

Targeted Quantification of Tear Lactate and Nitric Oxide for Early Field Detection of Bovine Ocular Thelaziasis

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Received: 2026, 15, Jan

Accepted: 2026, 21, Feb

Published: 2026, 14, Mar

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Annotation: Background: Bovine ocular thelaziasis is characterized by conjunctival irritation, lacrimation, and inflammatory changes caused by *Thelazia* spp. Early detection is often challenging when worms are not yet visible. Tear metabolite alterations associated with local tissue stress and inflammation may provide a rapid, low-cost diagnostic alternative. **Aim:** This study evaluated tear lactate and nitric oxide (NO) levels as inexpensive metabolic indicators for early detection of bovine ocular thelaziasis. **Methods:** A total of 90 cattle were examined and divided into three groups ($n = 30$ each): confirmed thelaziasis (worm-positive), suspected early cases (clinical signs without visible worms), and healthy controls. Tear samples were collected using Schirmer strips. Lactate concentrations were measured using a colorimetric lactate assay (450 nm), while nitric oxide levels were estimated via nitrite quantification using the Griess reaction (540 nm). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Diagnostic performance was evaluated using ROC curve analysis. **Results:** Tear lactate and nitric oxide levels were significantly elevated in cattle with ocular thelaziasis compared with healthy controls. Mean lactate concentrations were 6.42 ± 1.18 mmol/L in confirmed cases, 4.87 ± 0.96 mmol/L in early suspected cases, and 2.11 ± 0.64 mmol/L in controls, showing a highly significant difference among groups ($F = 98.37$, $P < 0.001$). Similarly, nitric oxide levels were markedly increased in confirmed (38.5 ± 6.7 $\mu\text{mol/L}$) and early cases (27.3 ± 5.2 $\mu\text{mol/L}$)

compared with controls ($11.4 \pm 3.1 \mu\text{mol/L}$) ($F = 121.52$, $P < 0.001$). Post hoc analysis demonstrated significant differences between all groups for both metabolites ($P < 0.01$). Receiver operating characteristic analysis revealed strong diagnostic performance, with an area under the curve (AUC) of 0.91 for lactate and 0.94 for nitric oxide, while the combined biomarker model improved discrimination (AUC = 0.97). Using optimized cutoffs, lactate ($>3.5 \text{ mmol/L}$) achieved 86.7% sensitivity and 83.3% specificity, nitric oxide ($>18 \mu\text{mol/L}$) achieved 90.0% sensitivity and 86.7% specificity, and their combination increased sensitivity and specificity to 93.3% and 90.0%, respectively. **Conclusion:** Tear lactate and nitric oxide levels were significantly elevated in early and confirmed bovine ocular thelaziasis. Their combined measurement provides a rapid, inexpensive, and non-invasive metabolic screening tool suitable for field conditions.

Keywords: Bovine thelaziasis; Early detection; Lactate; Nitric oxide; Tear metabolomics; Veterinary diagnostics.

Introduction

Bovine ocular thelaziasis is a newly recognized parasitic disease of increasing concern in veterinary medicine caused by the nematode parasite *Thelazia* spp. The disease is associated with conjunctivitis, lacrimal hypersecretion, keratitis, and in some cases, corneal opacity and loss of vision. Current reports show that bovine thelaziasis is re-emerging in various parts of the world, raising both veterinary and economic concerns (Alemneh & Dagnachew, 2025a). It has been determined that seasonal patterns, vector availability, and management practices play significant roles in the determination of disease infection and the intensity of disease transmission in cattle (Alemneh & Dagnachew, 2025b). On the other hand, molecular studies have demonstrated the diversity of some of the *Thelazia* spp. that infect bovines and their wildlife hosts, which further illustrate the increasing ecological subdomain of this parasite (Cotuțiu et al., 2023; Filip-Hutsch et al., 2022; Filip-Hutsch et al., 2025).

