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Anti-Recombinant Gametocyte 56 Protein IgY Protected Chickens from Homologous Coccidian Infection

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Abstract

Coccidiosis is caused by intra-cellular infection of Eimeria spp., which goes through a complex life cycle in the intestinal mucosa of infected hosts. Specific immunoglobulins (IgY) could be produced in egg yolk by immunizing hens with specific antigens. In the present study, we cloned the E. maxima gam56 gene, expressed the GST-GAM56 fusion protein and raised IgY to GST-GAM56 in hens. The anti-GST-GAM56 IgY antibody was isolated and used to treat chickens infected with E. maxima oocysts. Intramuscular injection of the antibodies provided minimal protection against parasite infection. However, oral dosing of the IgY 3 or 5 d after oocyst inoculation significantly improved body weight gain, reduced oocyst output and intestinal lesion score were reduced at 3 or 5 d after oocyst challenging, compared to the untreated control group. Our findings suggest that the IgY to gam56 could be an effective prophylactic or therapeutic agent against E. maxima infection in chickens and should have a practical application value.

Key words: Eimeria maxima, recombinant GST-GAM56, IgY, therapeutic agent

INTRODUCTION

Coccidiosis in poultry is caused by several species of apicomplexan parasites belonging to the genus Eimeria, which multiply in intestinal epithelial cells. Pathological changes associated with species infection are blood loss, shock syndrome and even death (Vermeulen et al. 2001). Annual economic losses, including the cost of prophylactic in-feed medication, alternative treatment (if the medication fails), mortality, reduction in egg production and poor feed conversion, due to coccidiosis are estimated to be in excess of £2 billion worldwide (Williams 1998).

Currently coccidiosis in poultry is controlled mainly by anti-coccidial drugs as in-feed medication. Alternative control strategies are necessary because of the rapid emergence of drug resistant field strains of Eimeria (Chapman 1997), high costs associated with the development of new drugs (Lillehoj 2000) and public concerns over potential adverse health effects from residual drugs in poultry products. Live vaccines containing virulent or attenuated strains of Eimeria are available, but these vaccines consist of several live Eimeria species, which are labour intensive and costly to produce (Vermeulen 1998), limiting their use in the poultry industry. New control methods such as vaccination with recombinant proteins and genetic improvement of poultry immunity are now actively pursued (Wallach et al. 1992; Min et al. 2001), but no effective recombinant vaccines are commercially available or offer acceptable protection.
Eimeria parasites undergo a complex life cycle in the intestinal mucosa of infected chickens. Transmission of coccidiosis occurs by ingesting oocysts which develops as a result of fertilization of macrogametes by microgametes (Wallach et al. 1992). Studies have shown that monoclonal antibodies raised against a 56 kDa gametocyte surface glycoprotein of the avian parasite *E. maxima* significantly reduced oocyst production in passively immunized chickens (Wallach et al. 1990). In addition, laying hens immunized with purified 56, 82 and 250 kDa *E. maxima* gametocyte antigens transferred very large amounts of maternal antiguametocyte antibodies to their offspring via the yolk (Wallach et al. 1992).

Egg yolk immunoglobulins (IgY) can be produced in eggs by immunizing hens with specific antigens. IgY antibodies can be isolated in large quantities from yolk by simple methods without distress to the birds (Yokoyama et al. 1992). Therefore, IgY as an efficient, cheap and easily produced vaccine has attracted much attention and is recognized to be efficient in therapy and prevention of infectious diseases (Carlander et al. 2000; Devi et al. 2002). It has been demonstrated that specific IgY can prevent or control infections caused by *Escherichia coli* in piglets (Erhard et al. 1996; Jin et al. 1998), rotavirus in calves (Kuroki et al. 1997), and *Salmonella* in mice (Sugita-Konishi et al. 2000). Oral administration of specific IgY was shown to protect mice from *Cryptosporidium parvum* infection (Cama and Sterling 1991).

The present study was conducted to characterize the specific IgY against a recombinant *E. maxima* gametocyte GST-GAM56 protein and, in particular, to evaluate the efficacy of the antibody in the treatment of coccidia in chickens inoculated with *E. maxima* oocysts.

