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ORIGINAL ARTICLE

Development of Two Rapid Diagnostic Tests for Detection *Cryptosporidium parvum*

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Abstract

Cryptosporidium is a protozoan parasite responsible for cryptosporidiosis, a gastrointestinal infection that affects both humans and animals. Transmission primarily occurs through contaminated water, food, and environmental sources, posing significant public health risks, particularly to immunocompromised individuals and malnourished children, in whom the infection can lead to severe or life-threatening complications. This study aimed to develop two rapid latex agglutination and immunochromatographic diagnostic tests for the efficient detection of *Cryptosporidium parvum* and check their activities with calve diarrheic fecal samples. Diarrheic fecal samples were collected from calves under two months of age and processed using the formalin-ether concentration method to isolate *Cryptosporidium* protozoa. *Cryptosporidium parvum* was used to immunize rabbit for the production of polyclonal antibody, which was subsequently purified. Polyclonal antibody was conjugated to latex particles and incorporated into latex and immunochromatographic tests. The activities of these tests were assessed using dot blot and sandwich ELISA assays. The polyclonal antibody was successfully produced in rabbit. The latex and immunochromatographic tests effectively identified *Cryptosporidium parvum*-positive fecal samples. The findings of this study underscore the utility of immunochromatographic and latex diagnostic tests as a viable alternative to conventional methods for *Cryptosporidium* detection. These tests offer cost-effective, accessible, and user-friendly diagnostic solutions for livestock farmers and veterinary professionals, while also contributing to the prevention and control of cryptosporidiosis in both human and animal populations.

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Introduction

Cryptosporidium parvum is a protozoan parasite that causes cryptosporidiosis, a gastrointestinal disease affecting both humans and a wide range of animal species. This parasite has a global distribution and demonstrates remarkable resilience, remaining viable under diverse climatic conditions (1). To date, Cryptosporidium has been identified in over 260 species across multiple taxonomic classes, including mammals, reptiles, birds, and fish. Infection occurs when a susceptible host ingests Cryptosporidium oocysts through contaminated water, food, or other sources exposed to fecal matter. These oocysts are highly resistant and can survive for extended periods up to six months in cool, moist environments and on inanimate surfaces (1).

Cryptosporidiosis is clinically characterized by watery diarrhea, abdominal cramps, vomiting, fever, and loss of appetite. Symptoms can persist for up to a month, with approximately one-third of cases experiencing relapse following apparent recovery (2). Transmission primarily occurs through the ingestion of sporulated oocysts via the fecal-oral route, including exposure to contaminated water and airborne particles (3). In immunocompetent individuals, the infection is typically self-limiting. However, in vulnerable populations such as malnourished children and immunocompromised individuals including those with HIV/AIDS or undergoing immunosuppressive therapy cryptosporidiosis can progress to a severe, life-threatening condition (4). Long-term sequelae include growth retardation and cognitive impairment, particularly in children. The disease is a leading cause of moderate to severe diarrhea in regions such as Africa and Southeast Asia, contributing significantly to morbidity and mortality in these populations (5).

Given the substantial public health and economic burden imposed by cryptosporidiosis in both human and animal populations, early and accurate detection is critical for identifying transmission pathways and implementing effective control measures. Conventional diagnostic methods, such as microscopic examination and staining techniques, remain the gold standard for detecting gastrointestinal parasites (6). However, these methods are labor-intensive, time-consuming, and require skilled personnel and specialized laboratory equipment (7). While molecular and immunological assays offer high sensitivity and specificity, their high cost and technical complexity limit their widespread use, particularly in low-resource settings where financial and educational barriers are prevalent (8).

To address these limitations, rapid diagnostic tests (RDTs) have emerged as a practical alternative, enabling simple, cost-effective, and timely detection of parasites without the risk of contamination associated with molecular techniques (9). Among these, latex agglutination test (LAT) and lateral flow immunochromatography assay represent promising diagnostic tools, offering rapid, affordable, and field-deployable detection without the need for advanced laboratory infrastructure or highly trained personnel (10). These assays serve as a viable alternative to conventional microscopic staining and molecular diagnostic methods.

