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Original Article

Isolation of Small Number of *Cryptosporidium parvum* Oocyst Using Immunochromatography

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Abstract

Background: *Cryptosporidium parvum* causes severe gastroenteritis in immunocompromised human and new borne animals. The organism can be transmitted through water. Since small number of *C. parvum* is infectious, the aim of the present study was to develop a chromatography method for the isolation of *C. parvum* oocyst in samples with limited number of oocysts.

Methods: Antibody was prepared against whole antigen from *C. parvum* oocysts, the achieved Ab bound to the sepharose 4B and used for the isolation of oocysts. Antibody against P23 bound to the sepharose 4B, used also for the isolation of *C. parvum* oocyst. In comparison to these both methods, 2 traditional methods (Salt floatation and 55% sucrose floatation) were also performed.

Results: Both chromatography methods could bind oocysts with capacity depends on the column size. The isolated oocysts were free of bacteria. Our results showed that the traditional methods are useful for the isolation of oocysts from feces, in its smear stained with ziehl-nelsen, at least 3 oocyts are detectable in each microscopic field under 1000 X magnification. In contrast to the chromatography methods, the bacterial contamination was always observed in oocysts isolated with traditional methods.

Conclusion: Immunochromatography could be used for the successful isolation of *C. parvum* oocysts from the samples containing limited number of oocysts.

Introduction

Cryptosporidium parvum is a coccidian protozoon that causes gastrointestinal illness in immunocompromised human and newborn animals (1). Billions oocysts of *C. parvum* can be released from the infected animals and can contaminate soil, food, water or surfaces (2). The oocysts can also remain viable for several months under a wide range of environmental stresses (1, 3). Unfortunately, there are no effective methods of treating or preventing *C. parvum* infection in animals or human (1).

A large number of waterborne outbreaks of cryptosporidiosis have been reported worldwide (4, 5). Since water is the most important source of infection and the number of oocysts required for initiation of infection is relatively low, a sensitive method is required for the detection of *C. parvum* oocysts in samples (6, 7). *Cryptosporidium* spp. oocysts present in small numbers in the water sources (5). One of the methods for detection of small amount of this parasite in water sources is based on filtration and immune magnetic separation (IMS) followed by immunofluorescence assays (IFA) detection (8, 9). This method has limitation by some difficulties like need of equipment for immune fluorescence and high cost.

Manouchehri Naeini et al. and Mahmoudi et al. detected *Cryptosporidium* in recreational and surface waters using SSU rRNA-based PCR-RFLP or nested- PCR technique followed by filtering the samples through a membrane filter (10, 11).

A number of conventional methods have been described for concentrating and isolating *C. parvum* oocysts from feces. These techniques include sucrose floatation (12), salt floatation (13), percoll or ficoll gradient centrifugation (14) and discontinuous sucrose gradient centrifugation in combination with percoll gradient isolation (15). Certain works (proteomics studies or cell culture) with *Cryptosporidium* require a large number of highly purified oocysts (16). The isolated oocysts from abovementioned methods are always contaminated with bacteria that make them inappropriate for such studies. Moreover, they are not suitable for samples with small number of oocysts.

The aim of this study was to develop a chromatography column method based on the binding of rabbit anti *C. parvum* oocyst antibody to the sepharose 4B for the isolation of the oocysts from samples with limited amount of oocysts.

Materials and Methods

Collection of C. parvum oocysts and experimental infection

Fecal samples from naturally infected calves with *C. parvum* were collected; prepared smear was stained with modified Ziehl Neelsen method (17) and purified as described by Petri et al. (13). To confirm that the collected oocysts belong to *C. parvum*, DNA was extracted from oocysts using DNA extraction kit (MBST, Tehran, Iran) according to the manufacturer’s instruction. DNA was amplified using two primers (F1=5'AAGCTCGTAGTTGGATTTCTG3' and R1=5' TAAGGAACAACCTCCAATCTC 3') derived from 18SrRNA gene of *Cryptosporidium* spp. The PCR product was purified using PCR-Purification Kit (MBST, Tehran, Iran) and amplified with C. *parvum*-specific primers (F2=5' CATATTACTATTTTTTTTTTTAG 3'and R1) in a semi-nested PCR reaction. 10×10^6oocysts were inoculated orally in a 1-day old *C. parvum* sero-negative calf. *C. parvum* oocysts were then collected from the feces of the calf during the days 5 to 11 post inoculation. The isolated oocysts were treated in 10% sodium hypochlorite and subsequently washed three times in double distilled water and stored at 4°C until use. The experimental infection was per-

formed with consent given according to institutional guidelines.

