Production of Specific Egg Yolk Antibody (IgY) against Cryptosporidium parvum Oocysts

Hossein Hashemzadeh Farhang (PhD)
Department of Pathobiology, Islamic Azad University, Tabriz Branch, Tabriz, Iran

Parisa Shahbazi (PhD)
Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Corresponding author: Parisa Shahbazi

Tel: +989143060963

Email: p-shahbazi@tabrizu.ac.ir

Address: Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

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ABSTRACT

Background and Objectives: *Cryptosporidium parvum* is a coccidian protozoan that causes diarrhea in immunocompromised humans and newborn animals. The severity of the disease depends on the immunological status of the affected. Cryptosporiosis can have lethal effects on immunocompromised individuals such as AIDS patients. About 10% of AIDS patients die following an infection with *C. parvum*. Since there is no efficient treatment for cryptosporidiosis, there is an urgent need to search for more effective and safer alternatives. IgY is an avian immunoglobulin found in egg yolks. Due to its several advantages, IgY technology has been successfully used in biomedical research on humans and animals in the recent years. In this study, the specific chicken egg yolk antibody (IgY) against *C. parvum* whole oocyst antigens was produced and examined.

Methods: The effect of specific chicken egg yolk antibody (IgY) against whole oocyst antigens was examined. IgY sample was obtained from eggs of chickens immunized with *C. parvum* whole oocyst antigens and analyzed with lysate *of C. parvum* oocysts by dot blot assay.

Results: The IgY was produced with concentration of 9.7 mg/ml. This antibody was able to recognize the whole oocyst antigens until the dilution of 1:1000, but the best dilution for other immunoassays was 1:500.

Conclusion: Since chicken egg yolk is a cheap and convenient source for mass production of specific antibodies, the use of IgY against whole oocyst antigens could be considered a suitable candidate for passive immunization against cryptosporidiosis in humans and animals.

Keywords: *Cryptosporidium parvum*, IgY, Oocysts.

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INTRODUCTION

Cryptosporidium parvum is a protozoan parasite and an important causative agent of diarrhea in humans and animals. It causes great economic losses to cattle industry and serves as a main cause of diarrhea in neonatal calves, lambs and newborn goats (1, 2). Cryptosporidiosis is one of the main causes of death in immunocompromised patients (10% of all death in AIDS patients) (3-5). In addition, *C. parvum* oocysts excreted from infected hosts pollute water and environment. The oocysts can remain viable for several months under a wide range of environmental temperatures (4). Currently, there is no effective method for treating or preventing *C. parvum* infection in animals or humans (6). Passive immunization with bovine and ovine hyper-immune colostrum or monoclonal antibodies has been used for treatment of cryptosporidiosis in both animals and humans (4, 7, 8). Although these antibodies reduce oocyst shedding and improve clinical signs, no antibody-based product has been marketed thus far. The main problems of developing these antibody products include high production cost and difficulty in achieving high and stable antibody titers. Chicken egg has been known as a cheap and convenient source for mass production of specific antibodies (9-12). Chicken antibodies offer advantages over traditional ones (e.g. rabbit antibodies) due to the evolutionary difference between these immunoglobulins. Chicken induces better antibody response against conserved mammalian antigens and does not react with rheumatoid factors, bacterial or mammalian Fc receptors , and can reduce background reactions due to cross-reactivity of anti-IgG antibodies. Egg yolk antibody (IgY) is highly stable under normal conditions. This antibody could be stored without any antibody loss for 10 years at 4°C, for 6 months at room temperature and for 1 month at 37 °C (13). Due to this advantage, IgY has been used extensively for treatment and prevention of various infections in animals and humans with mixed success (10, 14). Several methods of antibody extraction from egg yolk have been described (13), but the work of Tini *et al*. provided a method potentially superior to conventional techniques respecting purity, specificity, reproducibility and permanent availability of antibodies for use in any assay system. The aim of the present study was to produce and characterize specific IgY against *C. parvum* whole oocyst antigens.

MATERIAL AND METHODS

Oocysts were obtained from feces of a diarrheic lamb, and purified as previously described (15). They were counted using a Neubauer haematocytometer, suspended in 2.5% (w/v) aqueous potassium dichromate solution and stored at 4° C. Prior to use, the oocysts were washed with distilled water to remove potassium dichromate and then counted afresh using the Neubauer haematocytometer.

C. parvum oocysts were lysed in PBS/PMSF (1mM) buffer by freezing, thawing and sonication (amplitude 70%, 0.5 cycles, Dr. Hielscher GmbH, Germany). Debris was removed by centrifugation at 12,000 ×g at 4° C for 20 min. Supernatant obtained was collected and used for injection (16).

