ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 9 (11): 1031-1037, 2010
ISSN 1682-8356

Development and Evaluation of an ΔaroA / ΔhtrA *Salmonella enteritidis* Vector Expressing *Eimeria maxima* TRAP Family Protein EmTFP250 with CD 154 (CD 40L) as Candidate Vaccines against Coccidiosis in Broilers

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Abstract: Coccidiosis is caused by parasites of the genus *Eimeria*, belonging to phylum Apicomplexa. EmTFP250 is a high molecular mass, asexual stage antigen from *Eimeria maxima* (EM) strongly associated with maternal immunity in newly hatched chickens. Cloning and sequence analysis predict the antigen to be a novel member of the Thrombospondin-Related Adhesive Protein (TRAP) family. Three novel attenuated *Salmonella enteritidis* strains (ΔSE) expressing TRAP oligopeptides in association with a potential immune-enhancing CD 154 sequence, on the outer membrane protein lamB, were developed. Broiler chicks were grouped based on treatment and 10^8 cfu/chick of vectors expressing one of three sequences, or vehicle alone, was orally administered to each group. At 21 d of age, all groups were challenged with 10^4 sporulated oocysts/chick orally. Mortality at 5d post-challenge was markedly different ($p<0.05$) in chickens vaccinated with TRAP Upstream (US). To further evaluate the efficacy of TRAP US as a potential vaccine candidate, a similar study was conducted. Broilers were orally vaccinated with 10^8 cfu/chick vehicle with TRAP US and CD 154 or sham vaccinated with saline. Coccidia challenge was performed with 10^5 sporulated oocysts/chick at 22 d of age. Immunized chickens showed remarkable improvement in weight gain ($p<0.05$) and had reduced mortality ($p = 0.055$) when compared to non-immunized controls. These two studies underscore the potential of EmTFP250 as a potential candidate for a recombinant vaccine targeting coccidiosis in chickens.

Key words: *Eimeria*, *Salmonella*, coccidiosis, apicomplexa, TRAP, vaccination

INTRODUCTION

Coccidiosis continues to be one of the most important enteric diseases in the commercial poultry industry, causing large scale economic losses in the range of $800 million worldwide and $450 million in the United States annually (Allen and Fetterer, 2002). The disease is caused by parasites of *Eimeria spp.*, representing phylum Apicomplexa whose members are characterized by the presence of an "apical complex" (Escalante *et al.*, 1995; Li *et al.*, 2004). Coccidiosis manifests in the GIT, affecting performance with subsequent increases in feed conversion ratio and mortality. Each *Eimeria spp.*, colonizes specific areas of the gut depending on its specificity (Yun *et al.*, 2000).

The life cycle of *Eimeria spp.*, is complex and involves both intracellular and extracellular stages. The parasite is known to colonize the intestinal epithelium and hence, the primary line of host defense is Mucosal Associated Lymphoid Tissue (MALT) (Konjufca *et al.*, 2006). Immunity to the disease is complex and involves many facets of the host immune system. There is definite interplay between humoral and cell-mediated immunity, even though it is accepted that cell-mediated immunity is most important (Yun *et al.*, 2000; Lillehoj *et al.*, 2004; Hong *et al.*, 2006). Conventional approaches of disease control have employed prophylactic medications in the form of chemotherapy, antibiotics, anticoccidials and selection of disease resistant strains of chickens (Bumstead *et al.*, 1995; Yun *et al.*, 2000; Hong *et al.*, 2006; Konjufca *et al.*, 2006). However, with the ability of parasites to develop drug resistance, researchers are involved in designing alternative methods of disease prevention and control (Talebi and Mulcahy, 1994; Allen and Fetterer, 2002; Blake *et al.*, 2006). In this regard, vaccination against coccidiosis has become a key aspect of present research (Talebi and Mulcahy, 1994; Allen and Fetterer, 2002; Smith *et al.*, 1994).

Species of *Eimeria* are potently immunogenic and are capable of eliciting a strong immune response (Yun *et al.*, 2000; Allen and Fetterer, 2002). Recently, the use of recombinant vectored vaccines for disease control and protection has been well documented. Simple approaches to design and construction have been evaluated and used successfully in experimental

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models and a large number of parasite antigens have been employed as vaccine candidates to confer protection (Song *et al*., 2000). We have adopted a simple approach to design vectored vaccine candidates in our laboratory. Recombinant *Salmonella enteritidis* (SE) was designed by specific deletion of two virulence genes and insertion of parasite epitopes, along with a co-stimulatory molecule (CD 154). In the past, similar studies involving epitopes from *E. acervulina* and *E. tenella*, expressed in attenuated, recombinant *Salmonella spp*., have been carried out with a considerable amount of success (Konjufca *et al*., 2006; Konjufca *et al*., 2008).

