Iranian J Parasitol

Open access Journal at http:// ijpa.tums.ac.ir

Tehran University of Medical Sciences Publication http:// tums.ac.ir

Iranian Society of Parasitology http:// isp.tums.ac.ir

Original Article

Specific Egg Yolk Antibody against Recombinant *Cryptosporidium parvum* P23 Protein

P Shahbazi, P Shayan*, E Ebrahimzadeh, S Rahbari

Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran

(Received 6 Apr 2009; accepted 25 Jun 2009)

Abstract

Background: *Cryptosporidium parvum* is a parasitic protozoan that functions as important causative agent of diarrhea in human and animals. The host's immune response to surface antigens of *C. parvum* has been previously demonstrated. In this respect, the role of humoral immunity in the development of host protective immunity against this protozoon has been well demonstrated.

Methods: The effect of specific chicken egg yolk antibody (IgY) against recombinant *C. parvum* P23 was examined. IgY sample was prepared from eggs of chickens immunized with recombinant *C. parvum* protein p23 and analyzed with *C. parvum* lysate and recombinant P23.

Results: The anti P23 specific IgY was recognized a protein band with approximately 23 kDa in lysates prepared from the *C. parvum* oocysts. Also dot blot analysis of recombinant P23 showed that it could be recognized by the anti P23 specific IgY up to 1/1000 dilution of antibody. But the best antibody dilution for immunological studies was determined as 1:200.

Conclusion: Since P23 is an immunodominant surface glycoprotein expressed in the early phase of infection, specific IgY against recombinant p23 could be recommended as a favorable candidate for passive immunization against *C. parvum* infection in human and animals.

Keywords: Cryptosporidium parvum*, IgY; PCR, Recombinant protein p23, Western Blot*

* **Corresponding Author**: E-mail: pshayan@ut.ac.ir

Introduction

Cryptosporidium parvum causing cryptosporidiosis is a coccidian parasite that infects intestinal epithelium in a wide variety of mammalian including human.

Millions of oocysts of *C. parvum* can be released from infected human and animal into the environment and can contaminate soil, food, water or surfaces contaminating with the feces harboring oocysts. Due to this, *C. parvum* is a significant health care problem because no practical disinfectant is available for destroying the parasite and due to its small size the oocysts are difficult to remove from water using standard filtration procedures. The oocysts can also remain viable for several months under a wide range of environmental temperatures (1-5).

The disease causes a great economical loss in cattle industry and serves as one of the major causes of diarrhea in neonatal calves, lambs and goat kids (4-8). *Cryptosporidium* is rarely lethal but in immunocompromised patients such as AIDS patients can lead throughout to the death of patients. It is believed that infective dose of this agent is less than 10 oocysts and most probably one organism can initiate an infection. The incubation period is up to 12 days with the average of 7 days and asymptomatic infection is common (8). Until now, there are no effective methods of treating or preventing *C. parvum* infection in animals or human (6-10). Passive immunization with bovine hyperimmune colostrum or monoclonal antibodies has been used for treatment of cryptosporidiosis in both animals and human (4, 7). Although these antibodies resulted in reduction of oocyst shedding and improvement of clinical signs, no antibody-based products have been marketed until now. The main problems for development of these antibody products are high production cost and the difficulty in achieving high and stable antibody titers. Chicken egg has been known as a cheap and convenient source for mass production of specific antibody (11). Egg yolk antibody, or IgY, has been used extensively for treatment and prevention of various infections in animals and human with mixed successes (3).

The aim of this study was to produce specific IgY against recombinant p23 that recognized a protein band with approximately 23kDa in lysates prepared from the *C. parvum* oocysts.

