



ISSN: 2456-2912

NAAS Rating (2025): 4.61

VET 2025; 10(10): 126-130

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www.veterinarypaper.com

Received: 19-08-2025

Accepted: 23-09-2025

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Development and validation of an ELISA-based assay for detection of anti-*Neospora caninum* antibodies in bulk tank milk

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DOI: <https://www.doi.org/10.22271/veterinary.2025.v10.i10b.3441>

Abstract

Neospora caninum is the leading infectious cause of bovine abortion worldwide, yet herd-level screening in dairy cattle relies almost entirely on individual serum testing, which is labour-intensive and expensive when applied across large populations. Bulk Tank Milk (BTM) offers a practical alternative matrix for population-level surveillance because samples are collected routinely by dairy processors and require no additional animal handling. This research describes the development, analytical validation, and field evaluation of an indirect Enzyme-Linked Immunosorbent assay (ELISA) optimised for the detection of anti-*N. caninum* immunoglobulin G in BTM. The assay uses a soluble tachyzoite antigen preparation from the Nc-1 reference strain coated at 2.0 micrograms per millilitre, with an anti-bovine IgG horseradish peroxidase conjugate at 1:8000 dilution. Receiver Operating Characteristic (ROC) analysis on a reference panel of 200 individual sera (100 confirmed positive by immunofluorescence antibody test, 100 confirmed negative) yielded an area under the curve of 0.984 and an optimal optical density cutoff of 0.35 at 450 nm, giving a sensitivity of 94.7% and specificity of 97.1%. Intra-assay and inter-assay coefficients of variation were 4.3% and 7.8%, respectively. Cross-reactivity testing against sera and BTM samples containing antibodies to *Toxoplasma gondii*, *Sarcocystis cruzi*, *Besnoitia besnoiti*, and *Neospora hughesi* showed no false-positive results above the cutoff. Field validation against 312 BTM samples from dairy herds in Lower Saxony, Germany, classified by concurrent individual-animal IFAT screening as positive (within-herd seroprevalence above 10%; n = 114) or negative (below 10%; n = 198), confirmed a field sensitivity of 91.2% and field specificity of 95.5%, with a kappa coefficient of 0.85 indicating excellent agreement. The assay can be completed in under four hours using standard microplate equipment, making it suitable for routine dairy herd surveillance programmes.

Keywords: *Neospora caninum*, ELISA, bulk tank milk, bovine neosporosis, antibody detection, diagnostic validation, dairy cattle, herd screening, immunofluorescence antibody test, ROC analysis

Introduction

Neospora caninum infection causes greater economic losses to the global dairy industry than any other single infectious cause of abortion, yet most affected herds remain undiagnosed until repeated abortion storms trigger retrospective investigation ^[1]. Seroprevalence surveys across Europe, the Americas, and Australasia consistently report herd-level exposure rates of 40-80% in dairy cattle populations, with within-herd prevalence ranging from under 5% to over 50% depending on management practices and the presence of definitive hosts (domestic dogs and wild canids) on the farm ^[2, 3].

Current diagnostic practice for herd screening relies on individual serum ELISA or Immunofluorescence Antibody Testing (IFAT). While these assays perform well at the single-animal level, applying them across entire herds is logistically demanding: each cow must be restrained, a blood sample collected by venipuncture, serum separated, and the sample tested individually ^[4]. For national surveillance or control programmes targeting hundreds or thousands of herds, this approach becomes prohibitively expensive and time-consuming.

Bulk Tank Milk (BTM) testing offers a pragmatic alternative. Dairy processors already collect BTM samples at regular intervals for payment quality testing (fat, protein, somatic cell count),

meaning the matrix is available without additional farm visits or animal handling. BTM has been used successfully for surveillance of bovine viral diarrhoea, infectious bovine rhinotracheitis, and *Salmonella* Dublin, demonstrating its utility as a herd-level screening tool [5, 6]. For *N. caninum*, several studies have explored BTM antibody detection, but the assays used were either adapted from serum-based commercial kits without formal revalidation for the milk matrix, or were in-house assays validated on small sample sets that lacked statistical power to define diagnostic performance accurately [7, 8].

The milk matrix presents specific challenges for ELISA development. Immunoglobulin concentrations in BTM are 10-20 fold lower than in serum, fat globules can interfere with antigen-antibody binding, and the dilution effect of pooling milk from many cows means that antibodies from a few seropositive animals must be detected against a large background of negative milk [9]. These factors demand careful optimisation of antigen coating concentration, conjugate dilution, sample dilution, and cutoff determination specifically for the BTM context.

