Experimental Parasitology 121 (2009) 157–162

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Development and evaluation of a recombinant CP23 antigen-based ELISA for serodiagnosis of *Cryptosporidium parvum*

Chengmin Wang [a], Hongxuan He [a,*], Mingxing Duan [b]

[a] National Research Center for Wildlife Borne Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Datun RD, Chaoyang District, Beijing 100101, PR China
[b] State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, PR China

ARTICLE INFO

Article history:
Received 16 April 2008
Received in revised form 3 October 2008
Accepted 28 October 2008
Available online 7 November 2008

Keywords:
Recombinant CP23 protein
Cryptosporidium parvum
Indirect ELISA

ABSTRACT

The CP23 gene of *Cryptosporidium parvum* strain isolated from Changchun in China was expressed in *Escherichia* coli BL21 strain and was purified as a recombinant protein. An indirect ELISA assay (CP23-ELISA) for antibody detection was established using the purified recombinant CP23 protein. Antigen coating conditions and serum dilution for the CP23-ELISA were optimized. The S/P ratio of the absorbency value was calculated in the CP23-ELISA to evaluate the serum antibody level of the field cow samples. It indicated that the CP23-ELISA assay, which was more convenient and easier to prepare than traditional methods, was a good candidate for evaluation of *C. parvum* exposure to domestic animal in field.

1. Introduction

Cryptosporidium parvum is a widely distributed coccidian parasite that causes the enteric disease in humans and animals. Cryptosporidiosis is a cause of life-threatening disease in immunodeficient people and it has been estimated that 10–15% of patients with AIDS in the United States and considerably higher percentages in Africa, South America, and Haiti (Petersen, 1992) died as a result of infection with *Cryptosporidium*. In addition to humans, *C. parvum* has been reported as a common serious primary cause of outbreaks of diarrhea in farm animals, especially newborn ruminants, resulting in significant economic losses (De Graaf et al., 1998).

Endogenous stages and oocysts can be detected in stained impression smears from the ileac mucosa of the living body (O'Donoghue, 1995). Identification of oocysts directly in the feces can yield excellent results (Hall et al., 1988). However, microscopy is time consuming, has low specificity and can easily result in false positives. In 1991, Laxer developed a method with high specificity and sensibility for the specific detection of *C. parvum* with DNA sequences by the polymerase chain reaction (PCR) (Laxer et al., 1991). However, this new approach of diagnosing cryptosporidiosis could not easily be applied to clinical practice widely for reasons such as high cost and technique conditions (Moon and Woodmansee, 1986). Rapid immuno-chromatographic assays for detecting infections with *Cryptosporidium parvum* in calf fecal samples were evaluated using as gold standards a sedimentation–flotation technique, Sensitivity and specificity for detection of *C. parvum* in fecal samples were high (100% and 94.6%, respectively) (Luginbühl et al., 2005), but the sensitivity and specificity of the rapid assay for *C. parvum* were high compared to the sedimentation–flotation technique (100% and 94.6%, respectively). However, these results could not be confirmed conclusively by additional detection protocols (e.g. microscopic examination and PCR assay) (Daniela et al., 2008).

The immunological approach to the interaction between the host and the parasite provides valuable tools for serum epidemiological and immunological studies. Infection by *C. parvum* in both humans and animals elicits the development of characteristic serum and mucosal immunoglobulin G (IgG), IgA and IgM antibody responses against parasite antigens detectable by enzyme-linked immunosorbent assay (ELISA) (Riggs, 1997). Although detection of specific serum antibodies should not be regarded as indicative of active infection, some antigens identified by immunoblot analysis are recognized by IgA, IgG and IgM serum antibodies of many species, and are considered excellent markers of infection (Reperant et al., 1994). Additionally, the use of purified immunodominant antigens has been shown to improve the specificity and sensitivity of enzyme immunoassays for the detection of specific antibodies in epidemiologic studies (Priest et al., 1999). A review of the antigens recognized by immune serum/mucosal antibodies, hyper-immune serum/colostrum or monoclonal antibodies leads to the conclusion that some of them are neutralization-sensitive epitopes and may provide protection against infection (Riggs, 1997).

To date, the study of immune responses to specific antigens of *C. parvum* has been restricted to the characterization of serologic

* Corresponding author. Fax: +86 10 64807118.
E-mail address: hehx@ioz.ac.cn (H. He).

