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# Plant expressed EtMIC2 is an effective immunogen in conferring protection against chicken coccidiosis

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ABSTRACT

Coccidiosis is an economically important disease affecting poultry industry and remains one of the major problems globally. Developing a cost effective sub-unit vaccine may help mitigate loss in the industry. Here, we report expressing one of the microneme proteins, EtMIC2 from *Eimeria tenella* in tobacco using *Agrobacterium*-mediated transient expression. The ability of plant expressed recombinant EtMIC2 in eliciting both humoral and cell-mediated immune responses were measured in the immunized birds. The protective efficacy in the vaccinated birds against a homologous challenge was also evaluated. Birds immunized with plant expressed EtMIC2 showed good sero-conversion, reduced oocyst output and increased weight gain when compared to control birds. Our data indicate that use of plant expressed recombinant EtMIC2 in birds was safe and had the potential in imparting partial protection in chickens against homologous challenge.



## 1. Introduction

Avian coccidiosis is an economically important disease of poultry caused by protozoa of the genus *Eimeria*. *Eimeria tenella* is one of the seven different *Eimeria* species that infect chicken. The infection of Eimeria species causes damage to the intestinal epithelium with varying severity accompanied by reduction in body weight, reduced feed conversion efficiency and shedding of parasite oocysts in feces. Use of 'coccidiostat' and 'coccidiocidal' chemicals in poultry feed is the popular method of managing coccidiosis. However, the emergence of drug resistant *Eimeria* strains is of serious concern [1]. In order to contain the indiscriminate use of coccidiostat, European parliament has set a target of 2012 to phase out the use of coccidiostat in poultry feed [2]. Live vaccines containing either virulent or attenuated strains of *Eimeria* or affinity purified oocysts antigens are available as alternatives to chemo-prophylaxis. However, their use is restricted to Breeder and Layer stocks in the poultry industry as the vaccines are produced in very limited quantity [3]. *Eimeria* infection involves multiple stages of parasite invasion [4]. One of the approaches in developing a prophylactic vaccine, explored by many investigators, is to block the parasite invasion into gut epithelium. Microneme organelles are located at the apical tip of invading stage of all apicomplexan parasites and they harbor several proteins that are critical for motility of the parasite, identification and binding of the host cell-–surface proteins and invasion of host cells [5]. Thus, induction of neutralizing antibodies to one or several of these 'invasion proteins' presents a rational approach in developing a prophylactic vaccine. Earlier reports suggest that the recombinant microneme antigens might protect chickens against coccidiosis when used as vaccine [4,6].

Plants have been used to express a wide variety of proteins for therapeutic and diagnostic use. The plant expression platform has been particularly attractive because of the ease of transformation, low investment, high and controlled level of expression, easy scale up with no process optimization downtime, etc. Plants have the ability of performing post-translational modifications and complete absence of parasite/pathogens that may harm animals or humans makes plant particularly attractive expression host for therapeutic proteins [7–10]. We have explored the possibility of using plant expression system for production of prophylactic vaccine against poultry coccidiosis.

Here, we report transient expression of one of the microneme proteins, EtMIC2 from *E. tenella* as $His_6$-tagged fusion protein, in tobacco using Agro-infiltration. Chicken were immunized using the plant expressed EtMIC2 protein and humoral and cell-mediated immune responses in the immunized birds were measured. Protective efficacy of the plant-expressed EtMIC2 antigen was also evaluated. The findings of our studies hold promise for developing

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a low cost sub-unit coccidiosis vaccine for the poultry industry.

## 2. Materials and methods

### 2.1. Chicken

Day-old, coccidiosis free, male White Leghorn layer chickens (commercial breed—BV 300) were obtained from Sri Venkateswara Hatcheries (Hyderabad, India) and reared in clean brooder cages. The birds were provided with coccidiostat-free feed and water *ad libidum*. Birds were shifted to animal containment facility prior to challenge with *E. tenella* sporulated oocysts.

### 2.2. Coccidial oocysts

Wild type *E. tenella* oocysts were isolated from an Eimeria-infected farm in India. Oocysts were propagated in 3 weeks old birds by repeated passages [4]. The purity of oocyst suspension was assessed using species-specific nested-PCR for ribosomal Internal Transcribed Spacer I (ITS-I) region as described by us earlier [11].

### 2.3. Tobacco plant

*Nicotiana tabacum*, cultivar Petit Havana SR1, was cultivated in the greenhouse using vermiculate peat moss mixture. Leaves from 3 to 6 weeks old plants (5–6 leaf stage) were used for vacuum infiltration.

### 2.4. Cloning of EtMIC2 gene into plant expression vector and creating Agrobacterium clone

#### 2.4.1. Total RNA isolation from oocyst

Birds inoculated with 10,000 *E. tenella* sporulated oocysts were sacrificed on day 7 post-inoculation and oocysts were isolated from the cecal content of infected birds. The cecal content was filtered through a sieve to remove coarse food material and the filtrate was overlaid on saturated NaCl solution and centrifuged at 1000 × *g* for 10 min. Pure oocysts were aspirated from the salt water interphase. The purified oocysts were decontaminated by treating them with 4% (v/v) hypochloride solution. These oocysts were sporulated in 2% potassium di-chromate solution by constant shaking (180 rpm) at 25 °C overnight [12]. Total RNA was isolated from the sporocyst using Trizol® reagent (Invitrogen, USA). cDNA was synthesized from total RNA using Thermo-script™ reverse transcriptase (Invitrogen, USA) and gene specific reverse primers for EtMIC2.