In this study, we developed two rapid diagnostic tests for the early detection of *Cryptosporidium parvum*. The findings of this research have the potential to significantly enhance diagnostic efficiency, providing an effective alternative to staining-based methods while offering broad applicability in human and veterinary medicine, as well as related fields.

Materials and Methods

Sample Collection and Preparation

A total of 10 diarrheic fecal samples were collected from calves under two months of age on dairy farms in Kerman province. Upon arrival at the laboratory, each sample was divided into two portions: one was used for microscopic examination and the other was stored at -70°C for subsequent analyses.

Antigen Preparation

Fecal samples were prepared using the formalin-ether sedimentation method (11). Briefly, 10 mL of 10% formalin was added to approximately 1 g of fecal material and thoroughly mixed to create a homogeneous suspension. The mixture was filtered through a glass funnel, and 3 mL of diethyl ether was added. After vigorous mixing and centrifugation, four distinct layers formed. The sediment layer, containing the *Cryptosporidium* antigens, was collected and used for smear preparation.

Staining Procedure

The prepared smears were fixed with methanol and stained using the modified Ziehl-Neelsen technique. Carbol fuchsin was applied to the slides for 15 minutes, followed by rinsing with tap water and decolorization with 1% acid-alcohol. Counterstaining was performed using 0.4% methylene blue for 30 seconds. Under light microscopy, *Cryptosporidium* oocysts appeared as bright red structures against a blue background.

Polyclonal Antibody Production

A New Zealand White male rabbit (~3 kg) was intradermally immunized with 0.5 ml of the prepared antigen emulsified in Complete Freund's Adjuvant (CFA) (Sigma, USA) once and three extra doses of the antigen-adjuvant (incomplete Freund's Adjuvant) mixture with two weeks' intervals. Blood samples were collected before each immunization, and one week after the last injection. Serum was separated and stored at -20°C for further analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

To evaluate polyclonal antibody production, an indirect ELISA was performed. Microplate wells (Jet Biofil, Korea) were coated overnight at 4ºC with sonicated lysed of *Cryptosporidium parvum* antigen that was diluted 1:200 in phosphate-buffered saline (PBS). After washing and blocking with 3% bovine serum albumin (BSA) (Sigma, USA), diluted rabbit sera (1:100) were added to the wells. Following incubation 1 hours at 37 ºC and washing, goat peroxidase-conjugated anti-rabbit IgG antibody (Serotec, USA) was introduced. The reaction was developed using Tetramethylbenzidine (TMB) substrate solution (prepared by mixing 9 mL of phosphate-citrate buffer, 1 mL of TMB, and 4 µL of hydrogen peroxide). The reaction was terminated with 1 M sulfuric acid, and absorbance was measured at 450 nm and 620 nm using a plate reader (Epoch, USA).

Polyclonal Antibody Purification

Polyclonal antibody was purified using Proteus Protein a Mini Sample kit (Serotec, USA). The purification process involved column activation, equilibration, serum filtration, and antibody elution. The purified antibody was stored at -20°C for subsequent experiments.

Polyclonal Antibody Titration

To determine the optimal working dilution of the purified polyclonal antibody, serial dilutions ranging from 1:100 to 1:50000 were prepared. The ELISA was repeated as described above, with 100 µL of each antibody dilution used in place of serum. A negative control well containing 100 µL of 3% BSA/PBS solution was included.

Dot Blot Assay

The specificity of the polyclonal antibody against *Cryptosporidium* antigen was confirmed using a dot blot assay. A 5-µL aliquot of antigen was applied to a nitrocellulose membrane and allowed to dry. The membrane was blocked with 3% skim milk for 45 minutes, washed with PBS, and incubated with diluted polyclonal antibody (1:500) at 37 °C for 1.5 hours. After additional washes, HRP-conjugated anti-rabbit IgG antibody (1:2000) was applied, and the reaction was visualized using 4-chloro-1-naphthol substrate. Color development indicated antibody activity.