## RESULTS

### Expression of the recombinant GST-GAM56 protein

The *gam56* gene (912 bp) was amplified using the primers designed based on the coding sequence of *E. maxima* (Fig. 1), corresponding to 304 amino acids with a molecular weight of 34.9 kDa. The sequence shared 99.9% identity with *E. maxima* gam56 reported previously (GenBank accession no. AY129951). Only one base was changed, and the amino acid sequence was the same.

The *gam56* gene was ligated to pGEX-6p-1, and the recombinant plasmid was confirmed by PCR and restriction enzyme digestion. Maximal expression of GST-GAM56 protein was obtained after 6 h of incubation with 1 mmol L⁻¹ IPTG. SDS-PAGE of the whole cell preparation of the recombinant pGEX-gam56-BL21 strain showed a clear band of 60 kDa for the GST-GAM56 fusion protein (Fig. 2). This is consistent with the predicted molecular weight of gam56, which is estimated to be 34.9 kDa, plus GST (26 kDa). Only the GST protein was expressed in the bacteria transformed with the pGEX-6p-1 plasmid vector (Fig. 2, lane 2). The GST-GAM56 fusion protein was purified and confirmed by Western blotting with monoclonal antibodies directed to the GST protein (Fig. 3).

### Yolk anti-gam56 antibodies

Anti-GST-GAM56 IgY was raised in hens by intramuscular injection of the recombinant GST-GAM56 fusion protein with the Freund’s adjuvant. IgY was isolated from the egg yolk using a previously established method (Polson et al. 1985). IgY has a molecular weight of 120 kDa and is composed of two

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**Fig. 1** The *gam56* gene of *E. maxima* was amplified. 1, *gam56* (912 bp); M, marker. The same as below.
subunits, a heavy-chain of 65 kDa and light-chain of 35 kDa. Isolation and purification of IgY from the egg yolk by water dilution, salt precipitation and filtration were proven effective as shown by SDS-PAGE. Western blotting showed specific binding of the IgY to GST-GAM56 (Fig. 4), and the binding affinity of IgY was further confirmed by ELISA with a titer of 1:108.

**Passive immunoprotection**

There was no mortality in the control or immunized groups. Intramuscular injection of up to 1 mL IgY conferred some protection against *E. maxima* infection from inoculation of $1.5 \times 10^5$ sporulated oocysts. Chickens injected with 1 mL IgY 3 d after inoculation or 0.5 or 1 mL IgY 5 d after inoculation gained more weights than the challenged, untreated chickens (Table 1). Mean body weight gains in the groups injected with IgY 1 d after inoculation were small, ranging from 161 to 166 g, and were not significantly different from the challenged control group. There were no significant differences in intestinal lesion score or oocyst output between treated and untreated groups, but the mean lesion score of the chickens treated with IgY 5 d after oocyst inoculation tended to be lower than the challenged control group (2±0.3 cf. 3.4±0.1). No intestinal lesions were observed in the unchallenged control group.

Compared to the intramuscular injection treatment, oral dosing offered better protection against infection. Fecal oocyst output, body weight gain and intestinal lesions were significantly improved in all infected groups treated with 3 mL IgY irrespective of the time of treat-
ment compared to the challenged control group (Table 2), although body weight gains were below the unchallenged control value (mean body weights: 167-206 g cf. 258 g). Fecal oocyst output of the infected chickens treated with 3 mL IgY was 3-4 fold lower than the challenged control group, and the mean intestinal lesion score of the same IgY-treated groups was 1-1.8, compared with 3.4 for the challenged control group. In addition, chickens treated with 1.5 mL IgY 3 or 5 d after oocyst inoculation also had significantly higher body weight gain, and lower lesion score (3 d treatment group only) and oocyst output than the challenged control chickens. These parameters in the groups dosed with 0.3 mL IgY were not significantly different from the challenged controls.

