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In ovo vaccination with the *Eimeria tenella* EtMIC2 gene induces protective immunity against coccidiosis

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Abstract

An *Eimeria tenella* microneme recombinant gene (EtMIC2) and encoded protein were evaluated as potential vaccines against avian coccidiosis. *In ovo* inoculation with the EtMIC2 gene increased anti-EtMIC2 antibody titers at days 10 and 17 following *E. tenella* infection. In addition, vaccinated birds developed protective immunity against infection by *E. tenella* as assessed by significantly increased body weight gain and decreased fecal oocyst shedding compared with non-vaccinated controls. Vaccination with the EtMIC2 gene also led to protective immunity against infection by *E. acervulina*, but not *E. maxima*. Combined *in ovo* DNA vaccination plus post-hatch boosting with EtMIC2 DNA or protein did not improve antibody titers or protective immunity beyond that achieved with *in ovo* vaccination alone. These results provide evidence that *in ovo* immunization with a recombinant *Eimeria* microneme gene stimulates protective intestinal immunity against coccidiosis.
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1. Introduction

Important protozoan pathogens of humans and animals belonging to the phylum *Apicomplexa* include *Eimeria*, *Plasmodium*, *Toxoplasma*, *Crytosporidium*, *Neospora*, and *Sarcocystis*. Seven species of *Eimeria* are the etiologic agents of avian coccidiosis, an intestinal disease impairing the feed utilization and growth of infected animals [1]. Although anticoccidial drugs in poultry feed are good preventatives and convenient for large-scale use, alternative control strategies are needed due to the emergence of drug resistant parasites in commercial production settings [1–3]. Recent efforts to clone *Eimeria* genes as potential recombinant vaccines are directed toward this goal [4].

Apicomplexans possess a characteristic apical complex consisting of micronemes, rhoptries, dense granules, and structural elements such as the conoid, polar ring, and subpellicular microtubules. Micronemes are small membrane-bounded organelles located immediately beneath the cell membrane near the anterior end of the apical complex and releasing numerous soluble and transmembrane proteins [5]. Microneme proteins are involved in multiple interactions between the parasite and host cell, specifically in relation to motility, attachment, recognition, and penetration [6–10]. One microneme protein in particular, EtMIC2, was cloned from *Eimeria tenella* and shown during host cell invasion to be localized at the point of parasite entry and secreted

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from the host-parasite interface [6]. EtMIC2 represents one of nearly 30 *Eimeria* genes that have been cloned and characterized at the molecular level [3]. While many of these genes have been identified as potential vaccine candidates for immunization against coccidiosis, several technical and conceptual impediments remain to be solved before a recombinant subunit vaccine becomes commercially feasible. For example, a vaccination method producing optimum resistance to challenge infection has yet to be determined. Recently, *in ovo* immunization offers a promising new avenue for delivery of vaccines to chickens in a commercial setting [11,12].

Wolff et al. [13] discovered that direct administration of plasmid DNA (i.e. naked DNA) to the skeletal muscle of mice led to expression of the recombinant gene product. Over the past 10 years, substantial progress has been made in the design and formulation of DNA vaccines for control of pathogens of veterinary importance. While most of these are directed against viral pathogens, including bovine herpesvirus [14], foot and mouth disease virus [15], and porcine respiratory and reproductive syndrome virus [16], effective DNA vaccination against avian coccidiosis has also been reported [17–21]. However, no studies have examined *in ovo* delivery of *Eimeria* genes in an attempt to control coccidiosis. In the study reported here, vaccination of chicken embryos with the EtMIC2 *E. tenella* microneme gene was evaluated for protection against challenge infection with the homologous and heterologous parasites.

2. Materials and methods

2.1. Chickens and in ovo immunization

Specific pathogen-free embryonated eggs of white Leghorn SC inbred chickens (Hy-Vac, Adel, IA) were hatched at the Animal and Natural Resources Institute (Beltsville, MD) and chickens provided with feed and water ad libitum. For *in ovo* immunization, eggs were incubated for 18 days, candled to select fertile eggs, and injected with the EtMIC2 gene. All substances including EtMIC2-pcDNA were injected in 100 μl of sterile phosphate-buffered saline (PBS) pH 7.4 using an Intelliject system (AviTech, Easton, MD). Briefly, each egg is cleaned and positioned in a holder under the injecting needle with the large end up. With the help of a vacuum system, the needle penetrates the shell past the air cell, delivers the inoculum into the amniotic cavity [22], and is thoroughly disinfected after each inoculation. In addition, the proprietary system is designed not to create negative pressure inside the egg thus reducing the risk of cross-contamination. All experiments were performed according to guidelines established by the Beltsville Agriculture Research Center Small Animal Care Committee.

2.2. Parasites

The wild type strains of *E. tenella*, *E. acervulina*, and *E. maxima* were originally developed and maintained at the Animal and Natural Resources Institute (Beltsville, MD). Oocysts were cleaned by floatation on 5.25% sodium hypochlorite, washed three times with PBS, and enumerated by hemocytometry. Chickens were orally infected with 10,000 oocysts per animal and fecal oocyst shedding following experimental infections was calculated as described [23]. Prior to infection, all experimental birds were reared in brooder pens in *Eimeria*-free facility and transferred into small cages in separate location where they were infected and kept until the end of experimental period.

