theileria* in both acute and carrier stages, in cattle and ticks alike [12]. However, PCR requires expensive reagents and well-equipped laboratories, limiting its feasibility for large-scale epidemiological surveys. In contrast, serological tests are less costly, easier to perform, reproducible, and suitable for identifying carrier animals, making them ideal for extensive epidemiological studies [6]. The significantly elevated antibody titers observed during the later phases of infection enhance their utility [13]. Functional antibody activity has also been demonstrated, with immune sera and monoclonal antibodies able to neutralize sporozoites and reduce infectivity [14,15]. Antibodies against free merozoites appear following primary infection [16], and exhibit limited reactivity with infected erythrocytes or leukocyte surfaces [17].

Various antigens, including crude antigen extracts and stage-specific recombinant antigens, have been used to develop enzyme-linked immunosorbent assays (ELISAs) for the detection of *T. annulata* [13,18,19]. Serosurveillance employing a range of serological assays, such as indirect fluorescent antibody tests (IFAT), indirect ELISA (iELISA), dot-ELISA, and competitive ELISA (cELISA) has been extensively documented and routinely applied in epidemiological studies worldwide [13,18,19].

Given the challenges of detecting carrier animals and the limitations of existing diagnostic methods, this study aims to evaluate the performance of an in-house iELISA alongside PCR for the serodiagnosis and detection of *T. annulata* in both vaccinated and non-vaccinated cattle in Miankaleh, Iran. Through comparative analysis, we seek to provide insights into the diagnostic reliability, feasibility, and applicability of these methods for effective disease surveillance and control in endemic regions.

2. Materials and Methods

2.1 Study design

This study was conducted in the Miankaleh Peninsula, Mazandaran Province, Iran. The region's ecological characteristics provide favorable conditions for the occurrence of theileriosis epizootics. The Miankaleh Peninsula is influenced by a humid coastal climate with moderate seasonal variation, supporting wetlands, lagoons, sand dunes, and extensive pasturelands. These environments create suitable habitats for livestock and wildlife, while also sustaining large populations of *Hyalomma* ticks, the primary vectors of *T. annulata*.

2.2 Sampling

The study used 57 cattle from the Miankaleh peninsula, Mazandaran, Iran, comprising 28 animals vaccinated with the Razi Institute's live-attenuated *Theileria annulata* schizont vaccine (administered subcutaneously) and 29 age- and breed-matched non-vaccinated controls.

All vaccinated cattle were sampled one year after their final booster dose to evaluate the persistence of vaccine-induced antibody responses, while non-vaccinated control cattle were sampled concurrently during the seasonal peak of tick activity (May–July 2023) to ensure comparable exposure risk. Blood samples were collected via aseptic jugular venipuncture using sterile 18-gauge needles, with 5 mL aliquoted into EDTA tubes for PCR analysis and 5 mL into plain tubes for serum separation. EDTA samples were maintained at 4°C and processed within 24 hours for DNA extraction, while plain tubes were allowed to clot at room temperature for 1 hour before centrifugation at $3,000 \times g$ for 10 minutes to isolate serum, which was aliquoted and stored at -20°C until iELISA evaluation.

Strict inclusion criteria were applied: all animals were 1–4 years old (representing the demographic of peak disease susceptibility), showed no clinical signs of acute theileriosis at sampling, and had received no antiprotozoal treatments within the preceding 30 days. Non-vaccinated controls were verified through veterinary records and ear tag identification to confirm absence of prior vaccination. All sampling and handling procedures on animals comply with WOA animal welfare standards

2.3 Antigen preparation

2.4 Preparation of *T. annulata* cellular schizont antigen

The *T. annulata* (S15 vaccine strain) cellular schizont antigen was prepared from a monocyte and B-lymphocyte cell line persistently infected with the attenuated schizonts (Figure 1). The cells were cultured in modified Stoker's medium supplemented with 10% inactivated bovine serum and antibiotics at 37°C in a normal atmospheric incubator.

Cells were harvested at the late-log phase of growth, washed three times with sterile physiological saline, and concentrated to 25 million cells per mL in saline. A protease inhibitor (EDTA, 10 mg/mL) was added at 2 µL per mL of cell concentrate. The cell suspension was then sonicated on ice with 20 cycles of 20-second pulses, interspersed with 20-second rest intervals.

The sonicated antigen was aliquoted (2 mL) into 8 mL vials and lyophilized. The lyophilized vials were sealed with metal caps and stored at 4°C for short-term or -20°C for long-term preservation.