**DISCUSSION**

Wallach *et al.* (1990) demonstrated that monoclonal antibodies to *E. maxima* GAM56 and GAM82 antigens provided partial protection against *E. maxima* infection. Monoclonal antibodies against the GAM82 antigen cross-reacts with the GAM56 antigen, suggesting the two antigens have common epitopes (Wallach *et al.* 1990). Our laboratory identified the common epitope shared by the GAM56 and GAM82 antigens using the DNAMAN and DNAStar softwares and predicted the co-epitope of the two proteins (Zhang *et al.* 2007). In this study, we cloned the common epitope fragment of the gam56 gene from the purified *E. maxima* Beijing strain. The sequence has 99.9% identity to the reported sequence of gam56 from the same *Eimeria* species (GenBank accession no. AY129951). The cloned gam56 gene was transformed to *E. coli* BL21 (DE3) and a GST-GAM56 fusion protein was expressed with the predicted molecular mass of 34.9 kDa.

Humoral immunity plays an important role in the protection against infection by Eimerian parasites. Immunoglobulins in avian species can be obtained from serum (IgG) and egg yolk (IgY). Sera from chickens infected with *E. tenella* or *E. maxima* provide good

### Table 1 Efficacy of anti-gam56 IgY antibodies by intramuscular injection in the treatment of coccidiosis caused by *E. maxima* in chickens (n=8)

| Time of treatment | IgY dose (mL) | Body weight gain (g, mean±SD) | Lesion score (mean±SD) | Total oocyst output (×10^7, mean±SD) |
|-------------------|--------------|------------------------------|----------------------|--------------------------------------|
| 1 d after infection | 1            | 166.9±23.1                   | 2.7±0.2              | 9.8±1.1                              |
|                   | 0.5          | 163.6±15.7                   | 2.7±0.2              | 14.7±1.7                             |
|                   | 0.1          | 161.4±19.8                   | 3±0.3                | 10.8±2.3                             |
| 3 d after infection | 1            | 198.1±25.2                   | 2.5±0.4              | 14.5±3.1                             |
|                   | 0.5          | 181.9±22.4                   | 2.5±0.4              | 11.6±1.5                             |
|                   | 0.1          | 179.7±10.8                   | 3±0.3                | 15.8±1.3                             |
| 5 d after infection | 1            | 212.6±17.0                   | 2±0.3                | 12.2±2.2                             |
|                   | 0.5          | 215.4±11.0                   | 3±0.4                | 9.2±1.6                              |
|                   | 0.1          | 173.3±24.4                   | 3±0.1                | 13.7±2.7                             |
| Challenged control | -            | 121.9±21.1                   | 3.4±0.1              | 15.8±0.9                             |
| Unchallenged control | -          | 236.6±18.8                   | 0                    | 0                                    |

1) Body weight gain over 8 d after oocyst inoculation.
2) Intestinal lesion score 6 d after oocyst inoculation.

*, significantly different from the challenged control group, *P*<0.05.

The same as below.

### Table 2 Efficacy of anti-gam56 IgY antibodies by oral administration in the treatment of coccidiosis caused by *E. maxima* in chickens (n=8)

| Time of treatment | IgY dose (mL) | Body weight gain (g, mean±SD) | Lesion score (mean±SD) | Total oocyst output (×10^7, mean±SD) |
|-------------------|--------------|------------------------------|----------------------|--------------------------------------|
| 1 d after infection | 3            | 166.8±32.2                   | 1.8±0.4              | 3.2±1.1                              |
|                   | 1.5          | 128.9±24.3                   | 2±0.5                | 5.4±1.7                              |
|                   | 0.3          | 166.5±21.4                   | 3±0.2                | 7±2.4                                |
| 3 d after infection | 3            | 205.9±27.1                   | 1.5±0.3              | 3±2.1                                |
|                   | 1.5          | 169.4±13.7                   | 2±0.1                | 5±1.5                                |
|                   | 0.3          | 159.3±11.3                   | 3±0.3                | 6±1.3                                |
| 5 d after infection | 3            | 182.8±19.5                   | 1±0.2                | 3.8±2.0                              |
|                   | 1.5          | 192.1±15.6                   | 2.3±0.5              | 2.4±1.2                              |
|                   | 0.3          | 143.7±25.4                   | 3±0.6                | 5.8±0.9                              |
| Challenged control | -            | 117.6±9.5                    | 3±0.5                | 15.8±0.7                             |
| Unchallenged control | -          | 258.3±23.5                   | 0                    | 0                                    |
passive protection against homologous challenge infection (Rose and Long 1971). Passive protection against *E. tenella* infection has also been achieved using a monoclonal antibody to a surface antigen of *E. tenella* sporozoites (Crane 1988).