Materials and Methods

Preparation of recombinant protein P23

Formerly, the recombinant P23 cDNA-pGEX- 5X-2 was cloned in the Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran (12). In this study, the recombinant *E. coli* colons were separately grown and the plasmids were isolated using plasmid isolation kit (MBST, Tehran, Iran). The presence of insert DNA in pGEX- 5X-2 was controlled using primers F and R derived from the flanking region of multicloning site in vector. Moreover, the extracted plasmid was cut by the restriction endonucleasis BamH1 to control the size of the P23 cDNA in vector DNA. After that, this recombinant plasmid was transferred to the competent *E. coli* BL21 and incubated at 37 ºC overnight. Five hundred microliter 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 3h under shaking condition for expression of the recombinant protein . The GST – recombinant P23 protein was purified with purification module microspine GST kit and the P23 was then cleavaged from GST using factor Xa enzyme (Amersham, USA) according to manufacture's instruction. The GST- recombinant P23 protein and pure recombinant P23 protein were analyzed by

SDS- PAGE. The P23 was additionally analyzed with cryptosporidium positive serum by western blotting.

Anti P23 specific IgY production:

Twelve 28-week-old white leghorn hens (Tehran, Iran) were used. The hens were housed in two 6 hens groups in separated cages. One group was used for the immunization with P23 and the other group functioned as control group. Six microgram (137µl) of P23 was mixed with 113 µl PBS 7.2. The resulted solution was then emulsified with an equal volume (250µl) of Freund's complete adjuvant and injected into pectoral muscle of each chicken from test group.

The hens were boosted two weeks after the first immunization with the second injection composing of 250 µl P23 (6µg) in PBS 7.2 + 250 µl incomplete Freund's adjuvant. In control group same injections were done with PBS instead of P23 antigen. The hens were boosted again 2 days after the second immunization with the same condition as the second immunization. Finally, the eggs were harvested 7 days after the third immunization (13).

Purification of IgY from egg yolk

Egg yolks were separated from egg whites, washed with distilled water to remove as much albumen as possible, and rolled on paper towels to remove adhering egg white (14).

Based on Tini *et al.* (2002) the yolk was brought to 25 ml with sodium phosphate buffer (100 mM, pH 7.2) and vigorously mixed. Subsequently, chloroform (20 ml) was added and the mixed. The mixture was shaken until a semisolid phase was obtained. After centrifugation at 1200×g for 30 min, the supernatant was decanted and solid polyethylene glycol 6000 was added to a final concentration of 12% (w/v). Following centrifugation at 15700×g for 10 min, the pellet containing the antibody was resuspended in 2 ml of sodium phosphate buffer and examined in SDS-PAGE. The purified IgY concentration in egg yolk determined by spectrophotometer (Biorad,USA) and Bradford method. Finally, the IgY antibodies were stored at -20ºC until use (15).

Immunization of rabbits with chicken IgY

Two 4-6 month old rabbits (Razi Institute, Karaj, Iran) were immunized intramuscularly with 0.5ml purified IgY, 2 times with 2- weeks interval. The first immunization was performed using emulsion of 0.5 ml purified IgY and 0.5 ml complete Freund's adjuvant and the second immunization was performed with emulsion of 0.5ml purified IgY and 0.5ml incomplete Freund's adjuvant (16). Two days after the second immunization, serum was prepared from the immunized rabbits and stored at -20 ºC until used.

Evaluation of rabbit anti IgY

One micro liter of IgY, chicken's serum, goat serum and bovine serum were dotted on the one corner of each 1x 1 cm square nitrocellulose membrane. As negative control, purified *Theileria* sp. antigen was used and dotted on other site of the square diagonal to the first dots. Serum from the immunized rabbit with IgY was used in 1:200 dilutions for screening. Briefly, the dot blotted nitrocellulose membranes were incubated in 3% skim milk in TBS buffer (20 mM Tris base and 0.15 M NaCl in H_2O) containing 0.05% Tween 20 for 1h at RT to block the free binding sites on the membranes. Subsequently, the membrane was incubated in the prepared rabbit anti IgY (1/200 in TBS containing 0.05% Tween 20) for 1h at room temperature (RT). The membrane was then washed three times with TBS containing 0.05% Tween 20 for 5 min at RT. After that horseradish-conjugated goat anti-rabbit Ig (Dako, Denmark) (1:2000) were added to the washed membrane and incu-

bated for 1h 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.

