

ORIGINAL ARTICLE

Open Access



Anti-*Cryptosporidium* oocysts polyclonal antibodies for cryptosporidiosis diagnosis and protection

Alyaa Farid^{1*} , Silvia Aiad² and Gehan Safwat²

Abstract

Cryptosporidiosis is an intestinal infection that is triggered by the protozoan parasite *Cryptosporidium* spp. *Cryptosporidium* oocysts can spread from one host to another either through direct contact with infected hosts' faeces or through indirect means (consumption of contaminated water or food). Significant numbers of oocysts are produced as a result of the rapid growth of the parasite within the infected hosts. For proper care of cryptosporidiosis, a laboratory diagnosis is necessary. Therefore, this study aimed to produce anti-*Cryptosporidium parvum* (*C. parvum*) oocyst immunoglobulin (Ig)G polyclonal antibodies (pAbs). The produced pAbs were used in the detection of *C. parvum* oocysts antigens in stool and serum samples of infected calves. Moreover, pAbs were tested in protection of balb-c male mice from cryptosporidiosis infection. *C. parvum* oocysts were used in the preparation of antigens to be used in the immunization of New Zealand white rabbits. pAb was purified by ammonium sulphate precipitation method, caprylic acid purification method and diethylaminoethyl (DEAE) anion exchange chromatographic method. Sandwich enzyme-linked immunosorbent assay (ELISA) (using prepared pAb) scored higher sensitivity (85% and 95% for serum and stool samples) than that (80%) of microscopic examination of stool samples. Moreover, pAb significantly reduced the oocysts shedding, decreased inflammatory cytokines and enhanced the loss in the body weight of protected animals. The prepared pAb succeeded in the diagnosis of cryptosporidiosis in calves with high sensitivity either in the serum or stool samples. Our results indicated the usefulness of using the prepared pAb in protection against cryptosporidiosis.

Key points

1. Importance of pAb in the diagnosis of cryptosporidiosis in calves.
2. Protection of balb-c mice by anti-*Cryptosporidium* oocysts pAb.
3. Sandwich ELISA has a higher sensitivity than microscopy.

Keywords *Cryptosporidium parvum*, Polyclonal antibodies, Oocysts, Immunoglobulin A, Interferon- γ , Interleukin-4

*Correspondence:

Alyaa Farid
alyaafarid@cu.edu.eg

¹ Biotechnology Department, Faculty of Science, Cairo University, Giza, Egypt

² Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Giza, Egypt

Introduction

Livestock offers financial possibilities, draught power, revenue to rural communities, and an option of foreign finances for the country (Ebiyo and Haile 2022). The capacity to reproduce and the survival of calf are the two factors that mostly affect the cattle productivity. Worldwide, dairy farmers face serious issues with calf mortality



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

and morbidity (Gebremedhin 2014). The majority of calves' deaths are caused by infectious parasites. The most typical disease symptom in newborn calves is diarrhea, which is responsible for roughly 74% of the calves' mortality in the first two weeks after birth (Birhan et al. 2019). *Cryptosporidium* (*C.*), Rotavirus, *Salmonella*, *Escherichia coli* and *Giardia* are some of the most frequent causes of diarrhea in calves (Mullusew et al. 2020).

Calves are susceptible to the zoonotic disease cryptosporidiosis, which is brought on by a parasitic protozoan of the species *C.* According to Shafieyan et al. (2014), *C.* can grow and replicate in the intestinal tracts, causing diarrhea in the infected host. According to Wegayehu et al. (2013), this condition has a significant economic effect due to its poor performance, expensive treatment costs, high mortality and morbidity. According to Manzazewal et al. (2018), cryptosporidiosis is a serious public health problem. Cattle are thought to be significant potential reservoirs for human infection. According to Gharpure et al. (2019), infections are often spread by eating, inhalation, and collateral contact with infectious stages of fully sporulated oocyst. There are over forty different genotypes of *Cryptosporidium* and about twenty four different species, with *C. parvum*, *C. andersoni*, *C. ryanae*, and *C. bovis* being the most prevalent. Cryptosporidiosis affects a wide variety of hosts and is present throughout the world (Vohra et al. 2012). *C.* was initially discovered in immunocompromised Arabian horse foals in 1978 (Snyder et al. 1978). At first, none gave the parasite any attention. Cryptosporidiosis became well-known owing to the historical events behind the deaths of patients with AIDS who had contracted *C.* in the late nineteenth century (Li et al. 2022). Due to *C.*'s widespread distribution, infections with the organism have been reported in more than 70 different countries (Thompson and Ash 2016).

The identification of protozoan parasite can be done directly using parasitological methods or indirectly using serum circulatory antigens and antibody detection (Farid 2023). One of the most often employed methods, for cryptosporidiosis diagnosis, is the microscopical evaluation. In this method, the parasite's oocysts in stool samples are commonly examined using direct optical microscopy for the laboratory diagnosis of cryptosporidiosis. An experienced examiner is required to detect the presence of *Cryptosporidium* and the phase of its life cycle (McHardy et al. 2014). However, this method has a number of shortcomings, including the lengthy time frame needed, significant oocysts variability, and low infection rates (Van Dam et al. 2004). Contrarily, immunodiagnostic methodologies offer much higher sensitivity and usability in addition to a variety of antibody testing techniques that have been created for the early

identification of protozoan parasite (Maher et al. 2022). Immunological techniques can be used to diagnose protozoa by detecting antibodies [immunoglobulins (Ig) G or IgM] or antigens using enzyme-linked immunosorbent assay (ELISA), immunofluorescence assays, and/or western blots (Júlio et al. 2012). Although, IgM appeared to be a successful detector in severe infections, it has cross-reactivity with other protozoans. Moreover, IgG remains in the serum for 18 months while IgM amount decreases after 2 to 3 weeks of infection (Pacheco et al. 2020). As a result, antibody screening tests run the risk of returning a false positive result. With a specificity of 92.72% and a sensitivity of 97%, the sandwich ELISA approach with polyclonal antibodies (pAbs) has been successful in detecting several parasite types in feces samples (Maher et al. 2022). Therefore, antigen screening tests is more recommended due to the high sensitivity that it can offer, in addition to, its reliability in acute infection (Khurana and Chaudhary 2018).

