10.22059/IJVM.2022.339810.1005255

Iranian Journal of Veterinary Medicine
Volume 16- Issue 4

Original Article
Online ISSN: 2252-0554

Protection of Calves Against Cryptosporidiosis by Hyperimmunization of Pregnant Cattle Colostrum using Oocyst Whole Antigens

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Abstract

BACKGROUND: *Cryptosporidium parvum* is a coccidian parasite with worldwide distribution.

OBJECTIVES: It is considered one of the most important causes of diarrhea in many vertebrate species and immunocompromised humans. Due to the lack of effective treatment of cryptosporidiosis, protection strategy against this species can be focused on encouraging the immune system through vaccine development.

METHODS: For this aim, we prepared oocysts lysate as a whole antigen vaccine candidate (420 µg) and immunized 3 pregnant cows 4 times every 2 weeks from 70 days to parturition. As a control group, 3 unimmunized pregnant cows were used. After parturition, each calf was fed with colostrum of his dam and challenged at 12h of age with 1×10^7 *C. parvum* oocysts.

RESULTS: In contrast to the test group, the calves in the control group developed severe watery diarrhea with excretion of oocysts from 4 days post-infection. The calves in the test group, which received the hyperimmune colostrum, showed no clinical signs and a significant reduction in oocysts excretion.

CONCLUSIONS: The whole antigen prepared from oocysts of *C. parvum* can be considered a suitable candidate for immunizing pregnant cows producing hyperimmune colostrum.

KEYWORDS: *Cryptosporidium parvum*, Hyperimmune colostrum, Immunization, Oocyst whole antigen, Passive vaccine

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Received: 2022-02-28

Accepted: 2022-05-16

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How to Cite This Article
Mokhber Dezfouli, M R ., Zarghami, F., Rahbari, S., Ebrahimzadeh Abkooh, E., et al. (2022). Protection of Calves Against Cryptosporidiosis by Hyperimmunization of Pregnant Cattle Colostrum using Oocyst Whole Antigens. *Iranian Journal of Veterinary Medicine, 16*(4), 380-389.

Introduction

Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium parvum*, an apicomplexan parasite infecting the intestinal epithelium of humans and animals worldwide (Iness *et al.,* 2020). *C. parvum* belongs to the most opportunistic protozoa causing severe disease in immunocompromised humans, like acquired immunodeficiency syndrome (AIDS) patients. Cryptosporidiosis plays an important role in the health management in livestock industries because of the infection of newborn ruminants. It can cause high economic losses (Zarghami *et al*., 2015). Usually, cryptosporidiosis in newborn ruminants is accompanied by depression, anorexia, and diarrhea leading to weight loss and sometimes death (Radostits *et al*., 2006). *Cryptosporidium* oocyst is resistant to disinfectant agents, and there are no effective chemotherapeutic compounds against this parasite. Additionally, it is reported that even one oocyst can develop cryptosporidiosis in susceptible hosts (Ramirez *et al*., 2004). These mentioned facts make *C. parvum* an important zoonotic infection. It is believed that contaminated water and foodstuffs from infected animals can play a prominent role in the public health concern. Since the newborn ruminant are exposed to heavy infection in many countries, and the anatomy of cows (syndesmochorial placenta) do not allow antibodies to enter the fetus' bloodstream, the newborn calves, especially those whose dams have not been exposed to infection, are subject to a very high risk of infection. Therefore, passive immunization of calves right after birth through hyperimmune colostrum can help to prevent the infection (Askari *et al*., 2016). Many scientists have examined the immunization of calves using immunogenic proteins expressed in the parasite. The proteins GP900, CP15, GP15, GP40, CSL, and TRAP-C1 have been previously described as templates (Cevallos *et al.*, 2000; Tomley and Soldati, 2001), and there are many reports about different recombinant proteins protecting successfully against *Cryptosporidium* (Liu *et al.*, 2010; Askari *et al*., 2016). Previously, we have used P23 as a vaccine candidate to passively immunize newborn calves through colostrum (Askari *et al.*, 2016). This study aimed to produce hyperimmune colostrum in pregnant cows against oocysts of *C. parvum* and use it to prevent infection with oocysts of *C. parvum* in newborn calves. In this study, we used the crude antigens prepared from oocysts of *C. parvum* as a vaccine candidate and compared the achieved results with the results previously obtained from immunization of pregnant cows with P23 recombinant protein.

