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Brief Report

Evaluation of a Commercial Serum Competitive Enzyme-Linked Immunosorbent Assay for Detection of *Neospora caninum*-Specific Antibodies in Raw Milk of Ruminants

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Abstract: Bovine neosporosis is an infection caused by the protozoan parasite *Neospora caninum* and has substantial veterinary hazards. Neosporosis cannot be controlled by vaccination or chemotherapy. Thus, accurate diagnosis followed by isolation and culling of infected animals is regarded as the most efficient method of control. In vivo diagnosis often relies on serologic testing of the animals, and milk represents a non-invasive and easy-to-collect sample matrix. However, indirect enzyme-linked immunosorbent assay (ELISA) specifically designed for antibody detection in milk are sometimes not easily available and it is tempting to use ELISA kits that are originally designed for use in serum in milk samples instead. Herein, we evaluated a widely used commercial ELISA (ID Screen® *Neospora caninum* competition Multispecies ELISA (ID. Vet, Grabels, France)), developed for detection of *N. caninum* antibodies in serum samples, for its performance on milk samples. Milk samples from dairy ruminants (cows, buffaloes, sheep, and goats; n = 149) were tested in parallel with the serum ELISA and a commercial milk ELISA as a standard test (*Neospora caninum* Milk Competitive ELISA, ID. Vet, Grabels, France). The detected prevalence values were 28.2% (42/149), 17.4% (26/149), and 17.4% (26/149) using milk ELISA, serum ELISA, and both ELISAs, respectively. Sensitivity, specificity, positive predictive value, and negative predictive value for the serum ELISA used with milk samples were 61.9%, 100%, 100%, and 87%, respectively. The agreement and kappa value between the two ELISAs were 89.3% and 0.70, respectively, suggesting substantial agreement. High values of Pearson correlation coefficient (0.904, $p \geq 0.0001$) and area under the receiver operating characteristic (ROC) curve (0.789, $p \geq 0.0001$) demonstrated the high diagnostic performance of the serum ELISA in milk samples. Also, a Bland–Altman Plot and histogram describing the frequency of distribution of ELISA optical densities confirmed the high agreement of both serum and milk ELISAs. The current results revealed the high specificity but moderate sensitivity of the serum ELISA used for milk samples compared with the milk ELISA. However, the excellent positive predictive value of the serum ELISA makes it an alternative option in case of the unavailability of milk ELISAs. With this study, we provided additional evidence that a widely used serum ELISA test kit may also be used for the detection of *N. caninum* antibodies in milk samples.

Keywords: antibodies; cattle; ELISA; neosporosis; milk

Citation: Fereig, R.M.; Altwaim, S.A.; Frey, C.F. Evaluation of a Commercial Serum Competitive Enzyme-Linked Immunosorbent Assay for Detection of *Neospora caninum*-Specific Antibodies in Raw Milk of Ruminants. *Parasitologia* **2024**, *4*, 91–98. https://doi.org/10.3390/parasitologia4020008

Academic Editor: David Carmena

Received: 21 February 2024
Revised: 20 March 2024
Accepted: 22 March 2024
Published: 27 March 2024

1. Introduction

Worldwide, the protozoan parasite *Neospora caninum* causes sporadic, endemic, and epidemic miscarriages in cattle [1,2], and has also been identified as a cause of abortion in sheep [3]. The primary mode of transmission appears to be endogenous transplacental transmission from dam to calf during pregnancy [1,4]. It is also possible for cattle and other animals to be horizontally infected by ingesting oocysts that have been discharged by canines acting as the final hosts [5,6]. The reproductive disorders caused by *N. caninum* have a detrimental economic effect on dairy cow replacement and milk production [7,8]. In terms of public health, immunoglobulin G (IgG)- [9] or IgM [10]-specific antibodies against *N. caninum* have been found in female serum. Despite the placenta testing negative for this parasite, a recent study found that 2 samples (1%) out of 201 investigated human umbilical cord blood samples were Nc5 PCR-positive for *N. caninum* [11]. However, there has not yet been any proof of a clinical type of neosporosis in humans.