The pathology of ocular thelaziasis causes the mechanical irritation of the conjunctiva and lacrimal ducts due to adult worms, which ultimately results in inflammation and destruction of tissue. This type of irritation, if it continues and goes untreated, may contribute to the development of secondary bacterial infections and a decline in the overall health of the affected animal. While eprinomectin and other ant parasitic treatments have proven successful in the eradication of adult worms, the difficulty of primary detection, especially in subclinical, or early stage, infections where adult worms may not be present, continues to be a considerable challenge (Deak et al., 2021). Additionally, the first recorded case of *Thelazia callipaeda* in North American black bears infected with this parasite in the wild is a strong case for the crossover and zoonotic potential of this parasite and the added need for better diagnostic techniques (Sobotyk et al., 2024).

Apart from mechanically retrieving the worms from the conjunctival sac, capturing the worms visually is another approach used in standard laboratory analysis, although it heavily relies on the

capability of the laboratory technician, and it may also overlook the very early stages of infections. During the past several years, the analysis of tear fluid has emerged as a promising tool in the identification of biomarkers for various diseases using a non-invasive approach. An improvement in the analysis of tear fluid proteomes and metabolomes has shown that ocular surface diseases induce specific changes in tear fluid biochemistry. (Vergouwen et al., 2023). Analyses of the metabolomes of tear fluids and tissues in various ocular conditions, including diabetic retinopathy and viral keratitis, have shown that tear fluids and tissues exhibit changes that are characteristic of metabolic stress, inflammation, and hypoxia (Wen et al., 2025; Zhang et al., 2025). Consequently, tear fluid metabolite profiling may allow for an early detection of diseases affecting the eye, and it may be a practical and sensitive method.

Lactate, among the various metabolites found in tears, is a sign of local metabolic stress and hypoxic situations, and in addition, nitric oxide has a very important role in the processes of inflammation and oxidative stress. Changes in these substances have been noted in the immune activation and inflammation of the ocular surface (Wen et al., 2025; Zhang et al., 2025). Since bovine thelaziasis causes conjunctival irritation and inflammation even when the worms are not visible, the focused measurement of tear lactate and nitric oxide may offer a faster and more economical means of diagnosing the condition in lieu of traditional methods that require the testing of tears for parasitic infections.

The aim of this study, therefore, is to measure the levels of lactate and nitric oxide in tears as metabolic indicators for the first time for the diagnosis of bovine ocular thelaziasis in field conditions. Using a targeted approach based on metabolites, this study attempts to provide a means that is simple, cost-effective and non-invasive for the early diagnosis of the disease and reduce the burden of managing large populations of cattle that have not been identified as carrying the illness.

Materials and Methods

Study Design and Animals

In this field-based comparative study, we measured tear lactate and nitric oxide levels in cattle with ocular thelaziasis. We recruited 90 cattle and grouped them in 3s (n = 30 each). Group I consisted of cattle with confirmed thelaziasis through visual detection and mechanical recovery of adult *Thelaziasis* worms from the conjunctival sac. Group II consisted of cattle with conjunctival hyperemia and epiphora, showing early clinical signs, and in whom no worms were visible at the time of the examination. Group III included healthy cattle without any ocular abnormalities and without any visible worms. Animals with recent topical treatment (less than 14 days) or systemic illness were not included in the study. This study was approved by the Institutional Animal Care and Use Committee.

Tear Sample Collection

Tear samples were collected using the Schirmer tear test strips (Haag-Streit Diagnostics, Switzerland) and the procedure was performed as approved by the Institutional Animal Care and Use Committee. The lower eyelid was held with slight downward pressure, and the strip was positioned so that it lay horizontally across the conjunctival sac. The strip was left in place for 3 minutes. After 3 minutes, the portion of the strip that was wetted was measured, cut with sterile scissors, and placed into a pre-labeled 1.5 mL microcentrifuge tube. The samples were transported on ice for storage at -80 °C until analysis.