Antigen preparation from oocysts of C. parvum

C. parvum oocysts were isolated by method described by Winter et al. (18) with minor changes. Briefly, 50 ml of fecal sample were diluted with 3 volumes water and centrifuged at 3000xg for 10 min. This stage was repeated again. The pellet was suspended in 3 volumes ice-cold 1% (w/v) NaHCO3 solution, adding 1 volumes ice cold ether and centrifuged at 3000xg for 10 min. The supernatant was discarded and the pellet resuspended in 3 vol. ice-cold 1% (w/v) NaHCO3. The final pellet was resuspended in 40 ml ice-cold 55% (w/v) sucrose solution and 10 ml ice-cold H_2O was layered on to the surface. After centrifugation at 3000xg for 20 min, oocysts were collected from the interface between layers. Purified oocysts were washed in PBS (pH=7.2). The isolated oocysts were passed through the membrane filter (Cellulose Nitrate Filter, pure size 3.0μm, No. of units: 100, biotech, German) (19). 1×10^6 purified *C. parvum* oocyst in 300 μl PBS (pH=7.2) buffer were autoclaved by 121ºC for 20 min. The autoclaved oocysts were sonicated at 60% amplitude, and 0.5 cycles (Dr.Hielscher GmbH, Germany).

Immunization of rabbits with whole antigen of C. parvum oocysts

Two 4-6 months old rabbits (Pasteur Institute, Karaj, Iran) were immunized subcutaneously 3 times every two weeks with whole antigen of *C. parvum*. The first immunization was performed using emulsion of 250 μl antigen (500μg/ml) in PBS (pH=7.2) and 250 μl complete Freund's adjuvant. The second and third immunizations were performed with emulsion of 250 μl antigen (500μg/ml) in PBS (pH=7.2) and 250 μl incomplete Freund's adjuvant. Two days after the third immunization, serum was prepared from the immunized rabbits and maintained at -20ºC until use.

Preparation of serum IgG

The immune globulins precipitation was performed using saturated ammonium sulphate at 4 ºC. Diluted serum was mixed with equal volume of saturated ammonium sulphate by slow addition of the ammonium sulphate solution during gentle stirring for overnight. This material was subsequently centrifuged at10000xg for 20 min, and washed twice with 50% saturated ammonium sulphate solution. The precipitate was dissolved in distilled water and dialyzed against PBS (pH= 7.5) overnight at 4 °C.

Indirect fluorescent Antibody Test (IFAT)

The oocysts were stained with prepared antibody against whole antigen of *C. parvum* by IFAT method. After isolation of *C. Parvum* oocysts from feces of experimentally infected calf, oocysts number was determined by haemocytometer under light microscope at 40X magnification. One hundred microliter of mixture containing 10^6 oocysts was centrifuged in a 1.5 ml sterile tube and the supernatant was removed. The oocyst pellet was washed twice in PBS (pH=7.5) and incubated for 45 min in diluted serum prepared from immunized rabbit (1:200) or in negative serum (1:200). Subsequently, the oocysts were washed twice with PBS (pH=7.5) and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig (Dako, Denmark) (1:2000) for 45 min. After the oocysts were washed twice in PBS (pH= 7.5), they were analysed by fluorescence microscope (Olympus, Germany).

Affinity chromatography

Seven milliliters (about 100 mg) of sepharose 4B (Sigma, England) was dissolved in 5 ml double distilled water. The sepharose beads were placed on a magnetic stirrer and the pH was titrated to 11- 11.5 with 2 M sodium hydroxide. Five milliliters of cyanogen bromide with concentration of 50 mg/ml was used to activate sepharose 4B. Activated sepharose beads were first washed with 50 ml double distilled water, and then with 50 ml