Over a hundred medications have been investigated in the treatment of cryptosporidiosis, but none have yet been authorised for use in the prevention or treatment. Several studies used different approaches to protect and/or prevent calves from cryptosporidiosis infection. These results of Jenkins et al. (2004) suggested that inoculating calves with *C. parvum* oocysts exposed to gamma radiation (400-Gy) may be able to protect them against cryptosporidiosis. Focusing on the anti-P23 yolk antibody in animals and immunocompromised individuals, it would be able to develop a passive immunisation method against *C. parvum* (Omidian et al. 2014). In order to avoid *C. parvum* infection, Lefkaditis et al. (2020) demonstrated the significance of IgG levels in the colostrum given to newborn calves on their first day of life. However, given that numerous studies have shown the effectiveness of passive immunotherapy by hyperimmune bovine colostrum in decreasing the oocysts shedding and symptoms in humans, lambs, and calves (Fayer et al. 1989; Naciri et al. 1994; Perryman et al. 1999). Only two other investigations employed the pre-parturient sheep in the production of hyperimmune anti-*C.* colostrum (Naciri et al. 1994; Jenkins et al. 2004).

Therefore, the study aimed to produce anti-*C. parvum* oocyst IgG pAb. The produced pAb was used in the detection of *C.* oocysts antigens in the stool and serum samples of infected calves. Moreover, pAbs were tested in the protection of balb-c male mice from cryptosporidiosis infection.

Materials and methods

Preparation of *C. parvum* oocysts antigens

C. parvum oocysts (P102C @ 5×10/6) were purchased from Waterborne™, Inc. in the USA. The oocyst

suspension, containing 2×10^7 oocysts/ml of PBS, was sonicated (18,000 Hz) for twenty times at 20 s intervals for one hour (4 °C). More than 90% of the *C. parvum* oocysts were disrupted; and the undisrupted oocysts were removed by centrifuging the suspension at 1500 rpm for 15 min. The supernatant, containing oocysts antigens, was separated and divided into aliquots to be used in the immunization process.

Production of anti-*C. parvum* oocyst antigen IgG pAbs

Five healthy and parasite free male New Zealand white rabbits (weight from 2 to 2.5 kg) were used in the immunization process for the production of pAbs against prepared *C. parvum* oocyst antigens according to the method of Tendler et al. (1991). Animals were housed under conventional laboratory settings (temperature of 21 ± 2 °C, moisture of 15%, consuming filtered water and a balanced diet containing protein (15%), fat (3%) and fibres (22%). According to Hegazy et al. (2015), Maher et al. (2022) and Farid (2023), the immunization process was accomplished through three intramuscular injections. The first priming dose (one mg of prepared *C. parvum* oocyst antigen mixed with complete Freund's adjuvant by the ratio one to one) was followed by two boosting doses (half mg of prepared *C. parvum* oocyst antigen mixed with incomplete Freund's adjuvant by the ratio one to one) with two weeks intervals. For the purpose of determining the level of pAbs generated, blood samples were taken from the rabbit's ear vein after four days of each boosting. Rabbits were sacrificed at the end of immunization process in order to collect blood samples. Serum was divided and stored at -20 °C till used. Bradford method (Bradford 1976) was used to calculate the protein content of prepared antigen.

Purification of rabbit anti-*C. parvum* oocyst antigen IgG pAbs

IgG pAb was purified by ammonium sulphate precipitation method (Nowotny 1979), caprylic acid purification method (Nowotny 1979) and diethylaminoethyl (DEAE) anion exchange chromatographic method (Mckinney and Parkinson 1987). After each step protein content of the yield was measured (Bradford 1976) in addition to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the fractions (Thaumaturgo et al. 2002). The indirect ELISA techniques were used to evaluate the reactivity of prepared pAb (Engvall and Perlman 1971).

Ammonium Sulfate precipitation method

In 100 ml of distilled water, 100 g of pure ammonium sulphate salt was dissolved. After two days of full dissolution, the supernatant was collected, and concentrated

ammonia was added in drops to bring the pH level to 7–7.1. Rabbit serum was diluted to 50% saturation with saturated ammonium sulphate solution, continuously stirred for 20 min at 4 °C, and then centrifuged. The ammonium sulphate precipitation was again performed many times on the precipitate after the supernatant was discarded.

Caprylic acid purification method

After the ammonium sulphate precipitation method, anti-*C. parvum* oocyst antigen IgG pAb was diluted with two volumes of sodium acetate buffer (60 mM and pH 4.8). Dropwise addition of caprylic acid (7%) was conducted for 30 min at 4 °C with steady magnetic stirring. For thirty minutes, the mixture was centrifuged at 1000 rpm. Except for IgG pAb, other proteins other than albumin were precipitated. The supernatant, which contained almost pure IgG, was further refined while the precipitate was separated.

DEAE-sephadex A-50 ion-exchange chromatography

According to their charge, proteins can often be separated via DEAE chromatography. A counter ion, such as chloride ions, can balance out the +ve charge of the DEAE group. Antibodies attach to the DEAE group less strongly than albumin because they have a more basic isoelectric point than the bulk of other serum proteins. Additionally, since it contains more lysine and arginine than glutamate and aspartate residues, IgG is more basic than IgM. Chloride ions are going to compete with the bound proteins in the binding to the +ve DEAE group, and the proteins will be eluted if the ionic strength was raised by increasing the NaCl salt content in the eluting solution. IgM and albumin are eluted later than IgG molecules. Isolated IgG fractions using this method are very pure. After being diluted in 0.5 M Tris buffer (200 ml, pH 7), the DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) powder was washed four times with three-bed volumes of 20 M Tris buffer (pH 7). The suspension of swollen beads was poured into a Bio-Rad column (30 × 2.5 cm) using a glass rod to prevent air bubbles. After the beads were settled in the column and the surface was covered with the binding buffer, the estimated column binding capacity was calculated. Samples were dialyzed against the binding buffer (20 mM Tris–HCl, pH 7). The column's exit tube was sealed off after the buffer over the beads was taken out. Using a Pasteur pipette, an IgG sample with the proper volume and protein content (10% of column bed capacity) was administered to the column. After the sample had penetrated the beads, the outlet tube was closed once more for 10 min to allow IgG to attach to the beads. One cc of the fraction was collected in each tube when the outlet was opened and linked to an automatic

fraction collector (LKB, Dusseldoef, West Germany). Each fraction's absorbance (280 nm) was calculated using a spectrophotometer (Perkin-Elmer Lambda 1A). High absorbance initial peak fractions were gathered together.

Labeling of IgG pAb with horseradish peroxidase (HRP) (Periodate method)

The periodate method (Tijssen and Kurstak 1984) was used to label the prepared pAbs with HRP. After dissolving five mg of HRP in 1.2 mL of cold water, 0.3 ml of sodium periodate was added, and the mixture was allowed to sit at room temperature for 20 min. The HRP mixture was dialyzed several times against sodium acetate buffer (1 mM) at pH 4 (4 °C) overnight. The pAb IgG was diluted to 5 mg/ml and combined with 0.02 M carbonate buffer (pH 9.6). After mixing 0.5 ml of pAb solution with HRP solution, the mixture was incubated at room temperature for two hours. The solution was combined with 100 µl of sodium borohydride, which was then incubated for two hours at 4 °C. The HRP-conjugated IgG pAb was dialyzed in the presence of 0.01 M PBS at pH 7.