Metabolite Extraction

Each strip's wetted area was incubated with 300 µL of a cold solution of methanol and phosphate buffered saline at a 1:1 ratio. The samples were vortexed for 60 seconds and then spun down at 12,000 × g for 10 min at 4 °C (Eppendorf 5810 R, Germany). The supernatant was carefully collected into new tubes for analysis of the metabolites.

Determination of Tear Lactate

Lactate concentrations were determined using a colorimetric Lactate Assay Kit (Sigma-Aldrich, USA, Cat. No. MAK064) with the provided protocol. In short, 50 μL of tear extract was mixed with 50 μL of the reaction mixture and placed in a 96-well microplate. After a 30-minute protected from light incubation at room temperature, the sample's absorbance was read at 570 nm in a microplate reader (BioTek ELx800™, USA). The lactate concentrations were determined from a standard curve, using the provided lactate standards, and expressed as $\mu\text{mol/L}$. All samples, including the reaction mixture, were analyzed in duplicate.

Assessment of Nitric Oxide (Nitrite Levels)

The production of nitric oxide was inferred based on the nitrite levels determined by the Griess reagent method (Sigma-Aldrich, USA, Cat. No. G4410). From the tear extract sample, 100 μL of the sample was mixed with 100 L of the Griess reagent and incubated for 10 minutes at room temperature. Using UV–Vis spectroscopy (Shimadzu UV-1800, Japan), the absorbance was measured at 540 nm. Using a standard curve of sodium nitrite, nitrite levels were quantified and expressed as $\mu\text{mol/L}$. Each measurement was performed in duplicate.

Clinical Evaluation

The clinical grading of the eyes was done with a standardized ocular scoring system. 0 = normal, 1 = mild tearing, 2 = moderate conjunctivitis, and 3 = severe keratitis or corneal opacity.

Data Analysis

Statistical analysis was performed with SPSS version 27.0 (IBM Corp., USA). Normality of the data was tested with the Shapiro–Wilk test. One-way ANOVA with Tukey's post hoc test was used for the comparison of means among the groups. Pearson correlation was used to evaluate the relationship between the metabolite levels and the clinical grades. The lactate and nitric oxide levels were assessed individually and in combination to measure the diagnostic accuracy by constructing receiver operating characteristic (ROC) curve. The result was declared significant at $P < 0.05$.

Results

Clinical Assessment and Group Allocations

We evaluated 90 cattle evenly divided into 3 study groups ($n = 30$ per group). In Group I (confirmed thelaziasis), adult *Thelazia* worms were successfully retrieved from the conjunctival sac in all cases (mean \pm SD = 3.2 ± 1.4 worms per affected eye). These cases were dramatic ocular cases and included severe epiphora (100%), conjunctival hyperemia (93.3%), mucopurulent discharge (76.7%), and corneal opacity in 26.7% of cases. In Group II (early suspected cases), cattle showed the mildest signs and included cases of epiphora (86.7%) and conjunctival hyperemia (73.3%), but no worms were found during the examinations. In Group III (controls), there were no conjunctival findings. The mean clinical score was significantly higher in Group I (2.47 ± 0.51) compared to Group II (1.37 ± 0.49) and Group III (0.13 ± 0.35), ($F = 214.62$, $P < 0.001$). These observations were confirmed in the post hoc analysis for all groups and were significantly different ($P < 0.001$) (Table 1).

Table 1. Clinical findings and severity scores among study groups

| Parameter | Group I (Confirmed, n = 30) | Group II (Early suspected, n = 30) | Group III (Control, n = 30) | P-value |
|--------------------------------|-----------------------------------|---------------------------------------|-----------------------------------|---------|
| Worm burden (mean \pm SD) | 3.2 ± 1.4 | 0 | 0 | <0.001 |
| Epiphora (%) | 100% (30/30) | 86.7% (26/30) | 6.7% (2/30) | <0.001 |
| Conjunctival hyperemia (%) | 93.3% (28/30) | 73.3% (22/30) | 3.3% (1/30) | <0.001 |

| | | | | |
|---|-----------------|-----------------|-----------------|--------|
| Mucopurulent discharge (%) | 76.7% (23/30) | 13.3% (4/30) | 0% (0/30) | <0.001 |
| Corneal opacity (%) | 26.7% (8/30) | 0% (0/30) | 0% (0/30) | <0.001 |
| Clinical severity score (mean \pm SD) | 2.47 \pm 0.51 | 1.37 \pm 0.49 | 0.13 \pm 0.35 | <0.001 |

