Subunit Vaccine Based on Plant Expressed Recombinant *Eimeria* Gametocyte Antigen Gam82 Elicit Protective Immune Response against Chicken Coccidiosis

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Abstract

Avian coccidiosis is an intestinal tract infection caused by protozoan parasite of the genus *Eimeria* which is an economically important disease affecting poultry globally. In this study, E. maxima gametocyte antigen (Gam82) was expressed in tobacco leaves by agro-infiltration. The expression of Gam82 protein in plants was found to be 20 mg/kg fresh weight. The recombinant protein was purified by affinity column chromatography and its immunogenicity was evaluated in chickens. The birds immunized with plant purified plant expressed Gam82 protein showed 39% of increased weight gain and 69% of reduced oocyst output compared to control birds indicating that the plant expressed Gam82 antigen can elicit protective immune response in immunized birds. Our study demonstrated that the plant expressed Gam82 antigen can potentially be a candidate subunit vaccine against coccidiosis.

Keywords: Agroinfiltration; Eimeria gametocyte antigen; Recombinant protein; Subunit vaccine

Introduction

Coccidiosis is one of the major devastating protozoan diseases of chicken with severe weight loss, reduction in feed conversion and increase in mortality. The disease results in severe economic loss for the poultry industry worldwide [1]. Although preventive medications and anti-coccidial drugs are available to control coccidiosis in chickens, development of drug resistant organism and drug residues in meat and egg are the major limitations of the current control method [2,3].

Development of cost-effective recombinant subunit vaccine may help to reduce the loss in poultry industry. Gametocyte and microneme proteins of genus *Eimera* are reported to be possible vaccine candidates to control coccidiosis [4,5]. The efficacy studies of *E. coli* expressed *E. maxima* gametocyte antigen and plant expressed *E. tenella* microneme-2 proteins was reported earlier [6,7]. *Eimeria maxima* gametocyte antigens appears to have great potential as part of a maternally applied vaccine against coccidiosis [8,9]. Purified recombinant protein from *Eimeria maxima* gametocytes (Gam82) has the ability to stimulate immunity against experimental infection with live parasites.

Currently commercial protein production relies mainly on *E. coli*, mammalian, yeast and insect cell expression systems. Plant expression platform has been an alternative to conventional protein expression systems due to its ease of transformation, low initial investment, high recombinant protein expression, easy scale up and the plant expressed proteins can undergo post-translational modifications [10,11]. Earlier reports had also showed that the recombinant antigens expressed in plants could be used as subunit vaccines [7,12-14]. Present study was aimed at evaluating the plant expressed recombinant *E. maxima* gametocyte antigen Gam82 against chicken coccidiosis using a bird challenge experiment.

Materials and Methods

Chicken

One-day-old commercial layer breed-BV 300 was obtained from Sri Venkateswara Hatcheries, Hyderabad, India. Chickens were fed and maintained in a pathogen (*Eimeria*) free environment and reared in clean brooder cages.

Coccidial oocysts

Wild type *E. maxima* oocysts used in the present study were isolated from an *Eimeria* infected farm in India. Oocysts were propagated in 3 weeks old birds by repeated passages [15]. Purity of oocyst suspension was assessed by species-specific nested-PCR for ribosomal Internal Transcribed Spacer I (ITS-I) region as described earlier [16].

Cloning of *Gam82* gene into plant expression vector

E. maxima gametocyte antigen (Gam82) was amplified from a plasmid clone containing the full-length gene sequence of Gam82 [15] using Proof-start polymerase (Qiagen, USA). Gene specific primers flanked by BglII and NotI restriction sites (F-5'AAGCCAGATCTATGACGCGTGCGGCAGCGCTTG3' R-5'ATCTGCGGCCGCGTTGTATGTTTCCCATACAGT3') were used for the PCR amplification. The amplified products were ligated into plant expression vector pTRA-ERH and the plasmid clone was used to transform Agrobacterium cells (GV3101) by electroporation.