Application of IgG pAbs

Six-month-old male calves were enrolled in the study. The study was conducted on 20 *C. parvum*-infected calves, 10 healthy calves; and 30 calves infected with other parasites (*Giardia*, *Entamoeba histolytica* and *Escherichia coli*). Blood samples (for serum preparation) and fresh fecal samples from calves were collected for examination. All samples used in the study were collected by a Veterinarian.

Parasitological examination

Stool samples were concentrated by formalin-ether concentration method (FECT). Stool samples were carefully mixed with 10 ml of formalin (10%) before being filtered through a No. 10 mesh screen. The fecal suspension received three ml of ether before being vigorously shaken and centrifuged at 1500 rpm for 20 min. One g of the sediment was used as a fecal smear, and the supernatants were discarded. According to Farid et al. (2021), fecal smears were stained using a modified Ziehl Neelsen procedure.

Immunological examination using IgG pAb

After standardization of the sandwich ELISA, 15 and 1/10 µg/ml were the required concentrations of IgG pAb to be used as a coating and peroxidase-conjugated antibody, respectively.

Preparation of stool elutes

In a 25 ml polypropylene tube, each sample was combined with two parts of the dist. water and centrifuged for 15 min to create the aqueous stool elutes. To get the highest values with the least amount of background reactivity, the supernatant was serially diluted (in 2.5% FCS in PBS/T) before being tested by sandwich ELISA for antigen detection (Kamel et al. 2013).

Detection of *C. parvum* oocysts antigens in stool and serum samples

The purified IgG pAb was applied to the plate in 100 µl/well at a concentration of 15 µg/ml, and it was then left to sit at room temperature overnight. The washing buffer (PBS/Tween) was used to wash the plate four times. Blocking buffer (2.5% FCS in PBS/T, 200 µl/well) was added to the plate, which was then incubated for two hours at 37°C. Samples (100 µl/well) were added to the wells after the plates had been washed three times followed by two hours of incubation at room temperature. HRP-conjugated pAb IgG (1/10 µg/ml) was applied to the plates. The plates were incubated at room temperature for one hour before being washed four times. O-Phenylenediamine dihydrochloride (OPD) was used as a substrate in the ELISA test. OPD was dissolved in 25 ml of phosphate citrate buffer (0.05M and pH 5), then mixed with H₂O₂ (5 µl). Each well received 100 µl of OPD, and the plate was incubated for 30 min at room temperature in the dark. Next, 50 µl of H₂SO₄ was added to each well to terminate the reaction. The ELISA plates were read at an absorbance of 492 nm.

Application of anti-*C. parvum* oocysts IgG pAb in protection

Fifteen six-week-old healthy male balb-c mice (20–25 g) were divided in three groups (five/group); healthy uninfected control group I, *C. parvum* infected unprotected group II, *C. parvum* infected protected group III that received 0.5 ml of purified pAb with a concentration of 1/100 by subcutaneous injection. After three days of pAbs administration, mice were orally infected with 10⁵ *C. parvum* oocysts. Animals were monitored daily for symptoms of cryptosporidiosis; stool samples were collected from days 1–14 and used in detecting *C. parvum* oocysts by modified Ziehl Neelsen procedure. At the end of experiment, animals were anesthetized by an intraperitoneal injection of a combination of ketamine (30 mg/kg) and xylazine (10 mg/kg). Blood samples were collected by cardiac puncture for the preparation of serum samples (Farid et al. 2023a, b, c).

Immunological measurements

On days 3, 7 and 14 after infection, serum level of IgA was measured by mice ELISA kits (RAB0797-1KT, sigmaaldrich, USA). Furthermore, interferon (IFN)- γ , interleukin (IL)-4 and IL-6 serum levels were measured by mice ELISA kits (ab100689, ab100710 and ab222503, respectively, Abcam, UK) according to Farid et al. (2022), Amr and Farid (2023) and Alaa et al. (2023).

Statistical analysis

SPSS computer program (version 28) was used in the statistical analysis of the results, where, data were presented as mean (X) \pm standard deviation (SD) and the cut-off value = X of healthy animals + 2(SD). The % of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were measured for each test according to Farid (2023). For results of the protection experiment (oocysts shedding, body weight and immunological results), student's t-test was used (result was considered significant when $P < 0.05$).

Results

Protein content

After each step of purification, the protein content of the yield was measured. Protein content of prepared *Cryptosporidium* oocysts antigen was 4.3 mg/ml. After the immunization period, the protein content in rabbit's sera was 8.4 mg/ml. Finally, it became 3.2 mg/ml after the purification procedure.

Anti-*C. parvum* oocysts IgG pAb characterization

After each step of purification, SDS-PAGE was performed to analyze the fractions and determine the purity of prepared pAbs. After all purification steps, IgG pAb was pure and free from any other proteins; and was represented by two bands (53 and 31 KDa) that represented the heavy and light chains of immunoglobulin, respectively (Fig. 1).

Reactivity of pAbs

Indirect ELISA was used to check the reactivity of prepared pAbs against *C. parvum* oocyst antigens. The level of pAbs was elevated one week after the 1st booster dose; and 3 days after the 2nd booster dose, rabbits' sera gave a high titer against *C. parvum* oocyst antigens at OD₄₉₂ of 2.45.

Parasitological results

Ordinary microscopic examination of stool samples was used to determine the validity of the results when compared to those obtained by ELISA. When stool samples were examined under microscope, 16 of the 20

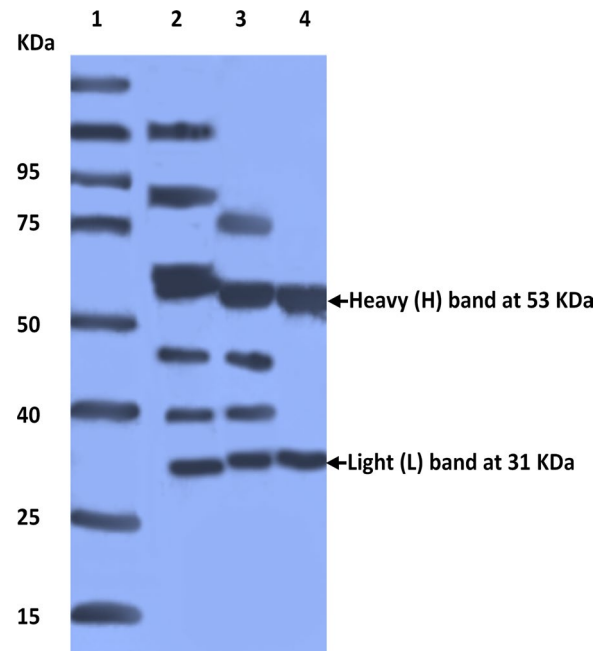


Fig. 1 photo of 12.5% SDS-PAGE of anti-*C. parvum* oocysts IgG pAb showing molecular weight of standard protein (lane 1), proteins after 50% ammonium sulfate precipitation (lane 2), IgG pAb after 7% caprylic acid treatment (lane 3) and purified IgG pAb after ion exchange chromatography (lane 4) (coomassie blue stain)

calves (infected with *C.*) tested positive for *C. oocysts*, while 4 of the calves produced false-negative results. So, the assay's sensitivity was 80% and its specificity was 100%. The NPV was 90.9%, and the PPV was 100%

