Detection of anti-cryptosporidium antibodies in colostrum of dairy cows in farms near Tehran: A study

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Abstract

Cryptosporidiosis, a common disease caused by a protozoan that can be transmitted between animals and humans, was the focus of our comprehensive and meticulously conducted study. The aim of this study was to detect the anti-Cryptosporidium antibodies in the colostrum of cows from several dairy farms near Tehran. We began by collecting fecal samples from diarrheic calves suspected of having *Cryptosporidium* infection. These samples were then subjected to modified Ziehl-Neelsen staining to confirm the presence of *Cryptosporidium* based on morphological structure and staining characteristics of the parasite oocysts. Subsequently, oocysts were isolated and purified from the fecal samples using modified 55% sucrose flotation to ensure the extraction of pure oocysts for further analysis. To investigate the prevalence of *Cryptosporidium* infection in cows around Tehran, we collected 100 colostrum samples from four dairy farms in the region from the first milking of 100 cows of different ages. After removing the fat content from the colostrum samples, the detection of anti-*Cryptosporidium* antibodies was conducted using the indirect immunofluorescence assay (IFAT). One of the key findings of this study is the potential protective role of anti-*Cryptosporidium* antibodies in colostrum. Of the 100 colostrum samples collected, 64 tested positive for these antibodies, while 36 were negative. Calves that received colostrum from the negative samples experienced severe diarrhea and excretion of oocytes. In contrast, calves that received colostrum from the positive samples showed no clinical symptoms and a significant decrease in oocyst excretion. These results strongly suggest that anti-*Cryptosporidium* antibodies in colostrum can provide significant protection against *Cryptosporidium* infection in calves. This finding could potentially revolutionize the health and well-being of cattle populations.

Keywords: Cryptosporidiosis, Colostrum, Indirect immunofluorescence method, Dairy cows.

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Introduction

Cryptosporidium parvum, a protozoan belonging to the Apicomplexa group, is a globally distributed parasite with significant implications for human and animal health. Its widespread distribution underscores the urgency of our research. It is known for its ability to invade the intestinal epithelial tissues of most vertebrates. Since its discovery by Tyzzer until now over 40 species have been described, from which at least 20 species were reported as zoonosis agents (Jennifer *et al*., 2021) Among these, *Cryptosporidium andersoni* and C. parvum are particularly important in cattle. *Cryptosporidium andersoni* affects the mammary gland's mucosal epithelial cells, reducing milk production in dairy cattle. *Cryptosporidium parvum*, on the other hand, is a significant cause of diarrhea in both humans and domestic animals (Radostits *et al*., 2007).

Recently, *Cryptosporidium parvum* has gained attention as an intestinal pathogen affecting individuals with compromised immune systems and children (Sagodira *et al*.,1999; de Graaf *et al*., 1999; Robinson *et al*., 2009; Jennifer *et al*., 2021). The parasite's life cycle occurs in the subepithelial space of the intestinal cells, primarily in the distal part of the small intestine. Its invasion results in atrophy of villi, alteration of epithelial cells, and reduction in intestinal microvilli size, which leads to diarrhea (Wee *et al*., 1996; Changizi *et al*., 2012; Zarghami *et al*., 2015).

The prevalence of *cryptosporidiosis* in immunocompromised individuals worldwide underscores the importance of this parasite in human health. In Iran, the rates of *cryptosporidiosis* in HIV and hemoglobinopathy-associated diarrhea patients are 33.4% and 11.1%, respectively. This disease is responsible for approximately 10% of HIV-related deaths (Nahrevanian and Assmar, 2006; Azizi *et al*., 2007). In addition to its impact on human health, *Cryptosporidium parvum* poses significant challenges for the dairy industry. It causes economic losses due to expenses related to treatment and veterinary services, diarrhea, weight loss, and mortality in calves (De Graaf *et al*., 1999; Mokhber Dezfouli *et al*., 2005). More than half of newborn calves are at risk of *Cryptosporidium* contamination (Fayer, 2008). Infected cows and calves have been identified as potential sources of human contamination, as they can contaminate drinking water by excreting parasite oocysts. The infective stage of this parasite can persist in moist environments for extended periods and exhibits resistance to many common disinfectants (Wee *et al*., 1996; Elfstrand *et al*., 2002; Nahrevanian and Assmar, 2006; Radostits *et al*., 2007: Fayer., 2008; Shayan *et al*., 2008).

