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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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Keywords: DNA immunization; Chicken; Oocyst; Microneme; *In ovo*; *Eimeria*

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 isopropyl-