

## Original Article

# Recombinant *Cryptosporidium parvum* p23 as a Candidate Vaccine for Cryptosporidiosis

E Ebrahimzadeh, P Shayan, MR Mokhber Dezfouli, \*S Rahbari

Dept. of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran Iran

(Received 11 Oct 2008; accepted 12 Feb 2009)

## Abstract

**Background:** *Cryptosporidium parvum* is a ubiquitous protozoan, which develops within the microvillous membrane of enterocytes in a wide variety of vertebrates, including man. Cryptosporidiosis is an important parasite causing severe diseases in the immunodeficient people especially AIDS patients. Cryptosporidiosis has been also reported as a common serious primary cause of outbreaks of diarrhea in newborn calves. The aim of this study was to confirm that P23 was an immunogenic antigen in domestic isolates of *C. parvum*.

**Methods:** We isolated cryptosporidial oocysts from the naturally infected calves. The oocysts were then purified and characterized as *C. parvum* by nested PCR. To obtain the recombinant P23 protein, we isolated the mRNA from oocyst of *C. parvum*, and synthesized the cDNA. The cDNA was then amplified using specific primers for P23 gene.

**Results:** Sequencing of PCR product showed 100% homology to the known P23 sequences in GenBank. The double strand P23-cDNA was then cloned in pGEX-5X-2 expression vector and P23-recombinant protein was prepared. Western blot analysis of recombinant P23 showed that it could be recognized by the positive *C. parvum* serum. Furthermore, serum from immunized goat with the recombinant P23 protein also recognized a protein band with approximately 23 kDa in lysates prepared from the oocytes.

**Conclusion:** Since P23 is an immunodominant surface glycoprotein expressed in the early phase of infection and the immunogenic epitopes are found in its residual chain of amino acid sequence, the recombinant P23 could be recommended as a favorable candidate for vaccination against *C. parvum* infection.

**Keywords:** *Cryptosporidium parvum*, Expression vector pGEX-5X-2, recombinant protein P23, PCR, RT-PCR, Western blot, Dot Blot

## Introduction

**C**ryptosporidium *parvum* is a widely distributed coccidian parasite that causes enteric disease in humans and animals; it has been reported as a common serious primary cause of outbreaks of diarrhea in newborn and young calves (1). These animals are mostly infected with *C. parvum* between day 4 and the third week of age (2). The main clinical signs in newborn ruminants are depression, anorexia, abdominal pain, diarrhea, weight loss, retarded growth during the first weeks of life and eventually high mortality rate which resulting significant economic losses (3,4). Since immunoglobulins cannot be transmitted

through placenta, transmission of *C. parvum*-specific antibodies through the colostrum is very important for the protection of the newborns (5, 6). It is believed that the antigens CP15/P60 and P23 are able to protect the newborn ruminants against cryptosporidiosis and therefore they were recommended as suitable tools for the immunization (7-9).

The aim of this study was to confirm that P23 is an immunogenic antigen in domestic isolates of *C. parvum* and to produce the recombinant P23 and evaluate the potential effect of this protein in the immunization.

## Material and Methods

### *Collection of C. parvum oocysts and experimental infection*

During 2007, oocysts of *C. parvum* from naturally infected calves in Tehran Province were collected and purified as described by Lorenzo et al (10). To confirm that the collected oocysts belong to *Cryptosporidium* spp., DNA was extracted from oocysts using DNA extraction kit (MBST, Tehran, Iran) and amplified using two primers (F1 and R1) derived from 18S rRNA gene of *Cryptosporidium* spp. (Table 1). The PCR product was amplified with *C. parvum*-specific primers F2 and common primer R1 either directly or after isolation and purification from the agarose gel using DNA isolation from agarose gel kit (MBST). After characterization, were inoculated orally in a 1-day old *C. parvum* seronegative calf. *C. parvum* oocysts were then obtained from the feces of the experimentally infected calf during 5 to 11 days post inoculation. The isolated oocysts were treated in 10% sodium hypochlorite and subsequently washed three times in distilled water and stored at 4°C until use. Sera before and after inoculations were collected from the calf, stored at -20°C and used as negative and positive controls.