The main clinical signs in calves include depression, anorexia, diarrhea, weight loss, and occasional fatalities (De Graaf *et al*., 1999; De Waele *et al*., 2010; Nourmohammadzadeh *et al*., 2010). Due to the syndesmochorial type of placental attachment in cattle, maternal antibody transfer to the fetus does not occur through the placenta, and a newborn calf

is devoid of gamma globulins. On the other hand, the mucosal immune system in this immature calf is underdeveloped. Therefore, a newborn calf relies on receiving maternal antibodies through colostrum, which should be acquired within 12 to 24 hours after birth (Arrowood *et al*., 1989; Shayan *et al*., 2008). The passive immunity acquired through colostrum ingestion is not limited to maternal antibodies (primarily IgG) but includes maternal lymphoid cells and immune-regulatory cytokines. Both humoral and cellular immune responses play roles in protection against *Cryptosporidium* infection. Interferon-gamma, CD4+ T cells, and Interleukin-12 play significant roles in resistance and protection against *cryptosporidiosis* (Shayan *et al*., 2008). This study aimed to detect anti-*Cryptosporidium* antibodies in cattle colostrum from farms near Tehran.

Material and methods

In this study, 100 first-milking colostrum samples were collected from various cows of various ages in four dairy farms around Tehran(Varamin and Karaj). The samples were collected in two containers, one with a capacity of one liter and the other with a capacity of 200 milliliters. The samples were stored near ice during transportation to the Parasitology Department of the Faculty of Veterinary Medicine at the University of Tehran to maintain their integrity. Upon arrival, the samples were promptly stored at -20°C until analysis. Simultaneously, when collecting colostrum samples, many diarrheic calf fecal samples were obtained from selected dairy farms around Tehran to isolate and purify *Cryptosporidium* oocysts. To ensure long-term preservation, potassium dichromate was added to the fecal samples before being transferred to the laboratory (Elfstrand *et al*., 2002; Zarghami *et al*., 2015).

Oocyst evaluation in fecal samples

The samples underwent meticulous procedures to detect *Cryptosporidium* oocysts in suspicious fecal samples. Firstly, the samples were washed two to three times using 7.2 pH PBS (phosphate-buffered saline) to remove potassium dichromate completely. This was achieved by centrifugation at 3000g for 10 minutes. Afterwards, prepared smears and subjected to the modified Ziehl-Neelsen staining method (Arrowood *et al*., 1989). Each slide was observed thrice under 1000x magnification, with an average of 20 fields per slide and counted (Anderson, 1981). Next, a purification process was performed on the fecal sample to isolate the oocysts. Specifically, 10 milliliters of the sample was thoroughly mixed with one volume of ether and three volumes of 1% sodium bicarbonate solution. The mixture was then centrifuged at 3000g for 10 minutes. The top layer contained lipids, while the sediment formed the bottom layer. From the obtained sediment, five milliliters were mixed with 40 milliliters of chilled 55% sucrose.

Additionally, 10 milliliters of cool distilled water was gently added, forming distinct layers. The container

was centrifuged at 3000g for 20 minutes, separating three layers: distilled water at the top, a middle layer containing suspended oocysts, and a sediment layer. The primary oocyst-containing solution was exposed to 70% alcohol for 30 minutes to minimize bacterial contamination and achieve disinfection. Oocysts collected from each stage were individually examined, counted, and enumerated using a Neubauer counting chamber (Winter *et al*., 2000).

Preparation stages of colostrum samples

The frozen colostrum samples were initially moved to the refrigerator at 4°C for thawing. Once thawed, they were further brought to room temperature. To extract fat from the colostrum samples and separate the soluble protein phase, the samples were carefully centrifuged at 10000 g for 30 minutes at 2°C. The lipid layer was then separated using a micropipette. The remaining contents were transferred to a sterile microtube, and 1µl of the enzyme renin (0.1units/mg)(sigma company) was added to the sample. The mixture was incubated in a water bath at 37°C for 1.5 hours. Following the incubation, it was centrifuged at 10000 g for 5 minutes at 25°C. The upper layer of the microtube, which contained the soluble proteins, was gently extracted and transferred to another sterile microtube. This microtube was then stored in a freezer at -20°C (Elfstrand *et al*., 2002; Askari *et al*., 2016; Mokhber Dezfouli *et al*., 2022).