Materials and Methods

Primary cryptosporidial oocysts were obtained from the feces of the naturally infected calf (10 days of age) with diarrhea manifestation, suspected for cryptosporidiosis. After purification, oocysts were analyzed by modified Ziehl-Neelsen staining, and PCR as described previously (35-40). Subsequently, a calf (1 day of age) was experimentally infected with 1×10^7 oocysts of *C. parvum* to obtain enough oocysts for further analysis. Oocysts of *C. parvum* were collected from experimentally infected calves isolated and purified from fecal debris as described previously (Shayan *et al.,* 2012; Ebrahimzade *et al.*, 2014; Omidian *et al*., 2014).

2.1. Preparation of the whole Antigen of *C. parvum* Oocysts

In order to prepare *C. parvum* whole antigen, 600×10^6 oocysts were divided equally into 12 sterile microtubes, as each microtube contained 50×10^6 oocysts. Then oocysts of each microtube were resuspended in 200 mL phosphate-buffered saline (PBS) and autoclaved at 121°C and 1 bar pressure for 20 minutes. Autoclaved oocysts were additionally disrupted by ultrasound treatment in an ice bath and then three cycles of freezing-thawing. The effect of the treatment was controlled using a light microscope, and the content of obtained protein was determined using the Bradford method.

2.2. Immunization of Pregnant Cows

All stages of immunization and evaluation of the vaccine efficacy procedure were performed in a dairy farm research center related to the Faculty of Veterinary Medicine of the University of Tehran.

In order to immunization and to induce hyperimmune colostrum, six healthy Holstein cows in their third–trimester of pregnancy were selected based on

low serum antibody titers against *C. parvum* determined by dot blot test (Askari *et al*., 2016). They, 70 days before parturition, were randomly divided into test (A) group and control (B) group (each group containing three cows). Subsequently, 420 µg of *C. parvum* whole antigen mixed with 1.5 mL PBS (pH: 7.2) was emulsified with an equal volume (1.5 mL) of Freund's complete adjuvant and was injected subcutaneously into the lateral aspect of the neck in the test group (pregnant cows). The test group was boosted 3 times after the first immunization with an injection of 420 µg antigen mixed in 1.5 mL PBS (pH:7.2) plus 1.5 mL incomplete Freund's adjuvant in 2 weeks intervals. The last injection was also 4 weeks prior to parturition. In control cows, PBS buffer was used instead of whole *C. parvum* antigen. Blood samples were collected from the test and control group via coccygeal vein before immunization (0 day) and 2 weeks before parturition to test the presence of antibodies against *C. parvum* antigens.

In order to minimize the environmental contamination, each cow was transported to the calving barn, which had been before cleaned with water and disinfectant and bedded with fresh straw. After parturition, the newborn calves were transported to the isolated box. Calves of both groups received 3 L of first milking colostrum within 2 h of birth, 2 L of colostrum at 12 h postpartum, and 1 L colostrum at 24 h postpartum using a nipple bottle. Then they received 4 L second milking colostrum on 2nd day of birth. After 48 h of age, calves were fed dam's milk at 10% of birth weight three times daily, and water was provided ad libitum. All calves of the test and control group were infected with 1×10^7 *C. parvum* oocysts mixed in 50ml colostrum at 12 h after birth. Prior to inoculation, fecal samples were collected from the rectum to evaluate the presence of *C. parvum*, and other known entero-pathogens, including Rotavirus, Coronavirus, *E. coli* K99, and Salmonella. Blood samples were collected from calves at birth, before taking colostrum, and again 48 h after birth. Serum was prepared and stored at -20°C for dot blot analysis.