Evaluation of hen anti P23 IgY

One micro liter of recombinant P23 was doted on one corner of a 1×1 cm square nitrocellulose membrane .As negative control purified *Theileria* sp. was used. After that the free binding sites on the membrane were blocked with 3% skim milk in TBS buffer (20 mM Tris base and 0.15 M NaCl in H2O) containing 0.05% Tween 20 for 1h at RT. Subsequently, the membrane was incubated in a corresponding diluted hen anti P23 IgY (1/100, 1/200, 1/500, 1/800, 1/1000, 1/10000 in TBS containing 0.05% Tween 20) for 1h at RT. The membrane was then washed three times with TBS containing 0.05% Tween 20 for 5 min at RT. Then membrane was incubated in diluted rabbit anti IgY (dilution 1/200 in TBS containing 0.05% Tween 20) for 1h at RT. The membrane was then washed three times with TBS containing 0.05% Tween 20 for 5 min at RT. After that horseradish-conjugated goat anti-rabbit Ig (Dako, Denmark) (1:2000) were added to the washed membrane and incubated for 1h 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 IgY from chickens before first injection and IgY from control group was used as negative control.

SDS-PAGE and Western blot analysis

C. parvum oocysts were lysed in PBS/PMSF (1mM) buffer by freeze and defreeze and sonication (amplitude 70%, 0.5 cycles, Dr. Hielscher GmbH, Germany). The debris was removed by centrifugation at 12,000 ×g at 4º C for 20 min. Supernatant was collected and used for SDS-PAGE and Western blotting (Lorenzo et al.1993). The prepared lysate from oocysts, 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 (0.1g Coomassie brilliant blue in 50% H2O/40% methanol/10% acetic acid) or transferred to the nitrocellulose membrane using Semi Dry trans-blotter (BioRad, USA). The western blot analysis was performed with 1:200 diluted anti P23 IgY as described above. In all experiments IgY from chickens before first injection and IgY from control group was used as negative control.

Results

PCR analysis of recombinant P23 cDNA-pGEX- 5X-2 with the primers F/R resulted in the expected PCR product of 495bp. BamH I restriction endonuclease analysis of recombinant P23 cDNA-pGEX- 5X-2 resulted in the expected DNA fragment of 342bp (P23 cDNA) in length (Fig. 1D). After induction of *E. coli* transfected with recombinant P23 cDNA-pGEX- 5X-2, the GST-recombinant P23 and recombinant P23 were isolated and analyzed on SDS-PAGE. The SDS-PAGE analysis showed the expected 43 kDa protein band for GST-P23 (Fig. 1B) and a 23kDa protein band for recombinant P23 (Fig. 1C). Fig. 1A showed the SDS-PAGE analysis of lysate prepared from *E. coli* transfected with recombinant P23 cDNA-pGEX- 5X-2 without induction.

After purifying of IgY from egg yolk, the IgY concentration in egg yolk determined by spectrophotometer (Biorad, USA) and Bradford method. The analysis showed 9.7 mg IgY in ml of egg yolk. Other investigators have determined the amount of specific IgY within egg yolk as 2 to 10 % (17, 18). The SDS-PAGE analysis of purified IgY under

reducing conditions (with 5% ß-mercaptoethanol) showed two bands of 67 and 25 kDa (Fig. 2B). Fig. 2A showed the SDS-PAGE analysis of crude yolk extract. Since horseradish-conjugated anti IgY was not available for Dot blot and western blot analysis, rabbit anti IgY was first prepared using isolated IgY to detect the P23 positive bands with horseradish-conjugated anti rabbit Ig in sandwich procedure. For this aim the rabbit anti IgY was first evaluated with serum from hen, goat and bovine, to ensure that the antibody can recognize specific the hen immunoglobulin. Fig. 3 part A, showed that the serum prepared from rabbit immunized with hen IgY could recognize the IgY and serum collected from hen (Fig. 3 a and b) and could not recognize the serum of goat and bovine (Fig. 3 c and d), which was analyzed by dot blot. The results showed that the serum from immunized rabbit can be used for further analysis. The IgY from egg yolk of immunized hens was prepared and analyzed for presence of specific antibody against P23. For this aim, different dilutions of IgY solution were used. Also dot blot analysis of recombinant P23 showed that it could be recognized by the anti P23 specific IgY up to 1/1000 dilution of antibody (Fig. 3B row 1 e) . But the best antibody dilution for immunological studies was determined as 1:200 (Fig. 3B row 1 b), which was decisive for the choosing this dilution for further analysis. Fig. 3B row 2 showed the positive and negative control. To ensure that the purified IgY can recognize the P23, lysate prepared from oocysts of *C. parvum* and recombinant P23 was analyzed by western blotting. Western blot analysis showed a 23 kDa protein band by both probes, which denote the presence of anti P23 specific IgY in isolated IgY from egg yolk collected from hens immunized with recombinant P23 (Fig. 3C).