The egg yolk IgY is a functional homologue and evolutionary ancestor of IgG (Warr et al. 1995). Oral administration of IgY obtained from chickens immunized with purified antigens was proven successful in the treatment of a variety of gastrointestinal infections, such as bovine and human rotaviruses, bovine coronavirus, *Yersinia ruckeri*, enterotoxigenic *E. coli*, *Edwardsiella tarda*, *Staphylococcus*, and *Pseudomonas* (Mine and Kovacs-Nolan 2002; Shin et al. 2002; Tini et al. 2002). Oral administration of anti-Cryptosporidium egg yolk from hens immunized with *C. parvum* oocysts resulted in parasite reduction in a mouse model (Cama and Sterling 1991).

Coccidian transmission occurs by the production of oocysts which develop from the fertilization of macrogametes by microgametes. The latter appear to be the major parasitic form causing intestinal pathology in coccidiosis. The *E. maxima* gametocyte GAM56 is an important component of the oocyst wall (Belli et al. 2002). Immunity to the gametocyte stage of *E. maxima* development may not only effectively block parasite transmission but also reduce the pathogenicity of the parasite. In our study, IgY to GST-GAM56 was raised by immunizing chickens with the recombinant GST-GAM56 fusion protein. Oral administration of the IgY conferred partial protection of chickens challenged with *E. maxima* oocysts. The anti-GST-GAM56 IgY administered orally 3 or 5 d post-infection improved body weight gain and reduced oocyst output and intestinal lesions. Administration of the IgY by the oral route 1 d after oocyst inoculation was less effective, compared to the oral dosing 3 or 5 d after challenge. Our study results are consistent with the development cycle of *E. maxima*. Gametocytes of *E. maxima* develop 4 or 5 d after oocyst inoculation, and gametocyte antibodies emerge late in the course of infection, generally between d 4 and 8 post infection (Rose and Long 1971).

Compared to the oral treatment, IM injection had minimal protective effects against infection. The better therapeutic effect by the oral administration than that by the IM route was probably due to direct delivery of the antibody to the site of infection (i.e., intestines) and binding of the antibody to the GAM56 protein on the gametocyte membrane, inhibiting the growth, development and/or fertilization of gametocytes (Wallach et al. 1990).

In order to effectively purify the recombinant protein, we used the pGEX-6p-1 vector which contain the GST tag sequence and produced the GST fusion protein. Our preliminary experiment with purified GST showed no protection of chickens from *E. maxima* infection (data not shown), which was in accordance to our previous results (Ding et al. 2008). These findings suggest that the immune response to *E. maxima* was conferred by IgY specific to the recombinant gam56, but not by GST.

We used total oocyst output, body weight gains and intestinal lesion scores to determine the therapeutic effect of the anti-GST-GAM56 IgY. While achieving a reduction in oocyst output is important in terms of preventing the spread of the disease, alleviation of intestinal lesions and improvement in body weight gain demonstrate healthier animals. In this study, reductions in oocyst output and intestinal lesion score and increases in body weight gain by anti-GST-GAM56 IgY suggest that the anti-GST-GAM56 IgY should have a practical application value as a therapeutic or prophylactic agent to control *E. maxima* infection and minimize the pathogenicity of *E. maxima* in chickens. Further studies are required to determine the best prophylactic/therapeutic regimen.

**MATERIALS AND METHODS**

**Animals**

One-day old broiler chickens were purchased from the Institute of Animal Science, Chinese Academy of Agricultural Sciences, and reared in wire cages in a coccidian-free environment. The chickens were provided with a non-medicated broiler diet and water *ad libitum*.