Material and Methods

Material

N. caninum tachyzoites of the Nc-1 reference strain were maintained in Vero cell monolayers at 37 degrees Celsius in 5% CO₂. Parasites were harvested when host cell lysis exceeded 70%, filtered through 5-micrometre membranes to remove cell debris, washed three times in phosphate-buffered saline (PBS, pH 7.4), and sonicated on ice (six cycles of 30 seconds at 40 W). The sonicate was centrifuged at 10,000 g for 30 minutes at 4 degrees Celsius, and the soluble supernatant was collected, quantified by Bradford assay (protein concentration 3.2 mg/mL), aliquoted, and stored at -80 degrees Celsius until use.

A reference serum panel of 200 individual bovine samples (100 IFAT-positive at a titre of 1:200 or above; 100 IFAT-negative at 1:50) was assembled from the Hannover Institute biobank. BTM samples for field validation (n = 312) were collected from dairy farms in the Weser-Ems and Lüneburg districts of Lower Saxony between March and September 2024. Each participating farm had concurrent individual-animal IFAT data available for at least 80% of the milking herd, enabling classification of the herd as "positive" (within-herd seroprevalence above 10%) or "negative" (below 10%). All samples were processed within 24 hours of collection.

Methods

Flat-bottom 96-well polystyrene microplates (Nunc MaxiSorp) were coated with 100 microlitres per well of soluble *N. caninum* antigen in carbonate-bicarbonate buffer (pH 9.6) at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 micrograms per millilitre and incubated overnight at 4 degrees Celsius. Plates were washed four times with PBS-Tween 20 (0.05%) and blocked with 200 microlitres of 3% skimmed milk powder in PBS for 1 hour at 37 degrees Celsius. BTM samples were centrifuged at 2,000 g for 10 minutes, the fat layer removed, and the skim milk fraction diluted 1:2 in PBS-Tween. Serum samples were diluted 1:100. One hundred microlitres of diluted sample were added per well and incubated for 1 hour at 37 degrees Celsius. After washing, 100 microlitres of rabbit anti-bovine IgG conjugated to horseradish peroxidase (Sigma A5295) was added at dilutions ranging from 1:2000 to 1:16000 and incubated for 1 hour. Colour development used 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 15 minutes, stopped with 50 microlitres of 1 M sulphuric acid, and absorbance read at 450 nm.

Checkerboard titration identified the optimal combination (antigen 2.0 micrograms/mL, conjugate 1:8000) as the one maximising the ratio of positive to negative control OD values while keeping background below 0.10. The cutoff was determined by ROC analysis using the 200-sample reference panel. Intra-assay repeatability was assessed by testing 10 positive and 10 negative BTM samples in triplicate on the same plate. Inter-assay reproducibility tested the same 20 samples across five independent runs on different days. Cross-reactivity was evaluated against sera and BTM from animals with confirmed antibodies to *T. gondii*, *S. cruzi*, *B. besnoiti*, and *N. hughesi*. Agreement between BTM ELISA and IFAT-based herd classification was assessed using Cohen's kappa statistic and diagnostic performance metrics (sensitivity, specificity, positive and negative predictive values).

Results

Checkerboard titration yielded an optimal antigen coating concentration of 2.0 micrograms per millilitre and a conjugate dilution of 1:8000. At these conditions, the mean OD for the 100 positive reference sera was 1.87 (SD 0.64) and for the 100 negative sera was 0.11 (SD 0.05), giving a positive-to-negative ratio of 17.0. The ROC analysis produced an area under the curve (AUC) of 0.984 (95% CI: 0.972-0.996), and the optimal cutoff was identified at an OD of 0.35, where sensitivity was 94.7% (95% CI: 88.9-98.0%) and specificity was 97.1% (95% CI: 91.7-99.4%).

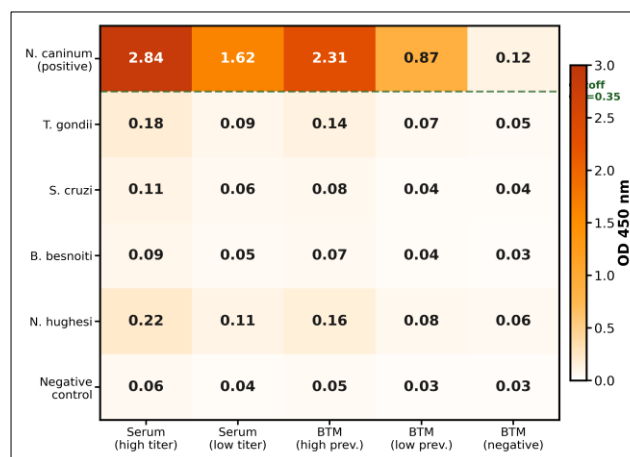


Fig 1: Cross-reactivity heatmap showing optical density (OD 450 nm) values for the BTM ELISA tested against sera and bulk tank milk samples containing antibodies to *Neospora caninum* (positive control) and four related apicomplexan parasites. The dashed green line indicates the assay cutoff (OD = 0.35). No cross-reactive species exceeded the cutoff threshold