Detection of *C. parvum* oocysts antigens in serum samples

The cut-off value of the test was 0.52. According to Table 1, the OD₄₉₂ values of the *C.* infected calves (1.9 ± 0.3) were greater than those of the healthy uninfected control group (0.3 ± 0.1), as well as, those of other calves groups infected with other parasites. Three calves out of 20 *C.* infected calves produced false

Table 1 Detection of *C. parvum* oocysts antigen in serum samples of calves

Groups	Positive cases		Negative cases	
	Number	Mean \pm SD	Number	Mean \pm SD
Healthy control (n=10)	–	–	10	0.3 \pm 0.11
<i>C. parvum</i> (n=20)	17	1.9 \pm 0.32	3*	0.4 \pm 0.03
<i>Giardia</i> (n=10)	1**	0.6 \pm 0.01	9	0.2 \pm 0.01
<i>Entamoeba</i> (n=10)	1**	0.6 \pm 0.12	9	0.3 \pm 0.01
<i>Escherichia coli</i> (n=10)	1**	0.7 \pm 0.11	9	0.4 \pm 0.02

SD: standard deviation; *: false negative result; **: false positive result

negative results. So, the sensitivity of the assay was 85%. The OD₄₉₂ values of three calves out of the thirty other parasites infected calves were larger than the cut-off value producing 92.5% specificity. The PPV was 85% and the NPV was 92.5%.

Detection of *C. parvum* oocysts antigens in stool samples

The cut-off value of the test was 0.44. One sample, only, from the *Cryptosporidium*-infected calves group gave a false negative result (Table 2) indicating 95% sensitivity and 95% PPV. One sample from *Giardia*-infected calves gave a false positive result with an OD₄₉₂ value (0.7) higher than the cut-off value (0.44) indicating a high specificity (97.5%) and NPV (97.5%).

Table 2 Detection of *C. parvum* oocysts antigen in stool samples of calves

Groups	Positive cases		Negative cases	
	Number	Mean ± SD	Number	Mean ± SD
Healthy control (n = 10)	–	–	10	0.2 ± 0.12
<i>C. parvum</i> (n = 20)	19	1.7 ± 0.21	1*	0.3 ± 0.21
<i>Giardia</i> (n = 10)	1**	0.7 ± 0.31	9	0.1 ± 0.01
<i>Entamoeba</i> (n = 10)	–	–	10	0.1 ± 0.01
<i>Escherichia coli</i> (n = 10)	–	–	10	0.1 ± 0.01

SD: standard deviation; *: false negative result; **: false positive result

Application of pAb in mice protection

Fifteen mice were divided in three groups; healthy uninfected control group I, *C. parvum* infected unprotected group II, *C. parvum* infected protected group III that received 0.5 ml of purified pAbs with a concentration of 1/100 by subcutaneous injection. After three days of pAbs administration, mice were orally infected with 10⁵ *C. parvum* oocysts and were monitored daily for symptoms of cryptosporidiosis. Infected unprotected group II showed severe diarrhea with high oocysts shedding (from day 3 to day 14) more than infected protected group III (Fig. 2). Group II was disinterested in eating and showed depression after the onset of diarrhea. Moreover, a significant reduction in the mice body weight was noticed in infected unprotected group II when compared to healthy control group I. In contrast, group III that received pAbs for protection showed no diarrhea along all experimental days and showed a non-significant reduction in body weight when compared to the healthy control group I (Fig. 3).

Serum levels of IgA and cytokines (IFN-γ, IL-4 and IL-6) were significantly elevated in infected unprotected group II when compared to healthy control group I on days 3, 7 and 14 after infection. Levels of IgA and cytokines, in infected protected group III, were significantly lower than those of infected unprotected group II (Figs. 4 and 5).

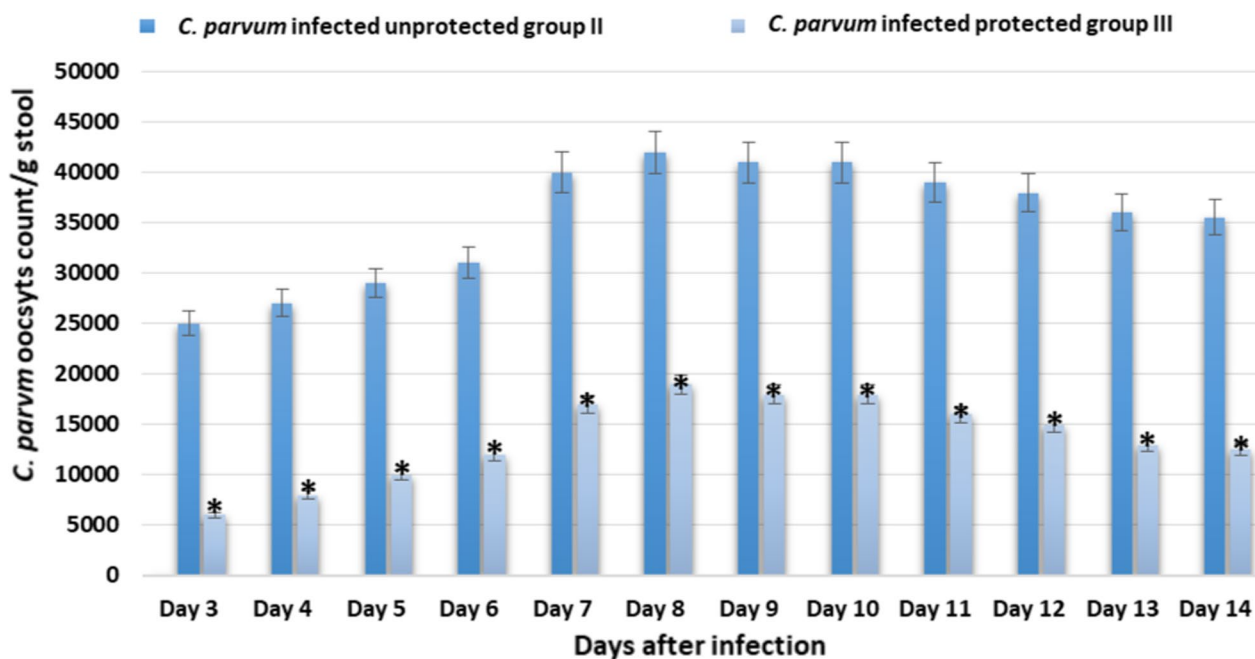


Fig. 2 oocysts shedding from day 3 to day 14 after infection with *C. parvum*. * indicated significance (p < 0.05) in comparison to group II