Thrombospondin related adhesive protein is a highly well-characterized protein that is part of the microneme complex of all apicomplexan parasites. Its most important function is to assist in sporozoite invasion, which are crucial phases of the parasite life-cycle. Sporozoites of *P. berghei* are known to leave a trail of TRAP molecules on a microscopic slide when incubated at 37°C (Spaccapelo *et al*., 1997). Specific antibodies generated against TRAP interfere with sporozoite motility and halt the progression of infection. Numerous TRAP family proteins have been identified in several apicomplexans including *T. gondii* and the *EtMIC4* protein described in *E. tenella* (Tomley *et al*., 2001; Witcombe *et al*., 2003). This is particularly important in species of *Eimeria* because, conventional vaccines do not offer protection against a mixed coccidial infection (Konjufca *et al*., 2006; Shirley *et al*., 2007). The conserved nature of TRAP sequences among apicomplexan members makes it a highly suitable vaccine candidate not only for coccidiosis, but may have implications in several other diseases such as malaria or toxoplasmosis.

The ability of oral *Salmonella*-based vectors to elicit mucosal immune responses has previously been observed (Ashby *et al*., 2005; Layton *et al*., 2009). With increasing importance being given to the active role of mucosal immunity against enteric pathogens, use of this vector system may be feasible to achieve optimum protection. The objective of these studies was to evaluate the efficacy of a potential TRAP epitope from *E. maxima* in combination with CD 154 as a suitable vaccine candidate against coccidiosis in broilers.

MATERIALS AND METHODS

Attenuation of *Salmonella* vaccine candidate strains: A poultry isolate of SE phage type 13A (SE13A) obtained from the USDA National Veterinary Services Laboratory (Ames, Iowa) was attenuated by introducing defined, irreversible deletion mutations in the *aroA* and/or *htrA* gene of the SE genome as previously described by Husseiny and Hensel (2005) and modified by Layton and co-workers (2009). Briefly, the target gene sequence in the bacterial genome was replaced with the kanamycin-resistant (Km^R) gene sequence. This was performed using 3S-PCR and electroporation of the 3S-PCR products into electrocompetent *Salmonella* cells containing the pKD46 plasmid. The resulting cell mixture was plated on Luria-Bertani agar (LB) plates (catalog no. 240110, Becton Dickinson, Sparks, MD) supplemented with Km (50 μg/mL-catalog no. K-1876, Sigma, St. Louis, MO) to select for positive clones containing a Km^R gene. The Km^R gene was inserted into the genomic region containing the genes of interest (*aroA* or *htrA*) by flanking the Km^R gene with sequences homologous to the genes of interest. Once Km^R mutants were obtained, deletion mutations were confirmed by PCR and DNA sequencing (data not shown).

Construction of recombinant TRAP-CD154 inserts: Recombinant strains of SE13A containing stable integrated copies of a TRAP-CD 154 insert, TRAP Upstream (TRAP US)-CD 154 insert and a TRAP Downstream (TRAP DS)-CD 154 insert were constructed using the method described by Cox *et al*. (2007). Briefly, an I-SceI enzyme site along with a Km^R gene was introduced into Loop 9 of the *lam*B gene by design of a PCR product which had the I-SceI enzyme site and Km^R gene flanked by approximately 200-300 base pairs of DNA on each side, homologous to the up and downstream regions of Loop 9. The PCR product was electroporated into electrocompetent attenuated *Salmonella* cells containing the pKD46 plasmid and the resulting cell mixture was plated on LB agar plates supplemented with Km to select for positive clones now containing a Km^R gene. After the I-Sce-I/Km mutation was made in Loop 9, this region was replaced by a codon-optimized TRAP-CD 154 DNA sequence (Burns and Beacham, 1985). This second 3S-PCR reaction produced a TRAP-CD 154 insert flanked by Loop 9 up and downstream regions and the resulting PCR product was electroporated into electrocompetent SE13A containing the I-Sce-I/Km mutation described above. Plasmid pBC-I-SceI was also electroporated into the cells along with the insert as the plasmid produces the I-SceI enzyme which recognizes and cleaves a sequence creating a gap at the I-SceI enzyme site in the Loop 9 region of the *lamB* gene where the previously described sequences were inserted into the SE13A genome (Kang *et al*., 2004). The plasmid also carries with it a chloramphenicol resistant gene (Cm^R) as the inserts that will replace the Km^R gene the mutations must have a new selection marker to counter-select against the previous I-SceI/Km mutation. After electroporation, cells were plated on LB agar plates containing chloramphenicol at 34 μg/mL (catalog no. C0378, Sigma, St. Louis, MO) for the selection of positive mutants. Once positive mutants/inserts were identified, PCR and DNA sequencing were performed to confirm that the insertion sequences were present and correct (data not shown).