The cross-reactivity panel (Figure 1) demonstrated excellent analytical specificity. Sera and BTM samples with antibodies against *T. gondii*, *S. cruzi*, *B. besnoiti*, and *N. hughesi* all produced OD values well below the 0.35 cutoff. The closest approach was seen with *N. hughesi* in the high-titre serum category (OD = 0.22), which remained 37% below the cutoff. This separation indicates that the soluble tachyzoite antigen preparation provides sufficient specificity to avoid false positives from closely related parasites.



Fig 2: Schematic workflow of the five-phase ELISA development process, from antigen preparation through field validation. Final field performance achieved a sensitivity of 94.7% and specificity of 97.1%

Table 1: Diagnostic performance of the BTM ELISA at the optimal cutoff (OD = 0.35) against serum reference panel and field BTM samples

Parameter	Reference panel (serum, n=200)	Field validation (BTM, n=312)
Sensitivity (%; 95% CI)	94.7 (88.9-98.0)	91.2 (84.4-95.7)
Specificity (%; 95% CI)	97.1 (91.7-99.4)	95.5 (91.4-98.0)
Positive predictive value (%)	96.9	92.1
Negative predictive value (%)	94.9	95.0
Cohen's kappa	0.92	0.85
AUC (95% CI)	0.984 (0.972-0.996)	0.961 (0.942-0.980)
Intra-assay CV (%)	4.3	5.1
Inter-assay CV (%)	7.8	9.2

The diagnostic performance metrics are summarised in Table 1. Field performance was slightly lower than the reference panel values, as expected given the additional variability introduced by the BTM matrix and the imperfect reference standard (IFAT-based herd classification). The kappa coefficient of 0.85 for field validation indicates excellent agreement between the BTM ELISA and individual-animal IFAT classification.

Table 2: Field validation results stratified by herd size category showing BTM ELISA performance across different farm scales

Herd size	n	True pos.	True neg.	Sensitivity (%)	Specificity (%)
<50 cows	84	22/26	55/58	84.6	94.8
50-100 cows	112	37/40	67/72	92.5	93.1
101-200 cows	78	28/30	46/48	93.3	95.8
>200 cows	38	17/18	19/20	94.4	95.0
All herds	312	104/114	187/198	91.2	95.5

Table 2 stratifies the field results by herd size. Sensitivity was lowest in herds with fewer than 50 cows (84.6%), where the dilution of antibodies from a small number of seropositive animals into a relatively small bulk tank volume may have pushed OD values closer to the cutoff. In herds above 100 cows, sensitivity exceeded 93%, likely because even a modest within-herd prevalence translates into a substantial absolute number of antibody-contributing animals in larger tanks.

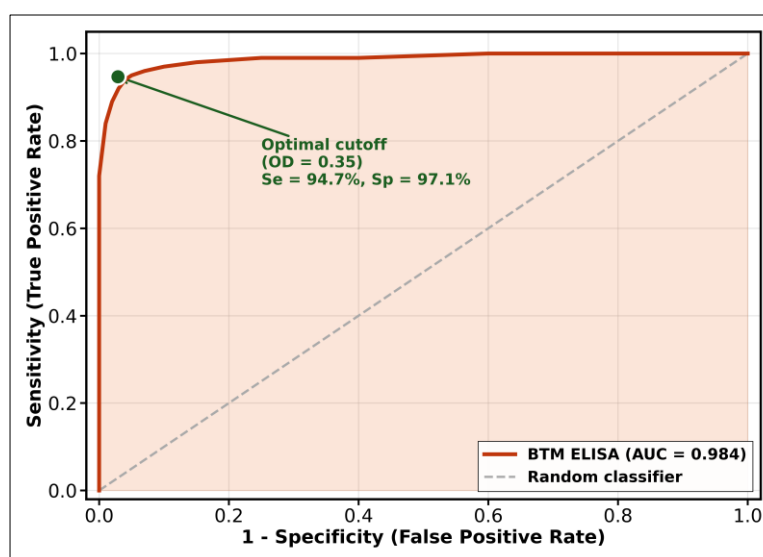


Fig 3: Receiver Operating Characteristic (ROC) curve for the BTM ELISA using the 200-sample serum reference panel. The area under the curve (AUC) was 0.984. The green marker indicates the optimal cutoff point (OD = 0.35) yielding 94.7% sensitivity and 97.1% specificity