Evaluation of anti-cryptosporidium antibody in colostrum indirect immunofluorescence antibody (IFA) assay

The indirect immunofluorescence antibody (IFA) test is a reliable and valuable serological method. The IFAT-test was performed as described by Dubey *et al*. (1988). For this aim, the dilutions of 1:200 were prepared from colostrum samples. To prepare each dilution, 1µl of colostrum sample was mixed thoroughly with 199µl of PBS solution with a pH of 7.4. *Cryptosporidium* oocysts were then placed on 12-well immunofluorescence slides and allowed to air dry. The samples were fixed by incubating them in acetone at laboratory temperature for 10 minutes. For each well on the slide, 20µl of the tested colostrum samples were added. One well was allocated for the positive control colostrum and another for the negative control colostrum without *Cryptosporidium* oocysts. The slides were placed in a humid chamber at room temperature (37°C) for 40 minutes. Afterwards, the slides were gently washed three times with PBS solution at a pH of 7.4 for half an hour. Once the area around the wells had dried, 20µl of conjugated anti-bovine immunoglobulins with fluorescein isothiocyanate (FITC) was added to each well at a dilution of 1:40 (Dubey *et al*., 1988; Mtambo *et al*.,

1995; Askari *et al*., 2016; Mokhber Dezfouli *et al*., 2022)

The slides were then placed in a dark location at room temperature for 40 minutes. After that, further washing was performed, and 50% glycerol solution was added to the wells. Finally, the slides were examined under a fluorescent microscope using ultraviolet light. They were initially observed at a lens of X40 and then at a lens of X100[19]. In this method, the FITC dye attached to the anti-bovine monoclonal antibody absorbs UV light at a wavelength between 490-495nm, which results in the emission of brilliant green fluorescence at this specific wavelength (Robinson *et al*., 2009).

Results

Evaluation of Cryptosporidium oocysts in fecal samples using modified ziehl-neelsen staining

The oocysts were visualized as round or oval bodies measuring approximately 4-6µm. They appeared bright red against a green background. Their cytoplasm displayed a granular appearance and often contained a prominent central spot, which could contain up to 6 very dark particles (Anderson, 1981) (Fig. 1).

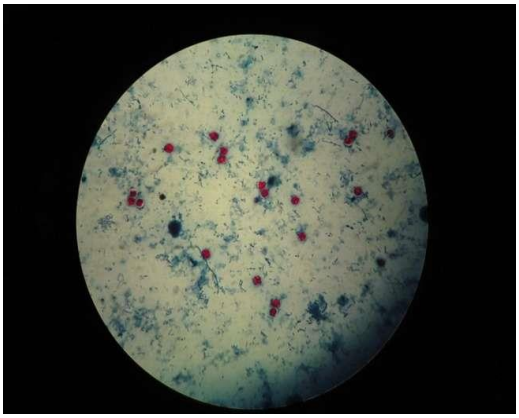

Figure 1: Positive sample of *Cryptosporidium parvum* using the modified Ziehl-Neelsen staining method.

Indirect immunofluorescence antibody (IFA) assay for anti-Cryptosporidium antibody evaluation

After the test, slides were carefully examined under X40 and X100 magnifications. This examination was repeated three times to ensure accuracy. In samples that tested positive for *Cryptosporidium*, the parasites were observed as round or oval bodies, measuring approximately 4-6µm. These parasites exhibited a distinctive yellowish-green fluorescence (Fig. 2).

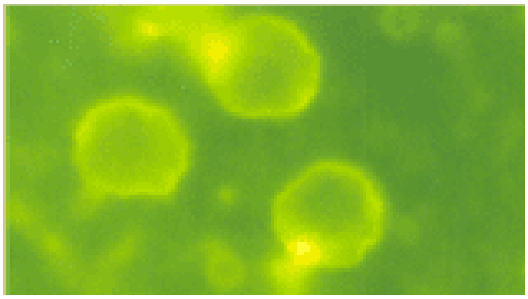

Figure 2: Immunofluorescent Indirect Staining of *Cryptosporidium parvum* Oocysts in Positive Sample.

However, in negative samples, *Cryptosporidium* parasites were present but did not display the bright yellowish-green fluorescence characteristic

(Arrowood *et al*., 1989; Robinson., 2009) (Fig. 3). Based on the analysis of 100 samples collected from 4 cattle farms near Tehran, 64 samples tested positive for *Cryptosporidium*, while 36 tested negative. The specific breakdown of these results can be found in (Table 1).

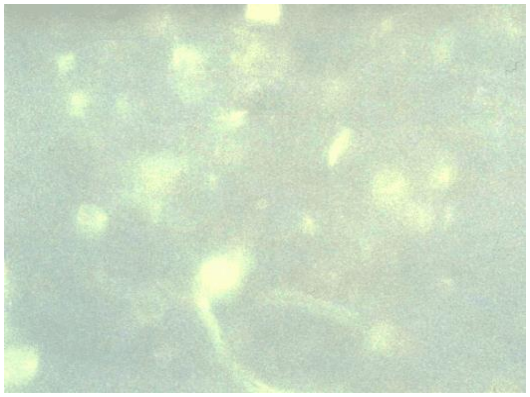

Figure 3: Immunofluorescent Indirect Staining of *Cryptosporidium parvum* Oocysts in Negative Sample.