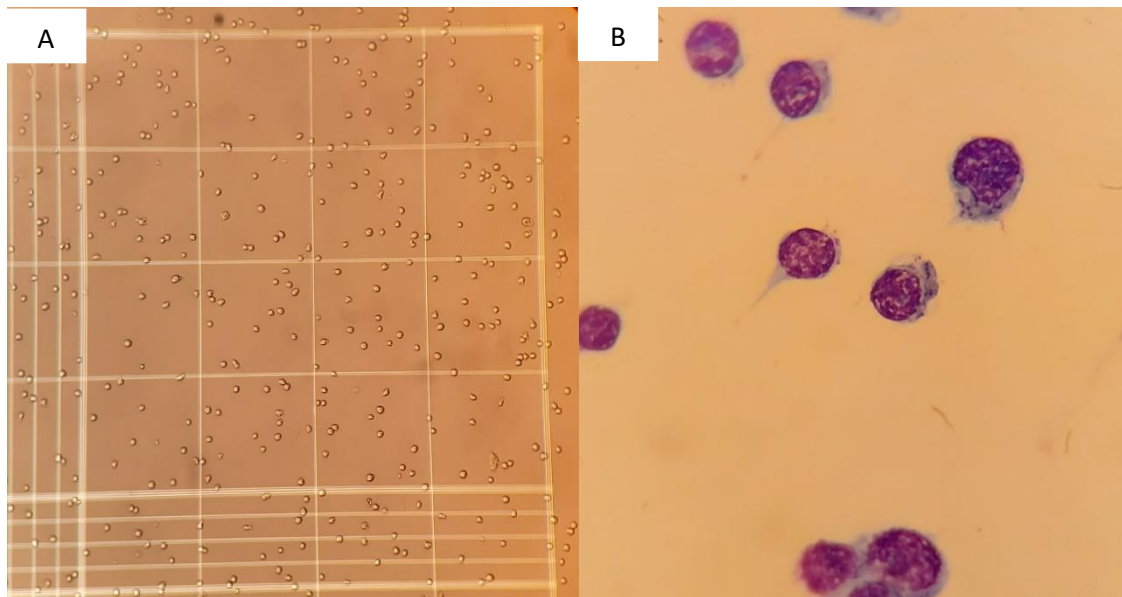


Figure 1: Morphology of *T. annulata* schizont-infected cells. (A) Attenuated vaccine strain cells observed under a Neubauer counting chamber (*in vitro* passage). (B) Schizont-infected lymphocytes from vaccine strain cells, exhibiting large, actively proliferating host cells with clearly identifiable schizont stages within the cytoplasm.

2.5 PCR

PCR targeting the Tams-1 gene was selected as the reference method for field detection of *T. annulata* infection because it directly detects parasite DNA and reflects active infection status. In this regard, genomic DNA was extracted using the proteinase K method, following the established protocol. Cell pellets were lysed with proteinase K, proteins were precipitated, and DNA was precipitated with cold ethanol, washed in 70% ethanol, dried, and dissolved in distilled water [20].

PCR amplification was performed using primers targeting the Tams-1 and Cytochrome b genes of *T. annulata*, with bovine GAPDH serving as an internal control (Table 1). The 25 μ L reaction mixture contained 12.5 μ L of 2 $\times$ Master Mix, 1 μ L of each primer (10 pmol/ μ L), 2 μ L of DNA template, and 8.5 μ L of nuclease-free water. Amplification conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were visualized on 1.5-2.5% agarose gels stained with redSafe. Each PCR run included a positive control containing *T. annulata* DNA, a negative control (DNA extracted from a confirmed uninfected animal), and a no-template control (nuclease-free water) to monitor assay performance and detect potential contamination. The results were visualized using an UVIDoc imaging system and recorded for comparison.

Table 1: Primer sets used in the present study

Primer	Sequence (5' - 3')	Expected product size (bp)	Reference
Cytochrome b	F: ATGTGCCAGCAAAAGGTATGG R: AAACCTCCCCTCCACTAAGCG	990	<i>T. annulata</i> cytochrome b (Tcytb) M63015
Tams-1	F: GAGACAAGGAATATTCTGAGT CC R: TTAAGTGGCATATAATGACTT AAGC	597	<i>T. annulata</i> merozoite surface glycoprotein Tams1 gene, U22888
Bovine GAPDH	F: CCA TCA CCA TCT TCC AGG AGC R: TCA TAA GTC CCT CCA CGA TGC	309	<i>Bos taurus</i> mRNA for glyceraldehyde dehydrogenase AJ000039

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174 2.6 Optimization and validation of the iELISA

175 2.6.1 Checkerboard titration

176 A checkerboard was applied to optimize the conditions of the assay by testing various
177 concentrations of antigen, positive serum, and conjugate. The optimization process started with
178 checkerboard titrations of antigens, tested against a reference positive serum and a negative
179 control.