2.3. Clinical Trial of Calves

All calves were checked by observing and examining twice daily for diarrhea and other clinical signs. Rectal body temperature, hydration, depression status, and vital sign (HR, BR) were evaluated, and fecal samples were collected from the rectum to evaluate the consistency and presence of oocysts from the start of the experiment (D0). Each day, a smear slide was made from each fecal sample and examined microscopically after modified acid-fast Ziehl-Neelsen staining to determine the presence of oocysts from D0 to D15 post-infection. The diarrhea severity was scored as follows: 0: normal, 1: solid 2: Fluid 3: watery. 0 and 1 scores were considered non-diarrheic, but 2 and 3 were taken as diarrheic. The start and end of the diarrheic period were recorded for each calf. The quantitative exanimation of oocysts was determined using collected 3 g feces using the method described by Askari *et al*. (2016). Purified oocysts from each feces were counted in an improved Neubauer Haematocytometer. Total oocysts were calculated by multiplying the number of oocysts per 1 gr of feces.

2.4. Analysis of Fecal Samples for the Presence of Salmonella, *E. coli* K99, Rota, and Corona Viruses

Fecal samples from all calves were cultured for salmonella and evaluated for the presence of Rotavirus Coronavirus and E. coli K99 in the Department of Microbiology, Faculty of Veterinary Medicine, the University of Teheran using the Pourquier Elisa kit.

2.5. Dot blot analysis of sera of cows and calves

Antibody detection against *C. parvum* in Sera of cows and calves and colostrum was performed by Dot blot test using recombinant C. parvum P23 antigen as described previously (Askari *et al.*, 2016).

2.6. Statistics Analysis

An analysis of repeated measures with a 95% confidence interval was carried out by general linear models (GLM) and independent samples t-test procedure of SPSS version 16.6 (SPSS Inc., Chicago, Ill., USA). Data were expressed as mean± SD.

Results

3.1. Determination of Antibody Against Recombinant P23 in Serum of Cows

Serum prepared from cows was analyzed for the presence of antibodies against *C. parvum* using recombinant P23 antigen in both groups by dot blot analysis. Figures 1 A(I) and 1 A (II) show that antibody against *C. parvum* was absent in serum prepared from cows in the control group before the experiment and two weeks after the 4th immunization of cows in the test group. Dot blot analysis of cows in the test group serum also showed the absence of antibodies against *C. parvum* before the start of the experiment [Figure 1B (I)], whereas, as expected, the serum titer against recombinant P23 was high (1:7000) two weeks after the 4th immunization [Figure 1B (II)].