borate- saline buffer [H_3BO_3 (3.1gr), Na_2B_4O7 10 xH_2O (4.75 gr), NaCl (2.2 gr), the final volume was adjusted to 0.5 liter (pH=8.4)]. Sepharose beads were settled and supernatant was removed. Twenty milligrams rabbit anti *C. parvum* oocysts antibody in 1 ml borate- saline buffer was added to the activated sepharose 4B and incubated under stirring condition at 4°C for 4 hours. Beads were washed 3 times with borate- saline buffer followed by 2 times with PBS (pH= 7.5). One molar glycine (pH=8) was used to block the unbounded sites on the beads. Different amount of *C. parvum* oocysts (10^6, 5×10^5, 4×10^5, 3×10^5, 2×10^5, 10^5) in 1 ml borate buffer was added to 500 µl anti *C.parvum* oocyst antibody bounded sepharose 4B beads and shacked for 30 min at RT. Subsequently the solution was transferred into the column, which was prepared with a 5 ml disposable syringe its outlet was blocked with nylon wool. The sepharose column was washed with PBS (pH=7.5) until all unbound *C. parvum* oocysts were exit completely by controlling under microscopic observation. Finally, oocysts were eluted with 500 µl of 2 M NaCl in multiple steps and each elution was collected in separate microtube. Elution was continued until at least in the continuous three elutions no oocyst was observed. Alternatively, oocysts were eluted first with 2 M NaCl followed by elution with PBS (pH=7.5). All unbound and eluted *C. parvum* oocysts were counted by haemocytometer.

The p23 gene of *C. parvum* and surface protein of *Theileria annulata* (TaSp) were previously cloned in pQE-32 vector at parasitology department of University of Tehran. The recombinant p23 of *C. parvum* and recombinant TaSp were prepared and antibody against these two recombinant proteins was produced in rabbits (20, 21). As negative controls, the column prepared with sepharose 4B or with rabbit anti TaSp antibody was simultaneously used.

All steps of affinity chromatography using antibodies against these 2 recombinant proteins were carried out in parallel with antibody against *C. parvum* oocysts. For this aim, 20 mg rabbit anti P23 Ig and 20 mg rabbit anti-TaSp Ig (*Theileria annulata* surface protein, as negative control) were used.

Traditional methods

Stool samples from calf that was infected experimentally with *C. parvum* was prepared by rectal examination, mixed to the same volume of potassium dichromate 3% and transferred to the parasitology department of Tehran University. Fecal sample was washed 3 times and sieved through a series of metal meshes to remove large debris. The resulting suspension was then centrifuged at 3000xg for 10 min. The two floatation procedures for isolation of *C.parvum* oocysts were performed. For this aim, salt floatation and 55% sucrose floatation techniques were performed. In salt floatation fatty materials was removed by suspending the pellet in 1/2 (V/V) ether-water and centrifuged at 3000xg for 10 min. The resulting pellet containing the oocysts was washed three times with water and treated with 10% sodium hypochlorite for 10 min. The pellet was suspended in 10 volumes of saturated NaCl solution. The supernatant containing oocyst was removed and washed with 10 volumes water. Further washing was performed and final pellet was diluted with PBS (pH=7.5).

In addition, 55% sucrose floatation method, which was formerly described by Winter et al. (18) with minor changes, was used. Briefly, 50 ml of fecal sample were diluted with 3 volumes water and centrifuged at 3000xg for 10 min. This stage was repeated again. The pellet was resuspended in 3 volumes ice-cold 1% (w/v) $NaHCO_3$ solution, adding 1 volumes ice cold ether and centrifuging at 3000xg for 10 min. The supernatant was discarded and the pellet resuspended in 3 vol. ice-cold 1% (w/v) $NaHCO_3$. The final pellet was resuspended in 40 ml ice-cold 55% (w/v) sucrose solution and 10 ml ice-cold H_2O was layered on to the surface. After centrifugation at 3000x g for 20 min, the oocysts were collected from the inter-

face between layers. Purified oocysts were washed in PBS (pH=7.5).

Results

The IFAT results showed that the oocysts had round morphology with 4 to 6 mic diameter exhibiting an apple-green fluorescence (Fig. 1).

The rabbit anti *C. parvum* oocysts antibody was bound to the activated sepharose 4B, and then transferred into the column and used for the oocysts separation. To determine the column capacity for the binding of oocysts, different amounts of oocysts (10^6, 5×10^5, 4×10^5, 3×10^5, 2×10^5, 1×10^5) were passed through the column. All unbound and eluted oocysts were counted by haemocytometer. The capacity of column was 50000 oocysts. The isolated oocysts were pure and without contamination with bacteria, which was determined by bacteriological examinations performed in Department of Microbiology of Faculty Veterinary Medicine, University of Tehran (Fig. 2).