To prepare the specific IgY, concentration of protein (oocyst lysate) was determined by spectrophotometer (Biorad, USA) and Bradford method. Briefly, for each chicken in test groups, antigen suspension (100µl whole oocyst antigens + 150µl PBS 7.2) was emulsified with an equal volume (250µl) of ferund's complete adjuvant (Razi institute, Karaj, Iran). Then, 0.5 ml of the suspension was injected into breast muscle of 27-weak old white leghorn chickens (Tabriz, Iran). The chickens were boosted by another two injections with 250µl antigen suspension mixed in PBS 7.2 and 250µl of incomplete ferund's adjuvant (Razi institute, Karaj, Iran) in a two-week interval. In control group, same injections were made without the antigen. Seven days after the third immunization (end of eighth week), eggs were harvested. Yolks were separated from egg whites, washed with distilled water to remove as much albumen as possible, and then rolled on paper towels to remove adhering egg white (17). The yolk was brought to 25ml with sodium phosphate buffer (100 mM, PH 7.2) and vigorously mixed. Subsequently, chloroform (20 ml) was added and 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 re-suspended in 2ml of sodium phosphate buffer, and then stored at -20 °C (18). For preparation of the anti-IgY antibody, two 6 months old rabbits (Pasteur institute, Karaj ,Iran) were immunized intramuscularly with 0.5ml of purified IgY four times with two-week intervals, with 0.5ml of Freund's complete (in first injection) and incomplete adjuvant (in other thee injections) (17). The antibody (anti-IgY) was later used for immunoblotting assay. Dot blot assay was performed to evaluate the IgY produced. First, 1 µl of antigen was doted on one corner of a 1×1 cm square nitrocellulose membrane. Purified theileria antigen (provided by the Department of Parasitology, Tabriz University) was used as the negative control. In order to determine the immunogenic reactions, 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 H_2O) containing 0.05% Tween 20 for 1h at room temperature (RT). Subsequently, the membranes were incubated in different dilutions of the specific IgY in TBS containing 0.05% Tween 20 (1:100, 1:200, 1:400, 1:800, 1:1000, 1:10000) for 1h at RT. The membranes were then washed three times with TBS containing 0.05% tween 20 for 5 min at RT. Then, the membranes were incubated in diluted rabbit anti-IgY (dilution 1:200 in TBS containing 0.05% Tween 20) for 1h at RT. The membranes were washed three times with TBS containing 0.05% tween 20 for 5 min at RT. After addition of horseradish-conjugated goat anti-rabbit Ig (Dako, Denmark) (1:2000) to the washed membranes, they were incubated for 1h at RT. After the incubation period, the membranes were washed three times as described above. Positive reaction was developed using 3, 3'-diaminobenzidine (Sigma, USA) as substrate under visual observation within 5 min.

RESULTS

The IgY concentration in egg yolk was determined as 9.7 mg IgY/ml of egg yolk by spectrophotometer in the Bradford method. Since horseradish-conjugated anti-IgY was not available for the dot blot analysis, rabbit anti-IgY was first prepared using isolated IgY to detect the positive bands with horseradish-conjugated anti-rabbit Ig in the sandwich procedure.

First, the rabbit anti-IgY was evaluated with serum from hen, goat and bovine to ensure that the antibody is able to

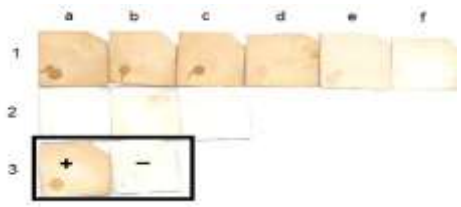
recognize the specific hen Ig. The results showed that the serum prepared from rabbit immunized with hen IgY could recognize the IgY, but the serum collected from hen could not recognize the serum of goat and bovine. Based on these findings, the serum from immunized rabbit could be used for further analysis.

The IgY from egg yolk of immunized hens was prepared and studied for presence of specific antibody against *C. parvum* whole oocyst antigens.

For this purpose, different dilutions of IgY solution were used.

Dot blot analysis of the antigen showed that it could be recognized by the specific IgY up to 1:1000 dilution of the antibody (Fig.1 row 1e). However, the best antibody dilution for immunological studies was determined as 1:500 (Fig. 1 row 1c), which was decisive for further analysis. To ensure that the purified IgY was capable of recognizing the antigens of *C. parvum* specifically and in the absence of non-specific reactions, the IgY from egg yolk of non-immunized hens was analyzed as well. Figure 1 (row 2) shows the IgY from control group that did not show any positive reaction with the *C. parvum* whole oocyst antigens. Figure 1 (row 3) shows the positive and negative controls.

Figure 1- Dot blot analysis of the IgY produced in different dilutions. *C. parvum* whole oocyst antigens were dotted on the lower left site of nitrocellulose membrane a, b, c, d, e and f, respectively. *Theileria sp.* antigen was dotted as negative control at the upper right side of the corresponding membranes. The membranes were analyzed with different IgY dilutions against whole oocyst antigens (lane 1a :1/100,b :1/200.c :1/500, d :1/800,e :1/1000.f :1/10000) and IgY from control group (Lane 2 a :1/100,b :1/200. c :1/500). Lane 3 represents the positive (positive cattle serum against *C. parvum*) and negative (chicken serum before immunization with whole oocyst antigens) controls.



CONCLUSION

A protective immunity could be achieved with the antibody against whole oocyst antigens. Given that such antibodies are easy to produce and chicken egg yolk is a cheap and convenient source for mass production of specific antibodies, the use of IgY against whole oocyst antigens could be considered a suitable candidate for passive immunization against cryptosporidiosis in animals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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