2.3. Cloning of EtMIC2 cDNA

E. tenella sporulated oocysts were excysted to sporozoites, washed with PBS, and lysed with 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate, and 0.1 M β-mercaptoethanol. Messenger RNA was purified on an oligo(dT) column (FastTrack 2.0 mRNA Isolation Kit, Invitrogen, Carlsbad, CA) and used as a template for cDNA synthesis (cDNA Synthesis Kit, Takara Bio, Shiga, Japan). EtMIC2 cDNA was amplified by PCR using the following primers: forward, 5′-CTTTGTATTCACATTCAAAATGGCTCG-3′; reverse, 5′-CGTCACTCTGCTTGAACCTCTTCC-3′ (GenBank accession number AF111839). Amplification was performed by an initial reaction at 94 °C (2 min) followed by 30 cycles of 94 °C (1 min), 55 °C (2 min), 72 °C (3 min), and final extension at 72 °C (10 min). The 1.1 kb PCR product was gel purified and subjected to a second round of amplification using the following primers: forward, 5′-GGGAATTCGGCACGAGCTTTGTATTCACATTC-3′; reverse, 5′-GGGTCGACACGTCTTTGCGTCACTCTGCTTGAACC-3′. The amplified fragment was digested with *Eco*RI and *Sal*I, cloned into pBluescript SK(−) phagemid (Stratagene, La Jolla, CA), and recombinant EtMIC2-pBL plasmids confirmed by nucleotide sequence analysis. A *Bam*HI site was inserted upstream of the EtMIC2 coding sequence by PCR using the following primers: forward, 5′-CAGCCGTTAGGATCCGTCCCAGGCG-3; reverse, 5′-GTAATACGACTCACTATAGGGC-3′. Amplicons were digested with *Bam*HI and *Sal*I, cloned into pGEX-6p-3 (Amersham Biosciences, Piscataway, NJ), and recombinant EtMIC2-pGEX clones confirmed by sequence analysis. The EtMIC2 coding sequence was subcloned into the *Bam*HI/*Sal*I sites of pcDNA3.1 (Invitrogen), transformed into *E. coli* DH5α, recombinant plasmids purified (Qiagen, Valencia, CA), and quantified spectrophotometrically.

2.4. Expression and purification of EtMIC2 recombinant protein

The EtMIC2 coding sequence was subcloned from EtMIC2-pGEX into the *Bam*HI/*Hin*dIII sites of the pMal4c vector with a NH_2-terminal maltose-binding protein tag, expressed in *E. coli* in TY broth (20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl) containing 100 μg/ml ampicillin, the bacteria grown to $OD_{600} = 0.5$, induced with 1.0 mM isopropylβ-D-thiogalactopyranoside for 3 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, NY). The EtMIC2 protein was isolated on an amylose affinity column (New England Biolabs, Beverly, MA) according to the manufacturer's instructions, digested with Factor Xa to release EtMIC2, and re-passed through the amylose column to remove maltose-binding protein. Final protein purity was confirmed by SDS-PAGE and Western hybridization.

2.5. EtMIC2 antibody ELISA

Flat-bottom, 96-well microtiter plates (Costar, Boston, MA) were coated with 100 μl of purified EtMIC2 protein (10 μg/ml) in 0.1 M sodium carbonated buffer, pH 9.6 at 4 °C overnight and washed twice with PBS, pH 7.2 containing 0.05% Tween-20 (PBS-T). Wells were blocked with 100 μl of PBS-1% BSA (Sigma) for 1 h at room temperature followed by 100 μl of serum for 2 h at room temperature. The wells were washed five times with PBS-T and incubated for 30 min at room temperature with 100 μl of horseradish peroxidase-conjugated anti-chicken IgG (Sigma) diluted 1:4,000 in PBS-1% BSA. The wells were washed five times with PBS-T, developed with 100 μl of 0.01% (w/v) tetramethylbenzidine (Sigma) in 0.05 M phosphate–citrate buffer, pH 5.0 for 10 min followed by 50 μl of 2N H_2SO_4, and OD at 450 nm determined with a microplate spectrophotometer.

2.6. Statistical analyses

Mean values for body weights and antibody titers were compared by the Tukey–Kramer Multiple Comparisons test. Mean values for fecal oocyst shedding were compared by Dunnett Multiple Comparisons test. Differences between means were considered significant at $p < 0.05$.

2.7. Experimental designs

2.7.1. Experiment 1

To assess anti-EtMIC2 antibody titers and protective immunity to coccidiosis following *in ovo* vaccination with the EtMIC2 gene, 75 fertile eggs were distributed into five groups (15/group) and non-injected or injected with 100 μl of sterile PBS, 50 μg/egg of the pcDNA empty vector, or 25 or 50 μg/egg of EtMIC2-pcDNA. At day 11 post-hatching, chickens were infected with 10,000 sporulated oocysts of *E. tenella*. Serum samples were collected at days 1, 10, and 17 post-infection (days 10, 21, and 28 post-hatching) and anti-EtMIC2 antibody titers determined by ELISA. Body weights were measured at days 0 and 5 post-infection, and fecal samples were collected between days 5 and 10 post-infection, pooled, and the number of oocysts counted.

