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Molecular Detection of *Neospora Canium* in Bovine Colostrum: A Possible Risk Factor for Neonatal Infection

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Abstract

Neospora caninum is an obligate intracellular parasite that is both vertically and horizontally transmitted in cattle leading to significant economic losses in the cattle industry. The aim of this study was to investigate the prevalence of colostrum contamination in Neospora positive cows as a potential route for horizontal transmission. To this end, whole blood and colostrum from 44 fresh cows were analyzed using polymerase chain reaction (PCR) and nested PCR methods to detect the presence of the parasite DNA. In addition, serum samples were tested with a whole cell-based enzyme-linked immunosorbent assay (ELISA) to identify antibodies against the parasite. The results showed that 19 out of 44 colostrum samples (43.18%) contained the parasite genome. In addition, 16 of 18 (88.9%) Neospora -positive cows and 13 of 14 (92.9%) seropositive cows were found to have secreted infected colostrum. The relative risk (RR) of colostrum infection was significantly higher in blood PCR-positive cows (RR=7.7, P=0.0002) and seropositive cows (RR=4.6, P<0.0001) compared to uninfected cows. Based on these results, it is suggested that colostrum may be considered as a potential source of horizontal transmission in newborn calves and that methods for removing the infection such as pasteurisation may be required to reduce the risk of transmission.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan protozoan, poses a significant threat to the cattle industry due to fetal losses (Dubey and Schares, 2011). The infection can be transmitted horizontally (exogenous transmission) or vertically (endogenous transmission) (Wouda *et al.*, 1998). The probability of abortion in infected cows is 3 to 7 times higher than in non-infected cows (Dubey and Schares, 2011). Infected fetuses may be aborted or, if delivered naturally, may be congenitally infected (Wouda *et al.*, 1998).

Colostrum is the primary source of passive acquired immunity, predominantly containing IgG1, as well as immune cells such as macrophages and neutrophils, which contribute to the secretion of complement factors, cytokines, and antimicrobial peptides (Stelwa-

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gen *et al.*, 2009). Studies on the contamination of colostrum with *Neospora* have been limited; however, recent research has focused on the mechanisms of the innate immune response, including Toll-like receptor molecules, which play a crucial role in the induction of pro-inflammatory and adaptive immune responses (Marin *et al.*, 2007; Rivera *et al.*, 2021).

Oocytes, tachyzoite or tissue cysts ingestion is reported as the main route for horizontal transmission. Neospora DNA was first detected in colostrum of infected cattle indicating the colostrum to be a route of the parasite transmission (Lindsay and Dubey, 1990). Based on a previous laboratory study showing a sensitivity for *N. caninum* tachyzoites in HCL-pepsin (Lindsay and Dubey, 1990), consumption of contaminated colostrum was considered to be uninfective. However, other studies reported oral transmission of *N. caninum* through colostrum. Experimental consumption of colostrum, milk or milk replacer, even one week after birth, can cause infection in calves (Moskwa and Cabaj, 2007).

The frequency of *Neospora caninum* tachyzoite contamination in bovine colostrum and milk may vary depending on the stage of lactation or dairy cow breed. Consequently, a considerable number of tachyzoites in colostrum or milk could result in infection of the calf (Moskwa and Cabaj, 2007).

Depending on the stage of lactation and the number of tachyzoites in the colostrum or milk, the contamination of colostrum and milk with tachyzoites may change The permeability of intestine in the first few hours after the birth is crucial for the efficient transfer of colostrum immunity to newborn calves. The high permeability of intestinal cells in newborn calves makes them highly susceptible to macromolecules and pathogens. Therefore, contaminated colostrum can serve as a potential source of pathogens for calves before their gut closure (Moskwa and Cabaj, 2007).

The horizontal role of colostrum in transmission of pathogens is also accounted as a topic of interest. The blood circulation of the parasite and the response of the immune system can probably affect the presence of the parasite in the breast tissue and its secretion in the colostrum. The primary objective of this study was to determine the prevalence of colostrum contamination in infected cows and to evaluate the correlation between the presence of the parasite in colostrum, the serological immune response, and the detection of parasite DNA in blood.

2. Materials and Methods

2.1. Animals and Sampling

Based on an effect size of 0.6 (Mohammed and Aaiz, 2025; Ayati *et al.*, 2023), α<0.05 level and a 1-ß=0.95, the sample size was determined. Sampling was performed in an industrial Holstein dairy cattle herd located in Qazvin Province, Iran, with cows evenly distributed across parities one through four. Blood and colostrum samples were collected from 44 full-term cows. Blood was collected using 10 ml EDTA K3 Venoject and plain 10 ml Venoject from the jugular vein, and colostrum in 70 ml sterile bottles (at least 10 ml from each quarter). The buffy coat and serum were separated by centrifugation at 1500×g for 10 minutes. All samples were stored at -20°C before analysis.