It has been established for dairy cows that milk is a useful substrate to detect antibodies directed against *N. caninum* [12]. The IgG immunoglobulin class is the predominant immunoglobulin class in cow's milk, and the antibodies contained in milk are carried from the serum into the mammary gland in a selective manner [13]. When compared to milk samples from *N. caninum* seronegative cows, those from seropositive cows had a considerably greater IgG level [14,15]. Also, a high agreement (95%) between serum and milk antibodies was recorded [16].

In the present study, we aimed at the evaluation of a widely available serum an indirect enzyme-linked immunoassay (ELISA) (ID Screen® *Neospora caninum* competition Multispecies ELISA) for the detection of antibodies against *N. caninum* in milk samples, as ELISAs developed specifically for milk samples are sometimes not widely available and as it would be convenient to test different sample types using the same ELISA. A previous study tested the same commercial serum ELISA for use with whole and skimmed cow's milk and found a high diagnostic correlation and agreement between serum and milk antibody levels [17]. However, this study validated the approach by comparing milk and serum samples obtained from the same animals. We wanted to assess the performance of the serum ELISA on milk samples previously tested with a milk ELISA [18], and to include milk from different ruminant species, namely cattle, buffaloes, sheep, and goats. Our results provide additional evidence for the utility of a commercially available and widely used serum ELISA test kit in the detection of *N. caninum* antibodies in milk samples.

2. Materials and Methods

2.1. Ethical Statement

This study was performed according to standard procedures identified by the Research Board of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. The study was approved by the Research Code of Ethics at South Valley University number 36 (RCOE-36).

2.2. Sample Collection and Preparation

Individual milk samples (total number = 149) from Sohag Governorate (dairy cows, n = 70) and Qena Governorate (dairy cows, n = 34), both in southern Egypt, and from Dakahlia Governorate (dairy buffaloes, n = 16; sheep, n = 18; goats, n = 11) in northern Egypt, were collected. For cows, samples were collected from large farms, while, for other animals, samples were collected from small farms and the numbers were representative of the whole animal population in the farm. Although the sample numbers for buffaloes, sheep, and goats were small, we included them in the current study because such samples showed high positive values in our previous study using milk ELISA and were also confirmed positive by PCR. Thus, we deemed that such a panel of samples would be appropriate for the evaluation of our tested method (serum ELISA kit, (ID. Vet, Grabels, France)) [18]. Generally, the availability of samples based on animal owners' cooperation determined the currently used numbers of samples which were collected randomly. De-

tailed information on the sample collection, tested animal cases, and epidemiology was fully described in our previously published paper [18].

In total, 5 mL of individual milk samples was obtained for laboratory testing. The milk samples were centrifuged at 1000× *g* for 10 min. Lactoserum was collected from the layer below the cream layer on the top and stored at −20 °C until used [18].

2.3. Detection of Antibodies against N. caninum in Milk Samples Using a Serum ELISA

Milk samples were tested for antibodies to *N. caninum* using the ID Screen® *Neospora caninum* competition Multispecies ELISA (ID. Vet, Grabels, France), marketed for use in serum and plasma samples, and referred to herein as serum ELISA.

Positive and negative controls provided in the kit and test milk samples were diluted twice [17]. Then, plates were incubated at 37 °C for 45 min. Afterwards, washing and all procedures were performed according to the manufacturer's instructions. The ODs obtained (read at 450 nm and measured with an Infinite R© F50/Robotic ELISA reader (Tecan Group Ltd., Männedorf, Switzerland) were used to calculate the percentage of sample (S) to negative (N) ratio (S/N%) for each of the test samples according to the following formula: S/N (%) = OD sample/OD negative control × 100. Samples with an S/P% greater than 60% were considered negative; if the S/P% was between 50% and 60%, the result was considered doubtful and considered positive if the S/P% was less than 50%. No doubtful values were obtained during the test.

2.4. Detection of Antibodies against N. caninum in Milk Samples Using a Milk ELISA

All samples were previously tested with the *Neospora caninum* Milk Competitive ELISA (ID. Vet, Grabels, France), a test explicitly developed for use in milk samples and referred to herein as milk ELISA. Testing was performed according to the manufacturer's instructions and as described previously [18]. Briefly, undiluted milk samples and positive and negative controls were added to the microplate and incubated at 5 °C for 20 h. The ODs obtained were used to calculate the percentage of sample (S) to negative (N) ratio (S/N%) for each of the test samples according to the following formula: S/N (%) = OD sample/OD negative control × 100. Samples with an S/P% greater than 50% were considered negative, and were considered positive if the S/P% was less than or equal to 50%.