180 2.6.2 iELISA

181 A reference serum panel comprising 62 bovine serum samples (41 *T. annulata*-positive sera and
182 21 negative sera) was used for iELISA optimization and diagnostic performance evaluation.

183 Native *T. annulata* schizont antigen was coated onto 96-well microplates at different dilutions
184 (1:100, 1:250, 1:500, and 1:1000) and incubated overnight at 4°C. Plates were washed three times
185 with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and blocked with 5% skim
186 milk in PBS-T for 1 h at 37°C. Positive and negative sera were evaluated at serial dilutions (1:100,
187 1:200, 1:400, and 1:800) to determine the optimal serum dilution. Following incubation for 1 h at
188 37°C, the plates were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit
189 anti-bovine IgG at dilutions of 1:10,000, 1:15,000, and 1:25,000 for 1 h at 37°C. After a final
190 washing step, tetramethylbenzidine (TMB) substrate was added, and the reaction was stopped with
191 2 M sulfuric acid. Absorbance was measured at 450 nm using a microplate reader.

192 Based on checkerboard titration, the optimal assay conditions were determined as an antigen
193 dilution of 1:500, a serum dilution of 1:200, and an HRP-conjugated rabbit anti-bovine IgG
194 dilution of 1:25,000.

195 The diagnostic cut-off value was subsequently determined by receiver operating characteristic
196 (ROC) curve analysis using OD450 values obtained from a reference serum panel comprising

confirmed positive and negative sera. Receiver operating characteristic (ROC) curve analysis was performed in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) using the OD450 values obtained from the reference serum panel. The area under the ROC curve (AUC), the optimal cut-off value, and the corresponding sensitivity and specificity were obtained from the ROC analysis. The diagnostic performance of the iELISA was further evaluated using MedCalc diagnostic test evaluation tools by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR). Based on this method, the optimal cut-off value was determined to be an OD of 0.475. At this cutoff, the assay achieved a sensitivity of 92.7% and a specificity of 76.2%. The PPV was 88.4%, while the NPV was 84.2%.

Approximately 24% of negative sera were falsely detected as positive (5 out of 21; 23.8%), which explains the relatively lower specificity (76.2%). Among all iELISA-positive results ($n = 43$), 38 were true positives (~88%), and 5 were false positives (~12%). The positive likelihood ratio (PLR) was 3.89, suggesting that a positive result was nearly four times more likely in infected cattle than in non-infected cattle, while the negative likelihood ratio (NLR) was 0.096, demonstrating that a negative result strongly rules out infection.

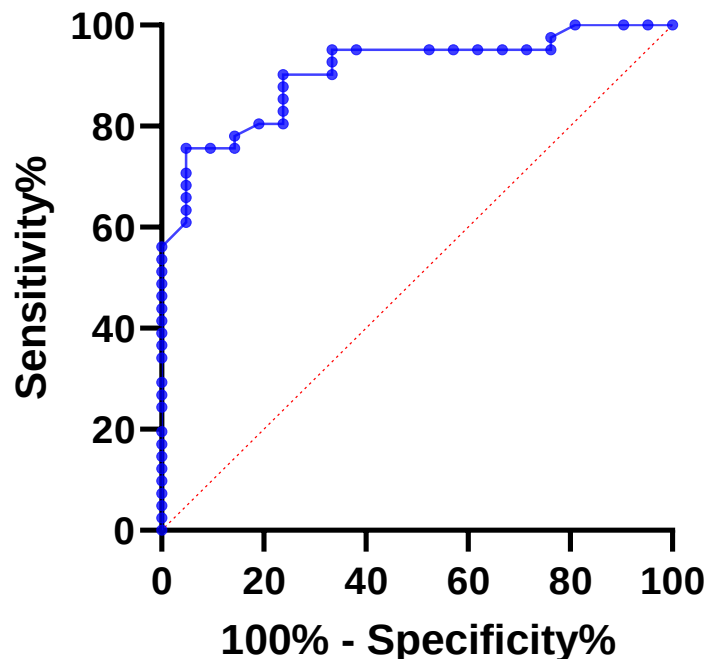


Figure 2: The ROC curve evaluated the in-house iELISA using native *T. annulata* schizont antigen. The curve rises sharply toward the top-left, with an AUC ~0.90, indicating high sensitivity at most specificity levels. A cut-off at OD 0.475 achieves ~92–93% sensitivity and ~76% specificity