2.7.2. Experiment 2

To determine the effects of post-vaccination boosting with the EtMIC2 gene, 150 fertile eggs were distributed into 10 groups (15/group) and non-injected or injected with 100 μl of sterile PBS, 50 μg/egg of the pcDNA empty vector, or 25 or 50 μg/egg of EtMIC2-pcDNA. At day 7 post-hatching, chickens were non-boosted or boosted with 25 or 50 μg/egg of EtMIC2-pcDNA and non-infected or infected with 10,000 sporulated oocyst of *E. tenella* at day 11 post-hatching. Anti-EtMIC2 antibody titers, body weight gains, and fecal oocyst shedding were measured as described above.

2.7.3. Experiment 3

To determine the effects of post-vaccination boosting with the EtMIC2 protein, 120 fertile eggs were distributed into eight groups (15/group) and non-injected or injected with 100 μl of sterile PBS, 50 μg/egg of the pcDNA empty vector, or 25 or 50 μg/egg of EtMIC2-pcDNA. At day 7 post-hatching, chickens were non-boosted or boosted with 100 μg of purified recombinant EtMIC2 protein and non-infected or infected with 10,000 sporulated oocyst of *E. tenella* at day 11 post-hatching. Anti-EtMIC2 antibody titers, body weight gains, and fecal oocyst shedding were measured as described above.

2.7.4. Experiment 4

To assess cross-protection against other *Eimeria* spp. after *in ovo* vaccination with the EtMIC2 gene, the same protocol was followed as in Experiment 1 with the exception that chickens were infected with 10,000 sporulated oocysts of *E. maxima* or *E. acervulina*. Body weights were measured at days 0 and 5 post-infection, and fecal samples were collected between days 5 and 10 post-infection, pooled, and the number of oocysts counted.

3. Results

3.1. Expression and tissue distribution of in ovo injected DNA

Initially, we determined the tissue distribution of *in ovo* injected DNA to verify that the EtMIC2 gene would be expressed in lymphoid organs. Eighteen-day-old embryos were injected with 10 or 25 μg/egg of the green fluorescent protein (GFP) gene expressed in the pcDNA vector (GFP-pcDNA) via the amniotic cavity as described in Section 2. Tissue samples were taken at 1, 2, and 3 days post-injection and single cell suspensions analyzed by flow cytometry. GFP-expressing cells were observed at 3 days post-injection, particularly in the lung, muscle, and spleen (Fig. 1).

3.2. Anti-EtMIC2 antibody responses following in ovo vaccination with the EtMIC2 gene

To determine humoral immunity following *in ovo* immunization with the EtMIC2 gene, eggs were injected with PBS, the pcDNA empty vector, or 25 or 50 μg/egg of the EtMIC2-pcDNA expression plasmid and either non-boosted

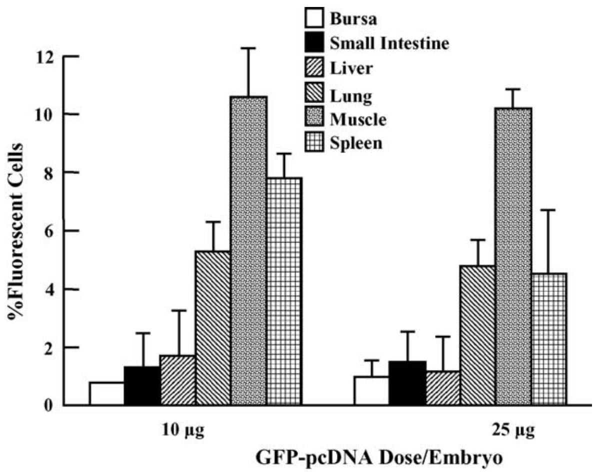

Fig. 1. Tissue distribution of *in ovo* injected GFP-pcDNA. Eighteen-day-old embryos were injected with either 10 or 25 μg/egg of GFP-pcDNA via the amniotic cavity. Tissue samples were taken at 3 days post-injection; single cell suspensions were prepared using cell strainers, and fluorescence of 10,000 cells was detected by flow cytometry. Each bar represents the mean ± S.D. ($N = 3$).