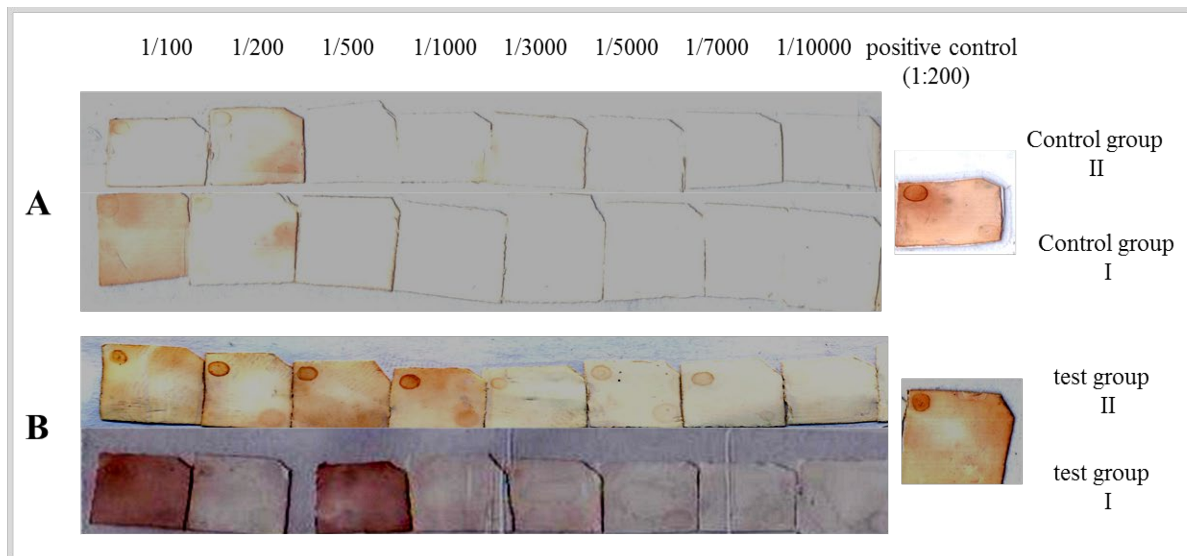


Figure 1. Determination of serum antibody titer of pregnant cows in control and test groups against recombinant P23 by dot blot analysis. **A (I)**: serum collected from the control group before starting the experiment. **A (II)**: serum collected from control group 2 weeks after 4th immunization of test group. **B (I)** serum collected from the test group before immunization. **B (II)**: serum collected from test group 2 weeks after 4th immunization.

3.2. Determination of antibody against recombinant P23 in colostrum

Colostrum collected from cows was analyzed for the presence of antibodies against *C. parvum* using recombinant P23 antigen in both groups by dot blot analysis. Figure 2A II showed that the antibody against *C. parvum* was nearly absent in colostrum prepared from cows in the control group. Only a very weak reaction (up to 1:500) could be detected in these samples, which most probably can be considered an unspecific reaction. As expected, high antibodies against recombinant P23 (1:10000) could be detected in colostrum collected from cows in the test group (Figure 2A I).

3.3. Clinical Signs of Infected Newborn Calves with Oocysts of *C. parvum*

12 h after parturition, the newborn Calves in both test and control groups were infected with 1×10^7 *C. parvum* oocysts mixed in 50 mL colostrum. Before inoculation, analysis of a fecal sample of all newborn calves showed the absence of oocyst of *C. parvum, Rotavirus*, *Coronavirus*, *E. coli K99,* and *Salmonella*. In newborn calves of the control group, the clinical signs could be depression, decreased appetite, and strong watery diarrhea from the 4th day after inoculation. The strong watery diarrhea continued for 6 to 7 days (6.66 ± 0.57). The color of the feces of these calves was yellow in some green-gray containing mucous masses. Stool consistency ranged from pasty to very watery. Following diarrhea, the control calves became dehydrated (9 to 10%) and had a weak suction reflex. Interestingly, a high temperature could not be observed in these calves. In contrast to the calves in the control group, the calves in the test group showed no clinical signs like depression, decreased appetite, diarrhea, and dehydration.

3.4. Determination of Antibody Against Recombinant P23 in Serum of Calves

Serum was prepared from the newborn calves before feeding with colostrum and 48 h after the first feeding with colostrum in both groups. The calves in both groups showed no detectable antibody titer against recombinant P23 (Figures 2B II and 2C II). In contrast to the serum prepared for calves from the control group (Figure 2B I), the calves from the test group showed a high titer of antibody against recombinant P23 48h after the first feeding with colostrum. The titer of the antibody was positive up to 1:7000 (Figure 2C I). As expected, the antibody against recombinant P23 could not be detected in the serum of calves 48h after the first feeding with the colostrum (Figure 2B I).