#### 2.4.2. Cloning into plant expression vector

The EtMIC2 gene was PCR amplified from the cDNA using forward primer 5′-AGCTCATGATGATGGCTCGAGCGTTGTCGCTGGT-3′ and reverse primer 5′-GCGGCCGCTCAGGATGACTGTTGAGTGTCACTCTC-3′. The RT-PCR amplified EtMIC2 coding sequence was cloned into plant expression vector, pTRA ERH (provided by Prof. Rainer Fisher) [13], downstream of the double 35S promoter using *Nco*I and *Not*I restriction enzyme sites. Since the EtMIC2 gene contains an internal *Nco*I site, the insert was digested using *Bsp*HI, which creates a compatible overhang for the vector digested with *Nco*I. pTRAEtM2, was sequenced from vector back-bone using the following primers, forward primer 5′-AAGACCCTTCCTCTATAT AAG-3′and reverse primer 5′-GAGCGAAACCCTATAAGAACC-3′ to confirm the cloning of EtMIC2. *Agrobacterium tumefaciens* strain GV3101 was electroporated with 5 μg of pTRA-ERH containing EtMIC2. The electroporation was performed using 2.5 kV, 200 ohms resistance and 25 μF capacitance in 2 mm cuvette (BTX, USA). Transformed *Agrobacterium* cells were plated on YEB plates [0.5% (w/v) peptone, 0.5% (w/v) beef extract, 0.5% (w/v) sucrose, 0.1% (w/v) yeast extract, 2 mM $MgSO_4$, 1.5% agar, pH 7.4] containing 100 μg/ml carbenicillin and 25 μg/ml rifampacin and incubated for 72 h at 28 °C. The Transformed *Agrobacterium* colonies were screened using gene-specific PCR to identify the recombinant clones.

### 2.5. Agrobacterium mediated transient expression of EtMIC2 protein

The recombinant *Agrobacterium* clone was grown overnight at 28 °C in YEB broth containing 100 μg/ml carbenicillin and 25 μg/ml rifampacin. Bacterial cells were harvested by centrifuging the overnight grown culture at 6000 rpm. The *Agrobacterium* pellet was inoculated in induction medium (YEB medium adjusted to pH 5.6 supplemented with 20 μM acetosyringone, 10 mM 2-N-morpholino-ethane-sulphonic acid) and incubated at 28 °C for 16 h. Following which the bacterial cells were harvested by centrifugation and the cells were resuspended in MMA medium containing (4.6 g/l Murashige and Skoog (MS) basal medium, 2% (w/v) sucrose, 10 mM MES, pH 5.6) supplemented with 200 μM acetosyringone to induce *vir* genes of *Agrobacterium*. The optical density of the suspension was adjusted to $OD_{600}$ 2 using the MMA medium and the bacterial suspension was incubated for 2 h at room temperature (24 ± 4 °C). Vacuum infiltration of the *Agrobacterium* culture was carried out as described by Kapila et al. [14]. The infiltrated leaves were incubated at 15 °C for 64 h under 16 h light and 8 h dark photoperiod. The leaves were stored at −80 °C until it was processed for protein extraction.

### 2.6. EtMIC2 gene expression in infiltrated tobacco leaves verified using RT-PCR

Total RNA was extracted from the infiltrated tobacco leaves using RNEasy plant-mini kit (Qiagen, USA) and the transcription specific mRNA was verified by RT-PCR using EtMIC2 specific primers. The total RNA was digested using RNase free DNase I to remove any contaminating DNA. A control reaction devoid of reverse transcriptase was used to rule out the PCR amplification from any residual contaminating DNA.

### 2.7. Extraction and purification of recombinant EtMIC2 protein from infiltrated tobacco leaves

The infiltrated tobacco leaves were ground into a fine powder after freezing the leaves in liquid nitrogen; the soluble proteins from the leaves were obtained by extracting in to two volumes of buffer containing (200 mM Tris–HCl, 5 mM EDTA, 0.1 mM DTT, 0.1% Tween20, pH 7.5). Cell debris from the leaf extract were removed by centrifugation at 20,000 × *g* for 30 min at 4 °C. A Solution of NaCl (500 mM) was added to the supernatant to prevent any non-specific interaction with the affinity matrix and pH of the extract was adjusted to 8.0 to precipitate plant cell proteins. The extract was incubated at 4 °C for 1–2 h and centrifuged at 15,000 × *g* for 30 min. The supernatant was filtered through Whatman filter paper (No-3) to trap any floating debris following which the extract was filtered through 0.45 μ filter and the filtrate was loaded to Hi-Trap metal chelating column with 0.5 ml/min linear flow rate using Akta prime plus (GE Healthcare, USA). Column was washed with 40 mM imidazole and protein was eluted using 500 mM imidazole. Six equal elution fractions of 0.5 column volume (2.5 ml) were collected. The purified protein was dialyzed extensively against PBS to remove imidazole. Yield of the purified protein was estimated using Bicinchoninic Acid kit (Sigma–Aldrich, USA). The purified EtMIC2 protein was stored in aliquots at −20 °C until further use.