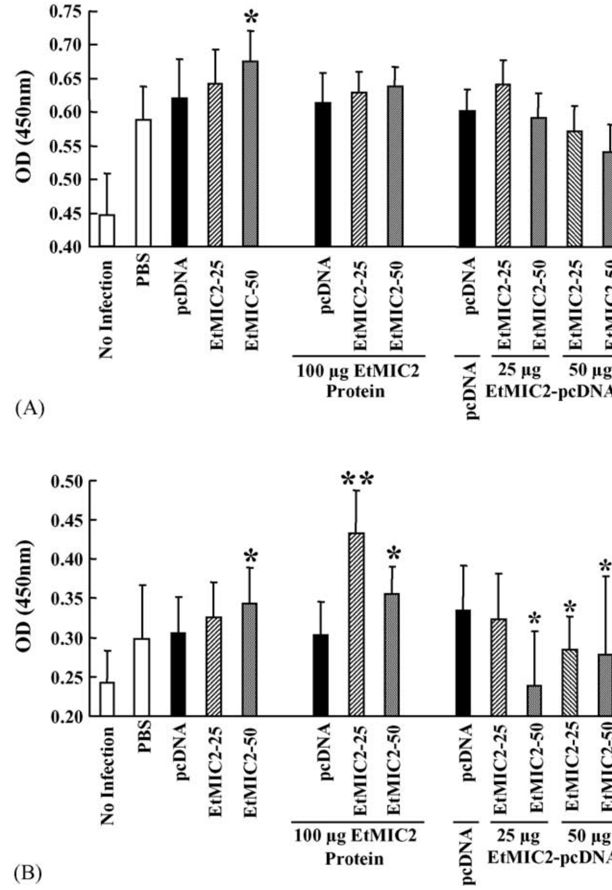

Fig. 2. Anti-EtMIC2 antibody responses following *in ovo* vaccination. Chickens were vaccinated *in ovo* with 100 μl of PBS, 50 μg of pcDNA vector alone, or 25 or 50 μg of EtMIC2-pcDNA at day 18 and either non-boosted or boosted with 100 μg of EtMIC2 recombinant protein or 25 or 50 μg of EtMIC2-pcDNA at day 7 post-hatching. Birds were non-infected or orally infected with 10,000 oocysts of *E. tenella* at day 11 post-hatching, bled at days 10 (A) and 17 (B) post-infection and anti-EtMIC2 antibody determined by ELISA. Each bar represents the mean ± S.D. ($N = 5$). Asterisks indicate significantly increased OD450 values compared with the pcDNA vector only group ($^{*}p < 0.05$; $^{**}p < 0.01$).

or boosted at day 7 post-hatching with the purified recombinant EtMIC2 protein (100 μg/chicken) or EtMIC2-pcDNA (25 or 50 μg/chicken). At day 11 post-hatching, animals were non-infected or infected with 10,000 *E. tenella* oocysts and EtMIC2-reactive antibody levels were determined by ELISA at days 1, 10, and 17 post-infection. Antibody levels in none of the vaccinated groups were increased at day 10 post-hatching compared with eggs given the pcDNA empty vector alone (data not shown). As shown in Fig. 2A, at day 10 post-infection antibody titers were significantly greater compared with controls only in the non-boosted group receiving 50 μg/egg of EtMIC2-pcDNA. By day 17 post-infection, animals receiving EtMIC2-pcDNA alone (25 and 50 μg/egg) and EtMIC2-pcDNA plus boosting with EtMIC2 protein developed significantly higher antibody titers compared with control groups given PBS or pcDNA vector alone (Fig. 2B). Interestingly, however, birds given EtMIC2-pcDNA plus boosting with EtMIC2-pcDNA displayed decreased antibody levels compared with pcDNA alone.

3.3. Protective immunity following in ovo vaccination with the EtMIC2 gene

To determine protective immunity following *in ovo* immunization with the EtMIC2 gene as described above (Section 3.2), body weight gain and fecal oocyst shedding were determined as parameters of coccidiosis. As shown in Fig. 3, control animals vaccinated with PBS or pcDNA vector alone and infected with *E. tenella* exhibited significantly reduced weight gain indicative of active intestinal disease compared with the non-infected group. In contrast, vaccination with 50 μg/egg of EtMIC2-pcDNA prior to infection restored weight gain to that seen in non-infected animals. In addition, vaccination with 25 or 50 μg/egg of EtMIC2-pcDNA followed by post-hatch boosting with 25 or 50 μg/chicken of EtMIC2-pcDNA significantly increased weight gain compared with animals given the pcDNA vector alone. In contrast, boosting with purified EtMIC2 protein, unlike the EtMIC2 gene, did not improve body weight gain of infected animals.

Similar results were observed with respect to fecal oocyst shedding. As shown in Fig. 4, chickens vaccinated with 25 or 50 μg/egg of EtMIC2-pcDNA demonstrated significantly reduced oocyst shedding compared with embryos receiving PBS or pcDNA empty vector. Additionally, three of the four groups receiving EtMIC2-pcDNA *in ovo* vaccination followed by post-hatch boosting with EtMIC2-pcDNA exhibited lowered oocyst shedding compared with pcDNA alone. Finally, vaccination with 50 μg/egg of EtMIC2-pcDNA and boosting with 100 μg/chicken of purified EtMIC2 protein also significantly decreased fecal oocyst numbers compared with the pcDNA vaccination group. However, oocyst shed-

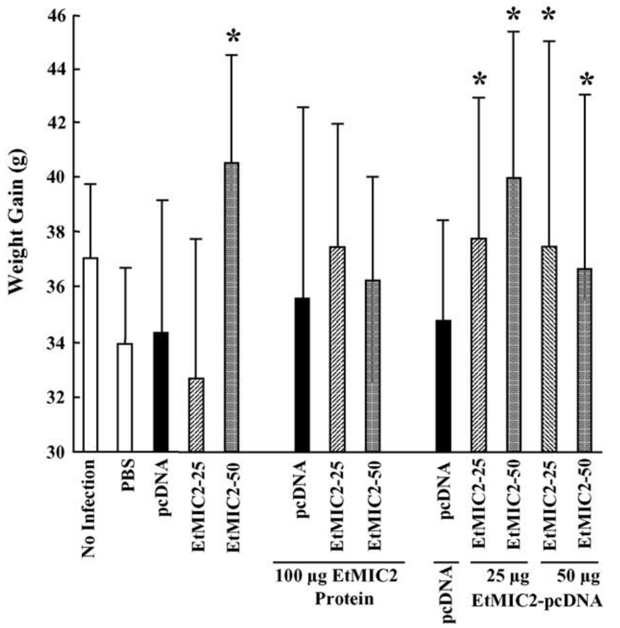

Fig. 3. Body weight gain following *in ovo* vaccination. Chickens were vaccinated *in ovo* with 100 µl of PBS, 50 µg of pcDNA vector alone, or 25 or 50 µg of EtMIC2-pcDNA at day 18 and either non-boosted or boosted with 100 µg of EtMIC2 recombinant protein or 25 or 50 µg of EtMIC2-pcDNA at day 7 post-hatching. Birds were non-infected or orally infected with 10,000 oocysts of *E. tenella* at day 11 post-hatching and body weight gain determined between days 0 and 5 post-infection. Each bar represents the mean ± S.D. ($N = 5$). Asterisks indicate significantly increased weight gain compared with the pcDNA vector only group ($^{*}p < 0.05$).