0014-4894/$ - see front matter
doi:10.1016/j.exppara.2008.10.015

reactivity. Humoral responses to antigens described by several groups recognize a number of immunodominant sporozoite antigens, including polypeptides of approximately 11, 15, 23, 44, 100, 180 and 200 kDa (Mead and You, 1998; Peeters et al., 1992; Hill, 1990). However, there is little knowledge regarding the nature of specific antigens that induce protective responses during natural infection or of the precise cellular responses that mediate protective immunity (Perryman et al., 1999; Sagodira et al., 1999). Data concerning the identity of *C. parvum* recombinant antigens which stimulate T lymphocytes remain scarce (Gomez-Morales et al., 1995; Iochmann et al., 1999).

CP23 antigen, a major target of humoral immunity (Arrowood et al., 1989, 1991) and one of the immunodominant antigens involved in the immune response to *Cryptosporidium parvum* infection (Ehigiator et al., 2007). It is present in both the sporozoite and merozoite stages and is considered a marker of infection because it is recognized by serum antibodies of humans and many animal species (Lumb et al., 1988; Riggs et al., 1994; Mead and You, 1998; Mead et al., 1988; Priest et al., 1999; Ehigiator et al., 2007). Previous studies on the development of CP23 antigen as a vaccine against cryptosporidiosis have been limited to immunization with the recombinant protein (Perryman et al., 1999; Hong et al., 2005). Hong et al. (2005) showed that immunization of young BALB/c mice with a CP15-23 fusion protein induced specific antibody responses to *C. parvum* oocyst lysate and reduced the level of *C. parvum* infection in the mice. Priest et al. (2001) reported also that CP23 and Triton antigen ELISAs are useful in epidemiologic studies of the prevalence of *Cryptosporidium* infection in the population. In the present study, we focused on 23 kDa recombinant antigen of *C. parvum* bovine expressed in *E. coli* BL21 strain and purified as specific antigen in developing an indirect ELISA.

2. Materials and methods

2.1. Parasite and serum samples

Cryptosporidium parvum was isolated originally from an outbreak of cryptosporidiosis in a cow farm and identified as the bovine genotype by the Wildlife Born Diseases Lab, CAS. Oocysts were obtained from calves orally infected with 10^6 parasites. Calf feces were kept at 4 °C in 2.5% potassium bichromate before use. Oocysts purification and parasite antigen preparation were performed as described previously (Jean et al., 1994).

Positive and negative serum was prepared as described previously (Hong et al., 2002). Briefly, three BALB/c mice (18–22 g) were inoculated by intraperitoneal injection with 0.2 ml of parasite antigen every 2 weeks each. Antigen suspensions were mixed Freund's complete adjuvant the first time, Freund's incomplete adjuvant the second, and PBS at the third and last time. Two weeks after the last treatment, the mice serum separated from venous plexus of eyes was regarded as positive serum.

2.2. Cloning, expression and purification of recombinant antigen

CP23 was PCR amplified from *C. parvum* genomic DNA with primers 5′-ATGGGTTGTTCATCATCA-3′ (forward primer) and 5′-TCGGTCGACTAC GGATT-3′ (reverse primer) designed from GenBank sequence U34390 and cloned in the expression vector pGEX-4T (Takara, Japan) in frame with Schistosoma japonicum Glutathione S-transferase (sj-GST) using standard techniques (Bonafonte et al., 2000). Finally, the culture was centrifuged at 7700g for 10 min, and the bacterial pellet was resuspended in 30 ml of ice-cold PBS and sonicated on ice until clear. The sonicate was centrifuged at 12,000g at 4 °C for 10 min and the supernatant was saved for purification.

The recombinant protein and the control GST were purified with a bulk GST purification module (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Thrombin protease was added to the concentrated samples to cleave GST. The *C. parvum CP23* protein was released by overnight cleavage with thrombin at room temperature and separated from uncleaved fusion protein and the GST cleavage product by passage over glutathione Sepharose 4B resin. Finally, the Bradford method was used to determine the concentration of the purified CP23 protein (Walker, 1996).

2.3. Western blot

The purified CP23 (5 μg) and GST proteins were separated on a 10% SDS–PAGE gel and electroblotted to a nitrocellulose membrane to assess the protein purity. Two identical blots were prepared and both were blocked with 5% milk buffer. One blot was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody (1:200) and the other was incubated with goat anti-GST (1:1000) (Amersham Pharmacia Biotech). The appropriate secondary antibodies conjugated to biotin were added at a 1:1000 dilution. The proteins were detected by incubation with streptavidin conjugated to HRP (1:1000) with tetramethylbenzidine. Biotinylated molecular weight markers (BioRad, CA) were detected with avidin–HRP.