Sandwich ELISA

A sandwich ELISA was performed using a monoclonal antibody as the capture antibody and a polyclonal antibody as the detection antibody. The procedure followed similar to the indirect ELISA, and absorbance was measured at 450 nm.

Antibody Conjugation to Latex Particles

A 200-µL aliquot of polyclonal (1:500 µL) antibody was mixed with 200 µL of 0.01 M glycine buffer (pH 8.2) and incubated at 4 °C for 1 hour. Subsequently, 12.5 µL of a 10% suspension of pink latex particles (Magsphere, USA) (300nm) was added to the antibody-buffer mixture and incubated on a shaker at room temperature for 2.5 hours. The mixture was centrifuged at 3000 × g for 15 minutes, and the resulting latex pellet was resuspended in 250 µL of storage buffer (0.01 M glycine, 10 µL Tween-20, 1% BSA). The latex suspension was stored at 4 °C for further use.

Latex Agglutination Assay

The functionality of the antibody-conjugated latex beads was assessed using a latex agglutination assay. 10 µL of conjugated latex beads were mixed with 10 µL of *Cryptosporidium parvum* antigen or samples, and the agglutination reaction was observed on a specialized test card for 5 min. The storage buffer served as a negative control. Results were evaluated visually based on the presence or absence of agglutination.

Lateral Flow Immunochromatography Assay

The sample pad and conjugate pad were pre-treated with PBS buffer containing 0.1% BSA and 0.5% PBS-Tween 20, followed by drying. Pink latex-conjugated with polyclonal antibody were applied to the conjugate pad. The nitrocellulose membrane was coated with anti-*Cryptosporidium parvum* monoclonal antibody (Santa Cruz, USA) (test line) and Protein A (control line), dried, and blocked with BSA. The immunochromatography test strips were assembled by integrating the membrane, absorbent pad, conjugate pad, and sample pad.
For analysis, diluted diarrheic bovine samples were applied to the test strip, allowing latex-conjugated antibodies to

migrate across the membrane. The presence of *Cryptosporidium* antigen was indicated by the appearance of both the test and control lines.

Results

Ziehl-Neelsen Staining

Ziehl-Neelsen staining identified *Cryptosporidium* oocysts as crescent-shaped structures, measuring 4–6 µm, which appeared bright red against a blue background (Figure 1). These findings confirmed the presence of *Cryptosporidium* infection in fecal samples collected from calves under two months of age.

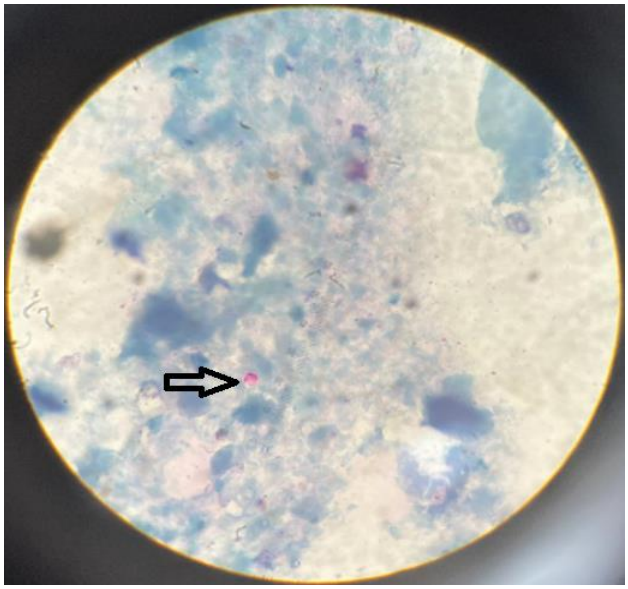

Figure 1. *Cryptosporidium* oocyst stained with Ziehl-Neelsen technique

ELISA

The production of polyclonal antibodies following rabbit immunization with *Cryptosporidium* antigen was evaluated using an indirect ELISA. The results demonstrated a progressive increase in antibody titers with each successive immunization, reaching peak levels after the final injection (Figure 2). In contrast, the negative control (BSA) showed no significant change in optical density (OD) values, confirming the specificity of the antibody response.