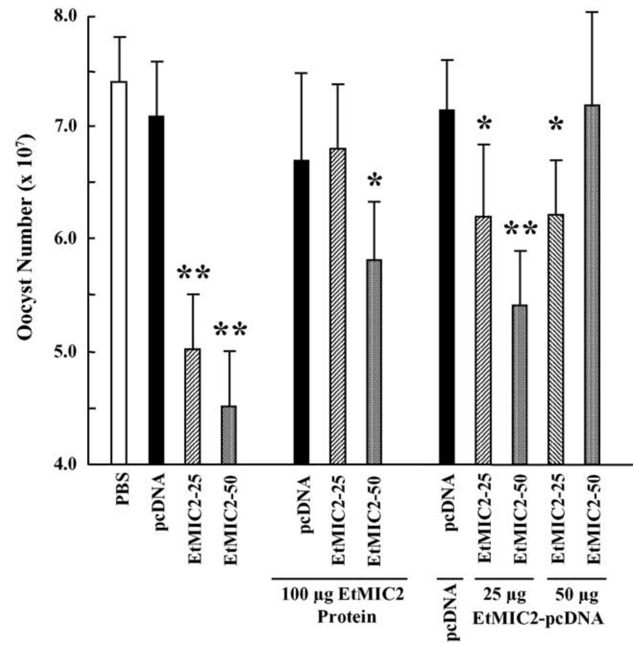

Fig. 4. Fecal oocyst shedding following *in ovo* vaccination. Chickens were vaccinated *in ovo* with 100 µl of PBS, 50 µg of pcDNA vector alone, or 25 or 50 µg of EtMIC2-pcDNA at day 18 and either non-boosted or boosted with 100 µg of EtMIC2 recombinant protein or 25 or 50 µg of EtMIC2-pcDNA at day 7 post-hatching. Birds were non-infected or orally infected with 10,000 oocysts of *E. tenella* at day 11 post-hatching and fecal samples were collected between days 5 and 10 post-infection, pooled, and the number of oocysts counted. Each bar represents the mean ± S.D. ($N = 5$). Asterisks indicate significantly decreased oocyst numbers compared with the pcDNA vector only group ($^{*}p < 0.05$; $^{**}p < 0.01$).

ding exhibited by both groups receiving secondary immunizations was not further reduced compared with the non-boosted groups indicating the lack of a booster effect.

3.4. Cross-protection against heterologous Eimeria spp. following in ovo vaccination with the EtMIC2 gene

Because multiple different species of *Eimeria* cause coccidiosis, we next investigated the effects of *in ovo* vaccination with the EtMIC2 gene on protection against infection by heterologous parasites. Eggs were either non-vaccinated and non-infected, or vaccinated with PBS, pcDNA alone, or 25 or 50 µg/egg of EtMIC2-pcDNA and infected with *E. tenella*, *E. acervulina*, or *E. maxima*. Body weight gain and fecal oocyst shedding were measured as parameters of disease. As shown in Fig. 5, body weight gain was significantly increased in chickens vaccinated with 25 µg/egg of EtMIC2-pcDNA and infected with *E. acervulina* compared with embryos receiving PBS or pcDNA alone. In contrast, EtMIC2 gene vaccination did not improve weight gain of *E. maxima*-infected birds. Further, chickens vaccinated with 50 µg/egg of EtMIC2-pcDNA and infected with *E. tenella* gained significantly more weight than those receiving PBS or pcDNA alone, consistent with findings of the first study (Fig. 3). As shown in Fig. 6, oocyst shedding was significantly reduced in birds vaccinated with 25 or 50 µg/egg of EtMIC2-pcDNA and infected with *E. acervulina* or *E. tenella* compared with negative controls. However, EtMIC2 gene vaccination did not reduce fecal oocyst numbers following infection with *E. maxima*. Taken together, these results indicated that animals vaccinated *in ovo* with the EtMIC2 gene developed cross-protection against challenge infection with *E. acervulina*, but not *E. maxima*.