Table 1: Positive and negative samples for antibody presence.

Farm	Positives	Negatives
1	38	17
2	15	9
3	7	6
4	4	4

Discussion

In cattle, the transfer of maternal antibodies to the fetus does not occur through the placenta, as the placenta is cotyledonary.This means a newborn calf is no in immunoglobulins, and its mucosal immune system is immature (Shayan *et al*., 2008). To compensate for this, a calf relies on receiving antibodies through colostrum, which should be ingested within 12 to 14 hours after birth (Arrowood *et al.*,1989; Shayan *et al*., 2008).

Colostrum intake provides passive immunity to the calf, which includes the transfer of maternal antibodies, primarily IgG, maternal lymphoid cells and immune-regulatory cytokines. Colostrum is rich in nutrients and contains immune and growth factors. Since vitamins and immunoglobulins do not pass through the placental barrier, colostrum plays a critical role in providing essential components for the health of neonatal calves (Arrowood *et al*., 1989; Mokhber Dezfouli *et al*., 2011). Since newborns are exposed to a contaminated and environmentally stressful setting during birth, they are vulnerable to numerous pathogens. Due to their insufficiently developed defence system, colostrum intake becomes vital for providing immunity and supporting the health of neonates. While there is no definitive treatment or prevention for *Cryptosporidiosis*, using colostrum as a protective measure against *Cryptosporidium* becomes crucial (Arrowood *et al*., 1989; Zarghami *et al*., 2015).

In recent years, using milk and colostrum to diagnose and monitor various cattle diseases has become common practice. Tests related to antibodies present in milk now play a significant role in controlling and eradicating cattle diseases. This highlights the importance and necessity of utilizing colostrum as a preventive measure against *Cryptosporidium* and other cattle diseases (Shayan *et al*., 2008). Given the widespread reports of *Cryptosporidium* prevalence in various countries, including the United States

(Ungar *et al*., 1988), Tanzania (Mtambo *et al*., 1997), Korea (Chai *et al*., 1996) and different regions of Iran, such as West Azerbaijan, East Azerbaijan (Nourmohammadzadeh *et al*., 2010), Shahrekord (Azizi *et al*., 2007), and Tehran (Mokhber Dezfouli *et al*., 2005; Nourmohammadzadeh *et al*., 2010), it is crucial to have accurate and rapid diagnosis of the infection, as well as preventive measures to minimize significant damages. The indirect immunofluorescence assay has been found to have advantages compared to other methods, making it a valuable complementary technique alongside serological methods for screening and eradicating *Cryptosporidium* infections (Stibbs and Ongerth,1986; Geurden *et al*., 2006).

Besides the direct harm caused by *Cryptosporidium* in calves, it also serves as a predisposing factor for viral (rotavirus and coronavirus) and bacterial diarrhea. Initial clinical diagnosis of *Cryptosporidiosis* involves identifying oocysts in fecal samples. Diagnostic methods such as flotation, sedimentation, and fecal smear staining (Bronsdon) yield positive results when a sufficient number of oocysts are present in the sample (Anderson.,1981; Zierdt, 1984).

To improve sensitivity and diagnostic specificity in detecting *Cryptosporidiosis*, researchers have expanded the use of enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFA) assay methods, utilizing monoclonal antibodies for detecting *Cryptosporidium* oocysts in fecal samplesTheir findings demonstrated that monoclonal antibody-based methods exhibit superior sensitivity and specificity, particularly when oocyst counts are low in fecal samples, compared to other methods (Beth *et al*., 1989; Rusnak *et al*., 1989). Furthermore, these monoclonal antibody-based methods help eliminate false positives and false negatives encountered in routine detection methods for *Cryptosporidium* in fecal samples. Among the various tests available, the immunofluorescence (IFA) assay has emerged as the preferred method for diagnosing *Cryptosporidiosis* in most veterinary diagnostic laboratories (Rusnak *et al*., 1989). This test not only serves as an efficient research tool for assessing the periodic prevalence of the disease but also offers advantages such as sensitivity and specificity (Mtambo *et al*., 1995; Geurden *et al*., 2006; Mokhber Dezfouli *et al*., 2011). Given these advantages and the absence of reports regarding the presence of anti-*Cryptosporidium* antibodies in colostrum, this study aimed to determine cases of *cryptosporidiosis* infection by detecting anti-*Cryptosporidium* antibodies in the colostrum of cows using the IFA method. However, it is worth noting that there are reports concerning the detection of antibodies against microbial agents in milk and colostrum, which will be discussed later (Geurden *et al*., 2006; Zarghami *et al*., 2015).