Agroinfiltration and RT-PCR

The seeds of Nicotiana tabacum, cultivar Petit Havana SR1 were germinated in the greenhouse. 4 to 6 weeks old plants were used for Agroinfiltration [17]. Total RNA was extracted from the infiltrated tobacco leaves using RNAEasy plant-mini kit (Qiagen, USA), three days post infiltration and the amplification of specific mRNA was verified by RT-PCR using gene specific primers.

SDS-PAGE analysis and immunoblotting

The plant expressed recombinant Gam82 protein was purified by affinity chromatography and resolved on SDS-PAGE. The protein was further confirmed by Western Blot probed either with anti-HIS antibodies (Qiagen, Germany) or rabbit polyclonal sera reactive against Gam82 protein. The protein was also electro-blotted on to PVDF membrane (Hybond-P; GE-Healthcare, USA). The tobacco leaves extract were used as negative control, while *E. coli* expressed recombinant Gam82 protein was used as positive control.

Immunization and efficacy study

The *in vivo* immunogenicity of plant produced recombinant antigen was evaluated in Chickens. The study consisted of three treatment groups. Birds in Group I were immunized intramuscularly with 50 µg of Gam82 protein with Freund's complete adjuvant whereas birds in Group II and III were used as control (PBS). Primary dose was followed by two booster doses on day 14 and 21. Number of birds in each group and treatment details are listed in Table 1.

Group	Immunogen (µg/bird)	Immunization (days)	Bleeding (days)	Challenge
Group-I (N=14)	Gam82 (50)	0, 7, 14, 21	0, 7, 14, 21, 28	20,000 oocyst/bird
Group-II (N=14)	PBS	0, 7, 14, 21	0, 7, 14, 21, 28	Unchallenged
Group-III (N=14)	PBS	0, 7, 14, 21	0, 7, 14, 21, 28	20,000 oocyst/bird

Table 1: Treatment groups and Immunization schedule

Humoral immune response

Blood was collected prior to each immunization and 7 days after final booster (28th day post primary immunization). Serum antibody titers of the immunized proteins were evaluated using *E. coli* expressed recombinant Gam82 protein to assess specific antibody titers.
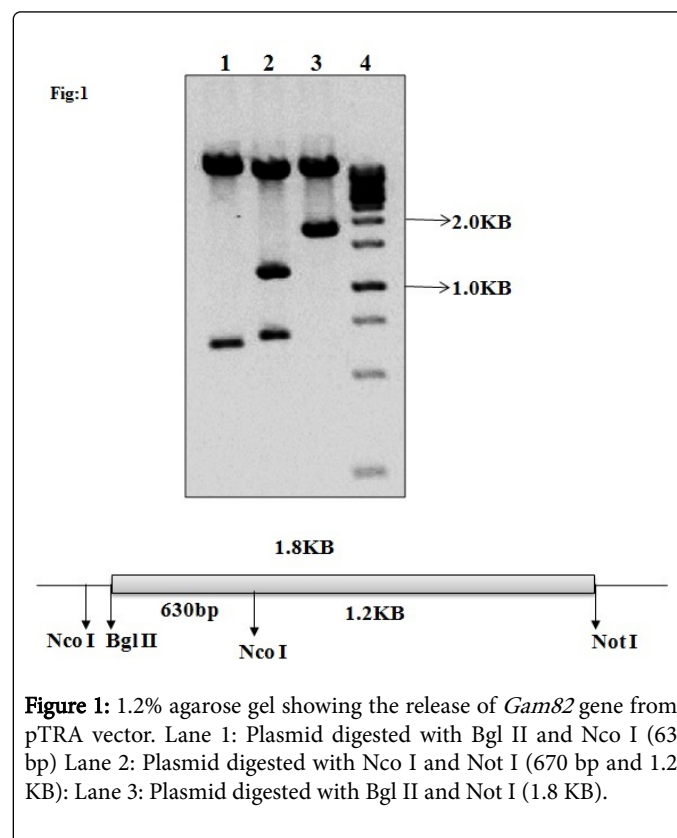
Animal challenge experiment

Nine days after second booster dose, treated and control groups were inoculated orally with 20,000 sporulated *E. maxima* oocysts. Birds were weighed prior to challenge and on 7th and 11th days post challenge to determine the weight gain. The average weight gain of birds and oocyst shedding per gram of fecal matter was determined as described earlier [15]. An average of three counts per group was taken to enumerate oocyst shedding. The percent decrease in oocyst output compared to the mock-immunized but challenged birds was estimated [15].