4. Discussion

The results presented in this study demonstrated that *in ovo* vaccination with the *E. tenella* recombinant gene EtMIC2 encoding a microneme protein stimulated protective immunity against challenge infection by the homologous parasite as well as against *E. acervulina*. Thus, EtMIC2 can be added to a growing list of *Eimeria* proteins that may offer promise as subunit vaccines to control coccidiosis. Danforth et al. [24] identified an *E. tenella* recombinant protein (5401) that elicited partial protection against coccidiosis. Jenkins et al. [17] reported that oral administration of live *E. coli* expressing a recombinant *E. acervulina* antigen (EAMZ250) was an effective means of inducing resistance to coccidiosis as assessed by reversal of weight loss and intestinal lesions after parasite challenge. Our laboratory first described naked DNA immunization against *Eimeria* infection [18–20]. Our studies

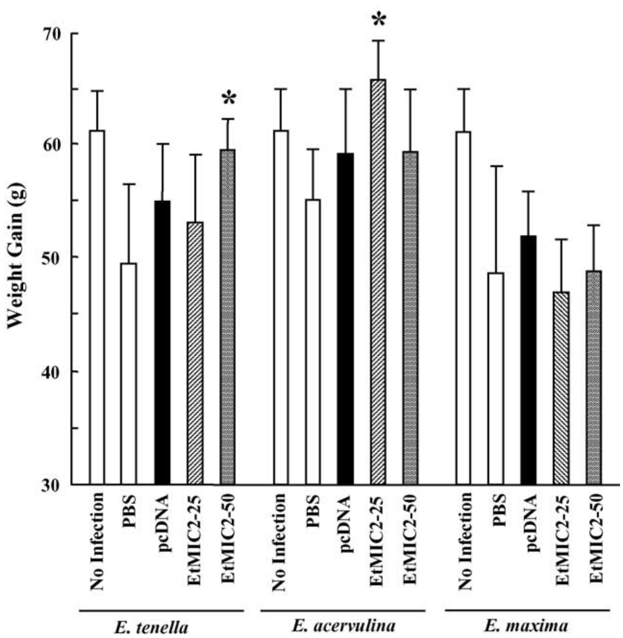

Fig. 5. Body weight gain following *in ovo* vaccination with EtMIC2 gene and infection with *E. tenella*, *E. acervulina*, or *E. maxima*. Chickens were vaccinated *in ovo* with 100 μl of PBS, 50 μg of pcDNA vector alone, or 25 or 50 μg of EtMIC2-pcDNA at day 18, orally infected with 10,000 oocysts of *E. tenella*, *E. acervulina*, or *E. maxima* at day 11 post-hatching, and body weight gain determined between days 0 and 5 post-infection. Each bar represents the mean ± S.D. ($N = 5$). Asterisks indicate significantly increased weight gain compared with the pcDNA vector only group ($^{*}p < 0.05$).

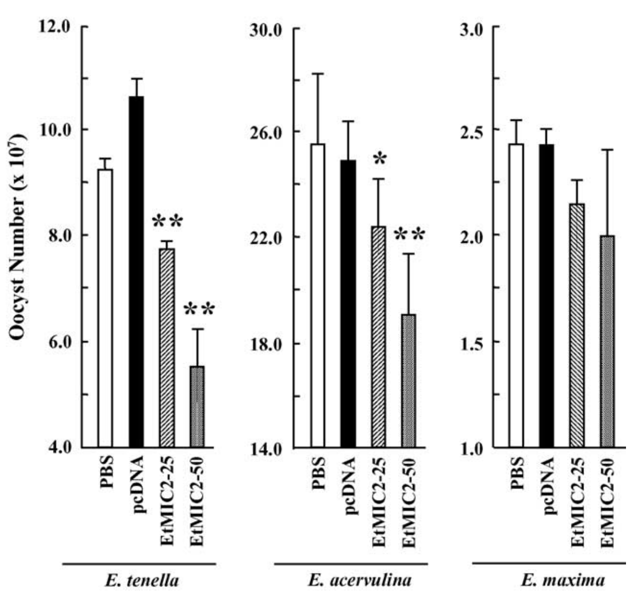

Fig. 6. Fecal oocyst shedding following *in ovo* vaccination with EtMIC2 gene and infection with *E. tenella*, *E. acervulina*, or *E. maxima*. Chickens were vaccinated *in ovo* with 100 μl of PBS, 50 μg of pcDNA vector alone, or 25 or 50 μg of EtMIC2-pcDNA at day 18, orally infected with 10,000 oocysts of *E. tenella*, *E. acervulina*, or *E. maxima* at day 11 post-hatching, and fecal samples were collected between days 5 and 10 post-infection, pooled, and the number of oocysts counted. Each bar represents the mean ± S.D. ($N = 5$). Asterisks indicate significantly decreased oocyst numbers compared with the pcDNA vector only group ($^{*}p < 0.05$; $^{**}p < 0.01$).

demonstrated that a recombinant gene (3-1E) and its corresponding protein expressed by sporozoites and merozoites of *E. tenella*, *E. acervulina*, and *E. maxima* were capable of stimulating protective immunity against coccidiosis. Immunization of chickens with either the *E. coli*- or baculovirus-expressed 3-1E protein in conjunction with adjuvant, or direct injection of the 3-1E cDNA, induced protective immunity against live *E. acervulina*. Kopko et al. [21] later reported DNA immunization against *Eimeria* infection using a gene encoding a recombinant refractile body protein (pcDNA3-SO7′) administered to 1-week-old chickens. Interestingly, while pcDNA-SO7′ reduced weight loss and intestinal lesions subsequent to challenge infection with live parasites, the corresponding recombinant protein (CheY-SO7′) was ineffective.