2.7 Optimization and validation of the Dot-blot ELISA

The dot-blot ELISA was standardized using a reference panel comprising 41 confirmed *T. annulata*-positive bovine sera and 21 negative sera. Cellular *T. annulata* schizont antigen was spotted onto nitrocellulose membranes and allowed to air-dry at room temperature. The membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) for 1 h. To optimize the assay, positive sera were tested at serial dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200), while negative sera were included to assess background reactivity and specificity. After incubation with the primary sera, membranes were washed with PBS-T and incubated with HRP-conjugated rabbit anti-bovine IgG (1:25,000) for 1 h at room temperature. Immunoreactive spots were visualized using 3,3'-diaminobenzidine (DAB) and hydrogen peroxide substrate. The reaction was terminated by rinsing the membranes with distilled water. Based on signal intensity and discrimination between positive and negative sera, the optimal assay conditions were determined as an antigen dilution of 1:250 and a serum dilution of 1:400, which were subsequently used for testing field samples.

Dot-blot ELISA for filed samples

Following optimization, the dot-blot ELISA was applied to serum samples collected from 28 vaccinated and 29 non-vaccinated cattle from the Miankaleh Peninsula. Cellular *T. annulata* schizont antigen (1:250 dilution) was spotted onto nitrocellulose membranes and allowed to air-dry. Membranes were blocked with 5% skim milk in PBS containing 0.05% Tween-20 (PBS-T) for 1 h at room temperature. Serum samples were diluted 1:400 in PBS-T containing 1% skim milk and incubated with the antigen-spotted membranes for 1 h at room temperature. After washing three times with PBS-T, HRP-conjugated rabbit anti-bovine IgG (1:25,000) was added and incubated for 1 h. Bound antibodies were visualized using DAB and hydrogen peroxide substrate. The reaction was terminated by rinsing the membranes with distilled water, and the membranes were air-dried before visual interpretation. Positive samples developed distinct brown-colored spots, whereas negative samples showed no color development. Positive and negative control sera were included in each assay. Dot-blot ELISA results were compared with the corresponding iELISA and PCR findings to evaluate diagnostic agreement and field performance.

2.8 iELISA for filed samples

The *T. annulata* schizont lysate antigen, prepared from the Razi strain, was coated onto polystyrene flat-bottom microtiter plates (1:500 dilution) carbonate-bicarbonate buffer (pH 9.6), and incubated overnight at 4°C in a humid chamber. Plates were washed three times with PBS-Tween (PBS-T) and blocked with 5% skim milk in PBS-T for overnight at 4°C.

Diluted sera (1:200) from 28 vaccinated animals and 29 non-vaccinated controls were added (100 µL/well) and incubated for 1 hour at 37°C. After washing five times with PBS-T, Horseradish peroxidase (HRP)-conjugated anti-bovine IgG secondary antibody (diluted 1:25,000) was applied and incubated for 1 hour at 37°C, followed by seven washes.

TMB substrate (100 µL) was added for 15 minutes in the dark; the reaction was stopped with 100 µL of 1 N sulfuric acid. Optical density was read at 450 nm using a microplate reader (TECAN Austria A-5082).

2.9 Data analysis

Data were analyzed using GraphPad Prism version 8 for Windows and online MedCalc diagnostic tools (https://www.medcalc.org/en/calc/diagnostic_test.php). Receiver operating characteristic (ROC) curve analysis was performed in GraphPad Prism using the OD450 values obtained from the reference serum panel (41 confirmed *T. annulata*-positive and 21 negative sera). The AUC, the optimal cut-off value, and the corresponding sensitivity and specificity were obtained from the ROC analysis.

Using PCR as the reference method, the diagnostic performance of the iELISA was evaluated by calculating sensitivity, specificity, PPV, NPV, PLR, and NLR with the MedCalc diagnostic test evaluation tool (https://www.medcalc.org/calc/diagnostic_test.php).