2.5. Statistical Analysis

The 95% confidence intervals (including continuity correction), estimated prevalence, sensitivity, specificity, positive predictive value, negative predictive value, concordance %, and kappa value were analyzed using the online statistical website www.vassarstats.net (accession dates: 1–2 October 2023) as described previously. *p*-values were estimated with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). The results were considered significant when the *p*-value was <0.05. Pearson's correlation coefficient was applied to test the correlation between OD values obtained in serum ELISA and in milk ELISA. Correlation coefficients were calculated using Pearson's correlation coefficient: $|r| = 0.70$, strong correlation; $0.5 < |r| < 0.7$, moderately strong correlation; and $|r| = 0.3$–0.5, weak-to-moderate correlation [18,19].

3. Results and Discussion

The growing human population demands safe and high-quality food, including milk and dairy byproducts. Some reports have revealed the presence of *N. caninum* DNA in raw milk samples from dairy cows [18,20,21]. This might suggest a potential risk of infection for sucking animals and thus the induction of additional economic losses. Another very important point is whether *N. caninum* may be infective for humans. This risk seems to be higher in cases of consuming raw milk from an individual animal rather than the consumption of bulk tank milk, in which the parasites would be greatly diluted [4].

The current control strategy for neosporosis relies on a test-and-cull approach. The use of milk samples instead of serum samples for the detection of anti-*N. caninum* antibodies

represents a non-invasive alternative method that might gain high importance in the control of and preventive strategies against *N. caninum* transmission. All of these reasons increase the demand for finding appropriate diagnostic ELISA tests for detecting and monitoring *N. caninum* antibodies in milk.

A previous study revealed the adequacy of a commercial serum antibody ELISA in screening *N. caninum* antibodies in cow milk using the same test against milk and serum samples from the same animal [17]. However, in the current study, we used a higher variety of animal species' milk and tested all samples with a milk antibody ELISA kit (standard) as well as with a serum antibody ELISA kit. We obtained 28.2% as the overall lacto-positive value (42/149, CI 95%; 21.3–36.2) using the milk antibody ELISA, and 17.4% (26/149, 11.9–24.7) using the serum antibody ELISA (Table 1). All 26 samples positive in the serum ELISA were also positive in the milk ELISA. The milk antibody ELISA revealed 29.8% positivity (31/104, CI 95%; 21.4–39.7) in cattle, 18.8% (3/16, CI 95%; 5–46.3) in buffaloes, 33.3% (6/18, CI 95%; 14.4–58.8) in sheep, and 18.2% (2/11, CI 95%; 3.2–52.2) in goats, respectively. When using the serum antibody ELISA, positive values were 21.2% (21/104, CI 95%; 13.2–29.4) in cattle, 6.3% (1/16, CI 95%; 0.3–32.3) in buffaloes, 11.1% (2/18, CI 95%; 2–36) in sheep, and 18.2% (2/11, CI 95%; 3.2–52.2) in goats, respectively (Table 1).

Table 1. Prevalence of *Neospora caninum* antibodies in raw milk of various ruminants.

Animal Species (No. of Examined)	Milk Kit			Serum Kit			Both		
	Negative (%)	Positive (%)	CI 95%	Negative (%)	Positive (%)	CI 95%	Negative (%)	Positive (%)	CI 95%
Cattle (n = 104)	73 (70.2)	31 (29.8)	21.4–39.7	83 (79.8)	21 (21.2)	13.2–29.4	83 (79.8)	21 (21.2)	13.2–29.4
Buffalo (n = 16)	13 (81.2)	3 (18.8)	5–46.3	15 (93.7)	1 (6.3)	0.3–32.3	15 (93.7)	1 (6.3)	0.3–32.3
Sheep (n = 18)	12 (66.7)	6 (33.3)	14.4–58.8	16 (88.9)	2 (11.1)	2–36	16 (88.9)	2 (11.1)	2–36
Goat (n = 11)	9 (81.8)	2 (18.2)	3.2–52.2	9 (81.8)	2 (18.2)	3.2–52.2	9 (81.8)	2 (18.2)	3.2–52.2
Total (n = 149)	107 (71.8)	42 (28.2)	21.3–36.2	123 (82.6)	26 (17.4)	11.9–24.7	123 (82.6)	26 (17.4)	11.9–24.7

CI 95%; confidence interval at 95% was calculated with Vassarstats.net; access date: 2 October 2023.