The novel finding of the current investigation is that a single, recombinant coccidia gene induced protection against clinical disease following *in ovo* vaccination. Successful chicken embryo vaccination with cloned pathogen genes has been reported recently for some viruses [25] but, to the best of our knowledge, not for *Eimeria* spp. While chickens immunized *in ovo* with whole pathogens, purified proteins, or viral genomes may develop humoral and cellular immunities, post-hatch protection against the infectious agent ultimately depends on the nature of the immunogen. Sharma and coworkers [22,26–28] demonstrated that vaccination of chicken embryos with viral genomes was an effective method to induce immunity to a variety of economically important diseases. Weber and colleagues [29,30] reported that *in ovo* immunization with *Eimeria* oocysts induced partial protection against subsequent challenge with the live parasite. In contrast, inoculation of embryonated chickens with *E. maxima* oocysts or sporocysts did not protect against coccidiosis [31] and *in ovo* vaccination with an oocyst extract of *Cryptosporidium baileyi* did not protect against infection with the homologous parasite [32].

Prior to *in ovo* immunization of 18-day-old embryos with EtMIC2 gene, we verified the expression and tissue distribution of *in ovo* injected DNA using a GFP gene expressed in the pcDNA vector via the amniotic cavity. GFP-expressing cells were observed at 3 days post-injection in all tested tissues, particularly in the lung, muscle, and spleen, proving that DNA injection via the *in ovo* route can be a successful immunization method. Further, induction of host immune responses to *in ovo* injected DNA was evidenced by serum anti-EtMIC2 antibody responses 10 and 17 days following *Eimeria* infection. Antibody levels in none of the vaccinated groups were increased at day 10 post-hatching compared with embryos given the pcDNA empty vector alone (data not shown). Interestingly, however, birds given EtMIC2-pcDNA plus boosting with EtMIC2-pcDNA displayed decreased antibody levels compared with pcDNA alone. Birds within those same groups exhibited better weight gains and reduced oocyst loads compared to their control counterparts. This is not an unusual observation whereby protection against eimerian infections was coupled with low antibody titers, as protective humoral immunity against coccidiosis remains debatable and a more concrete role of cellular immunity dominates protective mechanisms to the parasite [33].

Eimeria species possess complex life cycles, are host- and infection site-specific, and their pathogenicity varies in birds of different genetic background [33]. In the natural host, the immunity is species-specific, such that, chickens immune to one species of *Eimeria* are susceptible to others. These facts present major challenges in the development of effective vaccines that would protect against multiple *Eimeria* species. The work presented here provides a promising step towards achieving that goal as EtMIC2 immunization cross-protected chickens against a heterologous infection with *E. acervulina*. Microneme organelles are present in all apicomplexan protozoa and contain proteins that are essential for host cell adhesion and invasion making them attractive candidates as potential targets to inhibit infection. The EtMIC2 gene was originally cloned as one of a group of five different *E. tenella* microneme genes (EtMIC1-5) [34]. EtMIC2 encodes a 50 kDa acidic protein expressed in *Eimeria* sporozoites and merozoites [6]. During invasion, EtMIC2 was localized at the point of parasite entry but later became transiently dispersed over the entire surface of the infected cell. Although the function of EtMIC2 remains to be firmly established, it contains regions of homology to tropomyosin II and two known substrates of protein kinase C. The corresponding protein of *E. acervulina*, EaMIC2, possesses 94% sequence homology with EtMIC2 (Sato and Yasuda, unpublished observations), likely accounting for cross-protection against *E. acervulina* challenge infection following EtMIC2 immunization. Overall, these studies present a new outlook in the development of recombinant coccidiosis vaccines which, coupled with successful *in ovo* delivery, offers a promising means of controlling coccidiosis.

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References

[1] Williams RB. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathol 2002;31(4):317–53.

[2] Vermeulen AN, Schaap DC, Schetters TP. Control of coccidiosis in chickens by vaccination. Vet Parasitol 2001;100(1–2):13–20.

[3] Allen PC, Fetterer RH. Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin Microbiol Rev 2002;15(1):58–65.

[4] Shirley MW, Ivens A, Gruber A, Madeira AMBN, Wan K-L, Dear PH, et al. The *Eimeria* genome projects: a sequence of events. Trends Parasitol 2004;20(5):199–201.

[5] Wiersma HI, Galuska SE, Tomley FM, Sibley LD, Liberator PA, Donald RG. A role for coccidian cGMP-dependent protein kinase in motility and invasion. Int J Parasitol 2004;34(3):369–80.

[6] Tomley FM, Bumstead JM, Billington KJ, Dunn PP. Molecular cloning and characterization of a novel acidic microneme protein (Etmic-2) from the apicomplexan protozoan parasite, *Eimeria tenella*. Mol Biochem Parasitol 1996;79(2):195–206.

[7] Bumstead J, Tomley F. Induction of secretion and surface capping of microneme proteins in *Eimeria tenella*. Mol Biochem Parasitol 2000;110(2):311–21.

[8] Sonda S, Fuchs N, Gottstein B, Hemphill A. Molecular characterization of a novel microneme antigen in *Neospora caninum*. Mol Biochem Parasitol 2000;108(1):39–51.

[9] Rabenau KE, Sohrabi A, Tripathy A, Reitter C, Ajioka JW, Tomley FM, et al. TgM2AP participates in *Toxoplasma gondii* invasion of host cells and is tightly associated with the adhesive protein TgMIC2. Mol Microbiol 2001;41(3):537–47.

[10] Bromley E, Leeds N, Clark J, McGregor E, Ward M, Dunn MJ, et al. Defining the protein repertoire of microneme secretory organelles in the apicomplexan parasite *Eimeria tenella*. Proteomics 2003;3(8):1553–61.