Differences in PCR and iELISA positivity between vaccinated and non-vaccinated cattle were assessed using Pearson's Chi-square test. Risk ratios (RRs) with 95% confidence intervals (CIs) were calculated, and a two-tailed P value <0.05 was considered statistically significant. Agreement between PCR and iELISA results was assessed by calculating the overall percent agreement and Cohen's kappa coefficient (κ) (<https://www.graphpad.com/quickcalcs/kappa1/>). Kappa values were interpreted according to the Landis and Koch classification: <0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and >0.80, almost perfect agreement. All statistical tests were two-tailed, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 PCR detection of *T. annulata*

PCR analysis revealed *T. annulata* DNA in 64.3% (18/28; 95% CI: 44.1–81.4) of vaccinated cattle and 48.3% (14/29; 95% CI: 29.4–67.5) of non-vaccinated animals (Figure 3). Although the proportion was numerically higher in vaccinated animals, the difference was not statistically significant (RR = 1.33; 95% CI: 0.82–2.16; $P = 0.23$). All positive samples produced clear amplification of the expected 597 bp *Tams-1* fragment.

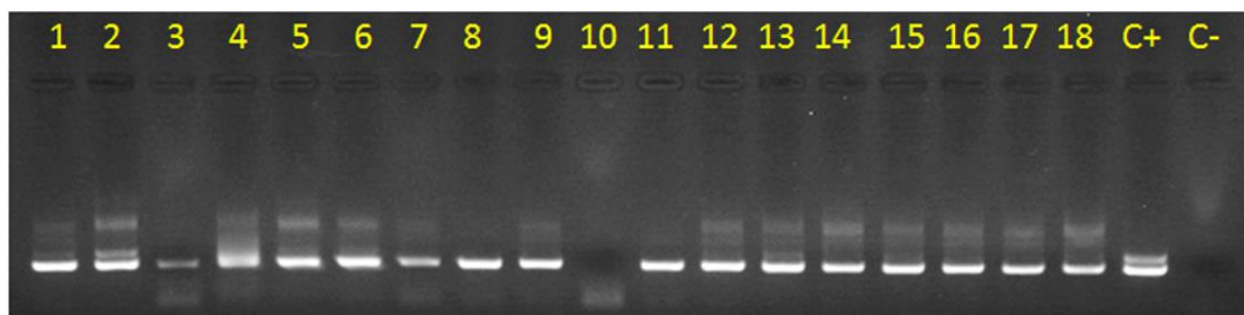


Figure 3. Gel electrophoresis of Tams1 gene PCR product was conducted on DNA samples isolated from cattle in Miankaleh. The samples numbered 1 to 18, all showed positive results, except for sample number 10. Lanes including both positive and negative controls are presented as C+ and C-.

3.2 iELISA seropositivity

iELISA demonstrated similar seropositivity between groups, with 82.1% (23/28; 95% CI: 63.1–93.9) of vaccinated and 72.4% (21/29; 95% CI: 52.8–87.3) of non-vaccinated cattle (RR = 1.13; 95% CI: 0.84–1.54; P = 0.40).

3.3 Concordance analysis between PCR and iELISA

To assess the level of agreement between PCR and iELISA, results were compared in both vaccinated and non-vaccinated groups (Table 2).

In the vaccinated group (n = 28), the majority of samples were double positive (PCR+/iELISA+, 18/28). No cases were detected as PCR positive but iELISA negative. Five samples were PCR negative yet iELISA positive, and another five were negative by both methods.

In the non-vaccinated group (n = 29), a similar pattern was observed. Fourteen animals were positive by both PCR and iELISA, while none were PCR positive, but iELISA negative. Seven samples tested iELISA positive despite being PCR negative, and eight were consistently negative in both assays.

When the two groups were analyzed together, 78.9% (45/57) of samples showed concordant results, being either double positive or double negative. In contrast, 21.1% (12/57) of cases were PCR-/iELISA+, highlighting instances where iELISA detected antibodies in the absence of PCR-detectable infection. Importantly, no samples were identified as PCR+/iELISA-, suggesting that iELISA did not fail to detect any PCR-confirmed infections.

The overall agreement between PCR and iELISA was 78.9% (45/57). Concordance analysis using Cohen's kappa yielded a coefficient of $\kappa = 0.55$ (95% CI: 0.33–0.75), which indicates moderate agreement between the two diagnostic methods.

Table 2. Concordance analysis between PCR and iELISA results in vaccinated and non-vaccinated cattle (n = 57).