Results

Construction of recombinant plant expression vector and *Agrobacterium* transformation

The Gam82 coding sequence (1.8 kb) was amplified from a plasmid clone by PCR and cloned into plant expression vector pTRA-ERH (Figure 1). Then the plasmid was transformed into *Agrobacterium* cells and the recombinant *Agrobacterium* clones were confirmed by PCR using gene specific primers.

Figure 1: 1.2% agarose gel showing the release of *Gam82* gene from pTRA vector. Lane 1: Plasmid digested with Bgl II and Nco I (63 bp) Lane 2: Plasmid digested with Nco I and Not I (670 bp and 1.2 KB): Lane 3: Plasmid digested with Bgl II and Not I (1.8 KB).

Transient expression of Gam82 in *N. tabacum*

The *Agrobacterium* harbouring pTRA-ERH vector was infiltrated into plants by vacuum infiltration. Three days post infiltration, the total RNA was extracted from the infiltrated leaves and used as a template for RT-PCR. In RT-PCR analysis, 1.8 kb amplicon was observed indicating the presence of mRNA in the infiltrated leaves (Figure 2).
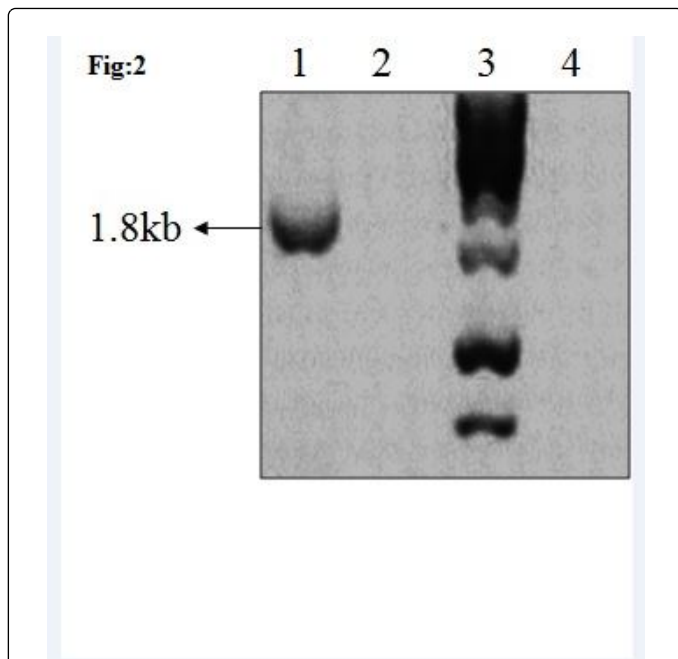
Figure 2: RT-PCR amplified product resolved on 1% agarose gel. Lane 1: Gam82 coding sequence of 1.8 KB from infiltrated leaves. Lane 2: RNA from Un-infiltrated leaves. Lane 3: Marker and Lane 4: Taq Polymerase control.

Figure 3a: PAGE showing plant expressed recombinant Gam82 protein stained with Coomasie blue.