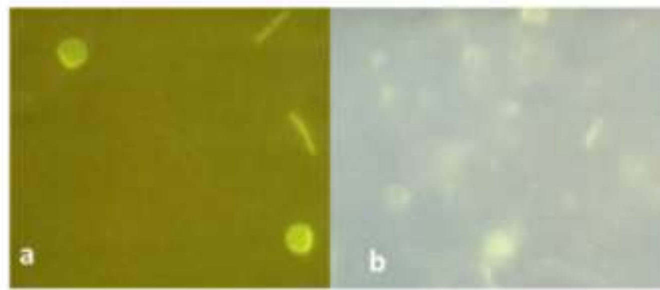

Fig. 1: a. Oocysts were analyzed with serum from rabbit immunized with whole antigen of *C. parvum* using the IFAT method. B. Negative control with serum from rabbit before immunization

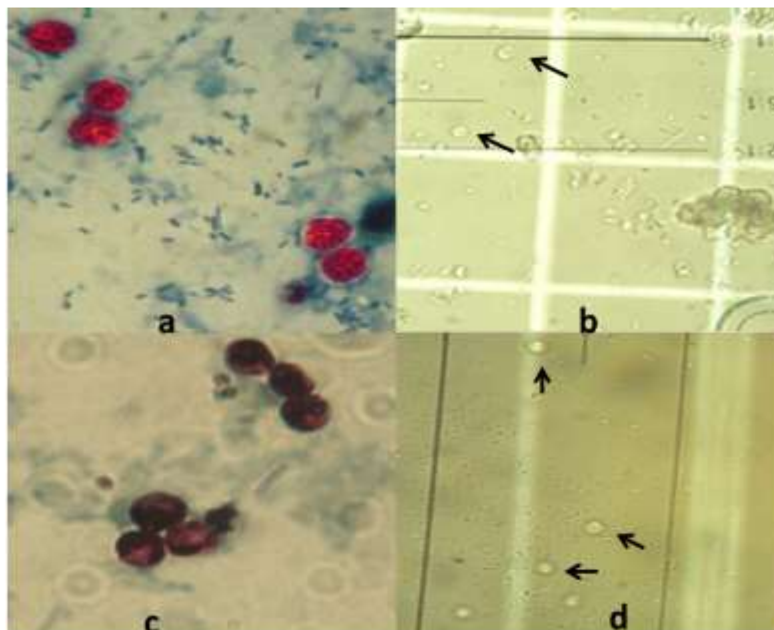

Fig. 2: a, b = Oocysts of *Cryptosporidium* before passing through chromatography column, a. Ziehl neelsen stained oocyst under 1000 x magnification. b. unstained oocysts under 40 x nagnification. C,d = Oocysts of *Cryptosporidium* after passing through chromatography column, a. Ziehl neelsen stained under 1000 x magnification b. unstained oocysts under 40 x magnification. Arrows show unstained oocysts of *Cryptosporidium*

Interestingly, the column prepared with antibody against p23 of *C. parvum* had comparable oocysts retention to the column prepared with Ab against oocysts, since the capacity of the columns prepared with Ab against p23 of *C. parvum* was approximately 40000 oocysts.

As negative controls, the column prepared with sepharose 4B or with rabbit anti TaSp (*Theileria annulata* surface protein) antibody, which was previously produced in our department, bound to the activated sepharose 4B were simultaneously used. In contrast to the results obtained with anti *C. parvum* column no retention of oocysts could be observed by columns prepared with rabbit anti TaSp bound to the activated sepharose 4B or with sepharose 4B alone.

Moreover, salt floatation and 55% sucrose flotation techniques were also used for the isolation of *C. parvum* oocysts. In the salt floatation producer, approximately 50% *C. parvum* oocysts did not float on the supernatant and retained in the pellet. Isolated *C. parvum* oocysts showed high contamination with bacteria, according to the bacteriological examinations performed by Department of Microbiology of Faculty Veterinary Medicine, University of Tehran. *C. parvum* oocysts, which were isolated by 55% sucrose flotation, showed lower contamination with bacteria in comparison to the salt floatation method.

In addition, we used 55% sucrose flotation followed by Cellulose Nitrate membrane filtration for isolation of *C. parvum* oocysts from feces. The isolated oocysts by filtration method were pure and without contamination with bacteria, but more than 70% of *C. parvum* oocysts were missed through membrane filtration.