Group	PCR+ / iELISA+	PCR+ / iELISA-	PCR- / iELISA+	PCR- / iELISA-	Total	PCR Positivity % (95% CI)	iELISA Positivity % (95% CI)	Discordant PCR-/iELISA+ % (95% CI)
Vaccinated (n=28)	18 (64.3%)	0 (0%)	5 (17.9%)	5 (17.9%)	28	64.3 (44.1– 81.4)	82.1 (63.1– 93.9)	17.9 (6.1–36.9)
Non- vaccinated (n=29)	14 (48.3%)	0 (0%)	7 (24.1%)	8 (27.6%)	29	48.3 (29.4– 67.5)	72.4 (52.8– 87.3)	24.1 (10.3–43.5)
Total (n=57)	32 (56.1%)	0 (0%)	12 (21.1%)	13 (22.8%)	57	32 (56.1%; 95% CI: 43.3– 68.2)	44 (77.2%; 95% CI: 64.8– 86.2)	21.1% (12/57; 95% CI: 12.5– 33.3)

3.4 Discordant results

Discordant PCR-/iELISA+ cases, consistent with seropositivity without detectable parasitemia, occurred in 17.9% (5/28) of vaccinated and 24.1% (7/29) of non-vaccinated cattle (RR = 0.74; 95% CI: 0.26–2.13; P = 0.58). Furthermore, iELISA positivity rates were comparable between vaccinated and non-vaccinated groups (82.1% vs. 72.4%, P = 0.40), indicating no significant difference in antibody presence between the two groups. Summary of diagnostic findings are present in Table 3.

Table 3. Summary of diagnostic findings in vaccinated and non-vaccinated cattle

Parameter	Vaccinated (n=28)	Non-vaccinated (n=29)	Risk Ratio (95% CI)	P-value
PCR positivity	64.3% (18/28; 44.1–81.4)	48.3% (14/29; 29.4–67.5)	1.33 (0.82–2.16)	0.23
iELISA positivity	82.1% (23/28; 63.1–93.9)	72.4% (21/29; 52.8–87.3)	1.13 (0.84–1.54)	0.40
Concordant PCR–iELISA	82.1% (23/28)	75.9% (22/29)	—	—
Discordant PCR–/iELISA+	17.9% (5/28)	24.1% (7/29)	0.74 (0.26–2.13)	0.58
Overall PCR–iELISA agreement	78.9% (45/57) ; κ = 0.55 (CI:0.33–0.75)			

3.5 Dot-blot ELISA

The dot-blot ELISA demonstrated comparable antibody detection patterns to the iELISA among the 57 field serum samples collected from vaccinated and non-vaccinated cattle. The assay showed consistent classification of seropositive and seronegative samples with the iELISA results, suggesting its potential application as a simplified serological screening method (Figure 4).

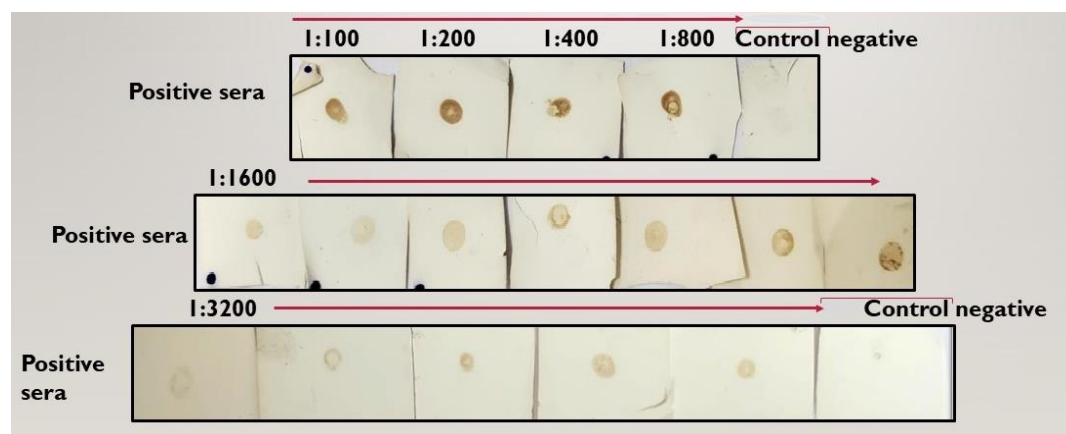


Figure 4: Dot-blot ELISA results showing antibody reactivity at different serum concentrations

4. Discussion

This study evaluates the performance of PCR and an iELISA for detecting *T. annulata* in vaccinated and non-vaccinated cattle in the endemic Miankaleh region. Our findings provide critical insights into the utility and limitations of molecular and serological tools for the surveillance of bovine theileriosis.

In this study, the iELISA using cellular *T. annulata* schizont antigen showed a sensitivity of 92.7% and a specificity of 76.2% under laboratory setup, suggesting strong screening ability but a considerable chance of false-positives. This is in line with prior reports that crude native antigens often exhibit cross-reactivity and batch variability. It likely reflects a (i) native-antigen heterogeneity or inherent limitations of the native antigen used, and (ii) cross-reactivity (e.g., *Babesia* spp.) [21,22].