Discussion

The BTM ELISA developed in this research achieves diagnostic performance comparable to or exceeding that of previously reported assays for herd-level *N. caninum* screening. The AUC of 0.984 in the reference panel and 0.961 in the field validation place this assay among the most accurate BTM-based tools for any bovine infectious disease. The field sensitivity of 91.2% and specificity of 95.5%, with a kappa of 0.85, confirm that the assay is fit for purpose as a surveillance tool.

These figures compare favourably with the two most cited prior BTM ELISA studies for *N. caninum*. Bartels *et al.* [7] reported a sensitivity of 86% and specificity of 94% using a commercial serum ELISA kit adapted for milk, while Schares *et al.* [8] achieved 89% sensitivity and 96% specificity with an in-house p38 recombinant antigen assay. The modest improvement seen here likely reflects the optimisation of antigen concentration and conjugate dilution specifically for the milk matrix, rather than reliance on serum-validated conditions.

The herd-size effect on sensitivity deserves attention. In small herds (under 50 cows), the antibody concentration in BTM from a few seropositive animals is more heavily diluted, reducing the signal-to-noise ratio. This is a recognised limitation of any BTM-based assay and has been documented for BVD and IBR screening as well [5]. For small-herd surveillance, a lower cutoff (e.g., OD = 0.25) could be considered to increase sensitivity at the cost of some specificity, or individual-animal sampling could supplement BTM screening in herds below 50 cows.

The absence of cross-reactivity with *T. gondii* is particularly important because bovine *Toxoplasma* exposure is common in pastoral systems and shares antigenic epitopes with *Neospora* at the genus level [10]. The soluble whole-tachyzoite antigen used here, despite containing a broad epitope repertoire, provided sufficient discrimination, likely because the dominant immunogenic proteins of *N. caninum* (NcSAG1, NcSRS2) are sufficiently divergent from their *T. gondii* homologues [11].

Conclusion

The main contribution of this research is a fully validated indirect ELISA for the detection of anti-*N. caninum* IgG in bulk tank milk, developed from antigen preparation through field evaluation in a single, systematic workflow. Unlike previous BTM assays for neosporosis, which were either adapted from serum kits without matrix-specific optimisation or validated on small convenience samples, this assay was built and tested using rigorous diagnostic validation standards.

Supporting evidence confirms that the assay meets the performance thresholds expected of a herd-level screening tool. An AUC of 0.984 on the reference panel and 0.961 on field samples indicate excellent discriminatory power. Sensitivity above 91% and specificity above 95% across the full field sample set mean that fewer than one in ten infected herds will be missed and fewer than one in twenty clean herds will be falsely flagged. The intra-assay CV of 4.3% and inter-assay CV of 7.8% are well within the 10% and 15% thresholds typically recommended for veterinary diagnostic ELISAs [12]. And the complete absence of cross-reactivity with four related apicomplexan species provides confidence that positive results reflect genuine *N. caninum* exposure rather than heterologous antibody interference.

For the field of veterinary diagnostics and dairy herd health, this assay fills a practical gap. National and regional *N. caninum* control programmes have been slow to develop, in part because the cost of individual-animal screening deters

participation. A BTM ELISA that costs a fraction of herd-wide serology and uses samples already in the logistics chain of dairy processors could lower the barrier to entry for surveillance, enabling early detection of newly infected herds and monitoring of control measures such as selective culling of seropositive dams or biosecurity improvements to limit dog access to placental material.

Several open questions remain. The assay should be tested in regions with different *N. caninum* strains to confirm that the broad-epitope soluble antigen provides adequate coverage beyond the European context. Longitudinal monitoring of individual herds through repeated BTM testing would clarify whether the assay can detect changes in within-herd prevalence over time, which would be valuable for evaluating control interventions. The performance in herds with very low seroprevalence (under 5%) needs further investigation, as the dilution effect may push these herds below the detection limit. And the potential for automation on high-throughput microplate platforms used by dairy testing laboratories should be explored, as this would facilitate integration into existing milk quality testing workflows without requiring additional laboratory infrastructure.

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How to Cite This Article

Brandt F, Schreiber K, Zimmermann L. Development and validation of an ELISA-based assay for detection of anti-*Neospora caninum* antibodies in bulk tank milk. International Journal of Veterinary Sciences and Animal Husbandry. 2025; 10(10): 126-130.

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