**Parasites**

*E. maxima* Beijing strain was maintained in the Parasitology Laboratory of the College of Veterinary Medicine, China Agricultural University. Purified sporulated oocysts of *E. maxima* were stored in 2.5% potassium dichromate.
solution at 4°C and passaged through chickens every 3 mon as previously described (Wu et al. 2004). Sporulated oocysts were cleaned by flotation in saturated sodium hypochlorite solution, washed three times with PBS, and counted using a hemocytometer before inoculation.

**Gametocytes**

For the preparation of gametocytes, chickens were inoculated with 10000 sporulated oocysts at 4 wk of age. At 134-136 h post-inoculation, the chickens were euthanized, and the intestine was removed and washed with cold SAC (170 mmol L⁻¹ NaCl, 10 mmol L⁻¹ Tris-HCl pH 7, 10 mmol L⁻¹ glucose, 5 mmol L⁻¹ CaCl₂, 1 mmol L⁻¹ phenylmethanesulfonyl fluoride (PMSF), 1 mg mL⁻¹ bovine serum albumin). The intestine was filled with SAC containing 0.5 mg mL⁻¹ hyaluronidase and incubated in PBS at 37°C in a water bath with constant shaking for 20 min. Then the intestine was slit open and the mucosa was washed with SAC. The washing was filtered successively through a 17 μm and a 10 μm mesh polymon filter. The gametocytes on the 10 μm filter were washed off with SAC and collected after centrifugation at 800×g for 5 min (Wallach et al. 1995).

**Construction of gam56 plasmid and expression of recombinant GST-GAM56 protein**

Total RNA was extracted from 2×10⁸ gametocytes using the Trizol reagent according to the manufacturer’s instructions (Invitrogen, USA). The first strand cDNA was synthesized by the conventional method using oligo(dT)₁₆ primers, MMLV and a ribonuclease inhibitor (RNasin, Promega, USA). Based on the known sequence of gam56 (GenBank accession no. AY129951), the following primer pairs were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd: forward primer 5′-TAG GAT CCC AGG TTC ACC CTT ACA GCG-3′ (bases for digestion by BamH I and EcoR I are underlined). The E. maxima gam56 gene was amplified using the Ex Taq PCR system (TaKaRa, Dalian, China) with the synthesized cDNA as the template. The amplified gam56 gene was purified and cloned into the pGEM-T Easy vector (Promega, USA). The resulting recombinant plasmid, named as pGEM-gam56 was transformed to Top10 competent cells for plasmid multiplication. The plasmid was digested with BamH I and EcoR I and ligated into the pGEX-6p-1 expression vector (Amersham Pharmacia Biotech, USA).

The recombinant plasmid, designated as pGEX-gam56, was transformed into E. coli BL21 (DE3), and one clone of the transformed E. coli was grown in LB broth supplemented with ampicillin (100 μg mL⁻¹). When the bacteria culture reached OD₆₀₀ of 0.5-0.6, 1 mmol L⁻¹ isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce GST-GAM56 protein expression and the culture was further incubated for 6 h. The expressed GST-GAM56 fusion protein in the whole cell preparation was separated on a 12% SDS-PAGE minigel (Bio-Rad, USA) according to the manufacturer’s instructions, and analysed by Western blot with mouse anti-GST monoclonal antibodies (NeoMarkers, Fermont, CA) and 3′,5′-tetramethylbenzidine (TMB; AMRESCO) as the substrate for detection of the immune complex.

The solubilized GST-GAM56 protein was purified by affinity chromatography using a Glutathione Sepharose 4B column (Amersham, USA) and 10 mmol L⁻¹ glutathione elution buffer according to the manufacturer’s instructions. The purity of the protein was assayed by SDS-PAGE and Western blotting, and the protein concentration was estimated by spectrophotometry at OD₂₈₀ nm and OD₆₆₀ nm⁻¹.