2.4. CP23-ELISA procedure

Mice antiserum to *C. parvum* antigen and SPF BALB/c mice negative sera were used to optimize the CP23-ELISA procedure. Ninety six-well ELISA plate (Corning Incorporated, USA) was coated with 100 μL appropriately diluted recombinant CP23 protein ranging from 16 mg/ml to 2 μg/ml and incubated at 37 °C for 2 h. The plate was washed three times with TBST buffer, blocked with 5% skim milk, antiserum diluted with 0.1% bovine serum albumin (BSA) were added and the plate was incubated at 37 °C for 1 h. After washing three times with TBST buffer, 100 μL HRP-labeled goat anti-mouse IgG was added to the plate and incubated at 37 °C for 1 h. After washing four times, 50 μL TMB was added to the plate followed by exposure for 10 min. The reaction was terminated with 2 M H_2SO_4 and the OD_{450} value was read with Elx800 Universal Microplate Reader (BioTek Instruments, USA). Every sample was repeated in three wells and the mean value was calculated. S/P ratio was used as a standard for judgment. S/P = (OD_{450} of sample−OD_{450} of negative control)/(OD_{450} of positive control−OD_{450} of negative control). S/P $\geqslant$ 0.2 was judged as positive and S/P $\leqslant$ 0.2 was judged as negative.

2.5. Stability and specificity of the CP23-ELISA procedure

To evaluate the stability of the CP23-ELISA procedure, three batches of CP23 protein prepared separately were used as coating antigen to detect a positive antiserum against *C. parvum*. In each experiment, six wells were repeated and the mean value was calculated for evaluation. To evaluate the specificity of the CP23-ELISA procedure, mice antiserum against *C. parvum* (three strains, two from cattle in Changchun city and Zhengzhou city and one from a human case in Xinxiang city), *Eimeria zurni, Giardia duodenalis, Balantidium coli* and *E. coli,* respectively were tested with the CP23-ELISA assay.

2.6. Field sample detection

One hundred and eighty serum samples and 180 fecal samples were collected from three separate cow farms (Farm 1, 2 and 3) in Xinxiang city of Henan province, China. These were examined with

both the CP23-ELISA assay and feces examination under light microscope.

2.7. Statistical analysis

Statistical analysis was performed with ANOVA method (SAS 1996). Difference was considered to be significant when $P < 0.05$.

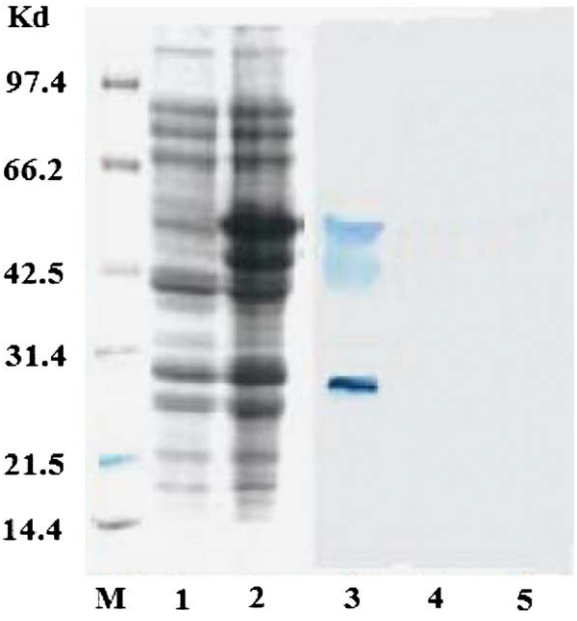

Fig. 1. The purified CP23 was separated on a 10% SDS–PAGE gel and electroblotted to a nitrocellulose membrane. The blot was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody. Lane M, molecular weight markers; lane 1, negative control *E. coli* BL21 strain which was transformed with pGEX-4T/CP23 vector; lane 2, recombinant *E. coli* BL21 strain presented the CP23 protein with a molecular weight of about 46 kDa; lane 3, 5 μg of CP23, anti-*Cryptosporidium parvum* polyclonal antibody reacted with proteins of 23 and 46 kDa; lane 4, negative control (media) for reactivity; lane 5, GST control, no reactivity of anti-*C. parvum* antibody was noted with the GST control.

3. Results and discussion

3.1. Expression and purification of CP23 protein

After expression, CP23 and GST control were purified and analyzed by SDS–PAGE and Western blot. Two identical blots were prepared; one was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody (Fig. 1) and the second blot was incubated with goat anti-GST (specific reactivity to the purified 27 kDa GST protein was observed, data not shown). Similar to Priest et al. (1999) and Bonafonte et al. (2000), who evaluated serologic specimens using the 23 kDa recombinant antigen, Fig. 1 demonstrates specific reactivity of anti-*Cryptosporidium* polyclonal antibodies with proteins of 23 kDa (cleaved protein) and of 46 kDa (purified fusion protein, GST/CP23 after partial thrombin cleavage). No reactivity of the *C. parvum-specific* serum was detected in the lane loaded with GST control (Fig. 1, lane 5).