[11] Johnston PA, Liu H, O'Connell T, Phelps P, Bland M, Tyczkowski J, et al. Application in *in ovo* technology. Poult Sci 1997;76(1):165–78.

[12] Coletti M, Del Rossi E, Franciosini MP, Passamonti F, Tacconi G, Marini C. Efficacy and safety of an infectious bursal disease virus intermediate vaccine *in ovo*. Avian Dis 2001;45(4):1036–43.

[13] Wolff JA, Malone RW, Williams P, Chong W, Ascadi G, Jani A, et al. Direct gene transfer into mouse muscle *in vivo*. Science 1990;247(4949 Pt 1):1465–8.

[14] Castrucci G, Ferrari M, Marchini C, Salvatori D, Provinciali M, Tosini A, et al. Immunization against bovine herpesvirus-1 infection. Preliminary tests in calves with a DNA vaccine. Comp Immunol Microbiol Infect Dis 2004;27(3):171–9.

[15] Wong HT, Cheng SC, Sin FW, Chan EW, Sheng ZT, Xie Y. A DNA vaccine against foot-and-mouth disease elicits an immune response in swine which is enhanced by co-administration with interleukin-2. Vaccine 2002;20(21–22):2641–7.

[16] Kwang J, Zuckermann F, Ross G, Yang S, Osorio F, Liu W, et al. Antibody and cellular immune responses of swine following immunisation with plasmid DNA encoding the PRRS virus ORF's 4, 5, 6 and 7. Res Vet Sci 1999;67(2):199–201.

[17] Jenkins MC, Castle MD, Danforth HD. Protective immunization against the intestinal parasite *Eimeria acervulina* with recombinant coccidial antigen. Poult Sci 1991;70(3):539–47.

[18] Lillehoj HS, Choi KD, Jenkins MC, Vakharia VN, Song KD, Han JY, et al. A recombinant *Eimeria* protein inducing interferon-gamma production: comparison of different gene expression systems and immunization strategies for vaccination against coccidiosis. Avian Dis 2000;44(2):379–89.

[19] Song KD, Lillehoj HS, Choi KD, Yun CH, Parcells MS, Huynh JT, et al. A DNA vaccine encoding a conserved *Eimeria* protein induces protective immunity against live *Eimeria acervulina* challenge. Vaccine 2000;19(2–3):243–52.

[20] Min W, Lillehoj HS, Burnside J, Weining KC, Staeheli P, Zhu JJ. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. Vaccine 2001;20(1–2):267–74.

[21] Kopko SH, Martin DS, Barta JR. Responses of chickens to a recombinant refractile body antigen of *Eimeria tenella* administered using various immunizing strategies. Poult Sci 2000;79(3):336–42.

[22] Sharma JM. Embryo vaccination of chickens with turkey herpesvirus: characteristics of the target cell of early viral replication in embryonic lung. Avian Pathol 1987;16(4):367–79.

[23] Lillehoj HS, Ruff MD. Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with *Eimeria tenella*, *E. acervulina*, or *E. maxima*. Avian Dis 1987;31(1):112–9.

[24] Danforth HD, Augustine PC, Ruff MD, McCandliss R, Strausberg RL, Likel M. Genetically engineered antigen confers partial protection against avian coccidial parasites. Poult Sci 1989;68(12):1643–52.
[25] Kapczynski DR, Hilt DA, Shapiro D, Sellers HS, Jackwood MW. Protection of chickens from infectious bronchitis by *in ovo* and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Dis 2003;47(2):272–85.
[26] Sharma JM, Burmester BR. Resistance to Marek's disease at hatching in chickens vaccinated as embryos with the turkey herpesvirus. Avian Dis 1982;26(1):134–49.
[27] Sharma JM, Witter RL. Embryo vaccination against Marek's disease with serotypes 1, 2 and 3 vaccines administered singly or in combination. Avian Dis 1983;27(2):453–63.
[28] Sharma JM. Embryo vaccination of specific-pathogen-free chickens with infectious bursal disease virus: tissue distribution of the vaccine virus and protection of hatched chickens against disease. Avian Dis 1986;30(4):776–80.
[29] Weber FH, Evans NA. Immunization of broiler chicks by *in ovo* injection of *Eimeria tenella* sporozoites, sporocysts, or oocysts. Poult Sci 2003;82(11):1701–7.
[30] Weber FH, Genteman KC, LeMay MA, Lewis Sr DO, Evans NA. Immunization of broiler chicks by *in ovo* injection of infective stages of *Eimeria*. Poult Sci 2004;83(3):392–9.
[31] Watkins KL, Brooks MA, Jeffers TK, Phelps PV, Ricks CA. The effect of *in ovo* oocyst or sporocyst inoculation on response to subsequent coccidial challenge. Poult Sci 1995;74(10):1597–602.
[32] Hornok S, Szell Z, Sreter T, Kovacs A, Varga I. Influence of *in ovo* administered *Cryptosporidium baileyi* oocyst extract on the course of homologous infection. Vet Parasitol 2000;89(4):313–9.
[33] Dalloul RA, Lillehoj HS. Recent advances in immunomodulation and vaccination strategies against coccidiosis. Avian Dis 2005;49(1):132–9.
[34] Ryan R, Shirley M, Tomley F. Mapping and expression of microneme genes in *Eimeria tenella*. Int J Parasitol 2000;30(14): 1493–9.