Using online software analysis (Vassarstats.net), the estimated prevalence was reported as 28.2 (CI 95%; 21.3–36.2), which was consistent with our manual calculated data from milk antibody ELISA-based testing (Table 2). The sensitivity, specificity, negative predictive value, and positive predictive value of the serum antibody ELISA compared to milk antibody ELISA for cow milk were found to be 61.4%, 100%, 87%, and 100%, respectively. Furthermore, our test method demonstrated a high concordance (89.9%) and a substantial kappa value (0.70) (Table 2).

Table 2. Comparison of serum ELISA test against milk ELISA test of *N. caninum*.

Parameter	Estimated Value	95% Confidence Interval	
		Lower Limit	Upper Limit
Estimated prevalence	28.2	21.3	36.2
Sensitivity (%)	61.9	45.7	76
Specificity (%)	100	95.7	100
Positive predictive value (%)	100	84	100
False positive	0	0	16
Negative predictive value (%)	87	79.4	92.2
False negative	13	7.8	20.6
Concordance (%)	89.3	82.9	93.5
Kappa value	0.70	0.59	0.81

Vassarstats.net access date: 2 October 2023. The strength of agreement was graded with kappa values of fair (0.21–0.40), moderate (0.41–0.60), and substantial (0.61–0.80).

The area under the curve was used to determine the accuracy of the immunoassays for ELISA (Figure 1A). The area under the receiver operating characteristic curve (AUC) was found to be 0.789 (CI 95%: 0.732–0.856), suggesting the high performance of the

serum antibody ELISA used on milk samples compared to the milk antibody ELISA. A similar interpretation was reported in a relevant study using our tested serum antibody ELISA against serum and milk samples from the same group of cow milk [17]. The correlation between the serum antibody ELISA and milk antibody ELISA OD values of tested milk samples was also analyzed. Scatter graphs show the correlation between the OD values recorded by the serum antibody ELISA and milk antibody ELISA from all tested samples (n = 149). A strong correlation for tested milk samples was observed between the serum antibody ELISA and milk antibody ELISA OD (Pearson's r = 0.904, $p \leq 0.0001$, R square = 0.817) (Figure 1B) [19].