3.2. Optimization of CP23-ELISA procedure

Recombinant CP23 protein (16 mg/mL) was immobilized onto ELISA plates in serial two-fold dilutions from 1:200 to 1:6400 and mice antiserum against *C. parvum* antigen or negative control mice serum was also diluted in serial two-fold dilutions from 1:50 to 1:400 for optimization. It was found that the highest P/N value was obtained when the CP23 protein was diluted to 1 μg/ml, and mice anti-*C. parvum* positive and negative sera were diluted to 1/100, respectively (Fig. 2B). To determine the optimal conjugate dilution in the CP23-ELISA, after the concentration of the CP23 protein, mouse anti-*C. parvum* positive and negative sera were fixed onto the ELISA plate, the different concentrations of HRP-labeled goat anti-mouse IgG were added onto the ELISA plate at the dilutions of 1:400, 1:600, 1:800, 1:1000 and 1:1200.

Data shown in Fig. 3A indicates that 1:1000 was the most appropriate dilution of the HRP-labeled goat anti-mouse IgG. To test the optimal coating buffer, NaOH (pH 13), 0.05 M Bicarbonate/Carbonate buffer(pH 9.6), 0.1 M Tris–HCl (pH 8.5), PBS (pH 7.4) and HCl (pH 4.6) was screened, it was shown that 0.1 M Tris–HCl (pH 8.5) was the best coating buffer for immobilization of the CP23 protein (Fig. 3B). In the experiment of blocking buffer

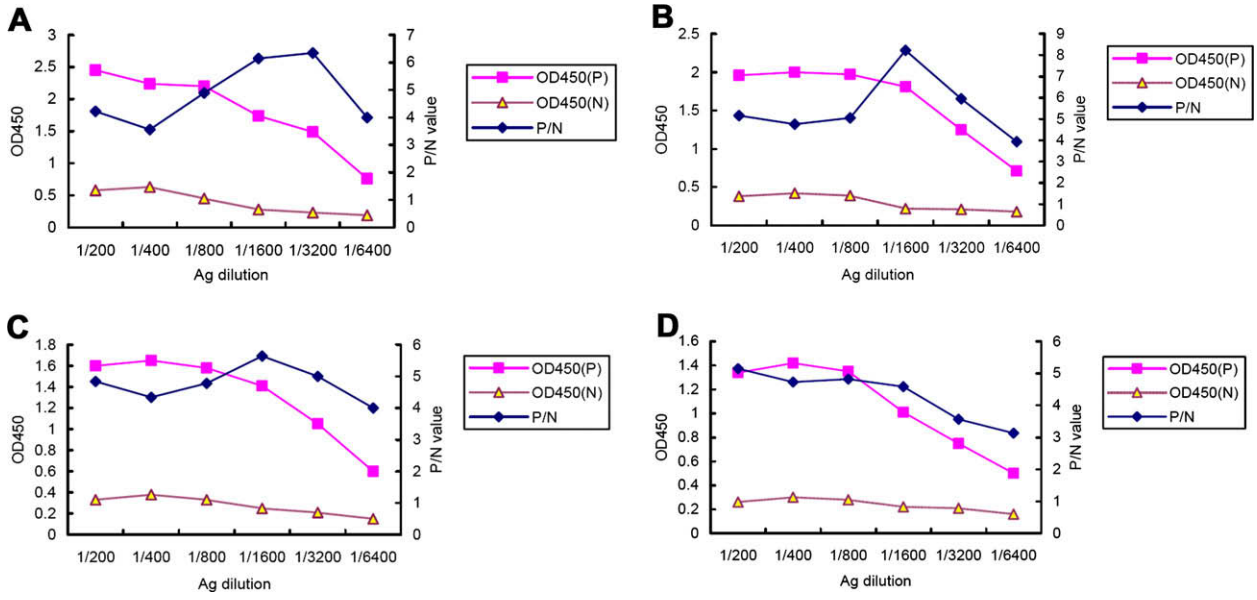

Fig. 2. Optimization of the CP23 protein and sera dilutions. The CP23 protein was diluted from 16 mg/ml to 0.5 μg/ml while antisera were diluted to 1:50 (A), 1:100 (B), 1:200 (C) and 1:400 (D). The optimal dilutions of the CP23 protein and sera were respectively 2 μg/ml and 1/100.