Positive and negative predictive values (PPV and NPV) are intrinsically influenced by disease prevalence. While the sensitivity and specificity of a diagnostic test are generally independent of prevalence, higher prevalence tends to increase the PPV and decrease the NPV [23]. The positive predictive value (88.4%) and negative predictive value (84.2%) reflect that while the assay is generally useful for serodiagnosis, results should be interpreted with caution, particularly in populations with lower disease prevalence or in cases where false positives could impact clinical or epidemiological conclusions.

LRs are considered one of the most informative measures of diagnostic test performance [24]. In this study, the PLR of 3.89 indicates a small to moderate increase in the probability of *T. annulata* infection when the iELISA test is positive, while the NLR of 0.096 reflects a substantial decrease in the probability of infection when the test is negative.

Comparative studies of native antigens (cellular/soluble schizont, soluble piroplasm) showed that piroplasm antigen elicits the highest antibody titers, followed by cellular and soluble schizont

antigens, with cellular schizont improving ELISA specificity and sensitivity relative to soluble schizont [22,25]. Recombinant Tams1 antigen produced positive responses in fewer samples (29 samples) compared with soluble piroplasm antigen (34 samples), and notably, the soluble piroplasm antigen cross-reacted with sera from *B. Bigemina*-infected cattle, highlighting its lower specificity [21]. However, the use of crude parasite antigens in ELISA faced major hurdles: high batch variability, lower diagnostic performance compared with IFAT, and quality and stability concerns [26,27].

In contrast, recombinant antigens (TaSP, Tams1) consistently improve specificity while maintaining high sensitivity [18,26,28]. These findings collectively suggest that, while native antigen iELISAs are valuable for surveillance, assays based on recombinant proteins significantly enhance specificity and diagnostic precision. This is demonstrated by established ELISAs for *T. annulata*, including Tams1-based ELISAs [26] and r-TaSP-based indirect ELISAs/Dot-ELISAs [13,18]. Therefore, we recommend using native iELISA as a high-sensitivity screening tool, supplemented by confirmatory PCR or transitioning to a recombinant antigen platform for improved specificity in diagnostic workflows.

PCR detected active *T. annulata* infection in 64.3% of vaccinated and 48.3% of non-vaccinated cattle, but this difference was not statistically significant. The overall infection rate across both groups was 56.1%, indicating more than half of all cattle carried active infection regardless of vaccination status.

The detection of *T. annulata* DNA in vaccinated cattle does not necessarily indicate vaccine failure but may reflect natural field exposure, persistent carrier status, or low-level parasitemia despite protection against clinical disease. In our study, PCR-positive cattle exhibited no clinical signs, consistent with subclinical *T. annulata* infection under field conditions, supporting the integration of molecular surveillance with vaccination and effective tick control programs in endemic areas.

Singh et al. (2001) conducted a 14-month field trial showing that although vaccinated cattle developed strong antibody responses and were protected against clinical disease, 36.7% still became asymptomatic carriers compared to 64.2% in unvaccinated controls [29]. A study demonstrated via sensitive reverse line blot (RLB) hybridization that certain vaccine lines (Cáceres and Sudanese strains) can establish a low-level carrier state, but they fail to transmit the parasite by Hyalomma ticks to naive calves, implying limited risk of the vaccine strains spread [26]. It is of note that live vaccines for *T. annulata* retain the potential to establish persistent, subclinical infections [30].

iELISA demonstrated seropositivity in 82.1% (23/28; 95% CI: 63.1–93.9) of vaccinated cattle and 72.4% (21/29; 95% CI: 52.8–87.3) of non-vaccinated animals capable of detecting seroconversion. The difference between groups was not statistically significant (RR = 1.13; 95% CI: 0.84–1.54; P = 0.40). When both groups were combined, the overall iELISA seropositivity was 77.2% (44/57; 95% CI: 64.8–86.2), reflecting the complex epidemiological dynamics of a hyperendemic region where both groups are likely to have encountered *T. annulata*, either through vaccination or natural infection (history of exposure) or having current carrier state.