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

Primer	Accesion No.	gene	sequence	Melting temperature
F	U13857	pGEX-5X-2	5′gcatggcctttgcagggctgg3′	70
R	U13857	pGEX-5X-2	5′cgaaacgcgcgaggcagatc3′	66

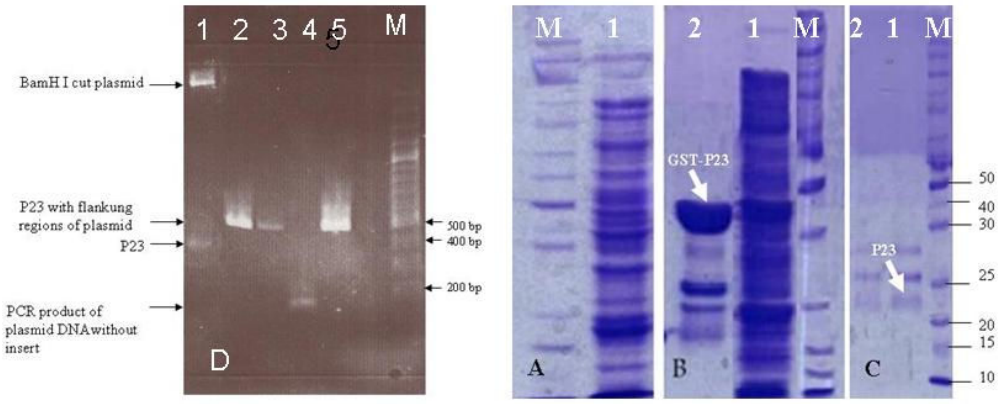

Fig. 1:

A: SDS-PAGE analysis of E. coli BL21 before expression of recombinant P23 (lane 1).
B: SDS-PAGE analysis of E. coli BL21 after expression of recombinant P23 (lane 1), GST-recombinant P23 (lane 2).
C: SDS-PAGE analysis of extracted P23 (lane 1 and 2).
M: unstained protein marker.
D: recombinant pGEX-5X-2-P23 was analyzed using restriction endonucleasis BamH I (lane 1), PCR fragments with primers F and R (lane 2 and 3). Lane 4 amplification of plasmid DNA without insert with primers F and R, Lane 5 positive control for recombinant pGEX-5X-2-p23. M is 100 bp marker.

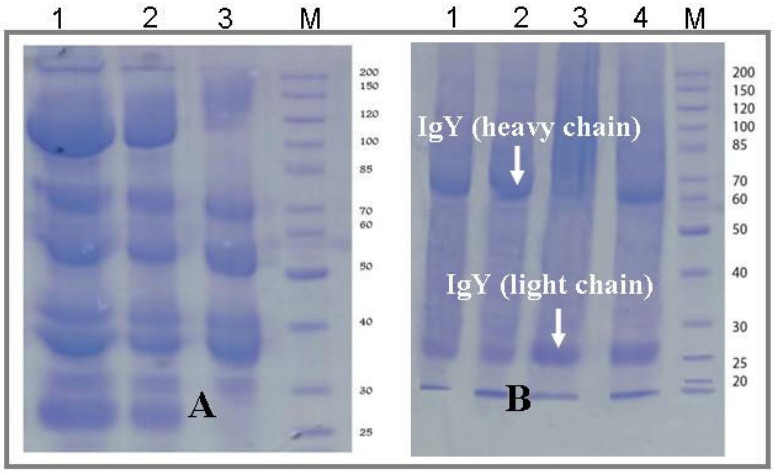

Fig. 2: A: Crude yolk extract (lanes 1, 2 and 3), **B:** reduced IgY extracted by chloroform plus PEG6000 (lanes 1, 2, 3 and 4). M: unstained protein marker