Upon confirmation of expression, the recombinant protein from the infiltrated leaves was purified by Nickel-affinity column chromatography. The purified proteins were resolved in SDS-PAGE and confirmed by western blotting probed either with rabbit polyclonal antibodies against Gam82 protein or anti-His antibody. A protein band of approximately 82 kDa was observed in SDS-PAGE, corresponding to the size of Gam82 protein (Figures 3a and 3b).
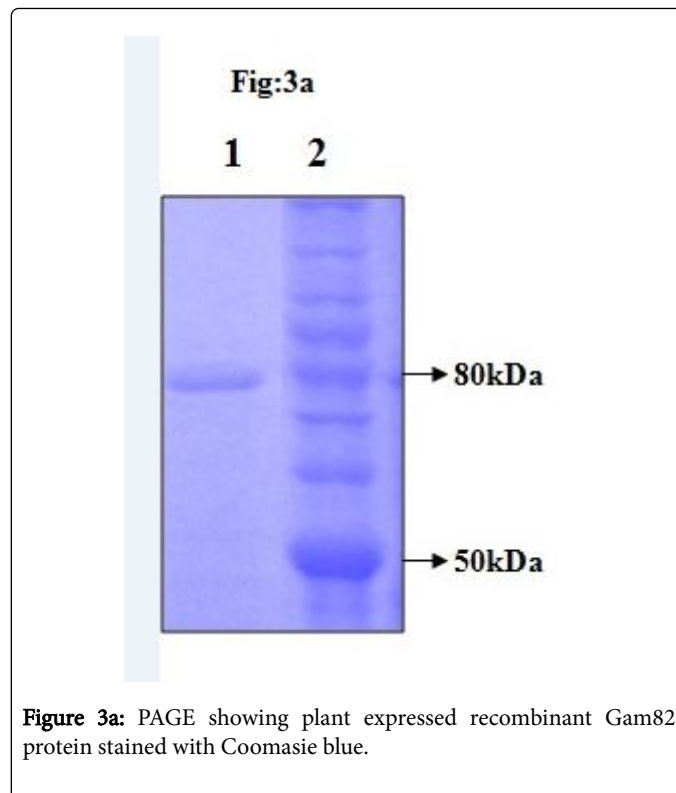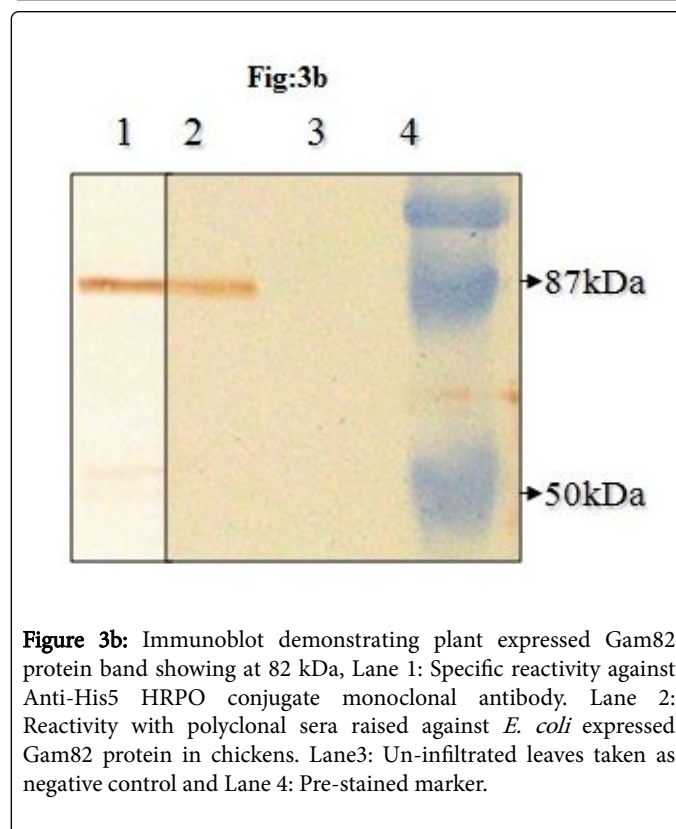

Figure 3b: Immunoblot demonstrating plant expressed Gam82 protein band showing at 82 kDa, Lane 1: Specific reactivity against Anti-His5 HRPO conjugate monoclonal antibody. Lane 2: Reactivity with polyclonal sera raised against *E. coli* expressed Gam82 protein in chickens. Lane3: Un-infiltrated leaves taken as negative control and Lane 4: Pre-stained marker.