Dot Blot

The specificity of both polyclonal and monoclonal antibodies was confirmed using a dot blot assay. The results indicated that both antibodies specifically bound to the *Cryptosporidium parvum* antigen (Figure 3), demonstrating their ability to accurately identify the target antigen.

Sandwich ELISA Test

The sandwich ELISA test was performed to further verify the ability of recognition of antigen by the polyclonal and monoclonal antibodies. The results confirmed that both antibodies specifically bound to the *Cryptosporidium* antigen, with OD readings for positive samples being significantly higher than those of the negative control (BSA) (Figure 4).

Latex Agglutination Test

The latex agglutination test revealed that latex particles conjugated with polyclonal antibody reacted with *Cryptosporidium parvum*-positive fecal sample, producing visible agglutination (Figure 5). No agglutination was observed in the negative control, which consisted of the storage buffer alone.

Lateral Flow Assay

The immunochromatographic test strips were designed. The results demonstrated that positive samples produced distinct colored lines in both the test and control regions, while negative samples displayed a line only in the control region (Figure 6). These findings confirm that the developed test is capable of rapidly and accurately detecting *Cryptosporidium parvum* in fecal samples.

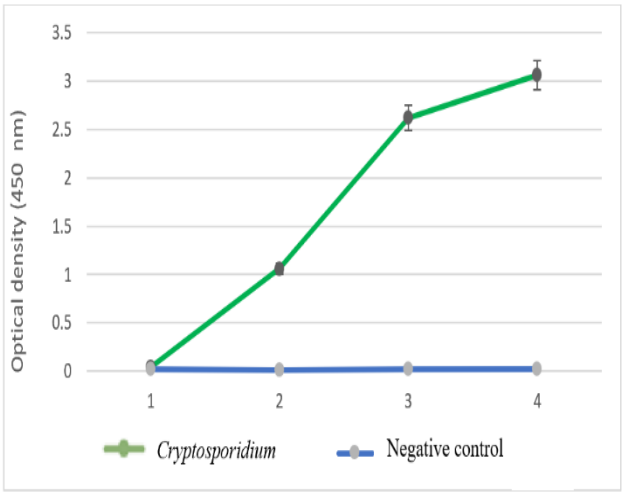

Figure 2. Production of rabbit polyclonal antibody against *Cryptosporidium parvum* surface antigens over four immunization phases. Green line: the reaction of immunized rabbit serum with *Cryptosporidium parvum* lysate, Blue line: the reaction of immunized rabbit serum with BSA as negative control. Histograms present the means from duplicate assays, the error bars showing standard deviations.

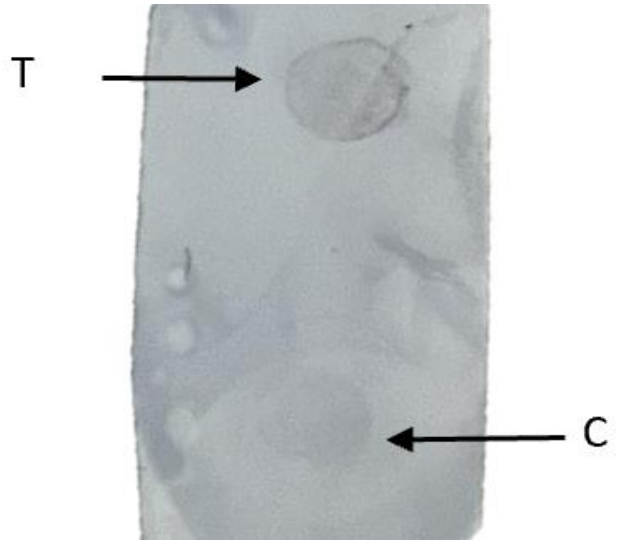

Figure 3. Dot blot assay to evaluate the reactivity of polyclonal antibody with *Cryptosporidium parvum*. T: *Cryptosporidium parvum* lysate, C: BSA as negative control.