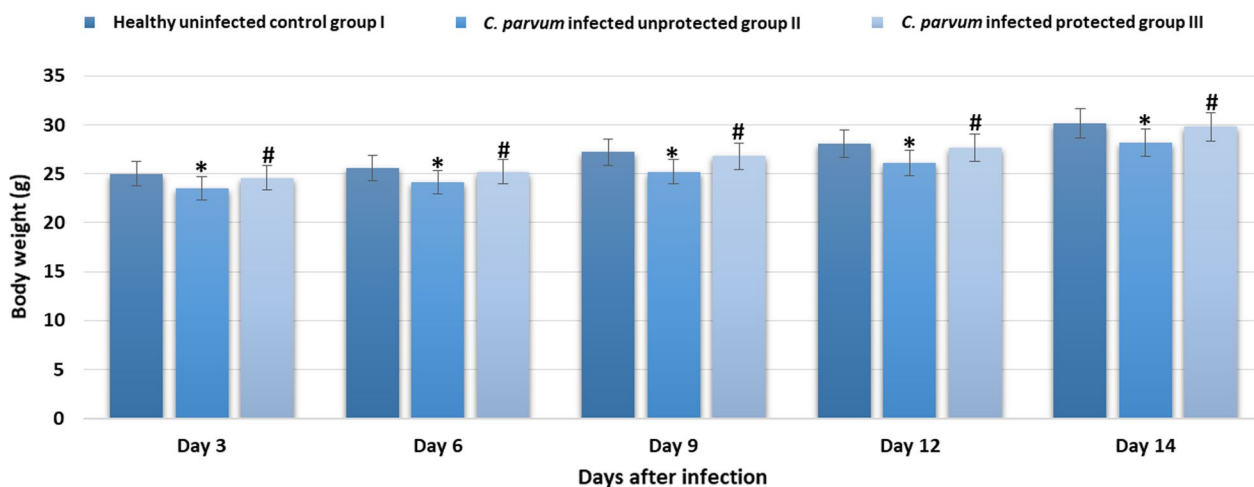


Fig. 3 body weight of mice in different experimental groups from day 3 to day 14 after infection with *C. parvum*. * indicated significance ($p < 0.05$) in comparison to group I and # indicated significance ($p < 0.05$) in comparison to group II

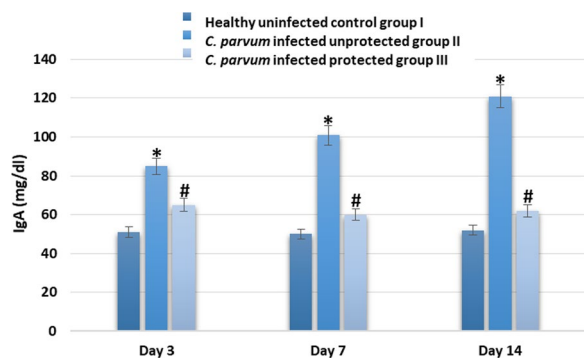


Fig. 4 serum IgA (mg/dl) level in different experimental groups on day 3, 7 and 14 after infection with *C. parvum*. * indicated significance ($p < 0.05$) in comparison to group I and # indicated significance ($p < 0.05$) in comparison to group II

Discussion

Calves cryptosporidiosis is widely prevalent and has been identified as a significant contributor to calf diarrhea in several countries worldwide (Featherstone et al. 2010; Cho et al. 2014). According to Hadfield et al. (2011), either the zoonotic species *C. parvum* or the human-adapted species *C. hominis* are responsible for the majority of human cases of cryptosporidiosis. Together, these two species are responsible for 96% of the reported cases in the UK and >90% of all human infections globally (Chalmers et al. 2011). Infection with *C. spp.*, often known as cryptosporidiosis, is well acknowledged as a major cause of chronic and severe diarrhoea in immunodeficient individuals and a substantial cause of morbidity and mortality. Additionally, it is one of the significant infections responsible for zoonotic or waterborne epidemics that are mostly linked to contaminating drinking water and recreational water sites (Thompson and Ash

2016). Particularly in countries with poor infrastructure, cryptosporidiosis is recognised as a significant cause of diarrhoea in children that is linked to chronic malnutrition and early death. The dissemination of infectious oocysts causes the parasite to spread via the fecal–oral pathway. According to Painter et al. (2015), it is usually linked to contamination of food and water.

For proper care, laboratory confirmation of cryptosporidiosis is necessary, whereas when dealing with environmental specimens it is often necessary for outbreak detection, source tracing, risk factor evaluation, and intervention. Some selection standards can be used to seek for *C.* especially without the existence of a particular request, as the majority of laboratories might not test for it unless expressly requested. Currently, a number of methods may be used to diagnose *C.*, such as microscopic inspection, wet mount preparation, smears staining with modified acid-fast stain, or fluorescence stains. There are immunological techniques for detecting both antigen and antibody. Additionally accessible are several molecular techniques for DNA detection as well as histological analysis of the biopsies.

The majority of laboratories regularly employ microscopic techniques to identify cryptosporidiosis. Unfortunately, this approach has a lot of drawbacks since it needs at least 5×10^4 oocysts per ml of faeces to be detected. Because the oocysts are shed irregularly, three samples taken on different days are thought to be optimum. Given that *C.* is a biosafety category II parasite, the sample must be handled carefully in a cabinet for safety (Khurana and Chaudhary 2018). Because of this, the parasite is difficult to be found with standard microscopy. Additionally, this method calls for advanced reporting skills. Antigen/antibody-based detection techniques like ELISA can be