Evaluation of humoral immune response

The serum samples were collected from the birds on 14, 21 and 28 days post vaccination and the samples were analyzed in ELISA for the presence of Gam82 specific serum antibodies. The mean serum antibody titers in immunized birds were 133.3 ($\pm$ 50), 344.4 ($\pm$ 206.8) and 1333.3 ($\pm$ 800) on 14, 21 and 28 DPV respectively (Figure 4).
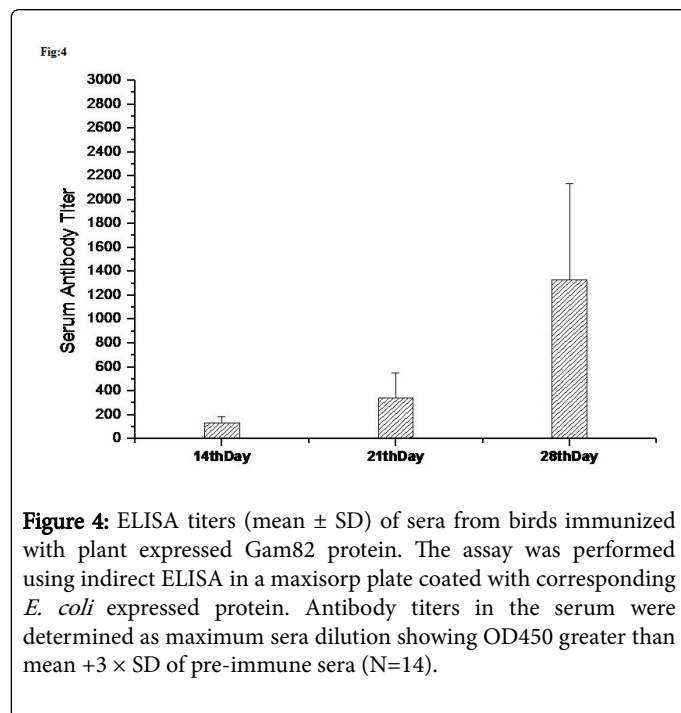

Figure 4: ELISA titers (mean $\pm$ SD) of sera from birds immunized with plant expressed Gam82 protein. The assay was performed using indirect ELISA in a maxisorp plate coated with corresponding *E. coli* expressed protein. Antibody titers in the serum were determined as maximum sera dilution showing OD450 greater than mean +3 $\times$ SD of pre-immune sera (N=14).

Bird challenge experiments

Weight gain: Birds in Groups I, and III were challenged with 20,000 virulent *E. maxima* oocysts. Weight gain was assessed on 7 and 11 days post-challenge (dpc). The percentage increase in weight gain in the immunized birds compared to control group (Group II and III) was determined. It was observed that the birds immunized with plant purified Gam82 had 37.2% ($\pm$ 52) and 20.6% ($\pm$ 27) increase in weight gain on days 7 and 11 post-challenge. The difference in the mean weight gain was subjected to Student's t-test. The mean weight gain was significantly different in all immunized birds on 7 dpc when compared with mock-immunized and challenged group (p<0.05*; N=14) (Figure 5).