the IFA method It was initially implemented by Dubey *et al*. (1988), and

has been widely used for serological investigations in various countries, especially in the context of epidemiology. The mentioned test was used as high sensitive in diagnosing protozoan diseases (Mtambo *et al*., 1995; Robinson *et al*., 2009) .One of the major advantages of the IFA test is its versatility in examining intact cells, tissues, and organisms. Additionally, the test exhibits a high level of sensitivity. Moreover, commercially available secondary antibodies that specifically bind to antibodies from different animal species make this test reliable, affordable, and easily accessible. The signals produced by this method are also brighter when compared to direct immunofluorescence. However, it is important to note that cross-reactions may occur, which is a limitation to consider (Riggs., 2002; De Waele *et al*., 2010).

In the current study, 64 out of 100 colostrum samples were found to have anti-*Cryptosporidium* antibodies using the IFA method. However, it is important to note that these antibodies, whether in blood or colostrum, do not necessarily indicate an acute or active infection. This is because oocysts, which are responsible for the infection, are excreted for a relatively short period. Therefore, antibodies without oocysts may indicate a past infection that occurred months or years ago . Researchers have also discovered that in male animals, anti-*Cryptosporidium* antibodies can persist for at least one to two years after a stable infection (Beth *et al*., 1989)

Additionally, studies have shown that over 50% of individuals without a history of *cryptosporidiosis* infection have been found to have anti-*Cryptosporidium* antibodies (Kenneth *et al*., 1985; Rahmatullah *et al*., 2000). Shayan *et al*., 2008 reported that the levels of anti-*Cryptosporidium* antibodies in the sera of 264 calves and 173 cows were 37% and 33%, respectively, using the Dot blot method (Shayan *et al*., 2008). This suggests that consuming colostrum containing anti-*Cryptosporidium* antibodies could confer immunity against this disease in animals, considering the importance of colostrum consumption by newborns in the early hours.

The present study found that calves that received negative colostrum experienced severe diarrhea and excreted oocysts. On the other hand, calves that received positive colostrum not only showed no clinical symptoms but also had a significant reduction in oocyst excretion. This supports the significance of IgG antibody levels in colostrum for preventing newborn calves from *Cryptosporidium parvum* infection, as Lefkaditis *et al*. (2020) demonstrated. Their study collected colostrum samples from 50 cows within 12 hours after calving, and the IgG antibody levels were evaluated using the single radial immunodiffusion method. The researchers observed cases of cryptosporidial diarrhea in calves when colostral IgG antibody levels ranged from 680-3680 mg/dl, indicating a negative correlation between antibody

levels and diarrhea incidence in calves(Lefkaditis *et al*., 2020).

Furthermore, Mokhber Dezfuli *et al*. (2022) demonstrated the effectiveness of vaccinating pregnant cows four times at two-week intervals with *Cryptosporidium parvum* oocyst lysates as a candidate complete antigen vaccine, starting at 70 days before calving. The resulting calves, nourished with colostrum known as hyperimmune colostrum, not only showed no clinical signs but also exhibited a significant reduction in oocyst excretion. This highlights the potential of vaccinating cows and providing their calves with hyperimmune colostrum as a preventive measure against *Cryptosporidium* infection (Mokhber Dezfouli *et al*., 2022).

Conclusion

To summarize, using milk and colostrum for diagnosing and monitoring cattle diseases has become increasingly popular. Testing milk and colostrum antibodies is crucial for disease control and eradication, with strong correlations observed between milk and serum antibody levels in various diseases. Also, sampling and storing colostrum is simpler, cost-effective, and less invasive than serum sampling. The indirect immunofluorescent assay in colostrum offers several advantages over other methods and can serve as a complementary serological tool for screening and eradicating *Cryptosporidium*. Administering colostrum-containing antibodies to newborn calves can confer immunity against *Cryptosporidiosis* and reduce associated damages. Implementing this approach could effectively minimize the impact of *Cryptosporidiosis* and its consequences. The present study found that calves receiving negative colostrum experienced severe diarrhea and excreted oocysts. In contrast, calves that received positive colostrum showed no clinical symptoms and significantly reduced oocyst excretion. These findings further support the potential benefits of using colostrum with antibodies to prevent and manage *Cryptosporidiosis* in calves.

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Conflict of interest

The Authors Declare That They Have No Conflicts of Interest

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