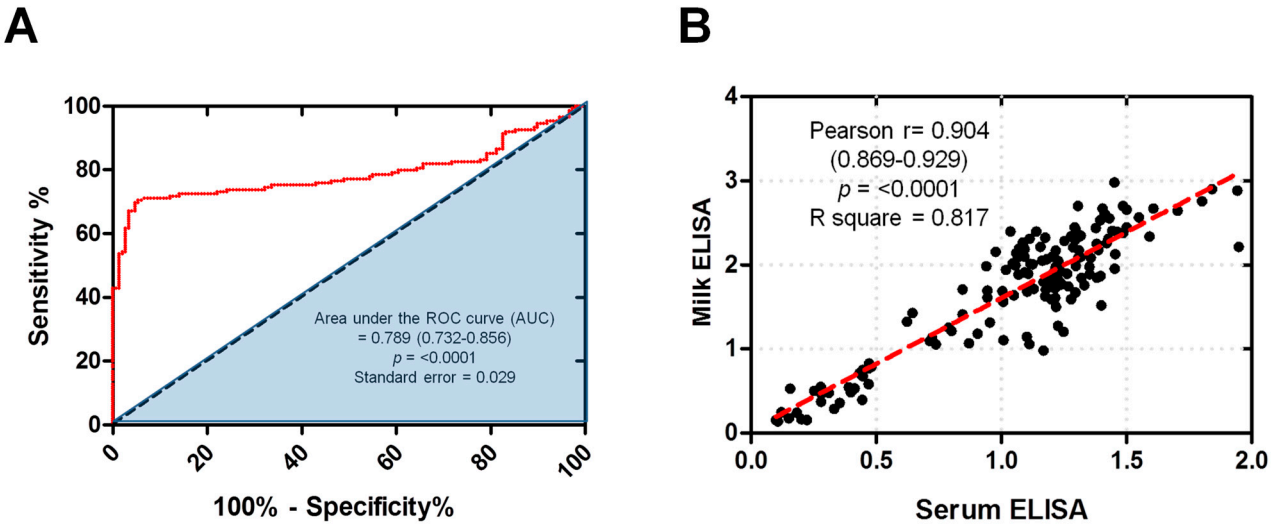

Figure 1. Evaluation of diagnostic efficiency of serum antibody ELISA against milk antibody ELISA. (**A**) Receiver operating characteristic (ROC) curve values were calculated using area under the curve (AUC) as a diagnostic accuracy test to validate the serum antibody ELISA using milk samples. ROC curve for the serum antibody ELISA against milk antibody ELISA shows an area under the curve of 0.789 (0.732–0.856 with a 95% confidence interval). (**B**) Correlation between serum antibody ELISA and milk antibody ELISA OD values of tested milk samples. Scatter graphs show the correlation between OD values recorded by serum antibody ELISA and milk antibody ELISA from all tested samples. The equation represents the approximation formula. The break line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: $|r| = 0.70$, strong correlation; $|r| > 0.5$–< 0.7, moderately strong correlation; and $|r| = 0.3$–0.5 weak-to-moderate correlation.

Figure 2A shows the Bland–Altman Plot of ELISA testing between the serum antibody ELISA and milk antibody ELISA. Dotted bluish lines are between 0.109 and −1.392 of the standard deviation 0.383 from mean (dotted red line). Almost all data points are between ±1.39 standard deviations (SDs), signifying good agreement between methods [22,23]. In the same context, the histogram of our tested milk samples using both methods showed a good correlation of the frequency distribution of the obtained data (serum antibody ELISA, 1.1 ± 0.4 SD; milk antibody ELISA, 1.7 ± 0.7 SD) for the total number of values (n = 149) [23]. However, the number of intervals illustrated at the x-axis was higher in the case of the milk antibody ELISA (n = 15) than that for the serum antibody ELISA (11) (Figure 2B).

To understand the obtained results, we assessed the antibody levels against *N. caninum* in milk samples by comparing % of inhibition using milk and serum antibody ELISAs. Percentage of inhibition of all samples was significantly lower in when using the milk antibody ELISA (standard method) compared to the serum antibody ELISA (test method) ($p \leq 0.0001$). According to the manufacturer's instructions, a lower % of inhibition indicates higher positivity and vice versa, where positive values are those of ≤50% in both test kits (Figure 3A). This result indicates the higher proficiency of the milk antibody ELISA than the serum antibody ELISA. For further analysis, positive and negative serum antibody

ELISA milk samples representing all positive samples using milk antibody ELISA were also compared. A significant difference was obtained, where dual-positive samples exhibited a lower % of inhibition than the positive samples in milk antibody ELISA only ($p \leq 0.0001$) (Figure 3B).