Confirmation of cell surface expression of recombinant inserts: Cell surface expression of the recombinant inserts were confirmed with a simple (+/-) antibody/antigen precipitation reaction using antisera generated against protein conjugates with the synthetic peptide. Aliquots of vaccine strains were made in Tryptic Soy Broth (TSB) (catalog no. 211822, Becton Dickinson, Sparks, MD) supplemented with 30% sterile glycerol and stored at -80°C until further use.

Eimeria dose titration: *Eimeria maxima* oocysts (Strain M6) were propagated *in vivo* according to previously published methods (Long *et al*., 1976; Martin *et al*., 1997). A preliminary dose titration study was carried out, offset by 1 week, to determine the *Eimeria* challenge selection for the present study. Briefly, 14 d old broilers were weighed, divided into three groups and challenged with three different doses of sporulated oocysts of EM by oral gavage. A fourth group of chicks were sham challenged with saline. One wk post-challenge, Body Weight (BW), Body Weight Gain (BWG) and lesion scores were determined. Based on the criterion that the challenge dose caused a 30-35% depression in weight gain when compared to saline-challenged controls, a single dose was chosen (Data not shown). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas.

Immunization study 1: For immunization, aliquots of vaccine strains of *Salmonella* were grown overnight in 10 ml of TSB at 37°C. This was followed by passaging the vaccine strains for a period of 24 h at intervals of 8h for each passage. The cells were recovered by centrifugation at 1864 x g for 15 min at 4°C (3X), the pellet was resuspended in 1 ml of sterile saline and the final volume made up to 25 ml with sterile saline. The concentration of the inoculum was measured spectrophotometrically (Spectronic 20 d) at 625 nm and kept on ice until further use. In addition, serial dilutions of vaccine strains were plated on Brilliant Green Agar (BGA) (catalog no. 228530, Becton Dickinson, Sparks, MD) plates supplemented with Novobiocin (NO- 25 µg/mL) (catalog no. N1628, Sigma, St. Louis, MO) and Nalidixic Acid (NA-20 µg/mL) (catalog no. N4382, Sigma, St Louis, MO) to count viable colonies.

In a preliminary immunization study, 280 day-of-hatch Cobb 500 broiler chicks were obtained from a local commercial hatchery and randomly assigned to one of four treatment groups: 1) Control, saline only, 2) SE13A Δ*aroA*/Δ*htrA* TRAP-CD 154, 3) SE13A Δ*aroA*/Δ*htrA* TRAP US-CD 154, or 4) SE13A Δ*aroA*/Δ*htrA* TRAP DS-CD 154; (N = 70/pen). Each treatment group was neck-tagged, housed in an individual floor pen on fresh pine litter and provided water and feed *ad libitum*. On day-of-hatch, all chicks in treatment groups 2, 3 and 4 were inoculated, via oral gavage, with 0.25mL/chick of a solution containing 1.2 x 10^8 cfu of the appropriate treatment. Chicks in group 1 were sham vaccinated with the same volume of saline. At 3d post-hatch, ten chicks from each treatment group were chosen randomly and euthanized humanely. The liver, spleen and cecal tonsils were aseptically removed and cultured for determination of organ invasion and recovery of the *Salmonella* vaccine strains as described previously (Layton *et al*., 2009). At 21 d post-hatch, all chickens were weighed and orally challenged with 1 x 10^4 sporulated oocysts of EM/chick and all treatments were co-mingled in a single, large pen.

The experiment was terminated at 26 d post-hatch and all chickens were weighed before termination and macroscopic lesions evaluated as per the standard Johnson and Reid scale (1970). The scoring pattern was as follows: 0- no lesions, 1- mild lesions with faint red petechiae on the mucosa, 2- moderate lesions with extensive petechiae formation, general redness of mucosa and thickened intestinal wall, 3- severe lesions with slime formation, orange mucus and inflammation and 4- hemorrhagic lesions with increased slime formation, reddish mucus and thickened mucosa.