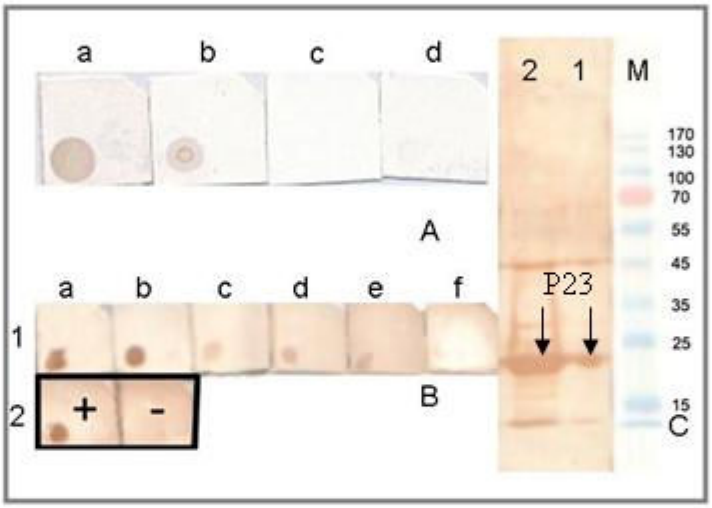

Fig. 3:

A: Purified IgY, serum of chicken, goat and cattle was dotted on the lower left site of nitrocellulose membrane a, b, c and d respectively. As negative control *Theileria* sp. antigen was dotted at the upper right side of the corresponding membranes. The membranes was analyzed using rabbit anti IgY

B: P23 was dotted on the lower left site of nitrocellulose membrane a, b, c and d respectively. As negative control *Theileria* sp. antigen was dotted at the upper right site of the corresponding membranes. The membranes was analyzed with different LgY dilutions against P23 (lane 1a: 1/100, b: 1/200, c: 1/500, d: 1/800, e: 1/1000, f: 1/10000). Lane 2 presents positive control analyzed with positive cattle serum against *Cryptosporidium parvum* and negative control analyzed with chicken serum before immunization with P23

C: Westren blot analysis of *Cryptosporidium parvum* oocysts lysate (lane 2) and P23 (lane 1) using anti-P23 specific IgY. M: prestained protein marker

Discussion

Passive immunization by specific antibodies has been an attractive approach against gastrointestinal (GI) pathogens including *C. parvum*, in both human and animals (1). Different studies have demonstrated that treatment with bovine colostrom, hyperimmune sera, and monoclonal antibodies provides protection against the cryptosporidiosis in mice, calves, lambs and humans (19-23).

Given the fact that no therapeutic products are available for cryptosporidiosis, chicken egg yolk is a cheap and convenient source for mass production of specific antibodies (24-26). Egg yolk contains 100-150 mg of IgY per yolk that 2-10% of it is specific IgY (17, 18). Oral administration of IgY has proved successfully for treatment of a variety of GI infections (25).

Kobayashi *et al.* (2004) have reported that the specific IgY against *C. parvum* whole oocyst antigens, caused morphological changes and damages of cryptosporidium sporozoites (1).