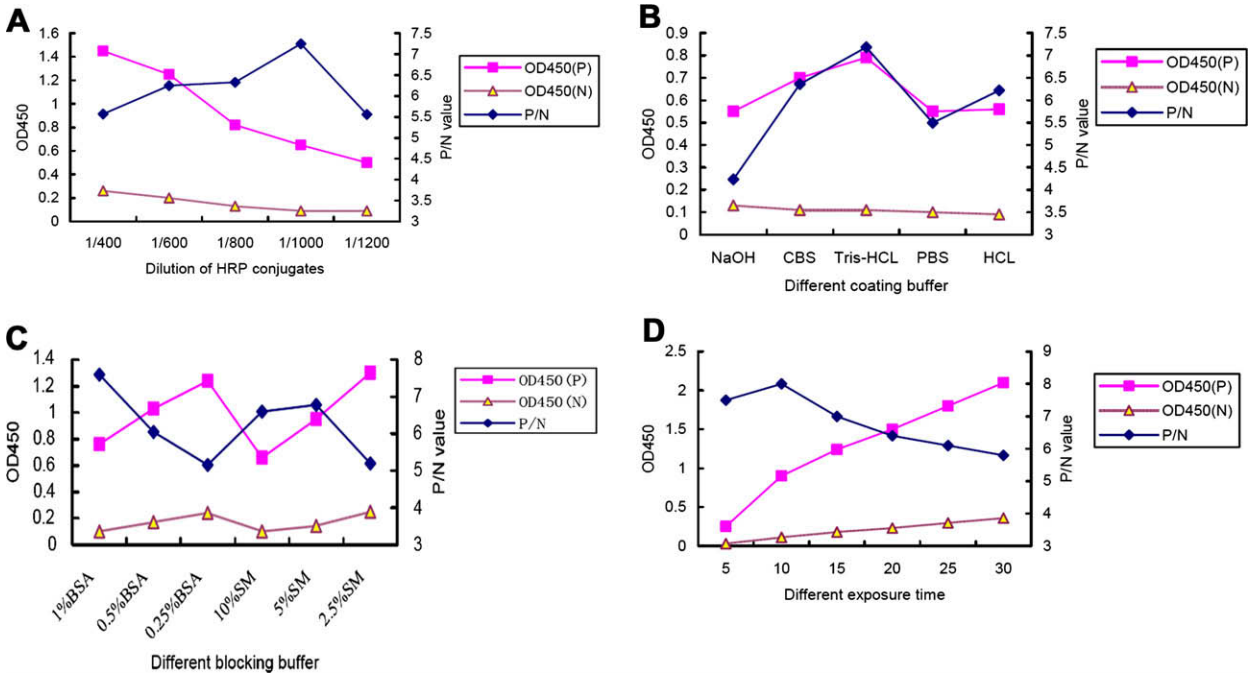

Fig. 3. Optimization of HRP conjugates dilution, coating buffer, blocking buffer, and exposure time. (A) Optimization of dilution of conjugates (HRP-labeled goat anti-mouse IgG). (B) Optimization of coating buffers: NaOH (pH 13), 0.05 M Bicarbonate/Carbonate buffer (CBS, pH 9.6), 0.1 M Tris–HCl buffer (pH 8.5), Phosphate buffer saline (PBS, pH 7.4) and HCl (pH 4.6). (C) Optimization of blocking buffer: BSA, SM. (D) Optimization of exposure time.

Table 1
Detection results of field samples by CP23-ELISA and light microscope from three cow farms in Xinxiang.

Farm number	Farm 1		Farm 2		Farm 3	
	Serum samples	Fecal samples	Serum samples	Fecal samples	Serum samples	Fecal samples
The total number	65	65	70	70	45	45
Positive samples	25	23	33	29	16	15
Negative samples	40	42	37	41	29	30

selection, six candidate blocking buffers (1% BSA, 0.5% BSA, 0.25% BSA, 10% skim milk, 5% skim milk and 2.5% skim milk) were compared, it was found that 1% BSA was the optimal blocking buffer for the CP23-ELISA (Fig. 3C). Finally, after the ELISA plates was exposed for from 5 to 30 min in TMB solution, the result shown in Fig. 3D indicated that the appropriate time of color development is 10 min with TMB solution in the CP23-ELISA procedure.

3.3. Specificity and stability of the CP23-ELISA assay

The S/P ratios ($\bar{x} \pm SD$) for three *C. parvum* strains were respectively 1.012 ± 0.021, 1.103 ± 0.022 and 0.987 ± 0.014, whereas the S/P ratio ($\bar{x} \pm SD$) for *Eimeria zurnii, Giardia duodenalis, Balantidium coli* and *E. coli* (O157) was respectively 0.113 ± 0.022, 0.165 ± 0.023, 0.140 ± 0.016 and 0.131 ± 0.014. The results above indicated that the CP23-ELISA assay was specific and appropriate for detection of antibodies against the different *C.* parvum strains including human case.