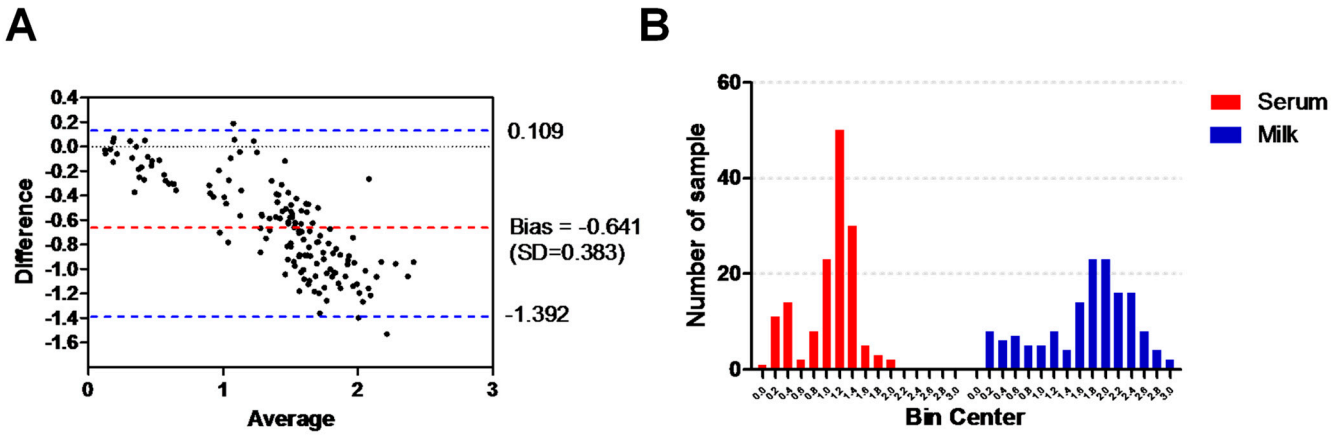

Figure 2. Comparison between the serum antibody ELISA and milk antibody ELISA using Bland–Altman Plot of ELISA and histogram. (**A**) Bland–Altman Plot of ELISA testing between the serum antibody ELISA and milk antibody ELISA. Dotted bluish lines between 0.109 and −1.392 of standard deviation 0.383 from mean (dotted red line). (**B**) Histogram of tested milk samples using serum antibody ELISA and milk antibody ELISA showing the frequency distribution of obtained data. The x-axis represents the OD values and the y-axis indicates the number of samples represented in each bar. Frequency distribution of obtained data using serum antibody ELISA was 1.1 ± 0.4 SD and using milk antibody ELISA was 1.7 ± 0.7 SD.

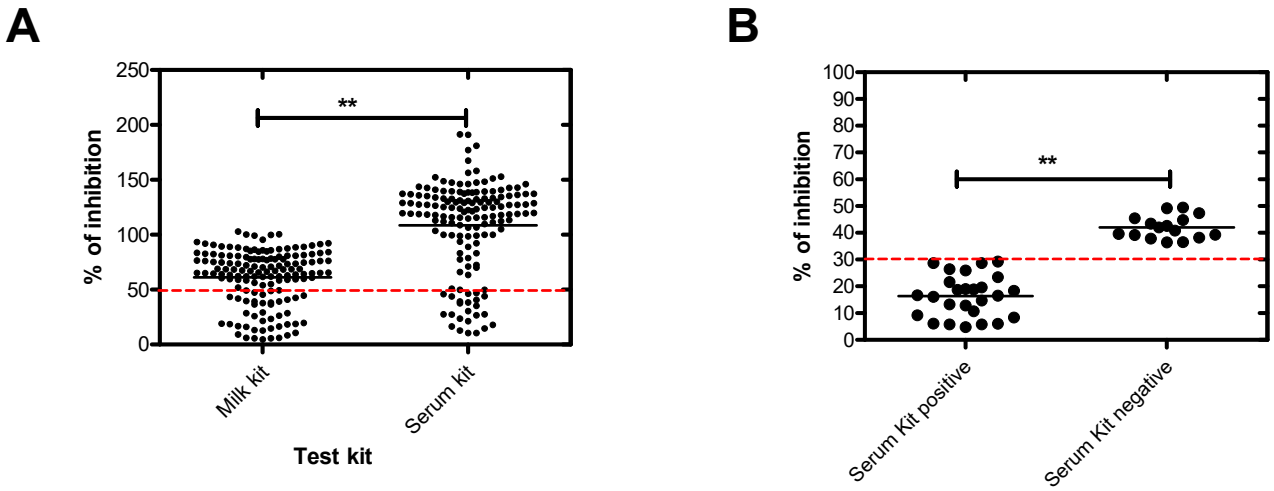

Figure 3. Comparison of the % of inhibition of *N. caninum* in milk samples using milk and serum antibody ELISAs. % of inhibition of all samples was significantly lower when using milk antibody ELISA (standard method) compared to serum antibody ELISA (test method) (** $p \leq 0.0001$). Positive values are those of ≤50% in both test kits (**A**). Positive and negative serum antibodies in ELISA of milk samples representing all positive samples and using milk antibody ELISA was also compared. A significant difference was obtained, where the dual-positive samples exhibited lower % of inhibition than the positive samples in the milk antibody ELISA only (** $p \leq 0.0001$) (**B**). Dashed red lines refer to the estimated cut-off values.