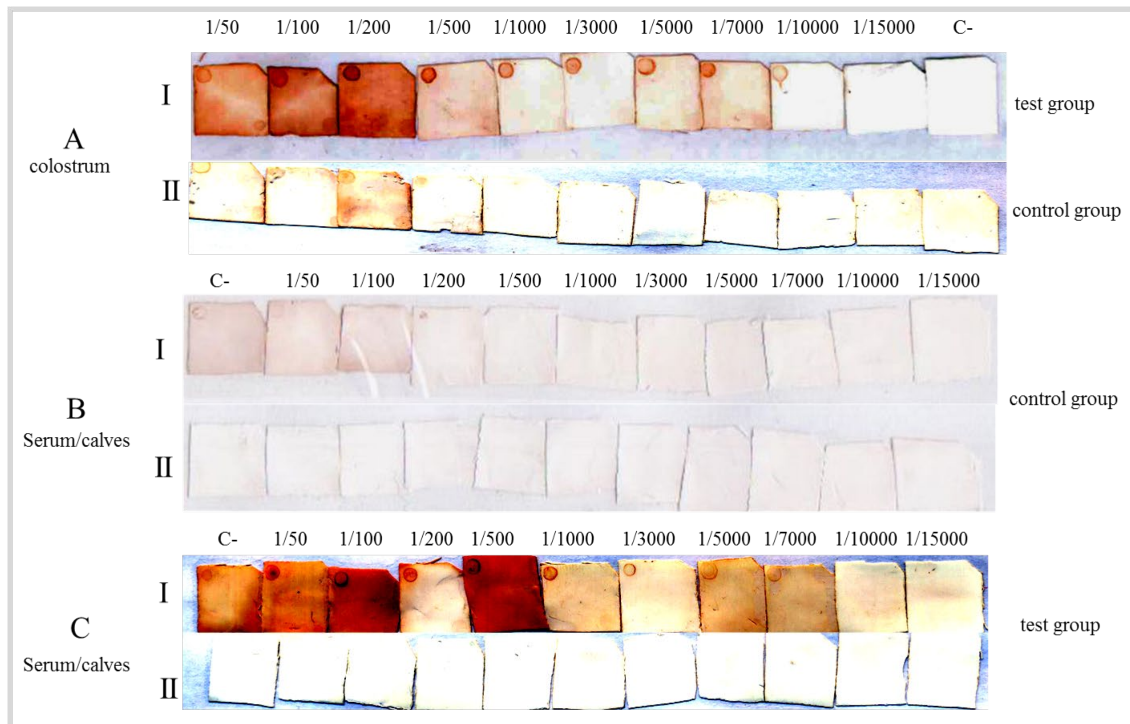


Figure 2. A: Colostrum collected from cows in both groups was analyzed for the presence of antibody against *C. parvum* by dot blot analysis using recombinant P23 antigen. A I: colostrum collected from cows in the control group. **A II:** colostrum collected from cows in the test group. C- is negative control. **B**: Serum was prepared from calves in control group and analyzed for the presence of antibody against *C. parvum* by dot blot analysis using recombinant P23 antigen. **B I**: before the feeding of colostrum. **B II**: after feeding of colostrum. C- is negative control. **C**: Serum was prepared from calves in the test group was analyzed for the presence of antibody against *C. parvum* by dot blot analysis using recombinant P23 antigen. **C I**: before the feeding of colostrum. **C II**: after feeding of colostrum. C- is negative control.

3.5. Parasitology Data

The pattern of the oocysts shedding is shown in Figure 3. The newborn calves in the control group shed *C. parvum* oocysts from day 4 post-infection, and the oocyst excretion reached a peak of $65.67 \times 10^6 \pm 13.85 \times 10^6$ oocyst /g feces on day 6 post-infection continued to decrease up to day 12 post-infection (Figure 3). The oocyst shedding in calves from the test group was detectable with a delay of 3 to 4 days and could be detectable at day 7 to 8 post-infection and continued until day 11 post-infection. The average duration of oocyst shedding was significantly (P<0.001) shorter in passive immune calves (4.33±0.57 days) compared to the shedding in control calves (8 days). According to the results, the calves received hyperimmune colostrum excreted significantly (P<0.05) fewer oocysts compared to control calves (Figure 3). The average peak of oocyst excretion in control calves ($65.67 \times 10^6 \pm 13.85 \times 10^6$) was significantly higher than in passively immunized calves ($1.87 \times 10^6 \pm 0.12 \times 10^6$)

(*P*=0.003). These results showed that the passive immunization of calves through the colostrum of his dam could reduce the shedding of oocyst by more than 99%.