To determine the stability of the CP23-ELISA assay, three batches of recombinant CP23 protein prepared separately were used as coating antigen and mice antiserum against *C. parvum* strain was used a reference positive serum, the result showed that the S/P ratio ($\bar{x} \pm SD$) was 1.112 ± 0.011, 1.076 ± 0.027 and 1.068 ± 0.035, respectively, indicating that the difference between the three groups was not significant ($P > 0.05$) and the CP23-ELISA assay was stable.

3.4. Field samples detection

One hundred and eighty field serum samples were tested with the CP23-ELISA assay and 180 fecal samples were treated and observed under light microscope. All data were shown in Table 1. The observation under light microscope could detect oocysts in the feces samples while the CP23-ELISA detected only specific antibodies against CP23 protein of *C. parvum*. The positive number of serum samples with the CP23-ELISA was 25, 33 and 16, while the positive results of fecal samples with etiological observation were 23, 29 and 15, respectively. However, our attempts were unsuccessful, and we presume that the degradation of the expressed products was due to proteolytic digestion in the host bacteria.

4. Discussion

CP23 is a surface pellicle glycoprotein in membranous and proteinaceous trails deposited by sporozoites during gliding locomotion (Riggs et al., 2002; Enriquez and Riggs, 1998). As with gp15, CP23 was initially reported to be a single antigen, although it now belongs to a family of proteins having similar MW (Arrowood et al., 1989; Arrowood et al., 1991; Riggs, 1997). CP23, known to be immunogenic in humans and cattle, is also present on the surface of whole oocysts (Arrowood et al., 1989; Arrowood et al., 1991). Two of the CP23 proteins have been cloned and are distinct at the gene level (Perryman et al., 1996; Riggs, 1997). The gene encoding of the CP23 peptides was sequenced by Perryman et al. (1996), who reported the predicted MW of its gene product to be 11.2 kDa (GenBank Accession no. U34390). However, on Western blots and polyacrylamide gels, this protein appeared to be 23 kDa, hence, the name CP23. They postulated that the discrepancy in size might

be due to physical properties of the protein or glycosylation. In the present study, the same phenomenon was observed.

In this experiment, to prevent the degradation of the expressed CP23 protein of *C. parvum*, the bacteria were cultured at 25 and 30 °C after induction (unpublished data). However, our attempts were unsuccessful, presuming that the degradation of the expressed products may be digested by the proteolytic in host bacteria. Purification of the CP23 protein is one of the critical steps for CP23-ELISA assay in the present study because the CP23 protein of *C. parvum* was produced in *E. coli.*

In this experiment, the recombinant protein and the control GST were purified with a bulk GST purification module (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Thrombin protease was added to the concentrated samples to cleave GST. The *C. parvum* CP23 protein was released by overnight cleavage with thrombin at room temperature and separated from uncleaved fusion protein and the GST cleavage product by passage over glutathione Sepharose 4B resin. It was found that more CP23 protein was recovered and the CP23 protein remained immunogenic. When the CP23 fusion protein was cleavaged, many inner epitopes were exposed, which might contribute to the immunoreaction of CP23 protein with antibodies. To evaluate the purity of CP23 protein, Western blotting was performed with antisera.

The protocol for the CP23-ELISA was optimized in the present study because many factors might affect the results of the ELISA (Fig. 3). As to the coating of CP23 protein, the use of Tris–HCl at pH 8.5 conferred the most efficient coating conditions despite the fact that bicarbonate/carbonate buffer at pH 9.6 (Fig. 3B) is used widely in previous reports. As to the standard for judgment in ELISA, the standard usually is absorbency value, S/N ratio (absorbency value of sample/absorbency value of negative sample) or S/P ratio (absorbency value of sample/absorbency value of positive sample). In present experiment, S/P ratio was chosen because it was more stable than other parameters. In fact, S/P ratio has also been chosen as standard in many previous reports (Wang et al., 2002; Chen et al., 2003).

The CP23-ELISA established in the present study showed that the CP23 protein of *C. parvum* strain was only able to react specifically with the antisera against *C.* parvum (Changchun strain, Zhengzhou strain, Human strain) and not with antisera against *Eimeria zurnii , Giardia duodenalis, Balantidium coli* and *E. coli (O157).* Subsequently, in field trails the CP23-ELISA assay showed much higher sensitivity than microscopy (Table 1), demonstrating that the CP23-ELISA may be used as a potential technique for evaluation of indicators of recent exposure to *C. parvum* for domestic animal and/or human exposed to *C. parvum*. In addition, sera samples of the 180 cows from Farm 1, 2 and 3, were also assayed for antibodies to CP23 recombinant antigens with the large-format Western blot, the results were in agreement with those of CP23-ELISA (unpublished data).