The protective immunity against cryptosporidiosis could be achieved using different antigens such as whole oocyst antigens (27), P23 (28-30), RC7 (31) and CP15/60 (32). The immune bovine colostrums induced by single *C. parvum* recombinant protein could reduce 99.8% of oocyst excretion in experimental calves (31). Since the surface protein P23 demonstrated to be involved in the infection process, its expression was determined in early and late stages of infection on sporozoites, and merozoites, P23 can play an important role in the infection. Indeed, it could be shown that P23 is expressed on diverse *C. parvum* isolates and also can induce protective immunity (14, 20, 21, 28-30). In this study, the specific IgY against recombinant P23 was produced in hens and examined. The anti P23 IgY could recognize the P23 in lysate prepared from *C. parvum* oocysts as well as recombinant P23. Interestingly, the IgY could recognize the P23 in 1:1000 dilution. We could isolate 9.7 mg IgY/ml yolk. In comparison with the other published data about the concentration of IgY in yolk (100-150 mg), we could obtain 194 mg per yolk. The difference between the amounts of IgY concentration in our study compared to the others, is probably due to the used modified PEG/chloroform procedure used in our study. It is known that an egg has approximately 20 ml yolk and approximately 2-10% of this belong to the antigen with which the hen is immunized (17, 18), therefore it is easy to produce enough specific IgY against antigens such as P23 in eggs. Since a protective immunity could be achieved with the antibody against P23 and the production of such antibodies is easy to perform and additionally chicken egg yolk is a cheap and convenient source for mass production of specific antibodies, the use of yolk IgY against recombinant P23 could be a suitable candidate for passive immunization against the cryptosporidiosis in human and animals.

Acknowledgments

We acknowledge Iranian national science foundation for financial support of Grant no. 84112/31 and financial support of Immunopathology central excellence of University of Tehran. We thank also Investigating Unit “Molecular Biological System Transfer (MBST)" for technical supports. We wish to express our special thanks to Dr. Nahrevanian in Institute of Pasteur in Iran and Poultry Section of Aminabad Research Center of University of Tehran and Narges Amini for technical support. The authors declare that they have no conflicts of interest.

References

1. Lorenzo MJ, 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.
2. Wyatt CR. Cryptosporidium parvum and mucosal immunity in neonatal cattle. Anim Health Res Rev. 2000; 1:25–34.
3. Kobayashi C, Yokoyama H, Neguyen S A, Kodama Y, Kimata T, Izeki M. Effect of egg yolk antibody on experimental *cryptosporidium parvum* infection in Scid mice. Vaccine. 2004; 23:232-5.
4. de Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE. A review of the importance of cryptosporidiosis in farm animals. Int J Parasitol. 1999; 29:1269– 87.
5. Harp JA, Goff JP. Protection of calves with a vaccine against *Cryptosporidium parvum*. J Parasitol. 1995; 81(1):54–7.
6. Jenkins MC, O‘Brien C, Trout J, Guidry A, Fayer R. Hyperimmune bovine colostrum specific for recom-