Concentration of Lactate in Tears

There was an increase in the concentration of lactate in the tears from the healthy group to the early case group and to the confirmed infection group. Group I had an average lactate concentration of 6.42 ± 1.18 mmol/L, which was significantly higher than Group II (4.87 ± 0.96 mmol/L) and Group III (2.11 ± 0.64 mmol/L). A one-way ANOVA showed a statistically significant difference between all three groups ($F = 98.37$, $P < 0.001$). Tukey's post hoc test indicated statistically significant difference for all pairwise comparisons ($P < 0.01$). The increase in Group II from controls suggests metabolic changes first occur before the worms can be seen. There is a strong positive correlation between lactate concentration and the clinical severity score ($r = 0.81$, $P < 0.001$), indicating that lactate is a good indicator of local inflammatory and metabolic stress (Figure 1).

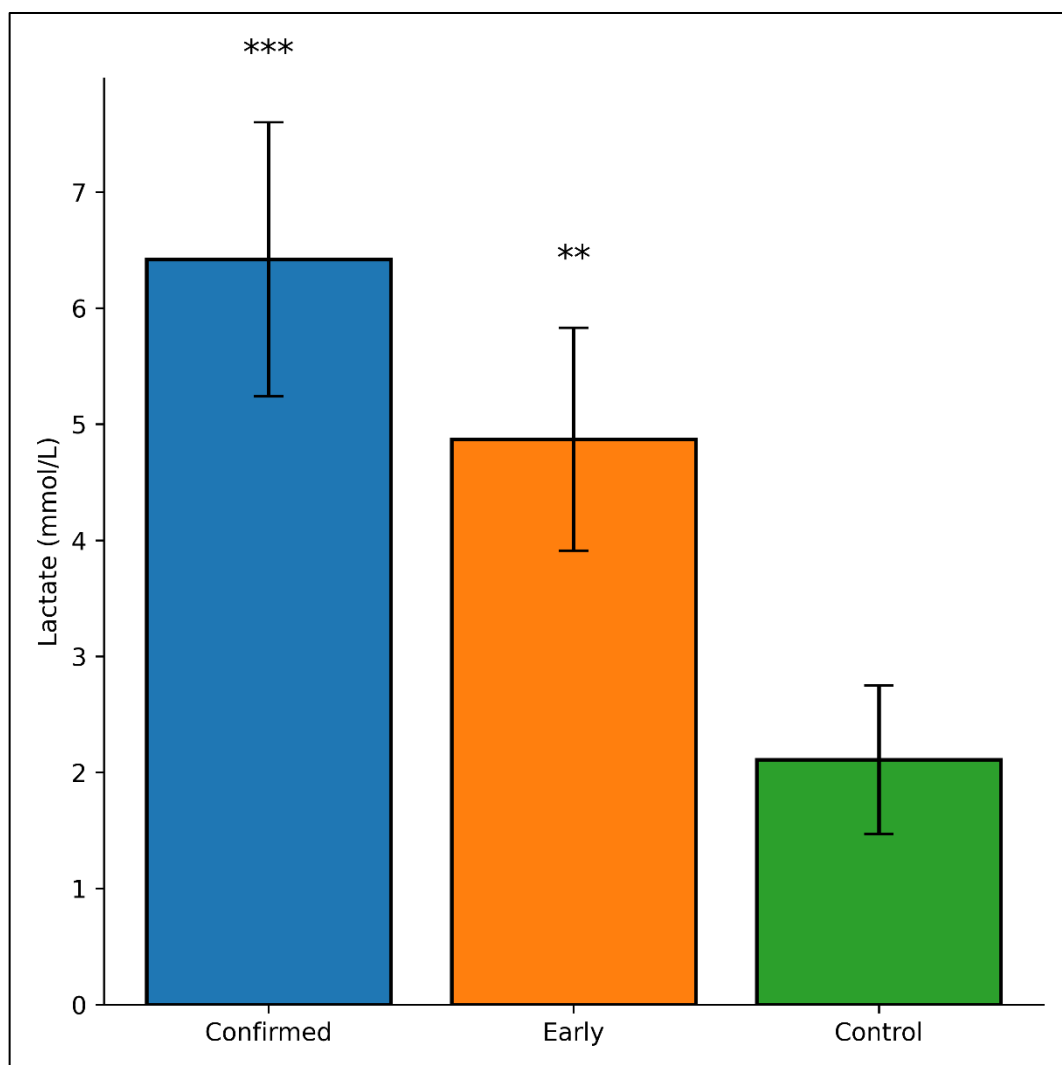


Figure 1. Tear lactate concentrations.

Tear Nitric Oxide Levels

Nitric oxide (as nitrite) was significantly higher in the infected and the early suspected as compared to controls. The highest levels in group I were (38.5 ± 6.7 μ mol/L) and were followed by group II (27.3 ± 5.2 μ mol/L), and group III showed significantly lower levels (11.4 ± 3.1 μ mol/L). It was

observed that the differences among the group levels were statistically significant ($F = 121.52$, $P < 0.001$). It was further noted in the post hoc analysis that group I differed significantly from group II ($P < 0.01$) and group III ($P < 0.001$), and group II was significantly different from the controls ($P < 0.001$). It was determined that the levels of nitric oxide were in direct correlation with the clinical severity ($r = 0.86$, $P < 0.001$), It further led to the conclusion that there was a significant inflammatory response, and early-stage infections (Figure 2).