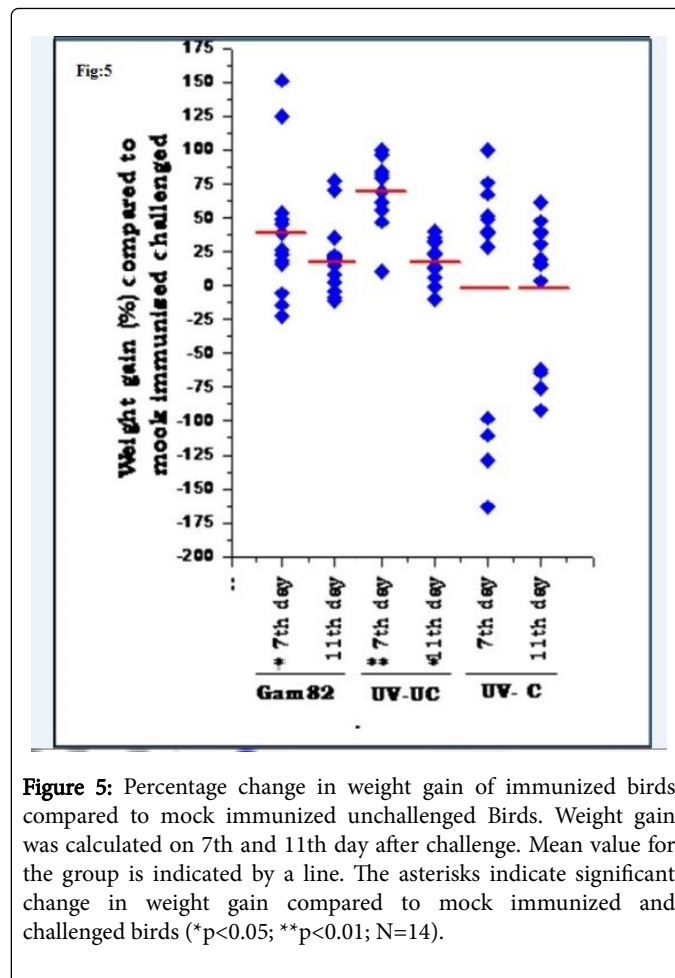**Figure 5:** Percentage change in weight gain of immunized birds compared to mock immunized unchallenged Birds. Weight gain was calculated on 7th and 11th day after challenge. Mean value for the group is indicated by a line. The asterisks indicate significant change in weight gain compared to mock immunized and challenged birds (*p<0.05; **p<0.01; N=14).

Evaluation of oocyst output: Oocyst output of the immunized birds was compared with the oocyst output of control birds (Group II and III) after live *Eimeria* challenge. Immunization of birds with plant produced recombinant Gam82 reduced the oocyst output by 69%. These results revealed that immunization of birds with the plant expressed recombinant gametocyte antigens imparted partial protection in chicken against parasite challenge (Figure 6).
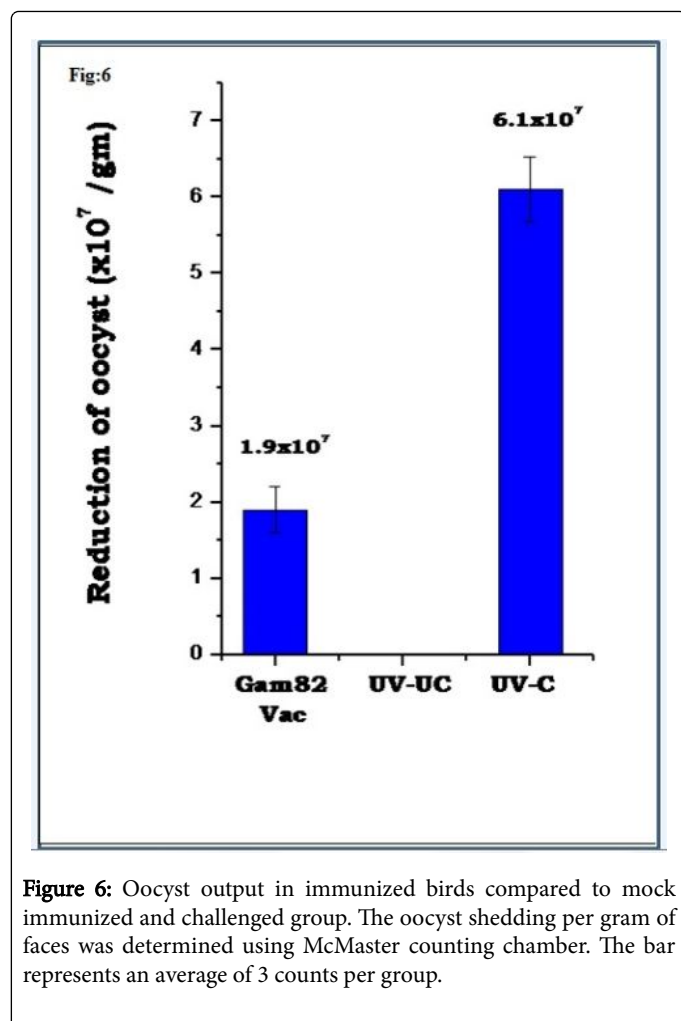
Figure 6: Oocyst output in immunized birds compared to mock immunized and challenged group. The oocyst shedding per gram of faces was determined using McMaster counting chamber. The bar represents an average of 3 counts per group.