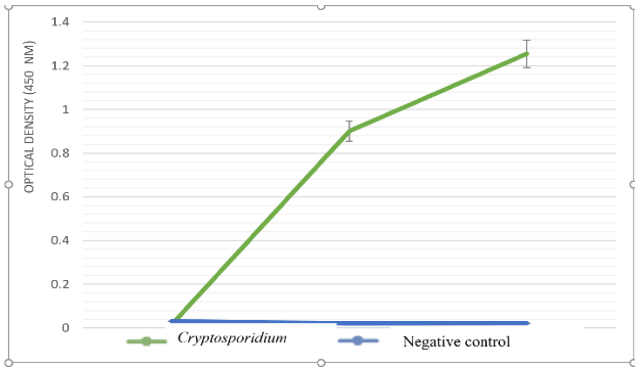

Figure 4. Sandwich ELISA for detection of *Cryptosporidium parvum* antigen by monoclonal antibody and rabbit-derived polyclonal antibody. BSA was used as negative control. Histograms present the means from duplicate assays, the error bars showing standard deviations.

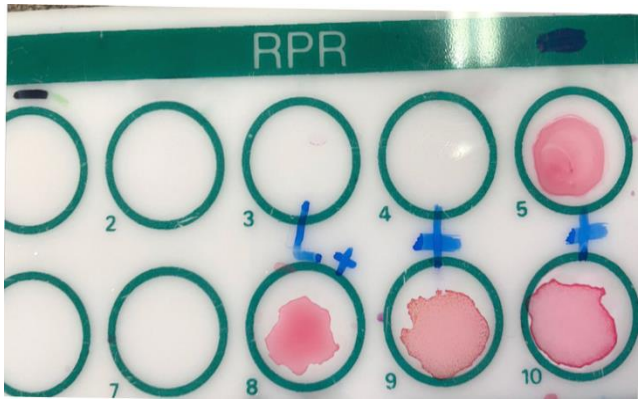

Figure 5. The reaction of latex particles coated with rabbit-derived polyclonal antibodies against *Cryptosporidium parvum*. (Well 5 serving as the negative control. well 10 serving as the positive control. wells 8 and 9 contained positive samples).

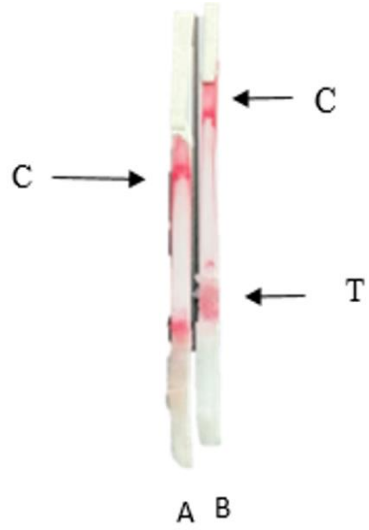

Figure 6. Lateral flow immunoassay for detection of *Cryptosporidium parvum*. A represents the negative result, while B corresponds to the positive result. In the positive strip, two distinct red spots are observed: the lower spot indicates the reaction of monoclonal antibody with sample, while the upper spot serves as control shows the reaction with protein A. For negative control strip PBS buffer was used as sample. T:test C: control.

Discussion

Cryptosporidium is a protozoan parasite responsible for cryptosporidiosis, a gastrointestinal illness affecting both humans and animals (12). In addition to its impact on human health, *Cryptosporidium* infections in livestock contribute to zoonotic transmission, underscoring the need for effective diagnostic and control measures in both veterinary and public health settings (4). Traditional diagnostic methods, including microscopic staining techniques and immunological assays, often require specialized laboratory infrastructure, trained personnel, and extended processing times. In regions with limited access to well-equipped diagnostic laboratories, the implementation of rapid diagnostic tests has the potential to significantly reduce the time and cost associated with *Cryptosporidium* detection. These assays facilitate point-of-care testing and minimize the dependency on highly trained personnel.