### *P23-cDNA cloning*

Total RNA was isolated from oocyst by RNA isolation kit (MBST). One microgram of total RNA was used for cDNA synthesis. For cDNA synthesis, 2 µl M-MULV enzyme (40 µM, Roche), 1 µl oligo d (T)<sub>15</sub> primer (Roche), 1 µl RNase free dNTP (20 µM, Gibco BRL), and 5 µl 5× buffer in an end volume of 25 µl were used. The cDNA synthesis was performed at 37°C for 1 h. The amplification of cDNA was performed using primers derived from the nucleotide sequence at the position 16 to 39 (F3) and from 340 to 357 (R2) of P23 mRNA sequences (Table 1). The nucleotide sequences of *Bam*HI restriction site were added to the 5' ends of primers (Table 1). After sequencing of RT-PCR product, P23 double strand cDNA was cloned in

the multicloning site (*Bam*HI site) of dephosphorylated pGEX-5X-2 using rapid DNA ligation kit (Roche, Germany). The recombinant P23 cDNA-pGEX-5X-2 was then transferred to the competent *Escherichia coli* BL21. The *E. coli* clones were separately grown and the plasmids were isolated using Plasmid Isolation Kit (MBST, Iran). The presence of insert DNA in pGEX-5X-2 was controlled using primers F4 and R3 (F4 and R3 were derived from the flanking region of multicloning site in vector) and the direction of P23 cDNA in vector DNA was determined using primers F3 and R3 (Table 1). Additionally, the nucleotide sequences of the insert cDNA was determined and compared to the known sequences in GenBank.

### *Preparation of positive serum against C. parvum*

Serum from a new borne uninfected calf was collected as a negative control. The calf was then inoculated orally with  $5 \times 10^6$  *C. parvum* oocysts. After three weeks, serum was collected and used as a positive control. To obtain, serum against recombinant P23, goat was inoculated sub cutaneously (s.c.) with recombinant P23. Briefly, 120 µg recombinant P23 protein in 500 µl PBS was mixed thoroughly with 500 µl complete Freund's adjuvant. The prepared emulsion was then inoculated s.c. to the goat. Three weeks after the first immunization, 60 µg protein in 500 µl PBS mixed with 500 µl incomplete Freund's adjuvant was inoculated s.c. to the goat and one week later, serum was collected from the goat and used as a positive control. Serum from naturally infected calves was also used as positive control.

### *SDS-PAGE, Western blot and DOT blot*

*C. parvum* oocysts were lysed in PBS/PMSF (1 mM) buffer by freezing, defreezing, and sonication (amplitude 70%, 0.5 cycles, Dr. Hielscher GmbH, Germany). The debris was removed by centrifugation at 12,000 ×g at 4°C for 20 min. Supernatant was collected and used for SDS-PAGE and Western blotting(10). 500 microliters of overnight-grown