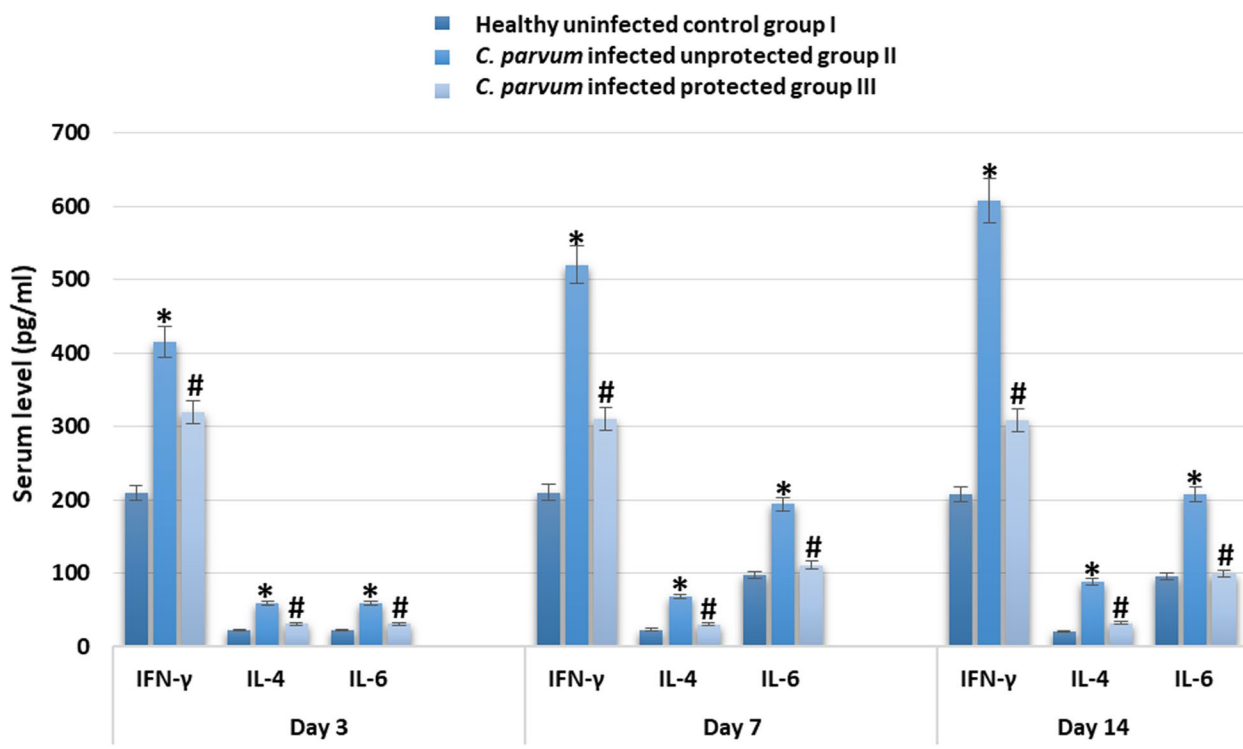


Fig. 5 serum IFN- γ , IL-4 and IL-6 (pg/ml) level in different experimental groups on day 3, 7 and 14 after infection with *C. parvum*. * indicated significance ($p < 0.05$) in comparison to group I and # indicated significance ($p < 0.05$) in comparison to group II

employed to get around all these disadvantages (Vanathy et al. 2017).

The present study aimed to prepare pAbs against *C. parvum* oocysts to be used in the diagnosis of cryptosporidiosis in serum and stool samples of infected calves. Furthermore, the prepared pAbs were tested in protection of balb-c mice from *C.* infection. The efficacy of pAbs in protection was evaluated by monitoring oocysts shedding, animals’ body weight and immunological measurements of IgA and cytokines.

For antigen preparation, *C.* oocysts were sonicated twenty times at 20 s intervals for one hour (4 °C) followed by centrifugation to obtain supernatant to be used in the immunization process. Three intramuscular shots were required to complete the immunization procedure. After the initial priming dose of one mg of prepared *C. parvum* oocyst antigen mixed with complete Freund’s adjuvant in a one-to-one ratio, two boosting doses of half mg of prepared *C. parvum* oocyst antigen mixed with incomplete Freund’s adjuvant were administered at intervals of two weeks. Blood samples were obtained from the rabbit’s ear vein four days after each boosting to measure the amount of pAbs produced. At the end of the immunization procedure, rabbits were sacrificed in order to collect blood for serum preparation. IgG pAb was purified by ammonium

sulphate precipitation method, caprylic acid purification method and DEAE anion exchange chromatographic method. After each step protein content of the yield was measured in addition to SDS-PAGE to analyze the fractions. Indirect ELISA technique was used to evaluate the reactivity of prepared pAbs. Prepared pAbs were labelled with HRP to be used in the sandwich ELISA technique for cryptosporidiosis diagnosis in calves.

Our results showed that the protein content of prepared *Cryptosporidium* oocysts antigen was 4.3 mg/ml. After the immunization period, the protein content in rabbit’s sera was 8.4 mg/ml. Finally, it became 3.2 mg/ml after the purification procedure. After the purification steps, IgG pAb was free from any other proteins and was represented by two bands (53 and 31 KDa) that represented the heavy and light chains, respectively. The level of pAbs was elevated one week after the 1st booster dose; and 3 days after the 2nd booster dose, rabbits’ sera gave a high titer against *C. parvum* oocyst antigens at OD₄₉₂ of 2.45. For calves’ serum and stool samples, the cut-off value of the test was 0.52 and 0.44, respectively. The sensitivity of the ELISA test was 85% and 95% for serum and stool samples; and the specificity was 92.5 and 97.5% for serum and stool samples. It was obvious that sandwich ELISA (using prepared pAbs) scored higher sensitivity

than that of microscopic examination of stool samples (80%). The NPV of ELISA test (92.5% and 97.5% for serum and stool samples) was higher than that (90.9%) of the microscopic examination.

Commercially accessible kits for enzyme-labeled antibody-based ELISA for antigen detection are available. According to reports, coproantigen detection ELISA has varying detection limits of 3×10^5 – 10^6 , which is comparable to microscopy. The sensitivity of the kit, however, may vary based on the antibody employed in some instances. Khurana et al. (2012) reported that a large number of samples can be processed quickly in the ELISA, in addition to, an excellent specificity range of 98% to 100%. Khurana and Chaudhary (2018) added that fresh, frozen, or samples that have been formalin-preserved can all be used for these assays. These benefit from quick test times and multiple findings in a single response device made the ELISA technique more superior to microscopic examination of stool samples.

Because of the oocyst stage's resilience and the absence of medications that have been licensed to prevent or treat illness, cryptosporidiosis continues to pose a serious danger to both human and animal health (Madbouly et al. 2021). It is quite common in calves, posing a risk to their health and serving as a large environmental contaminants (Riggs 2002). Passive immunotherapy using monoclonal antibodies, immune serum, or hyperimmune colostrum specific for *C. parvum* antigens has demonstrated some effectiveness against cryptosporidiosis. Although *C. parvum* oocysts continue to be shed in large quantities, passive immunotherapy using hyperimmune bovine colostrum has decreased clinical symptoms in people or dairy calves (Jenkins et al. 2004).