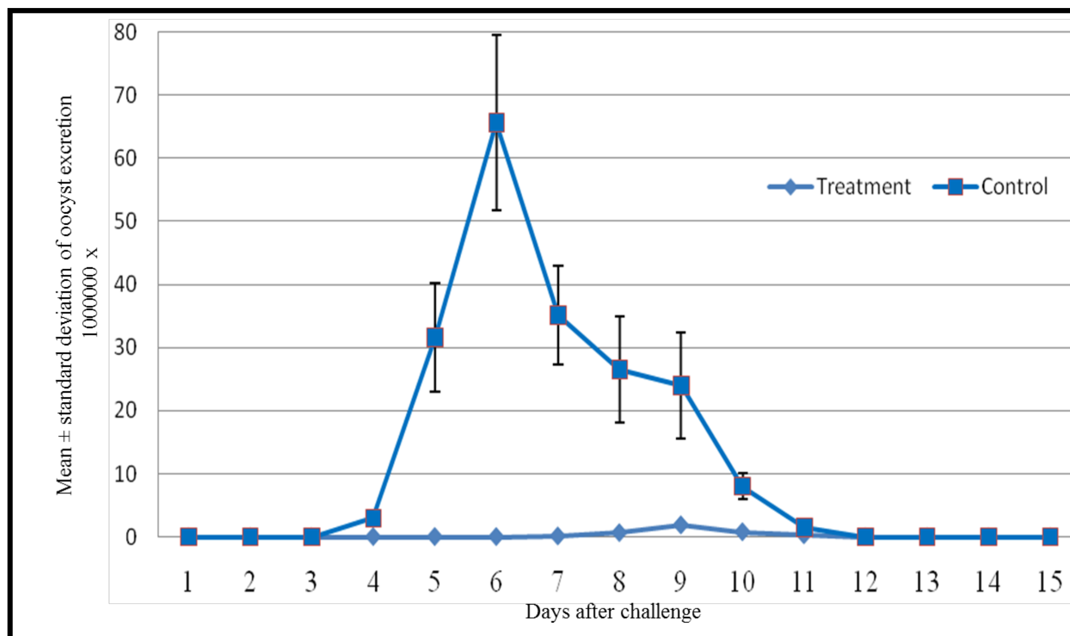
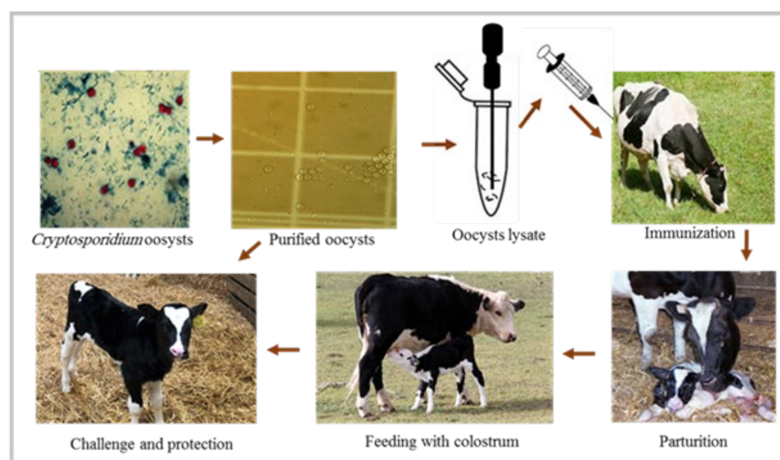


Figure 3. Oocyst shedding was determined in the faces of newborn calves from both (control and test) groups daily for up to 15 days.