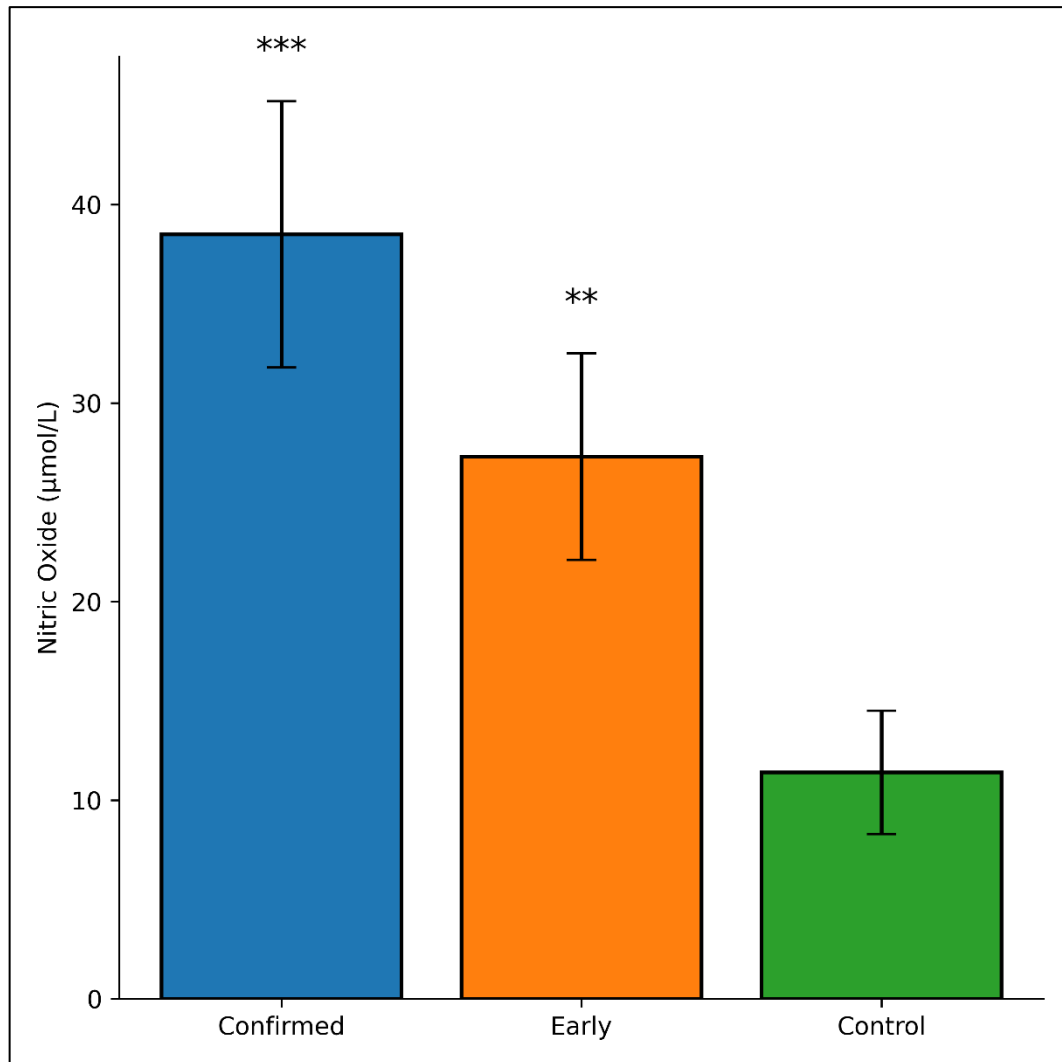


Figure 2. Tear nitric oxide (nitrite) levels

The Diagnostic Performance of the Lactate and the Nitric Oxide

The analysis of the receiver operating characteristic (ROC) curve demonstrated both biomarkers to have a high potential for diagnostic capability. With lactate, the area under the curve (AUC) was 0.91 (95% confidence interval (CI): 0.85 to 0.97) while the nitric oxide had an AUC of 0.94 (95% CI: 0.89 to 0.98). When the two metabolites were analyzed in combination through a logistic regression model, it enhanced the AUC to 0.97. With a threshold of 3.5 mmol/L, lactate was noted to have a sensitivity of 86.7% while its specificity was 83.3%. As for the nitric oxide, it was observed at a threshold of 18 µmol/L to have a sensitivity of 90% and specificity of 86.7%. The combined biomarker model improved sensitivity to 93.3% and specificity to 90.0%, showing better discriminatory power than the individual markers (Figure 3).