In addition to using pAb in diagnosis, the study evaluated the possibility of using it in protection of balb-c mice. Fifteen six-week-old healthy male balb-c mice were divided in three groups (five/group); healthy uninfected control group I, *C. parvum*-infected unprotected group II, *C. parvum* infected protected group III that received 0.5 ml of purified pAbs with a concentration of 1/100 by subcutaneous injection. After three days of pAbs administration, mice were orally infected with 10^5 *C. parvum* oocysts. Animals were monitored daily for symptoms of cryptosporidiosis; stool samples were collected from days 1–14 and used in detecting *C. parvum* oocysts by modified Ziehl Neelsen procedure. On day 3, 7 and 14 after infection, serum levels of IgA and cytokines (IFN- γ , IL-4 and IL-6) were measured. Our results showed that infected unprotected group II showed severe diarrhea with high oocysts shedding, reduction in body weight and significant elevation in serum levels of IgA and cytokines (IFN- γ , IL-4 and IL-6) more than infected protected group III. These

results indicated the usefulness of using prepared pAbs in protection against cryptosporidiosis.

To our knowledge this is the first study to use pAbs in protection against cryptosporidiosis. The prepared pAbs succeeded in the diagnosis of cryptosporidiosis in calves with high sensitivity either in serum or stool samples. The pAbs can be prepared in large amounts at low cost and used in the diagnosis and protection. We recommend the test of anti-*C. parvum* IgG pAb in protection of calves in the future. However, the study has some limitations such as the use of single concentration of pAbs (1/100) and small number of animals in the protection experiment.

Abbreviations

C	<i>Cryptosporidium</i>
DEAE	Diethylaminoethyl
ELISA	Enzyme-linked immunosorbent assay
FECT	Formalin-ether concentration method
H	Heavy
HRP	Horseshoe peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
L	Light
NPV	Negative predictive value
OPD	O-Phenylenediamine dihydrochloride
pAb	Polyclonal antibodies
PPV	Positive predictive value
SD	Standard deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

Acknowledgements

Not applicable.

Author contributions

AF: conceptualization, Methodology and Writing the manuscript. SA and GS: data curation, Visualization and Investigation. GS: software and Validation. All authors have read and approved the manuscript.

Funding

Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

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

Declarations

Ethics approval and consent to participate

All experimental procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals, and the study was conducted in accordance with the guide for the care and use of laboratory animals, Eighth edition (2011). The study has been approved by the ethics committee of Faculty of Biotechnology, October University for Modern Sciences and Arts.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 3 August 2023 Accepted: 26 October 2023

Published online: 09 November 2023

References

- Alaa H, Abdelaziz M, Mustafa M, Mansour M, Magdy S, Mohsen S, El-Karamany Y, Farid A (2023) Therapeutic effect of melatonin-loaded chitosan/lecithin nanoparticles on hyperglycemia and pancreatic beta cells regeneration in streptozotocin-induced diabetic rats. *Sci Rep* 13(1):10617
- Amr M, Farid A (2023) Impact of cow, buffalo, goat or camel milk consumption on immune response in male and female Sprague dawley rats post weaning time. *Sci Rep*
- Birhan M, Misganaw A, Gessese T (2019) A cross-sectional study on the prevalence of cryptosporidiosis and its associated risk factors in calves in Gondar town and its suburbs, NW Ethiopia. *J Animal Feed Res* 9(4):162–168
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:245–254
- Chalmers RM, Smith R, Elwin K, Clifton-Hadley FA, Giles M (2011) Epidemiology of anthroponotic and zoonotic human cryptosporidiosis in England and Wales, 2004–2006. *Epidemiol Infect* 139:700–712
- Cho YI, Yoon KJ (2014) An overview of calf diarrhea—infected etiology, diagnosis, and intervention. *J Vet Sci* 15:1–17
- Ebiyo A, Haile G (2022) Prevalence and factors associated with *Cryptosporidium* Infection in Calves in and around Nekemte Town, East Wollega Zone of Ethiopia. *Vet Med Int* 2022:1468242
- Engvall E, Perlman P (1971) Enzyme linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. *J Immunochem* 8:871–874
- Farid A (2023) Preparation of polyclonal anti-*Schistosoma mansoni* cysteine protease antibodies for early diagnosis. *Appl Microbiol Biotechnol* 107(5–6):1609–1619
- Farid A, Tawfik A, Elsioufy B, Safwat G (2021) In vitro and in vivo anti-*Cryptosporidium* and anti-inflammatory effects of Aloe vera gel in dexamethasone immunosuppressed mice. *Int J Parasitol Drugs Drug Resist* 17:156–167
- Farid A, Haridyy H, Ashraf S, Ahmed S, Safwat G (2022) Co-treatment with grape seed extract and mesenchymal stem cells in vivo regenerated beta cells of islets of Langerhans in pancreas of type I-induced diabetic rats. *Stem Cell Res Ther* 13(1):528
- Farid A, Michael V, Safwat G (2023a) Melatonin loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles reduce inflammation, inhibit apoptosis and protect rat's liver from the hazardous effects of CCL4. *Sci Rep* 13:16424
- Farid A, Ooda A, Nabil A, Nasser A, Ahmed E, Ali F, Mohamed F, Farid H, Badran M, Ahmed M, Ibrahim M, Rasmy M, Saleeb M, Riad V, Ibrahim Y, Madbouly N (2023b) Eobania vermiculata whole-body muscle extract-loaded chitosan nanoparticles enhanced skin regeneration and decreased pro-inflammatory cytokines in vivo. *J Nanobiotechnol* 21(1):373
- Farid A, Mohamed D, Mostafa D, Tarek R, Sherif V, Safwat G (2023c) Novel grape seed extract nanoparticles attenuate amikacin-induced nephrotoxicity in rats. *AMB Express*
- Fayer R, Andrews C, Ungar BL, Blagburn B (1989) Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. *J Parasitol* 75(3):393–397
- Featherstone CA, Giles M, Marshall JA, Mawhinney IC, Holliman A, Pritchard GC (2010) *Cryptosporidium* species in calves submitted for postmortem examination in England and Wales. *Vet Rec* 167:979–980
- Gebremedhin R (2014) Major causes of calf mortality in intensive dairy farms. *Int J Livestock Res* 4(3):9–16
- Gharpure R, Perez A, Miller AD, Wikswo ME, Silver R, Hlavsa MC (2019) Cryptosporidiosis outbreaks—United States, 2009–2017. *MMWR Morb Mortal Wkly Rep* 68(25):568–572
- Hadfield SJ, Robinson G, Elwin K, Chalmers RM (2011) Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. *J Clin Microbiol* 49:918–924
- Hegazy S, Farid A, Rabae I, El-Amir A (2015) Novel IMB-ELISA assay for rapid diagnosis of human toxoplasmosis using SAG1 Antigen. *Jpn J Infect Dis* 68(6):474–480
- Jenkins M, Higgins J, Kniel K, Trout J, Fayer R (2004) Protection of calves against cryptosporiosis by oral inoculation with gamma-irradiated *Cryptosporidium parvum* oocysts. *J Parasitol* 90(5):1178–1180
- Júlio C, Vileas A, Oleastro M, Ferreira I, Gomes S, Monteiro L, Nunes B, Tenreiro R, Angelo H (2012) Prevalence and risk factors for *Giardia duodenalis* infection among children: a case study in Portugal. *Parasit Vectors* 5:22
- Kamel D, Farid A, Ali E, Rabia I, Hendawy M, El Amir AM (2013) Diagnostic potential of target *Giardia lamblia* specific antigen for detection of human giardiasis using coproantigen sandwich ELISA. *World J Med Sci* 9(2):113–122
- Khurana S, Chaudhary P (2018) Laboratory diagnosis of cryptosporidiosis. *Trop Parasitol* 8(1):2–7
- Khurana S, Sharma P, Sharma A, Malla N (2012) Evaluation of Ziehl-Neelsen staining, auramine phenol staining, antigen detection enzyme linked immunosorbent assay and polymerase chain reaction, for the diagnosis of intestinal cryptosporidiosis. *Trop Parasitol* 2:20–23
- Lefkaditis M, Mpaïramoglou R, Sossidou A, Spanoudis K, Tsakiroglou M, Györke A (2020) Importance of colostrum IgG antibodies level for prevention of infection with *Cryptosporidium parvum* in neonatal dairy calves. *Prev Vet Med* 176:104904
- Li XM, Geng HL, Wei YJ, Yan WL, Liu J, Wei XY, Zhang M, Wang XY, Zhang XX, Liu G (2022) Global prevalence and risk factors of *Cryptosporidium* infection in Equus: a systematic review and meta-analysis. *Front Cell Infect Microbiol* 12:1072385
- Madbouly N, El Amir A, Abdel Kader A, Rabee I, Farid A (2021) The immunomodulatory activity of secnidazole-nitazoxanide in a murine cryptosporidiosis model. *J Med Microbiol* 70(3)
- Maher A, Atallah D, Hassan M, Hammad M, Galal M, Mohamed SE, Abdelkafy Y, Farid A (2022) Role of anti-*Giardia* recombinant cyst wall protein IgG polyclonal antibodies in diagnosis and protection. *AMB Express* 12(1):147
- Manyazewal A, Francesca S, Pal M, Gezahegn M, Tesfaye M, Lucy M, Teklu W, Getachew T (2018) Prevalence, risk factors and molecular characterization of *Cryptosporidium* infection in cattle in Addis Ababa and its environs. *Ethiopia Vet Parasitol Reg Stud Rep* 13:79–84
- McHardy IH, Wu M, Shimizu-Cohen R, Couturier MR, Humphries RM (2014) Detection of intestinal protozoa in the clinical laboratory. *J Clin Microbiol* 52(3):712–720
- Mckinney MM, Parkinson A (1987) A simple, non-chromatographic procedure to purify immunoglobulins from ascites fluid. *J Immunol Meth* 96:271–278
- Mullusew G, Negesse W, Dinka A, Waktole H (2020) Study on eimeria and *Cryptosporidium* infection in dairy cattle farms of holeta, west shoa zone, Oromia. *Ethiopia J Am Sci* 16(8):44–60
- Naciri M, Mancassola R, Reperant JM, Canivez O, Quinque B, Yvore P (1994) Treatment of experimental ovine cryptosporidiosis with ovine or bovine hyperimmune colostrum. *Vet Parasitol* 53(3–4):173–190
- Nowotny A (1979) Basic exercises in immunochemistry. Springer-verlag, Berlin, New, pp 232–234
- Omidian Z, Ebrahimzadeh E, Shahbazi P, Asghari Z, Shayan P (2014) Application of recombinant *Cryptosporidium parvum* P23 for isolation and prevention. *Parasitol Res* 113(1):229–237
- Pacheco FTF, de Carvalho SS, Santos SA, Das Chagas GMT, Santos MC, Santos JGS, Da Costa-Ribeiro H, Júnior Ribeiro TCM, de Mattos ÂP, Gomes MA, Soares NM, Teixeira MCA (2020) Specific IgG and IgA antibody reactivities in sera of children by enzyme-linked immunoassay and comparison with *Giardia duodenalis* diagnosis in feces. *Ann Lab Med* 40(5):382–389
- Painter JE, Hlavsa MC, Collier SA, Xiao L, Yoder JS (2015) Cryptosporidiosis surveillance—United States, 2011–2012. *MMWR Surveill Summ* 64(Suppl 3):1–4
- Perryman LE, Kapil SJ, Jones ML, Hunt EL (1999) Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. *Vaccine* 17(17):2142–2149
- Riggs MW (2002) Recent advances in cryptosporidiosis: The immune response. *Microbes Infect* 4:1067–1080