Schematic figure

Discussion

Cryptosporidium parvum is a protozoan parasite that infected a broad spectrum of mammalian species and caused high economic losses in the livestock industry worldwide. *C. parvum* is a zoonotic parasite that plays an important role in public health, especially in immunocompromised humans (acquired and congenital). It is reported that even one oocyst of this species can cause human disease (Ramirez *et al*., 2004). The oocysts are resistant to disinfectant agents, and there are no effective chemotherapeutic compounds against this parasite. Currently, Hassan *et al*. (2021) used the methionyl-tRNA (*Cryptosporidium parvum* MetRS [*Cp*MetRS]) synthetase inhibitor 2093 against cryptosporidiosis in the calf,

but they reported the development of spontaneous resistance (Hasan *et al*., 2021). It seems that passive immunization of newborn calves could be considered a suitable strategy to protect newborn calves against infection (Askari *et al*., 2016). The use of oocysts as a candidate for vaccine development goes back many years ago. Harp and Goff (1995) used lyophilized *C. parvum* oocysts as an oral vaccine in calves short after birth (Harp and Goff, 1995). The calves were then orally administered 10^4 viable *C. parvum* oocysts at one week of age and reported that this kind of vaccine could reduce diarrhea and oocyst shedding.

Frayer et al. (1989) used hyperimmune colostrum as a passive immunization strategy for calves and could observe partial protection against cryptosporidiosis. Jenkins *et al*. (2004) reported about the gamma-irradiated *C. parvum* oocyst as a protective oral vaccine for 1-day-old dairy calves. Harp and Goff (1998) also immunized passive calves with pooled hyperimmune colostrum like that we performed in our work but could not show any protective effect against cryptosporidiosis. As they mentioned in their report, they used pooled hyperimmune colostrum with specific antibody titer against *C. parvum* up to 10, 240, which was prepared years ago reported in their previous work published by Harp et al. 1989. In contrast, in our experiments, the titer of specific antibody against *C. parvum* in sera of pregnant cows after 4th immunization in the test group measured using P23 was 1:7000. In their colostrum 1:10000. The calves fed with hyperimmune colostrum also showed a high titer of antibodies in their sera (1:7000) after feeding the hyperimmune colostrum. We believe that for the protection of calves, the hyperimmune colostrum has to have a high titer of neutralizing antibodies against *C. parvum*. Many investigators used immunogenic proteins like GP900, CP15, GP15, GP40, CSL, TRAP-C1, and P23, to protect animals against cryptosporidiosis and have reported successful protection (Cevallos *et al*., 2000; Tomley and Soldati, 2001; Shayan e*t al*., 2008; Ebrahimzadeh *et al*., 2009). Shayan *et al*. (2008) have also prepared recombinant P23 and reported that this protein is suitable for screening cryptosporidiosis in calves and cows (Shayan *et al*., 2008). In the next step, they could show that IgY prepared against P23 could protect mice against *C. parvum* (Shahbazi *et al*., 2009; Omidian *et al*., 2014). Askari *et al.* (2016) could show, in continue, that hyperimmune colostrum generated in cows can protect newborn calves against cryptosporidiosis (Askari *et al.*, 2016). In the present study, we used lysate prepared from oocysts of *C. parvum* as a vaccine to produce hyperimmune colostrum against *C. parvum* infection in pregnant cows. Our results showed that the hyperimmune colostrum against oocysts could protect newborn calves against *C. parvum* infection. Compared the results achieved in the present study with the results achieved by Askari *et al*. (2016), in which the authors used recombinant P23 instead of oocyst lysate as a vaccination tool, we can observe that oocyst lysate could cause the same antibody titer (10^{10}) in colostrum of immunized cows(Askari *et al*., 2016). The titer of antibodies against P23 two weeks after the 4th vaccination was in cows immunized with oocyst lysate was $1:10^7$, whereas this was higher (10^{10}) in serum of cows immunized with P23. Interestingly, the same titer of antibodies against P23 (10^7) could be measured in newborn cows in both studies after the first milking colostrum. More interestingly, although the time span from start to end of shedding of oocyst was comparable in both studies, the number of oocysts in this time was significantly higher in calves taking the colostrum from cows immunized with oocyst lysate. The results achieved by Askari *et al.* (2016) showed significantly less excretion of oocysts by feeding newborn calves with hyperimmune colostrum against P23 compared to the present study (Askari *et al*., 2016). These results showed that although the lysate prepared from oocysts of *C. parvum* can be used for hyperimmune colostrum in dams, the growing of oocysts in animals is expensive and against the moral principle of animal rights, and is recommended that it should be replaced through other approaches. Arrowood *et al*. (1994) developed an *in vitro* culture system to grow oocysts of *C. parvum*. Still, in their system, it was not possible to develop a significant number of mature oocysts (Arrowood *et al*., 1994). Morada *et al*. (2016) developed a culture system in which they could culture the oocysts over 6 months producing 10^8 oocysts per day, which can be considered a possible strategy for the replacement of animals (Morada *et al*., 2016). As described above, recombinant proteins like P23 can also be successfully used for producing hyperimmune colostrum,