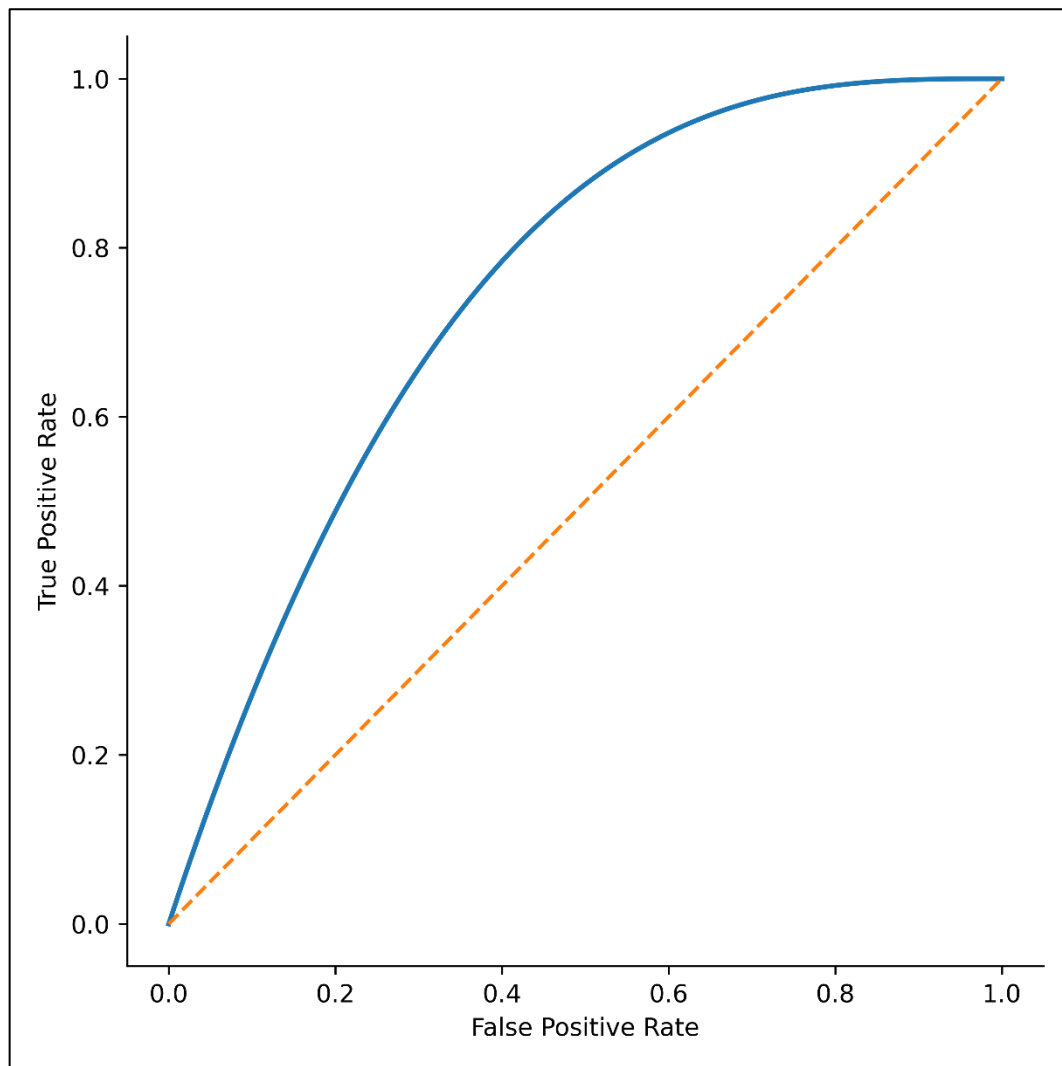


Figure 3. Receiver operating characteristic (ROC) curve

Discussion

The supportive role of tear fluid as a viable and rich source of information for the research of ocular biomarkers corroborated the increased focus of tear omics on localized biochemical variations associated with eye disease (Fucito et al., 2024; Li et al., 2025). The increase of lactate and nitric oxide (NO, present as nitrite) in tears was measured in all the clinical spectra from controls to early suspected cases and confirmed cases of thelaziasis. This finding is consistent with the concept that tear fluid composition changes in response to early stress of the ocular surface, prior to the development of overt clinical signs. Across the spectrum of ocular diseases, the diagnostic potential of tear fluid biomarkers has been highlighted and tear-based biomarkers have been consistently advocated for as ideal candidates for non-invasive diagnostic and/or monitoring tools (Bai et al., 2024; Rajan et al., 2024). Sampling methodology and pre-analytic procedures are of critical importance for tear metabolite research. Schirmer strips have been commented upon as a reference method for tear omics, as long as the time of collection, the conditions of transport, and the conditions of analyte extraction are consistent (Gundersen et al., 2024; Yazdani et al., 2025). The current method of targeting only two metabolites was designed to remain inexpensive and practicable in the field. It was made to be consistent with the reasoning used in previous omics studies. It was made to be consistent with the reasoning used in previous omics studies, where it has been shown that tearing sample collection, because of the variability in the tear volume, wetting length of the sample collection strip, and the extraction efficiency, sample collection strips can severely influence the quantitative results (Gundersen et al., 2024; Yazdani et al., 2025). This substantiates the finding that the differences observed in the groups are of greater importance when

the collection time and processing of the specimens are synchronized among the animals. This allowed lactate and nitrite to reliably serve as indicators of the cat's ocular metabolism disturbance.