2.2. Indirect ELISA Test

Two million (2×10^6) parasite tachyzoites were cultured at room temperature for three days. 300 L of blocker buffer (100 ml PBS + 5 g powdered milk) were added to each well of the plate and incubated at 37°C for one hour. The parasites were washed, and 100 L of the serum samples were added diluting at a ratio of 1:100. HRP-conjugated sheep anti-bovine IgG heavy chain was added to the wells and incubated at 37°C for one hour. After three washes and a thirty-minute incubation, 100 L of substrate was added. Finally, 50 L of 2.5 M sulfuric acid was added as the stop solution. The optical density (OD) was measured at a wavelength of 450 nm. The S/P ratio was calculated as follows: S/P= (sample-negative control) / (positive control-negative control). An S/P value less than 0.5 (the cut-off point for negative ELISA) was considered as negative (Schares *et al.*, 2004).

2.3. The Polymerase Chain Reaction (PCR) Conforming

DNA extraction from colostrum and blood buffy coat was performed using the Tissue Genomic DNA Extraction Mini Kit and the Blood Genomic DNA Extraction Mini Kit, respectively, both from FAVORGEN Company (Meiri-Bendek *et al.*, 2002). The extracted DNA was stored at -20°C until used.

PCR: NC5 gene from *N. caninum* was detected in buffy coat samples using Np6 forward (5′-CTCGCCAGTCAACCTACGTCTTCCT>-3′) and Np21 reverse (5′ -CCCAGTGCGTCCAATCCTGTAA CC >-3′) primers (Muller *et al.*, 1996). The PCR was programmed as 10 minutes at 95C for primary denaturation and 35 cycles of 95C for 1 minute, 65°C 1 min and 72°C for 1 minute and a final extension at 72°C for 10 min.

Nested-PCR: 1 μl of the PCR products was subjected to nested-PCR for confirming the presence of *N.caninum* NC5 gene using 5′-GTGTTGCTCTGCTGACGTGT-3′ forward and 5′-TACCAACTCCCTCGGTTCAC-3′ reverse primers (Ayati *et al.*, 2023). The nested PCR was programmed as 10 minutes for primary denaturation at 95°C and 35 cycles of 1 minute at 95°C for denaturation, 45 sec-

onds at 54°C for annealing and 1 minute at 72°C for extension. Finally, the reaction was completed with a final extension at 72°C for 10 minutes.

Statistical Analysis: Chi-square test was used to describe the relationship between variables and logistic regression to the model and predict the factors determining the presence of parasites in colostrum. Analyzes were performed at $\alpha<0.05$ level by IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, N.Y., USA).

3. Results

Neospora caninum DNA was detected in 19 out of 44 colostrum samples (43.18%). *Neospora caninum* DNA was also detected in colostrum of 16 out of 18 (88.9%) blood positive and 13 out of 14 (92.9%) seropositive cows (Table 1).

Table 1
Correlation between blood PCR and serum ELISA-Ab with colostrum PCR results

		Colostrum PCR		*P*-value	Relative Risk (CI 95%)
		Positive	Negative		
Blood PCR	Positive	16 88.9%	2 11.1%	0.0002	7.7 (2.6-22.6)
	Negative	3 11.5%	23 88.5%		
ELISA -Ab	Positive	13 92.9%	1 7.1%	<0.000	4.6 (2.2-9.6)
	Negative	6 20.0%	24 80.0%		

Table 2
Reliability of blood PCR and serum ELISA-Ab for detection of *N. canium* infection

		ELISA-Ab		*P*-value	*kappa*
		Positive	Negative		
Blood PCR	Positive	13 72.2%	5 27.8%	<0.001	0.708
	Negative	1 3.8%	25 96.2%		