The observed data indicate that vaccination is followed by transient PCR positivity (two weeks after vaccination) and antibodies against *Theileria* spp. are generally detectable 2–3 or 4–5 weeks

after the initial exposure [2,31] with high levels remaining stable for 30–60 days post-recovery. These antibodies can be traced for 4–6 months and, if the animal is naturally re-exposed, may persist for over a year [31]. The duration and timing of these antibodies can be influenced by factors such as carrier status, drug treatments, and the intensity of natural challenges in endemic areas. In endemic regions, seroprevalence in cattle populations can fluctuate widely, depending on how frequently and consistently animals encounter the parasite (e.g., through tick bites). Even in areas with seasonal transmission cycles, serological tests may sometimes fail to detect antibodies despite their high accuracy [31]. Therefore, a negative serology result does not necessarily mean that an animal has never been exposed to the parasite. It may simply reflect a long interval since the last exposure, waning antibody levels, lack of natural re-exposure, or limitations of the test itself.

No cases were PCR-positive but iELISA-negative, highlighting the iELISA's high diagnostic sensitivity and its ability to reflect antibody responses following *T. annulata* exposure [13,14,18]. Some animals, however, were PCR-negative but iELISA-positive (21.1%), more commonly among non-vaccinated cattle (24.1% vs. 17.9% in vaccinated animals), likely reflecting antibodies from resolved infections (previous exposure) or vaccine-induced seropositivity. This difference was not statistically significant ($P = 0.58$).

Overall, PCR and iELISA results agreed in 78.9% of cases, with a moderate Cohen's kappa coefficient ($\kappa = 0.55$). Such concordance is consistent with expectations when comparing molecular and serological methods under field conditions, where infection stage, immune status, and prior exposure influence results. While PCR detects active infection, iELISA provides a complementary view of the host's antibody response and exposure history, explaining the observed PCR-/iELISA+ discordances. Although slightly lower than the agreement reported for recombinant antigen-based ELISAs ($\kappa \approx 0.82$) [6], the correlation observed here aligns with the intrinsic differences between molecular and serological tests.

However, this does not exclude the possibility of iELISA+/PCR- cases, which may result from antibody persistence after parasite clearance, vaccine-induced seropositivity, or potential cross-reactivity with other *Theileria* or *Babesia* species. Several studies have demonstrated that other *Theileria* species, particularly *T. orientalis*, circulate in northern Iran [32,33] and may lead to serological cross-reactions [34]. Additionally, the presence of *Babesia* spp. (*B. bigemina* and *B. bovis*) in cattle in Iran may be another potential reason of serological cross-reactivity [35,36]. Cross-reactivity between *T. annulata* and *T. parva* may occur in the IFA test, but this is of little practical importance because *T. parva* does not occur in regions such as Iran where *T. annulata* is present.

High seropositivity, combined with substantial PCR positivity among vaccinated cattle, indicates ongoing transmission pressure in Miankaleh, underscoring the need for integrated control strategies encompassing vaccination, vector control, and carrier detection. The Miankaleh peninsula's climatic conditions, characterized by warm temperatures and high humidity, sustain year-round activity of *Hyalomma* ticks, the primary vector of *T. annulata*. This ecological backdrop likely contributes to the high infection proportion observed. Persistent exposure to infectious ticks could induce repeated subclinical infections, leading to endemic stability, especially in vaccinated herds.

Limitations of the study are as follow: First, the sample size was relatively small and limited to cattle from a single endemic region. Factors such as tick burden, management practices, or prior subclinical infections were not directly measured but may have influenced results, as animals were free-grazing, allowing tick attachment and ongoing transmission of the parasite.

5 Conclusion

High PCR and iELISA positivity in both vaccinated and non-vaccinated cattle confirm active circulation and exposure to *T. annulata* in the Miankaleh Peninsula. The cellular schizont antigen-based iELISA showed high sensitivity but moderate specificity, indicating its value as a population-level screening tool. However, as iELISA detects host antibody responses that may persist after parasite clearance or vaccination and does not necessarily indicate active infection, combining iELISA with PCR improves interpretation by integrating exposure surveillance with detection of active *T. annulata* infection. These findings highlight the need for integrated control strategies in the peninsula's highly favorable ecological conditions that combine vaccination, vector management, and carrier detection.

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Authors' Contribution

Study concept and design: G.H., S.F.

Acquisition of data: A.S, A.A., Q.K., B.A.T.

Analysis and interpretation of data: G.H., S.F.

Drafting of the manuscript: S.F., A.A., G.H.

Critical revision of the manuscript for important intellectual content: G.H.

Administrative, technical, and material support: A.A., Q.K., B.A.T., M.H.F.M.

Ethics

Blood samples were collected as part of routine veterinary procedures, and no experimental interventions were performed on the animals. All sampling was conducted by trained veterinarians with minimal distress to the cattle and with the informed consent of the owners, in accordance with institutional and national guidelines for animal welfare.

Conflict of Interest

The authors declare no conflict of interest.

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Data Availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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