Acknowledgments

This study was supported by grants from the National Key Basic Research and Development Program of China (9732007BC109103), the CAS Innovation Program (KSCX2-YW-N-063) and The National Natural Sciences Foundation of China (30671576).

References

Arrowood, M.J., Mead, J.R., Mahrt, J.L., Sterling, C.R., 1989. Effects of immune colostrum and orally administered antisporozoite monoclonal antibodies on the outcome of *Cryptosporidium parvum* infections in noenatal mice. Infection and Immunity 57, 2283–2288.

Arrowood, M.J., Sterling, C.R., Healey, M.C., 1991. Immunofluorescent microscopial visualization of trails left by gliding *Cryptosporidium parvum* sporozoites. Journal of Parasitology 77, 315–317.

Chen, H., Coote, B., Attree, S., Hiscox, J.A., 2003. Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. Avian Pathology 32, 519–526.

Daniela Kleina., Angela Kernb., Gudrun Lapanc., Viviane Benetkad., Karin Möstld., Andreas Hassle., Walter Baumgartner., 2008. Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. The Veterinary Journal. doi:10.1016/j.tvjl.2008.07.016.

De Graaf, D.C., Walravens, K., Godfroid, J., Peeters, J.E., 1998. A *Cryptosporidium parvum* oocyst low molecular mass fraction evokes a CD41 T-cell-dependent IFN-gamma response in bovine peripheral blood mononuclear cell cultures. International Journal for Parasitology 28, 1875–1880.

Enriquez, F.J., Riggs, M.W., 1998. Role of immunoglobulin A monoclonal antibodies against P23 in controlling murine *Cryptosporidium parvum* infection. Infection and Immunity 66, 4469–4473.

Ehigiator, H.N., Romagnoli, P., Priest, J.W., Secor, W.E., Mead, J.R., 2007. Induction of murine immune responses by DNA encoding a 23 kDa antigen of *Cryptosporidium parvum*. Parasitology Research 101 (4), 943–950.

Gomez-Morales, M.A., Ausiello, C.M., Urbani, F., 1995. Crude extract and recombinant protein of *Cryptosporidium parvum* oocysts induce proliferation of human peripheral blood mononuclear cells in vitro. Journal of Infectious Diseases 172, 211–216.

Hall, G.A., Reynolds, D.J., Parsons, K.P., Bland, A.P., Morgan, J.H., 1988. Pathology of calves with diarrhea in southern Britain. Research in Veterinary Science 45, 240.

Hong, X.H., Zhang, X.C., Ouyang, H.S., 2002. Construction of prokaryotic expression vector containing gene encoding sporozoite surface protein of *C. parvum* and its expression in *E. coli*. Chinese Journal of Preventive Veterinary Medicine 24 (6), 75–78.

Hong, X.H., Lei, C., Cheng, M.W., Kai, Z., Yi, T., Xi, M.Q., Ming, X.D., 2005. Expression of the recombinant fusion protein CP15-23 of *Cryptosporidium parvum* and its protective test. Journal of Nanoscience And Nanotechnology 5 (8), 1292–1296.

Hill, B.D., 1990. Enteric protozoa in ruminants: diagnosis and control of *Cryptosporidium*, the role of the immune response. Revue Scientifique et Technique 9, 423–440.

Iochmann, S., Sagodira, S., Mevelec, M.N., Reperant, J.M., Naciri, M., Coursaget, P., Bout, D., 1999. Comparison of the humoral and cellular immune responses to two preparations of *Cryptosporidium parvum* CP15/60 recombinant protein. Microbial Pathogenesis 26, 307–315.

Jean, M.R., Muriel, N., Sophie, I., Micheal, T., Daniel, T.B., 1994. Major antigens of *Cryptosporidium parvum* recognized by serum antibodies from different infected animal species and man. Veterinary Parasitology 55, 1–13.

Laxer, M.A., Timblin, B.K., Patel, R.J., 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. American Journal of Tropical Medicine And Hygiene 45 (6), 688–694.

Luginbühl, A., Reitt, K., Metzler, A., Kollbrunner, M., Corboz, L., Deplazes, P., 2005. Feldstudie zu Pralenz und Diagnostik von Durchfallerregern beim neonaten Kalb im Einzugsgebiet einer schweizerischen Nutztierpraxis. Schweizer Archiv für Tierheilkunde 147, 245–252.