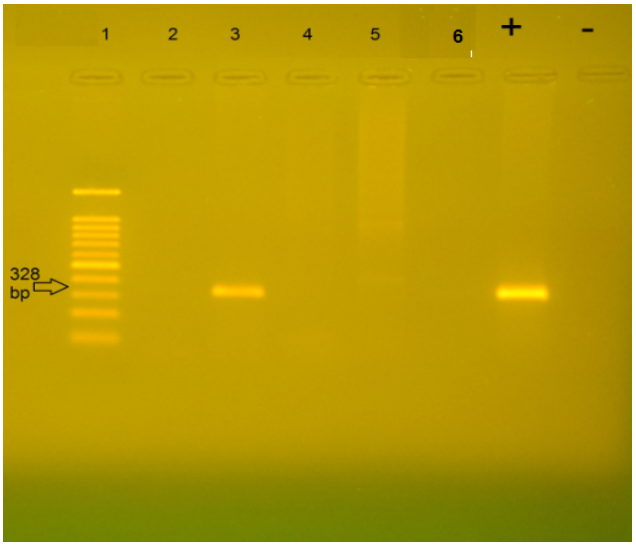

Fig. 1. PCR results with primers NP6 and NP21, bp 328 (1: standard DNA, 2 to 6: tested samples, +: positive control, -: negative control)

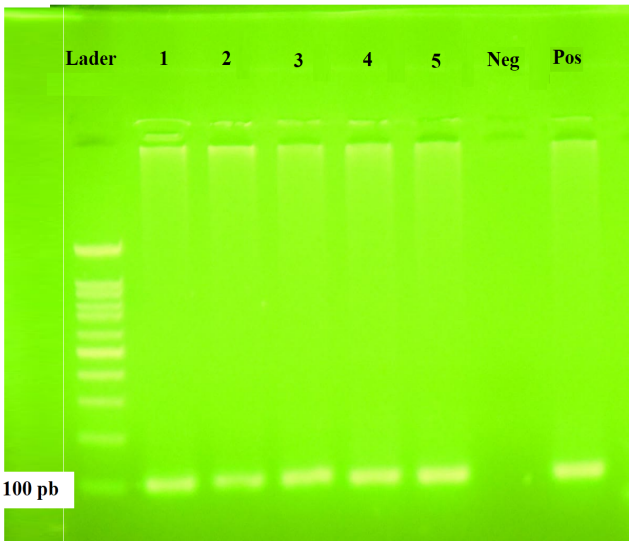

Fig. 2. Nested-PCR results with bp100 primer (Standard DNA (Lader), 1 to 5: positive samples, positive: positive control, negative: negative control)

4. Discussion

Neospora caninum-DNA was detected in 18/44 (40.90%) and 19/44 (43.18%) blood and colostrum samples, respectively. As presented in Table 1, Neospora DNA was detected in the colostrum of 88.9% (16/18) of cows positive by blood PCR and 92.9% (13/14) of cows seropositive by ELISA. These findings indicate that a substantial proportion of cows testing positive for the parasite-either through molecular detection in blood or serological assays-produce colostrum containing parasite DNA.

This point becomes more significant when despite the antibody response, Neospora parasite can still be detected in the bloodstream of infected cows (Ayati *et al.*, 2023; Okeoma *et al.*, 2004). Therefore, because of the high rate of blood circulation mammary glands can be considered as a target for the parasite in newborn cows.

The genome of *Neospora caninum* was first identified in the colostrum of infected cattle in 2007 (Moskwa *et al.*, 2007). Previous studies reported the mammary glands as one of the target tissues of the parasite. They noted that the parasite was present in the mammary glands during the acute phase of the disease and it was cleared when the disease became chronic (Lopez-Perez *et al.*, 2006). The presence of the parasite in the colostrum in the early lactation is due to the natural hyperaemia of the mammary glands in this lactation stage (Gotze *et al.*, 2010).

Genomic detection methods such as PCR are capable of identifying DNA from both viable and non-viable pathogens, whereas bioassay techniques are required to detect only live pathogens. Therefore, assays targeting specific pathogens must be meticulously designed and rigorously validated to enable accurate interpretation of results and definitive conclusions (Valasek and Repa, 2005). With this understanding, if genomic detection by PCR is assumed as the criterion for identifying live parasites, then the data suggest that oral infection with *N. caninum* through colostrum could serve as a potential mechanism for horizontal transmission, resulting in the infection of newborn calves shortly after birth (Uggla *et al.*, 1998). It is shown that one-week-old calves can be experimentally infected by ingesting *N. caninum*-infected colostrum or milk replacer (Davison *et al.*, 2001). The susceptibility of *N.caninum* tachyzoites to HCL-pepsin is reported. However, certain factors, such as encapsulation of the parasite by biological materials like placenta, may shield the tachyzoites from the acidic gastric environment, facilitating their safe passage into the intestine and subsequent infection of the host animal (Dijkstra *et al.*, 2001). Moreover, concentration of the parasite in colostrum and milk can significantly vary among the host cows and during stages of lactation and it can be influenced by immunological conditions. Thus, calves may potentially contract the infection through the colostrum under specific circumstances, particularly when high numbers of tachyzoites are present in the colostrum or milk during the lactation period.