This result also indicates that the serum antibody ELISA could mainly detect samples of strong reactivity at the % of inhibition of ≤30% instead of the ≤50% samples reported in cases of serum or plasma use. Thus, low sensitivity and high specificity should be

considered in case of future usage of serum antibody ELISA in testing for *N. canium* antibodies in milk instead of serum or plasma. Also, an increase in cut-off value to 60% or considering doubtful samples as positive ones might be another prospect for positive milk sample judgments using serum antibody ELISAs.

Collectively, based on a previous study and the current data, serum antibody ELISA test kit might be used as an alternative method of detecting *N. caninum* antibodies in milk samples. The utility of the serum antibody ELISA is highly indicated because of its higher geographical distribution and easier preparation and use.

Author Contributions: Conceptualization and design, R.M.F. and C.F.F.; experiments, R.M.F., formal analysis, investigation, R.M.F., S.A.A. and C.F.F.; resources and shared materials, R.M.F., S.A.A. and C.F.F., writing—original draft, R.M.F., S.A.A. and C.F.F.; writing—review and editing, R.M.F. and C.F.F. Project administration and funding acquisition; R.M.F. and C.F.F. All authors have read and agreed to the published version of the manuscript.

Funding: This study did not receive any special funding.

Institutional Review Board Statement: The protocols were approved by the Research Code of Ethics at South Valley University number 36 (RCOE-36).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated and analyzed during this study are included in this published article. Raw data supporting the findings of this study are available from the corresponding author on request.

Acknowledgments: We would like to appreciate the great help of our colleagues at the Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena for their cooperation and technical assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Dubey, J.P. Review of *Neospora caninum* and neosporosis in animals. *Korean J. Parasitol.* **2003**, *41*, 1–16. [CrossRef] [PubMed]
2. Dubey, J.P.; Schares, G. Neosporosis in animals-the last five years. *Vet. Parasitol.* **2011**, *180*, 90–108. [CrossRef] [PubMed]
3. Lindsay, D.S.; Dubey, J.P. Neosporosis, Toxoplasmosis, and Sarcocystosis in Ruminants: An Update. Vet. Clin. *North Am. Food Anim. Pract.* **2020**, *36*, 205–222. [CrossRef] [PubMed]
4. Davison, H.C.; Otter, A.; Trees, A.J. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *Int. J. Parasitol.* **1999**, *29*, 1683–1689. [CrossRef]
5. McAllister, M.M.; Dubey, J.P.; Lindsay, D.S.; Jolley, W.R.; Wills, R.A.; McGuire, A.M. Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* **1998**, *28*, 1473–1478. [CrossRef]
6. Gondim, L.F.; McAllister, M.M.; Pitt, W.C.; Zemlicka, D.E. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* **2004**, *34*, 159–161. [CrossRef] [PubMed]
7. Hemphill, A.; Gottstein, B. *Neospora caninum* and neosporosis—Recent achievements in host and parasite cell biology and treatment. *Acta Parasitol.* **2006**, *51*, 15–25. [CrossRef]
8. Moore, D.P.; Cantón, G.J.; Louge Uriarte, E.L. Editorial: Infectious diseases affecting reproduction and the neonatal period in cattle. *Front. Vet. Sci.* **2021**, *8*, 679007. [CrossRef] [PubMed]
9. Ibrahim, H.M.; Huang, P.; Salem, T.A.; Talaat, R.M.; Nasr, M.I.; Xuan, X.; Nishikawa, Y. Short report: Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in northern Egypt. *Am. J. Trop. Med. Hyg.* **2009**, *80*, 263–267. [CrossRef] [PubMed]
10. Duarte, P.O.; Csordas, B.G.; Oshiro, L.M.; Higa, L.O.S.; Zimmermann, N.P.; Martins, K.R.; Barros, J.C.; Andreotti, R. Serological evaluation of *Neospora caninum* in pregnant women treated at referral center for prenatal screening in Mato Grosso do Sul, Brazil. *Rev. Bras. Parasitol. Vet.* **2020**, *29*, e010820. [CrossRef]
11. Duarte, P.O.; Oshiro, L.M.; Zimmermann, N.P.; Csordas, B.G.; Dourado, D.M.; Barros, J.C.; Andreotti, R. Serological and molecular detection of *Neospora caninum* and *Toxoplasma gondii* in human umbilical cord blood and placental tissue samples. *Sci. Rep.* **2020**, *10*, 9043. [CrossRef] [PubMed]
12. Björkman, C.; Johansson, O.; Stenlund, S.; Holmdahl, O.J.; Uggla, A. Neospora species infection in a herd of dairy cattle. *J. Am. Vet. Med. Assoc.* **1996**, *208*, 1441–1444. [CrossRef]
13. Hurley, W.L.; Theil, P.K. Perspectives on immunoglobulins in colostrum and milk. *Nutrients* **2011**, *3*, 442–474. [CrossRef] [PubMed]
14. Björkman, C.; Holmdahl, O.J.; Uggla, A. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Vet. Parasitol.* **1997**, *68*, 251–260. [CrossRef] [PubMed]