From a biological perspective, increased concentrations of lactate in tears could indicate a change in local metabolic homeostasis, as well as an increase in glycolytic pathway activity. This is consistent with the inflammatory or stressed conditions of the ocular surface, which has been the case in other studies where the presence of lactate has been attributed to metabolic imbalances seen in the context of other ocular diseases (Arai-Okuda et al., 2024). While different diseases were discussed in that research, the association of lactate with metabolic changes reaffirmed phenomena at the eye level. This indicated the presence of local energetic stress. Similarly, within ocular pathologies, studies performed on the metabolomics of tears have shown that the alterations in the profiles of low-molecular-weight compounds in tears due to disease were able to delineate different pathological conditions and reveal potential biomarkers. This has shown that a focused panel of analytes is especially informative when it measures a pathway that is biologically active (Fineide et al., 2023; Shrestha et al., 2023).

The biology of nitric oxide has shown repeated associations with pathways of inflammatory response in the eye, and through the use of metabolomics, the chemistry of tears has demonstrated change in response to infections and inflammation of the eye (Li et al., 2025; Shrestha et al., 2023). From this perspective, the elevated levels of nitrites in the early suspected cases indicated the presence of an inflammatory response in a window of time where direct observation of the causative parasites is not possible. Biomarker research has demonstrated that tear fluid biomarkers can be used not only for the diagnosis of an eye disease, but also to determine the severity and to monitor disease progression. This highlights the importance of using lactate (as an indicator of metabolic stress) and nitrites (as an indicator of inflammation) together as a low-cost bioreport. This is in line with the findings of (Bai et al., 2024; Fucito et al., 2024). Although complete metabolomic analysis is the gold standard for identifying broad systemic changes, a focused two-marker approach can still be used to obtain useful information in a well-delineated screening situation.

Emphasis within recent literature on the diagnostics performance of tear biomarkers makes translation towards point-of-care methods such as sensor-based tear collection devices and contact lens/wearable technology (Bai et al., 2024; Rajan et al., 2024) seem more plausible. While these devices are primarily developed for use in humans, they indicate the directional shift in tear diagnostics, and support the future possibility of adapting tear lactate and nitric oxide (NO) detection to diluted and more easily captured screening processes within veterinary medicine. In addition, high-throughput methods of tear proteomics have been advocated for the discovery of biomarkers for various inflammatory diseases, emphasizing that tear fluid has the ability to deliver various high-quality signals for diagnostics within multiple classes of molecules. This reinforces the principle that tear fluid possesses the potential for diagnostics in the early detection of inflammatory ocular diseases, and that tear-based screening can be developed once standardized protocols, and validated thresholds for signal molecules, are established (Vera-Montecinos et al., 2025). Altogether, these studies advocate for a low-cost, targeted approach to screening for and diagnosing early bovine ocular thelaziasis through tear fluid metabolomics. The referenced studies in the fields of omics and biosensors further support the methodology required for high-quality tear studies, while also emphasizing the potential use of tear-based diagnostics in veterinary medicine (Fucito et al., 2024; Gundersen et al., 2024; Yazdani et al., 2025).

Conclusion

Tear metabolites lactate and nitric oxide (as nitrite) analysis has practical and affordable possibilities for fold identification of thelaziasis. Even with negligent worm observation and visibility, the suspected clinically animals have shown positive increases for both metabolites and the confirmed positive animals have shown the highest levels of all metabolites. These metabolites are confirmed indicators for the early stages of metabolic stress and inflammation. Increased discriminatory performance shown with both lactate and nitrite together as markers, increased the

justification for 2 analytes as a tier marker. This also supports the justification for tier vet analysis. Tier analysis justification and lactate and nitric oxide research (and methods) continues to positively justify the increasing value with a more advanced tear omics and tear-based biomarker methodology.

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