The high intestinal permeability and incomplete function of the HCL-pepsin system in the abomasum of new born calves increase the absorption of macromolecules from the colostrum within the first 24 hours of life. This phenomenon guarantees the effective absorption of maternal immunity (Lopez and Heinrichs, 2022). Nevertheless, there is still a potential risk of infection for newborn calves due to environmental exposure or the ingestion of colostrum contaminated with pathogens.

The immune function of periparturient cows may be suppressed in various ways due to metabolic, hormonal and immunological disturbances. Consequently, clinical mastitis and other diseases are more common during this period (Vlasova and Saif, 2021). The pathogens are likely not only present in the secretions of the mammary glands but also in the circulatory system. Umbilical endothelial cells are immunoreactive when they encounter parasites, stimulating the transcription of genes encoding for inflammatory and immunomodulatory molecular genes, which in turn lead to innate and acquired immune responses (Taubert *et al.*, 2006). A previous study indicated the presence of *N. caninum* DNA in the milk of seropositive cows, which can be followed by lactogenic transmission. Other researchers have observed the absence of *Neospora* DNA in colostrum samples, peripheral blood mononuclear cells (PBMC) of heifers and calves, as well as in the umbilical cord (5), which is attributed to the infection occurring prior to delivery. In this case, the bradyzoite form of the parasite may enter the tissues due to the weak immune responses. Furthermore, the expression of genes and the production of anti-inflammatory cytokines such as IL-10 in the umbilical cord may also contribute to the observed weakness of the immune system (Taubert *et al.*, 2006).

Moskwa *et al.* (2007) reported that due to the presence of high levels of specific anti-Neospora IgG antibodies in the colostrum, it is unlikely that the infection will be transmitted through colostrum. Consequently, the presence of contamination in colostrum cannot be the definitive reason for the transmission of the parasite due to the presence of antibodies. The mechanism of this neutralisation is not yet clear, but colostrum can be considered as a source of contamination and transmission (Moskwa *et al.*, 2007).

Immunomodulatory agents suppress the responses of the immune system and lead to colonization of the parasite in the tissue, such as the mammary glands. TH2 lymphocytes stimulate antibody production by the humoral immune system, which leads to the production of specific antibodies, including IgG. These antibodies have the capacity to neutralize the parasite.

However, although they neutralize the parasite in the tissue and colostrum of the cow, they still have the potential to transmit the infection to calves (Taubert *et al.*, 2006).

The relative risk of colostrum contamination was 7.7 (95% CI: 2.6-22.6, p-value <0.0002) and 4.6 (95% CI: 2.2-9.6, p-value<0.000) for dams that tested positive for *N. caninum* by PCR and ELISA, respectively (Table 1). These results indicate that the risk of colosterum contamination was significantly higher in mothers that tested positive than in those that tested negative (7.7 and 4.6 times, respectively). Therefore, both the PCR and ELISA tests are useful markers of the risk of colosterum contamination in cattle.

The Kappa coefficient is a statistical measure used to evaluate the agreement between two diagnostic tests and is a standard method for assessing the consistency and reliability of classified data. In this study, a Kappa value of 0.708 (P-value<0.001) between PCR and ELISA indicates good agreement between the results of the two tests detecting *N. caninum* infection. This value means that their diagnostic agreement is beyond chance and is reliable. Generally, a Kappa between 0.61 and 0.80 indicates acceptable and reliable agreement (Cohen, 1960). Therefore, these two tests provide similar and dependable results in diagnosing the disease in question. (Table 2).

The reliability of blood PCR and serum ELISA-Ab tests for detecting *N. caninum* infection is highlighted in Table 2. Although both tests demonstrate a good level of agreement, there are instances where their results differ. The blood PCR test appears to be more accurate in identifying positive cases, but the ELISA-Ab test may be more susceptible to producing false positive results. These findings suggest that using both tests for combination can lead to a higher level of diagnostic accuracy in *N. caninum* infection.

5. Conclusion

If the presence of the parasite genome is interpreted as the presence of viable parasites, colostrum may be considered a significant potential source of horizontal transmission in newborn calves. Therefore, it is recommended to implement biosecurity measures, such as pasteurization, to reduce this potential risk. Furthermore, studying the distribution of the parasite in mammary gland tissue and its correlation with parasitemia and the immune response status may contribute to a better understanding of the disease pathogenesis. It can be suggested to design an observational study to investigate the potential risk of transmission through colostrum and milk to calves, as well as to assess the viability of the parasite in these samples.

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