transfected *E. coli* was incubated in 10 ml LB medium containing 1 mM IPTG and 10 µl ampicillin (100 mg/ml) for 3 h under shaking condition. Recombinant P23 protein was then extracted using Microspin GST purification Module kit and Factor Xa (Amersham, USA) according to the manufacturer's instruction. Briefly, the fusion protein was first bound to the carrier placed in the Microspin GST-column and the cell debris and other undesired contaminants were then washed from the column using PBS buffer. Finally, the P23 protein was cut from the GST using factor Xa and collected in a sterile tube. The oocyst lysate, the P23-GST fusion protein, and recombinant P23 were separated in 12% SDS-PAGE. The protein bands were either stained with Coomassie brilliant blue solution (0.1 g Coomassie brilliant blue in 50% H<sub>2</sub>O/40% methanol/10% acetic acid) or transferred to the nitrocellulose membrane using Semi-Dry trans-Blot (BioRad, USA). To determine the immunogenic bands, free binding sites on the membrane were first blocked with 3% bovine serum albumin in TBS buffer (20 mM Tris base and 0.15 M NaCl in H<sub>2</sub>O) containing 0.05% Tween 20 for 1 h at room temperature (RT). Subsequently, the membrane was incubated in a positive serum (1:200 dilution) obtained from serum collected from infected calf with *C. parvum* and with positive serum against recombinant P23 protein obtained from immunized goat, for 1 h at room temperature (RT). The membrane was then washed three times with TBS containing 0.05% Tween 20 for 5 min at RT. Horseradish-conjugated rabbit anti-bovine Ig (Dako, Denmark) (1:1,000) were added to the washed membrane and incubated for 1 h at RT. After incubation, the membrane was washed three times as described above. The positive reaction was developed using DAB (Sigma, USA) as substrate under visual observation within 5 min. In all experiments, serum from newborn calf before first feeding with colostrum was used as negative serum control. Optimized serum dilution (1:200) was then selected for the test sera. Dot blot analysis of re-

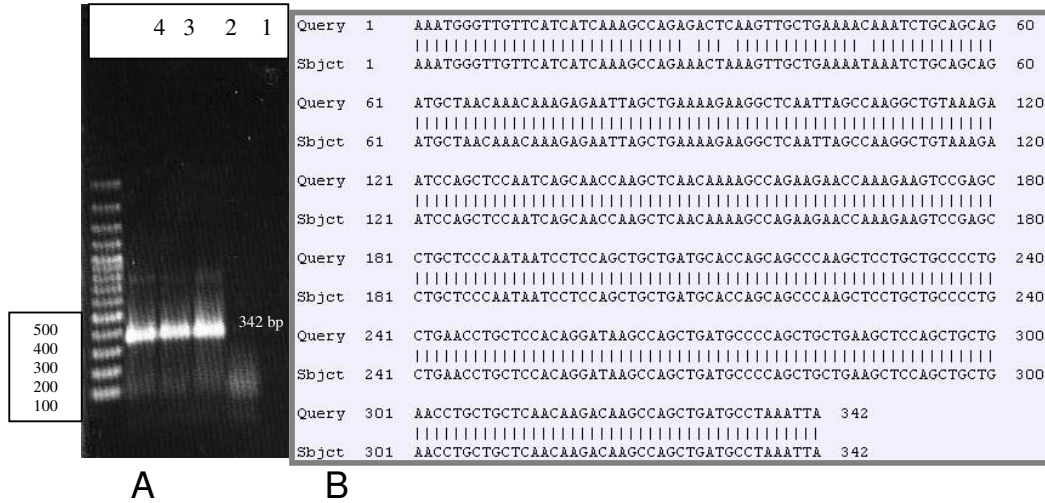
combinant P23 with positive serum against *C. parvum* was performed (11).