and it seems to be cheaper than the cultivation of oocysts *in vitro* and can replace also the animal use for developing a vaccine against *C. parvum*.

Authors Contribution

M.D.MR, S.P, and R.S; The study design, supervise the project, and the revision and finalization of the manuscript. **S.P, Z.F, E.A.E and L.S;** Carried out the experiments. **S.P, E.A.E and S.C.S:** Analysis of the data and prepared the initial manuscript.

Ethical Statement

We hereby declare all ethical standards have been respected in preparation for the submitted article. This research was ethically approved by the ethical committee of the Faculty of the Veterinary Medicine, University of Tehran, under ethical code 7508006/6/12, 2019.

Acknowledgments

None

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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10.22059/IJVM.2022.339810.1005255

Iranian Journal of Veterinary Medicine

Abstracts in Persian Language

Online ISSN 2252-0554

مجله طب دامی ایران، ۱۴۰۱، دوره ۱۶، شماره ۴، ۳۸۹-۳۸۰

حفاظت از گوساله‌ها در برابر کریپتوسپوریدیوزیس با آغوز گاو آبستن ایمن‌شده با آنتی‌ژن‌های کامل اووسیست

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(دریافت مقاله: ۰۹ اسفند ماه ۱۴۰۰، پذیرش نهایی: ۲۶ اردیبهشت ۱۴۰۱)

زمینه مطالعه: کریپتوسپوریدیوم پارووم یک انگل کوکسیدیایی با انتشار جهانی است. این انگل به‌عنوان یکی از مهم‌ترین عوامل اسهال در اکثر مهره‌داران و انسان‌های دارای نقص ایمنی محسوب می‌شود

هدف: با توجه به عدم درمان موثر کریپتوسپوریدیوزیس، استراتژی حفاظتی در برابر این انگل می‌تواند از طریق واکسینه کردن در حیوان مادر بر ضد انگل قبل از زایمان باشد.

روش کار: برای این منظور لیزات از اووسیست‌های کریپتوسپوریدیوم پارووم به‌عنوان کاندیدای واکسن آنتی‌ژن کامل (۴۲۰ میکروگرم) استفاده شد و ۳ گاو آبستن را ۷۰ روز مانده به زایمان ۴ بار هر ۲ هفته واکسینه شدند. به‌عنوان گروه شاهد از ۳ گاو آبستن غیر ایمن‌شده استفاده شد. پس از زایمان، هر گوساله با آغوز مادر خود تغذیه شد و در ۱۲ ساعت پس از تولد با 1×10^{7} اووسیست آلوده گردید.

نتایج: برخلاف گروه آزمایش، گوساله‌های گروه کنترل دچار اسهال شدید آبکی همراه با دفع اووسیست از ۴ روز پس از عفونت شدند. گوساله‌های گروه آزمایش که آغوز هایپرایمنی دریافت کردند، نه تنها علائم بالینی نشان ندادند، بلکه کاهش قابل توجهی در دفع اووسیست داشتند.

نتیجه‌گیری نهایی: آنتی‌ژن کامل تهیه‌شده از اووسیست‌های *C. parvum* را می‌توان به‌عنوان یک کاندیدای مناسب برای ایمن‌سازی گاوهای باردار تولیدکنندهٔ آغوز هایپرایمن در نظر گرفت.

واژه‌های کلیدی: کریپتوسپوریدیوم پارووم، آغوز هایپر ایمن، واکسیناسیون، آنتی‌ژن‌های کامل اووسیست، ایمنی پسیو

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