To address these limitations, latex agglutination and immunochromatographic-based rapid diagnostic tests have been developed, offering a faster and more accessible means of detecting *Cryptosporidium* antigens. These rapid tests are particularly advantageous for preliminary screening and emergency diagnostics due to their ease of use and ability to deliver quick and reliable results (6, 13).

In the present study two nano-biosensor-based rapid detection assays were developed. These tests could offer substantial commercial potential and could be widely

implemented for *Cryptosporidium* detection in both human and veterinary medicine. Beyond their affordability for livestock producers, their broader use could help mitigate the spread of cryptosporidiosis within human populations, addressing an important global public health concern.

This study focused on the development of rapid diagnostic kits for detecting *Cryptosporidium parvum* in ruminants (cattle and sheep) using latex agglutination and immunochromatographic techniques. Polyclonal antibody against surface antigens of *Cryptosporidium parvum* was generated through the immunization of rabbit, followed by purification and conjugation of it to latex particles. This antibody was also immobilized onto glass pads of immunochromatographic test strips. The results demonstrated the ability of the test strips to accurately detect *Cryptosporidium*-positive samples, with clearly visible diagnostic bands corresponding to monoclonal antibody and Protein A control. These findings suggest that the developed these diagnostic tests could offer rapid and effective alternative assays for detecting *Cryptosporidium parvum* in fecal samples.

For detection *Cryptosporidium parvum* in calf feces by latex agglutination assay several studies have been developed previously. One of these assays is employing a quantitative latex agglutination test (QLAT) by Nussbaum et al. (1999) for detection of *Cryptosporidium parvum* oocysts in cattle stool samples with enhanced sensitivity (14). Their results demonstrated high sensitivity (98.7%) and specificity (98.2%) for detecting *Cryptosporidium parvum* in bovine feces.

Several studies have supported the use of immunochromatographic techniques as viable alternatives to conventional diagnostic methods.

A comparative study evaluating three commercially available rapid diagnostic kits—FASTest® CRYPTO Strip, FASTest® CRYPTO-GIARDIA Strip, and TETRASTRIP®—demonstrated their effectiveness in detecting *Cryptosporidium parvum* in diarrheic calf fecal samples. The study concluded that these kits provide a precise, rapid, and practical approach to diagnosing cryptosporidiosis in neonatal calves, even in mild cases of diarrhea (15).

Another study comparing diagnostic approaches for *Cryptosporidium* detection in lambs indicated that immunochromatographic test kits offered higher sensitivity compared to traditional carbol-fuchsin staining (16).

A more recent study by Mohamed et al. (2025) focused on the development of a home-based immunochromatographic rapid test for *Cryptosporidium parvum*. Their findings demonstrated that this immunochromatographic assay had greater sensitivity and specificity compared to the sandwich ELISA method, establishing it as a more reliable, cost-effective, and accurate diagnostic tool for *Cryptosporidium* infections (17).

Manouana et al. (2020) evaluated an immunochromatographic rapid test for *Cryptosporidium* detection in diarrheic children in African communities, demonstrating its effectiveness and practicality as an alternative to conventional diagnostic techniques (9).

Conclusion

In this study, immunochromatographic and latex agglutination methods were developed for the detection of *Cryptosporidium parvum*. The findings demonstrated that the designed assays effectively identified the protozoan, confirming their suitability for detecting infected samples. These methods provide a reliable approach for diagnosing *Cryptosporidium* infections, emphasizing the importance of collecting suspected samples for accurate detection.

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Authors' Contributions

Asma Alipour Kermani: Conceptualization, Data curation, Investigation, Writing – original draft preparation. **Mehdi Golchin:** Conceptualization, Methodology, Validation, Resources, Project administration, Writing – review & editing. **Neda Eskandarzade:** Conceptualization, Visualization, Validation, Formal analysis, Writing – review & editing. **Saeid Reza Nourollahi Fard:** Methodology, Validation, parasitological evaluation.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

All animal experiments were assessed and approved by the Research Ethics Committees of Faculty of Shahid Bahonar University of Kerman.

Conflict of Interest

The authors affirm that there are no competing interests or

potential conflicts of interest associated with the publication of this work.

Consent for Publication

Not applicable.

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