## Results

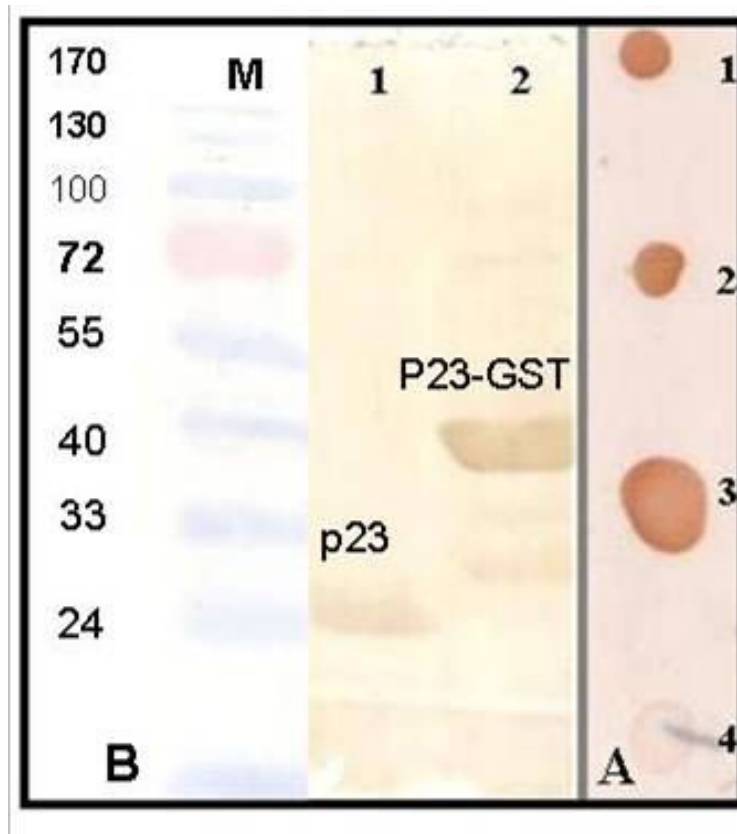
The genomic DNA from oocysts was first amplified with the primer pair (F1/R1) specific for cryptosporidial agents, resulting in an expected PCR product of 412 bp in length. The amplicate was then amplified with the primer pair (F2/R1) specific for *C. parvum* resulting in the expected PCR product of 354 bp in length. Therefore, the purified oocytes from naturally infected calf were characterized as *C. parvum*. To obtain sufficient material for the SDS-PAGE and western blot analysis, a total volume of 200 µl of oocytes in a concentration of the 5 x 10<sup>7</sup>/ml oocysts were collected from experimentally infected calf. SDS-PAGE analysis of lysate from purified oocysts showed beside other protein bands, a protein band with the approximately 23 kDa (Fig 4A). The western blot analysis revealed that the 23 kDa protein band was also recognized with the serum collected from *C. parvum* infected calf and the serum from immunized goat with the recombinant P23 protein (Fig 2B and Fig. 3).

To obtain the recombinant P23 protein, we isolated the mRNA from oocyst of *C. parvum* and amplified the cDNA of P23 gene by reverse transcriptase PCR. The amplicate with 342 bp in length (Fig.1A) was then sequenced. The Sequencing of the P23 cDNA showed 100% homology to the known P23 sequences registered in the GenBank (Fig.1B).

The double strand P23-cDNA was then cloned in pGEX-5X-2 expression vector and the recombinant plasmid was transfected in *E. coli*. Induction of transfected *E. coli* revealed GST-P23 fusion protein from which P23 could be isolated (Fig 4B). Dot blot analysis of recombinant P23 with positive serum against *C. parvum* showed positive reaction (Fig. 2A). Western blot analysis of recombinant P23 showed it could be recognized by the positive *C. parvum* serum (Fig. 2B).



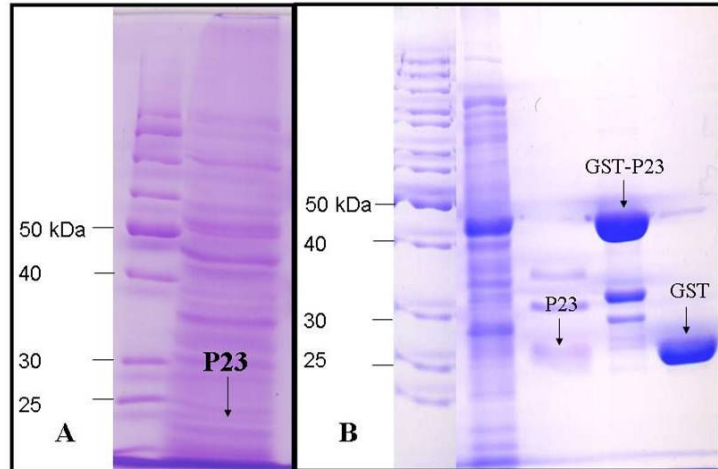
**Fig 1.** A: RT-PCR product of cDNA with F3 and R2 primers. Lane1 is negative control, lanes 2, 3 and 4 are RT-PCR with P23- cDNA samples. B: sequencing of RT-PCR product and comparison with Gene bank