**Production of anti-GST-Gam56 antibodies**

Hens were immunized according to the method of Shin et al. (2002). The recombinant protein was emulsified with an equal volume of complete and incomplete Freund’s adjuvant for the first and subsequent booster immunizations, respectively. Six 4-mon-old White Leghorn laying hens housed in wire cages in a coccidian-free environment were injected intramuscularly at three sites with a total volume of 1.0 mL for the first dose (d 0) and 0.5 mL for each booster dose on d 10 and 20, respectively. Eggs were collected daily from the immunized hens 20 d after the first injection and were stored at 4°C before isolation of IgY antibodies.

**IgY antibody isolation**

The egg yolk was mixed 1:1 with PBS buffer supplemented with 0.3% Tween-20, 0.02% sodium azide and 0.05% bovine hemoglobin, and then frozen at -20°C overnight. The thawed yolk-PBS solution was centrifuged at 10000×g for 10 min at 4°C. IgY was purified from the PBS supernatant using PEG 8000 according to Polson et al. (1985). Briefly, pulverized PEG 8000 was added to the supernatant to a final concentration of 3.5% (w/v). Following centrifugation at 2000×g for 30 min, the solution was separated into a semi-solid precipitate and a clear liquid fraction with a yellow fatty layer floating on the surface. The liquid supernatant and the fatty layer were decanted through a fine muslin to remove the fatty layer. The liquid fraction containing water soluble IgY was collected. The protein content of the egg yolk-PBS solution and the IgY fraction was estimated by UV spectrophotometry at OD₂₈₀ nm and OD₆₆₀ nm⁻¹.

The IgY fraction was electrophoresed in a 15% gel on a Pharmacia Phast System and stained with Coomassie brilliant blue according to the manufacturer’s instructions.
The proteins were transferred to a polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked with 5% skim milk powder and incubated with the isolated IgY fraction diluted 1:200 in Tris-buffered saline (TTBS, 0.1 mol L\(^{-1}\) Tris-HCl, pH 7.5, 0.15 mol L\(^{-1}\) NaCl, and 0.1% Tween 20). The membrane was incubated with the mouse anti-IgY antibody coupled to horseradish peroxidase and then dianamobenzidine (DAB).

**Determination of antibody titers**

The antibody titer was measured by ELISA. The purified antigen was coated on a microtiter plate at 1 µg/well. Following overnight incubation at 4°C, 100 µL serial dilutions 1:100 to 1:51 200 of IgY were added. Then mouse anti-chicken IgY horseradish peroxidase conjugate (KPL Co., USA) diluted to 1:3000 was added and incubated for 1 h. The reaction was visualized using TMB as the substrate. The reaction was stopped with 2 mol L\(^{-1}\) sulfuric acid, and the plates were read at 450 nm. The titer was defined as the reciprocal of the highest dilution showing a positive result.

**Passive immunoprotection experiments**

One-day-old chickens (AA broiler) were fed a non-medicated broiler diet ad libitum and raised in wire-floored cages under coccidia-free conditions. At 10 d of age, the chickens were leg-banded and grouped with 8 chickens per cage. Each chicken was orally inoculated with \(1.5 \times 10^6\) sporulated *E. maxima* oocysts. The chickens were treated with 0.1, 0.5 or 1 mL IgY solution by intramuscular injection to the pectoral muscle or with 0.3, 1.5 or 3 mL by oral gavage at 1, 3 or 5 d after oocyst inoculation. One group as the unchallenged control was not inoculated or treated with IgY, and one group as the challenged control was inoculated but not treated with IgY. Six days after infection, 3 chickens of each group were euthanized and examined for examination of oocyst production (no lesions) to 4 (severe lesions). The remaining chickens were maintained for examination of oocyst production between 6 and 8 d after infection using a previously described method (Talebi 1995). The chickens were weighed immediately prior to oocyst inoculation and 8 d post inoculation to determine weight gains.

**Statistical analysis**

Data were statistically analyzed by analysis of variance (ANOVA) and Student’s *T* test. Difference between groups was considered significant if the *P* value was less than 0.05 (*P*<0.05).

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