binant *Cryptosporidium parvum* antigen confers partial protection against cryptosporidiosis in immunosuppressed adult mice. Vaccine. 1999; 17:2453- 60.
7. Xiao L, Fayer R, Ryan U, Upton SJ. Cryptosporidium Taxonomy: Recent Advances and Implication for public health. Clin Microbial Rev. 2004; 17(1):72-97.
8. Fayer R . Cryptosporidium and cryptosporidiosis. 1st ed. CRC: NewYork; 1997.
9. Pohlenz J, Moon HW, Cheville NF, Bemrick WJ. Cryptosporidiosis as a probable factor in neonatal diarrhea of calves. J Am Vet Med Assoc. 1978; 172:452–7.
10. Perryman LE, Kapil SJ, Jones ML, Hunt EL. Protection of calves against cryptosporidiosis with immune bovine colostrums induced by a *Cryptosporidium parvum* recombinant protein. Vaccine. 1999; 17(17):2142–9.
11. Kuroki M. Oral passive immunization using chicken egg yolk Immunoglobulin against bovine rotavirus and coronavirus infection. Recent Res Dev Virol. 1999; 1:95-106.
12. 12. Shayan P, Ebrahimzade E. Recombinant *Cryptosporidium parvum* P23 as a target for the detection of cryptosporidium-specific antibody in calf sera. Parasitol Res. 2008; 103:1207-11.
13. Lee DY, Cho YW, Kang SG, Shin SJ, Yoo HS. Development of a novel antigen capture-ELIZA using IgY against porcine interleukin-6 and its application. J Vet Sci. 2004; 5(4):337-43.
14. Bizanov G, Jonauskiene I. Production and purification of IgY from egg yolk after immunization of hens with pig IgG. Bull Vet Inst Pulawy. 2003; 47:403-10.
15. Tini M, Jewel UR, Camendisch G, Chilov D, Cassmann M. Generation and application of chicken egg-yolk antibodies. Comparative biochemistry and physiology (CBP). 2002; 131:569-74.
16. Raj GD, Latha B, Chandrasekhar MS, Thiagarajan V. Production, characterization and application of monoclonal antibodies against chicken IgY. Vet Archive. 2004; 74:189-199.
17. Schade R, Staak C, Hendrikson C, Erhard M, Hugl H, Koch G, Larsson A, Pollmann W, van Regenmortel M, Rijke E, Spielmann H, Steinbush H, Straughan D. The production of avian (egg yolk) antibodies: IgY. ATLA. 1996; 24:925-34.
18. Gottstein B, Hemmeler E. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Z Parasitenkd. 1985; 71:273-6.
19. Nord J, Ma P, DiJohn D, Tzipori S, Tacket CO. Treatment with bovine hyperimmune colostrum of Cryptosporidial diarrhea in AIDS patients. AIDS. 1990; 4:581-4.
20. Perryman LE, Kegerries KA, Mason PH. Effect of orally administered monoclonal antibody on persistent *Cryptosporidium parvum* infection in scid mice. Infect Immun. 993; 61:4906-8.
21. Reperant JM, Naciri M, Iochmann S, Tilley M, Bout DT. Major antigens of *Cryptosporidium parvum* recognized by serum antibodies from different infected animal species and man. Vet Parasitol. 1994; 55:1–13.
22. Ebrahimzade E, Shayan P, Mokhber-Dezfouli R, Rahbari S. Recombinant P23 as a candidate vaccine for

Cryptosporidiosis. Iran J Parasitol. 2009; 4(1):6-12.

23. Riggs MW, Cama VA, Leary HL, Sterling CR. Bovine antibody against *Cryptosporidium parvum* elicites a circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistant cryptosporidiosis in Scid mice. Infect Immun. 1994; 62:1927-39.
24. Bizanov G, Normantine T, Jankauskiene I. Development of antibodies to Sendai virus in chickens and their isolation. Biologi J A. 2006; 2:68-71.
25. Mine Y, Kovacs-Nolan J. Chicken Egg yolk antibodies as therapeutics in enteric infectious disease: A review. Journal of Medicinal food. 2002; 5(3):159-169.
26. Ruan GP, Ma L, He XW, Meng MJ, Zhou MQ, Hu ZM, Zhu Y, Wang ZN. Efficient production, purification, and application of egg yolk antibodies against human HLA-A*0201 heavy chain and light chain (β2m). Prot Exp and Purific. 2005; 44:45-51.
27. Harp JA, Gogg JP. Protection of calves with a vaccine against *Cryptosporidium parvum*. J Parasitol. 1995; 81:54-7.
28. Shirafuji H, Xuan X, Kimata I, Takashima Y, Fukumoto S, Otsuka H, Nagasawa H, Suzuki H. Expression of p23 *Cryptosporidium parvum* in *Toxoplasma gondii* and evaluation of its protective effects. J Parasitol. 2005; 91(2):476–9.
29. 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(2):276–82.
30. Wang H, Swain J, Besser T, Jamser D, Wyatt C. Detection of antibodies to a recombinant *Cryptosporidium parvum* P23 in serum and feces from neonatal calves. J Parsitol. 2003; 89:918-23.
31. Perryman LE, 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.
32. 32. Jenkins MC, Fayer R, Tiley M, Upton SJ. Cloning and expression of a cDNA encoding epitopes shared by 15- and 60- kilodalton proteins of *Cryptosporidium parvum* sporozoites. Infect Immun. 1999; 61(6):2377–82.