Discussion

Coccidiosis is one of the major hindrances in the growth of poultry industry, as it affects the birds in both clinical and subclinical forms. Though medications are available to control the disease, the excessive usage of drugs may cause some potential side effects, such as drug residues in the product, emergence of resistant strains of *Eimeria* etc., which can be overcome by vaccination strategies. Recombinant subunit vaccines are used as a substitute to whole cell vaccines. However, large scale production of recombinant subunit proteins is essential to meet the global vaccination demand. Plant expression system is preferred as an attractive alternative for the large scale production of recombinant proteins in a short period at much reduced cost. Many vaccine candidates are expressed in plants and shown that plant purified recombinant proteins elicit potent immune response in animal models [7,18-20].

Gam82 is 82kDa tyrosine-rich sexual stage glycoprotein of *E. maxima*, which is responsible for oocyst wall formation. Affinity purified gametocyte antigens including Gam82 from the sexual stage of *E. maxima* have been proved as potential vaccine targets in inducing transmission-blocking immunity [21]. Earlier studies reported that the gametocyte antigens are immunogenic and can be used as vaccine candidates to control coccidiosis [21,22]. The study conducted to evaluate the effects of *in vivo* vaccination with an *E. maxima*

gametocyte recombinant protein, Gam82, on host immunity following live-challenge infection, promoted cell mediated immunity against experimental coccidiosis and reduced fecal oocyst shedding [23].

In the present study, we attempted to evaluate the *in vivo* immunogenicity of plant expressed *Eimeria maxima* gametocyte antigen Gam82. The *Gam82* gene was cloned in pTRA-ERH expression vector and the protein was expressed in the leaves of *N. tabacum* by agro-infiltration. The recombinant protein was purified from the plant leaves by nickel affinity chromatography. The purified protein was confirmed in SDS-PAGE and western blot probed with anti-HIS antibody and anti-Gam82 polyclonal sera. The yield of purified protein was found to be 20 mg/kg of leaves. Plant expressed recombinant Gam82 protein had induced high serum antibody response on 14, 21 and 28 days of post immunization in chickens when given as monovalent vaccine. Serum antibody response raised against plant expressed Gam82 protein was evaluated in ELISA using *E. coli* produced Gam82 as coating antigen. A challenge experiment was performed with live parasite in chickens after immunization. The average weight gain in vaccinated birds was higher both on 7th day and 11th day post challenge, compared to unvaccinated birds (Figure 5). We observed that there was reduction of up to 69% of oocyst output from birds immunized with Gam82 protein (Figure 6). Our earlier work showed that the recombinant EtMIC1 and EtMIC2 protein expressed in plants elicit potent immune response which can be used as a subunit vaccine to control coccidiosis [7,24]. Earlier studies showed that the antibodies raised against gametocyte antigens play a major role in protection against homologous challenge [25,26]. The results obtained by immunizing *E. coli* produced recombinant Gam82 protein [23] are consistent with our results.

In summary, our study showed that plant produced recombinant gametocyte protein Gam82 was immunogenic and induced partial protection against live parasite challenge. This study explores the possibility of using plant produced recombinant Gam82 as a subunit vaccine against avian coccidiosis. As, coccidiosis is a complex disease that includes multiple stages of parasite invasion; inclusion of two or more recombinant subunit proteins as multivalent vaccine may help in complete protection against the disease.

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