Discussion

Cryptosporidium parvum is a zoonotic coccidian parasite, which plays an important role in the human and animal health management. One oocyst can also cause disease in immunosuppressed individual (6, 7), which speaks for the aggressiveness of this parasite. It is also known that an infected calf with *C. parvum* can shed billions of oocysts through the feces and can contaminate the soil, water, food and surface areas (2). The oocysts of *C. parvum* are very resistance with robust nature and no effective treatment can be achieved by conventional drugs or practical disinfectants (22, 23). Diagnosis of cryptosporidiosis can be performed by serological methods or by detection of oocysts in the feces of the suspected animals; the latter is more practicable by routine diagnosis. The detection of oocysts in the samples with limited number of oocysts is much more difficult.

One of the most used method for detection of *Cryptosporidium* species in samples with small number of oocysts is the immunomagnetic separation (IMS) (24- 26). The recovery efficiency of the IMS procedure for the isolation of *Cryptosporidium* oocysts was evaluated as 82.3% - 86.3% (24). This method has limitation by some difficulties like need of equipment for immunofluorescence and high cost.

In the present study, we developed an affinity chromatography method for the isolation and detection of *C. parvum* oocysts. For this aim, membrane filteration was used to achieve highly pure *C. parvum* oocyst. The isolated oocysts were pure and without contamination with bacteria, but large number (70%) of *C. parvum* oocysts were lost through the physical pore in the filter. As we showed previously (19), filtration could not isolate limited number of *C. parvum* oocysts. In another approach, antibody prepared in rabbit against the pure oocysts obtained from filtration was used for isolation of *C. parvum* oocysts in immunochromatography system. The antibody was bound to the sepharose 4B, transferred in to the column and used for isolation of oocysts. The capacity of column was determined as 50000 oocysts.

Genes encoding *C. parvum* surface antigen like p23 are involved in the invasion of oocsyst to the host cells and caused immune response (27). The p23 recombinant protein

of *C.parvum* oocyst was previously produced and analyzed at Parasitology Department of University of Tehran. Shayan et al. (28) showed that recombinant *C. parvum* P23 was an important target to screen the serum of pregnant dams and their calves for detection of *Cryptosporidium* specific antibody. Moreover, they showed that the specific IgY against recombinant P23 provides protection against cryptosporidiosis in a mouse model (21). Previously, we also developed an affinity chromatography method based on antibody against p23 protein. The capacity of prepared column was 40000 oocysts. The isolated oocysts were pure and without contamination with bacteria (21). Our results showed that the affinity chromatography based on antibody against oocysts was comparable to the affinity chromatography based on antibody against p23. Since immunochromatography methods separated oocysts specifically, the use of mentioned methods could be recommended for detection of oocysts in samples with limited amount of oocysts.

A number of methods have been described for isolating of *C. parvum* oocysts from feces. These include sucrose floatation (12), salt floatation (13, 29), percoll or ficoll gradient centrifugation (14, 30), and discontinuous sucrose gradient centrifugation in combination with percoll gradient isolation (31, 32). In this study, traditional methods such as salt and 55% sucrose floatation, which separate oocysts unspecifically, were also used. Our results showed that beside loss of high number of oocysts, the isolated oocysts were always contaminated with high amount of bacteria. Therefore, we believe that such methods are not suitable for detection of oocysts in the samples with low number of oocysts. Furthermore, due to the bacteria contamination, the isolated oocysts cannot be used for at least proteomic studies. However, Koompapong et al.(33) reported a detection system based on sucrose floatation with FA and they are the opinion that this method is efficient enough and economical compared to the IMS method.

Our results showed both immunochromatography methods could bound oocysts with capacity depend on the column size. Increase the size of column will increase its capacity.

Conclusion

The ice-cold 55% (w/v) sucrose solution followed by anti *C. parvum* oocyst antibody column-based chromatography or anti p23 antibody column-based chromatography could be recommended for isolation of *C. parvum* oocyst from bacterial contaminated samples like feces. These combination methods could give us highly pure oocysts for proteomics studies or cell culture. Furthermore, anti *C. parvum* oocyst antibody column-based chromatography or anti p23 antibody column-based chromatography could be recommended for the isolation of *C. parvum* oocysts from samples with small number of oocysts like drinking or recreational water.

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