**Fig. 2 :** A: recombinant P23 (No 1= 0.1 µg, No 2 = 0.2 µg, No 3 = 0.4 µg) and negative antigen (No 4) were analyzed by dot blot using the positive serum against *Cryptosporidium parvum*. B: recombinant P23 (lane 1) and fusion protein GST-P23 (lane 2) were screened with the positive serum against *Cryptosporidium parvum*. M is prestained protein marker



**Fig. 3 :** Western blot analysis of lysates prepared from oocysts of *Cryptosporidium parvum* with p23-*C.parvum* positive goat serum



**Fig. 4:** SDS-PAGE analysis of lysate from oocyst of *C. parvum* (A) and from lysate of recombinant *E. coli*, extracted P23, GST-P23 fusion protein and GST (B)

**Table1:** Primers used in this study were derived from P23 mRNA and pGEX-5X-2 sequences

primer	Accession No.	gene	sequence	Melting temperature
F1	AF093489, AF093496, AF112575, AF112574, AF093495, AF112573	18 S rRNA genes	5` aagctcgtagttggatttctg 3`	60
R1	AF093489	18 S rRNA genes	5` taaggaacaacctccaatctc 3`	60
F2	AF093489, AF093496, AF112575, AF112574, AF093495, AF112573	18 S rRNA genes	5` catattactatttttttttag 3`	52
F3	Y16243,VersionY16243, 1	P23	5'acggatccaaattgggttgcacatcaaaagc3'	82.5
R2	Y16243,VersionY16243, 1	P23	5'acggatcctaatttaggcacagctg3'	76
F4	Amersham biosciences	pGEX-5X-2	5'gcattggcctttgcagggtgg3'	70
R3	Amersham biosciences	pGEX-5X-2	5'cgaaacgcgcgaggcagatc3'	66

## Discussion

*C. parvum* is a coccidian parasite that infects microvillous membrane of enterocytes of newborn and young calves causing severe diseases accompanied with diarrhea, dehydration and weight loss. The role of humoral immunity in development of resistance is not known, although specific antibody against *C. parvum*, has been demonstrated in the sera of infected normal and immunodeficient individuals. It is known that the immunized cattle can protect calves through colostrum (8). The protective immunity could be achieved using different antigens such as whole oocyst antigens (12), P23 (13), rC7(8) and CP15/60 (14). Since P23 involved in the infection process, it is almost expressed in early and late stages of infection and conserved on diverse *C. parvum* isolates and also can induce protective immunity (8,13,15), thus it could be considered as a suitable candidate for protection. The analysis of the P23-PCR product revealed a 100% homology to the p23 mRNAs published in GenBank. This sequence similarity suggests that the P23 protein may belongs to the conserved proteins of all *C. parvum* world wide. Western blot analysis of induced P23-pGEX-5X-2 transfected *E. coli* BL2 compared to the induced pGEX-5X-2 transfected *E. coli* BL2 showed that the fusion protein GST-P23 as well as purified recombinant P23 could be detected with the *Cryptosporidium parvum* positive serum (Fig.2B). It is very important to use the pure P23 for further analysis, since *C. parvum* positive serum can also react with GST, which may be expressed by so many parasites infecting calves. It can be concluded that this protein is an important antigen that stimulates host immunoresponses, resulting in a specific antibody production under natural infection.

There are a lot of works, which confirm the use of recombinant proteins as vaccines against causative agents of human and animal diseases. Perryman used recombinant P23 for the immunizing of the pregnant cattle to protect the calves against cryptosporidiosis with immune bovine colostrums (8). Taka-

shima have also emphasized the *C. parvum* P23 as a potential vaccine for prevention(16). Shayan showed that recombinant *C. parvum* P23 was an important target to screen the serum of pregnant dams for detection of cryptosporidium specific antibody and in their calf sera. They revealed that 67% newborn calves was negative in antibody screening test sera(11).