Lumb, R., Lanser, J.A., O'Donoghue, P.J., 1988. Electrophoretic and immunoblot analysis of *Cryptosporidium* oocysts. Immunology and Cell Biology 66, 369–376.

Bonafonte, M.T., Smith, M.L., Mead, J.R., 2000. A 23 kDa recombinant antigen of *Cryptosporidium parvum* induces a cellular immune response on in vitro stimulated spleen and mesenteric lymph node cells from infected mice. Experimental Parasitology 96, 32–41.

Mead, J.R., Arrowood, M.J., Sterling, C.R., 1988. Antigens of *Cryptosporidium* sporozoites recognized by immune sera of infected animals and humans. Journal of Parasitology 74, 135–143.

Mead, J.R., You, X., 1998. Susceptibility differences to *Cryptosporidium parvum* infection in two strains of gamma interferon knockout mice. Journal of Parasitology 84, 1045–1048.

Moon, H.W., Woodmansee, D.B., 1986. Cryptosporidiosis. Journal of The American Veterinary Medical Association 89, 643–646.

O'Donoghue, P.J., 1995. *Cryptosporidium* and cryptosporidiosis and animals. International Journal for Parasitolology 25, 139–195.

Peeters, J.E., Villacorta, I., Vanopdenbosch, E., Vandergheynst, D., Naciri, M., Ares-Maza, E., Yvore, P., 1992. *Cryptosporidium parvum* in calves: kinetics and immunoblot analysis of specific serum and local antibody responses (Immunoglobulin A [IgA], IgG, and IgM) after natural and experimental infections. Infection and Immunity 60, 2309–2316.

Perryman, L.E., Kapil, S.J., Jones, M.L., Hunt, E.L., 1999. Protection of calves against cryptosporidiosis with immune bovine colostrums induced by a *Cryptosporidium parvum* recombinant protein. Vaccine17, 2142–2149.

Perryman, L.E., Jasmer, D.P., Riggs, W., Bohnet, S.G., McGuire, T.C., Arowood, M.J., 1996. A cloned gene of *Cryptosporidium parvum* encodes neutralization-sensitive epitopes. Molecular and Biochemical Parasitology 80, 137–147.

Petersen, C., 1992. Cryptosporidiosis in patients infected with the *human immunodeficiency virus*. Clinical Infectious Diseases 15, 903–909.

Priest, J.W., Kwon, J.P., Moss, D.M., Roberts, J.M., Arrowood, M.J., Dwokin, M.S., 1999. Detection by enzyme immunoassay of serum immunoglobulin G antibodies that recognize specific *Cryptosporidium parvum* antigens. Journal of Clinical Microbiology 37, 1385–1392.

Priest, J.W., Li, A., Khan, M., Arrowood, M.J., Lammie, P.J., Ong, C.S., Roberts, J.M., Isaac-Renton, J., 2001. Enzyme immunoassay detection of antigen-specific immunoglobulin g antibodies in longitudinal serum samples from patients with

cryptosporidiosis. Clinical and Diagnostic Laboratory Immunology 8 (2), 415–423.

Reperant, J.M., Naciri, M., Iochmann, S., Tilley, M., Bout, D.T., 1994. Major antigens of *Cryptosporidium parvum* recognised by serum antibodies from different infected animal species and man. Veterinary Parasitology 55, 1–13.

Riggs, M.W., 1997. Immunology: host response and development of passive immunotherapy and vaccines. In: Fayer, R. (Ed.), Cryptosporidium and Cryptosporidiosis. CRC Press, Boca Raton, FL, pp. 129–161.

Riggs, M.W., Cama, V.A., Leary Jr, H.L., Sterling, C.R., 1994. Bovine antibody against *Cryptosporidium parvum* elicits a circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistent cryptosporidiosis in SCID mice. Infection and Immunity 62, 1927–1939.

Riggs, M.W., Schaefer, D.A., Kapil, S.J., Barley-Maloney, L., Perryman, L.E., 2002. Efficacy of monoclonal antibodies against defined antigens for passive immunotherapy of chronic gastrointestinal cryptosporidiosis. Antimicrobial Agents and Chemotherapy 46, 275–282.

Sagodira, S., Iochman, S., Mevelec, M.N., Dimier-Poisson, I., Bout, D., 1999. Nasal immunization of mice with *Cryptosporidium parvum* DNA induces systematic and intestinal immune responses. Parasite Immunology 21, 507–516.

Walker, J.M., 1996. The Protein Protocols Handbook. Humana Press Incorporation, New Persey. 15–20.

Wang, C.H., Hong, C.C., Seak, J.C., 2002. An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. Veterinary Microbiology 85, 333–342.