15. Moskwa, B.; Cabaj, W.; Pastusiak, K.; Bien, J. The suitability of milk in detection of *Neospora caninum* infection in cows. *Acta Parasitol.* **2003**, *48*, 138–141.
16. Chanlun, A.; Näslund, K.; Aiumlamai, S.; Björkman, C. Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand. *Vet. Parasitol.* **2002**, *110*, 35–44. [CrossRef] [PubMed]
17. Byrem, T.M.; Bartlett, P.C.; Donohue, H.; Voisinet, B.D.; Houseman, J.T. Performance of a commercial serum ELISA for the detection of antibodies to *Neospora caninum* in whole and skim milk samples. *Vet. Parasitol.* **2012**, *190*, 249–253. [CrossRef] [PubMed]
18. Fereig, R.M.; Abdelbaky, H.H.; Mazeed, A.M.; El-Alfy, E.-S.; Saleh, S.; Omar, M.A.; Alsayeqh, A.F.; Frey, C.F. Prevalence of *Neospora caninum* and *Toxoplasma gondii* Antibodies and DNA in Raw Milk of Various Ruminants in Egypt. *Pathogens* **2022**, *11*, 1305. [CrossRef] [PubMed]
19. Fereig, R.M.; Abdelbaky, H.H.; Nishikawa, Y. Comparative evaluation of four potent *Neospora caninum* diagnostic antigens using immunochromatographic assay for detection of specific antibody in cattle. *Microorganisms* **2021**, *9*, 2133. [CrossRef] [PubMed]
20. Moskwa, B.; Pastusiak, K.; Bien, J.; Cabaj, W. The first detection of *Neospora caninum* DNA in the colostrum of infected cows. *Parasitol. Res.* **2007**, *100*, 633–636. [CrossRef] [PubMed]
21. Gharekhani, J.; Yakhchali, M.; Afshari, A.; Adabi, M. Herd-level contamination of *Neospora caninum*, *Toxoplasma gondii* and *Brucella* in milk of Iranian dairy farms. *Food Microbiol.* **2021**, *100*, 103873. [CrossRef] [PubMed]
22. Raez-Bravo, A.; Granados, J.E.; Serrano, E.; Dellamaria, D.; Casais, R.; Rossi, L.; Puigdemont, A.; Cano-Manuel, F.J.; Fandos, P.; Pérez, J.M.; et al. Evaluation of three enzyme-linked immunosorbent assays for sarcoptic mange diagnosis and assessment in the Iberian ibex, *Capra pyrenaica*. *Parasit. Vectors* **2016**, *9*, 558. [CrossRef] [PubMed]
23. Bogan, J.E., Jr. Analytical and Clinical Evaluation of Two Methods for Measuring Erythrocyte Sedimentation Rate in Eastern Indigo Snakes (*Drymarchon couperi*). *Animals* **2023**, *13*, 464. [CrossRef] [PubMed]