Therefore, we concluded that colostrums enriched with polyclonal specific antibody, could be the substitute solution to achieve a sufficient protection of calf against cryptosporidiosis.

## Acknowledgements

We acknowledge Iran National Science Foundation for financial support of Grant no. 84112/31 and Investigating Unit "Molecular Biological System Transfer" (MBST) for technical supports. The authors declare that there is no conflict of interests.

## References

1. Wyatt C. *Cryptosporidium parvum* and mucosal immunity in neonatal cattle. *Anim Health Res Rev.* 2000;1:25-34.
2. Pohlenz J, Moon H, Cheville N, Bemrick W. Cryptosporidiosis as a probable factor in neonatal diarrhea of calves. *J Am Vet Med Assoc.* 1978;172:452-7.
3. Fayer R, Speer C, Dubey J. The general biology of *Cryptosporidium*: CRC Press., 1997.
4. de Graaf D, Vanopdenbosch E, Ortega-Mora L, Abbassi H, Peeters J. A review of the importance of cryptosporidiosis in farm animals. *Int J Parasitol.* 1999;29:1269-87.
5. Tennant B, Harrold D, Reina-Guerra M, Laben R. Neonatal alterations in serum gamma globulin levels of Jersey and Holstein-Friesian calves. *Am J Vet Res.* 1969;30:345-54.

6. Weaver D, Tyler J, VanMetre D, Hostetler D, Barrington G. Passive Transfer of Colostral Immunoglobulins in Calves. *J Vet Internal Med.* 2000;14:569-77.
7. Reperant J, Naciri M, Iochmann S, Tilley M, Bout D. Major antigens of *Cryptosporidium parvum* recognised by serum antibodies from different infected animal species and man. *Vet Parasitol.* 1994;55:1-13.
8. Perryman L, Kapil S, Jones M, Hunt E. Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. *Vaccine.* 1999;17:2142-49.
9. Wang H, Swain J, Besser T, Jasmer D, Wyatt C. Detection of antibodies to a recombinant *Cryptosporidium parvum* p23 in serum and feces from neonatal calves. *J Parasitol.* 2003;89:918-23.
10. Lorenzo-Lorenzo M, Ares-Mazas E, Villacorta Martinez de Maturana I. Detection of oocysts and IgG antibodies to *Cryptosporidium parvum* in asymptomatic adult cattle. *Vet Parasitol.* 1993;47:9-15.
11. Shayan P, Ebrahimzadeh E, Mokhber-Dezfouli M, Rahbari S. Recombinant *Cryptosporidium parvum* p23 as a target for the detection of *Cryptosporidium*-specific antibody in calf sera. *Parasitol Res.* 2008;103:1207-11.
12. HARP J, GOFF J. Protection of calves with a vaccine against *Cryptosporidium parvum*. *J Parasitol.* 1995;81:54-57.
13. Shirafuji H, Xuan X, Kimata I, Takashima Y, Fukumoto S, Otsuka H, Nagasawa H, Suzuki H. Expression of P23 of *Cryptosporidium parvum* in *Toxoplasma gondii* and Evaluation of its Protective Effects. *J Parasitol.* 2005;91:476-79.
14. Jenkins M, Fayer R, Tilley M, Upton S. Cloning and expression of a cDNA encoding epitopes shared by 15- and 60-kilodalton proteins of *Cryptosporidium parvum* sporozoites. *Infect Immunol.* 1993;61:2377-82.
15. Arrowood M, Mead J, Mahrt J, Sterling C. Effects of immune colostrum and orally administered antiparasite monoclonal antibodies on the outcome of *Cryptosporidium parvum* infections in neonatal mice. *Infect Immunol.* 1989;57:2283-88.
16. Takashima Y, Xuan X, Kimata I, Iseki M, Kodama Y, Nagane N, Nagasawa H, Matsumoto Y, Mikami T, Otsuka H. Recombinant bovine herpesvirus-1 expressing p23 protein of *Cryptosporidium parvum* induces neutralizing antibodies in rabbits. *J Parasitol.* 2003;89:276-82.