- Shafieyan H, Alborzi A, Ahmadinejad H, Tabandeh MR (2014) Prevalence of *Cryptosporidium* species in ruminants of Lorestan province. *Iran J Parasitic Dis* 12:63–94
- Snyder SP, England JJ, McChesney AE (1978) Cryptosporidiosis in immunodeficient Arabian foals. *Vet Pathol* 15(1):12–17
- Tendler M, Almeida MSS, Pinto RM, Noronha D, Katz N (1991) *Schistosoma mansoni*-New Zealand rabbit model: resistance induced by infection followed by active immunization with protective antigens. *J Parasitol* 77:138–141
- Thaumaturgo N, Vilar MM, Edelenyi R, Tendler M (2002) Characterization of Sm14 related components in different helminthes by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blotting analysis. *Mem Inst Oswaldo Cruz* 97:115–116
- Thompson RCA, Ash A (2016) Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. *Infect Genet Evol* 40:315–323
- Tijssen P, Kurstak P (1984) Highly efficient and simple methods for the preparation of peroxidase and active peroxidase-antibody conjugate for enzyme immunoassays. *Anal Biochem* 136:451–457
- Van Dam GJ, Wichers JH, Ferreira TM, Ghati D, van Amerongen A, Deelder AM (2004) Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. *J Clin Microbiol* 42(12):5458–5461
- Vanathy K, Parija SC, Mandal J, Hamide A, Krishnamurthy S (2017) Detection of *Cryptosporidium* in stool samples of immunocompromised patients. *Trop Parasitol* 7(1):41–46
- Vohra P, Sharma M, Chaudhary U (2012) A comprehensive review of diagnostic techniques for detection of *Cryptosporidium parvum* in stool samples. *IOSR J Pharmacy* 2(5):15–26
- Wegayehu T, Adamu H, Petros B (2013) Prevalence of *Giardia duodenalis* and *Cryptosporidium species* infections among children and cattle in North Shewa Zone, Ethiopia. *BMC Infect Dis* 13(1):419

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► [springeropen.com](https://www.springeropen.com)

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH (“Springer Nature”).

Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users (“Users”), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use (“Terms”). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.

These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.

We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.

While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval, sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.

These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.

To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.

Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.

If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com