Immunization study 2: Based on the results of the first immunization study, another study was carried out to assess the efficacy of TRAP US-CD 154 as a potential vaccine candidate against coccidiosis. Vaccine strains were prepared as described previously. 240 day-of-hatch Cobb 500 broiler chickens were obtained from a local hatchery and randomly assigned to one of four groups (N = 60/pen): 1) Saline only (Non-challenged) - NV, NC, 2) SE13A *aro*A/*htr*A TRAP US-CD 154 (Non-Challenged) - V, NC, 3) Saline only (Challenged) - NV, C and 4) SE13A *aro*A/*htr*A TRAP US-CD 154 (Challenged) - V, C. Immunization and vector recovery procedures were consistent with study 1. At 22d post-hatch, all chickens were weighed and groups 3 and 4 were orally challenged with 1 x 10^5 sporulated oocysts of EM/chick. Groups 1 and 3 were sham challenged with saline and co-mingled in one, large pen 24 h post-challenge. At 7dpi, all chickens were weighed and the gut was examined for the presence of macroscopic lesions.

Statistical analysis: BW, BWG and lesion score data from the immunization studies were subjected to ANOVA using JMP7 (© 2007, SAS institute, Cary, NC), partitioned and treatment means were deemed significant if the p-value was less than or equal to 0.05 ($p \leq 0.05$). Mortality data were compared using the chi-square test of independence testing all possible group combinations to determine significance ($p \leq 0.05$) for these studies (Zar, 1984).

Table 1: Peptide sequences used to construct recombinant *Salmonella* vectors[1]

Construct	Peptide sequence
TRAP-CD154	GGGFPTAAVA-WAEKGYYTMS
TRAP US-CD154	AAPETRAVQPKPEEGHERPEPEEEEEKKEEGGGFPTAAVA-WAEKGYYTMS
TRAP DS-CD154	GGGFPTAAVAGGVGGVLLIAAVGGGVAAFTSGGGGAGAQE-WAEKGYYTMS

[1]Cox *et al*. (2007)

Table 2: Organ invasion, colonization and clearance of attenuated recombinant *Salmonella* vaccine vectors expressing TRAP-CD 154[†] after oral immunization in broilers evaluated at 3 days post-hatch (Experiments 1 and 2)

	3 days post-hatch (%)	
Treatment	L/S	CT
Experiment 1		
Control	0/10	0/10[b]
TRAP-CD154	0/10	10/10 (100)[a]
TRAP US-CD154	0/10	9/10 (90)[a]
TRAP DS-CD154	0/10	9/10 (90)[a]
Experiment 2		
NV, NC	0/10	0/10[b]
V[‡], NC	0/10	10/10 (100)[a]
NV, C	0/10	0/10[b]
V[‡], C	0/10	9/10 (90)[a]

Incidence of the vaccine strains is represented as the percentage of the number of positive Liver and Spleen (L/S) and Cecal Tonsils (CT) samples. Liver and spleen were analyzed as pooled samples. All immunized chicks received an oral dose of 1.2 x 10^8 cfu of the respective vector at day of-hatch (N = 10/trt).

[†]Table 1.

[‡]TRAP US-CD 154. NV, NC: Non-vaccinated, non-challenged; V, NC: Vaccinated, non-challenged; NV, C: Non-vaccinated, challenged; V, C: Vaccinated, challenged.

[a,b]Means with different letters within the same column indicate difference ($p<0.05$)

Table 3: Body Weight Gain (BWG), lesion scores and percent mortality in broilers immunized with a recombinant vaccine expressing TRAP-CD154[†] vectors against coccidiosis (Experiments 1 and 2)

Experiment 1			
Treatment	BWG D21-26	Lesion scores	Mortality
Control	265.4±13.6[a]	2.04±0.3[a]	21.7[a]
TRAP	250.2±17.1[a]	2.25±0.12[a]	16.3[ab]
TRAP US	261.5±14.5[a]	2.14±0.11[a]	2.2[c]
TRAP DS	252.9±18.5[a]	2.33±0.11[a]	11.0[b]
Experiment 2			
Treatment	BWG D22-29	Lesion scores	Mortality
NV, NC	393.2±17.2[b]	1.7±0.3[b]	0
V[‡], NC	484.4±17.3[a]	1.4±0.1[b]	0
NV, C	273.0±10.2[c]	2.4±0.1[a]	5.5*
V[‡], C	446.2±13.0[ab]	2.4±0.1[a]	0

BWG (g) and lesion scores expressed as means±standard error. All chicks were immunized with the respective vector at hatch and *Eimeria* challenge was performed either at 21 or 22 d of age. BWG was evaluated during the challenge period in both experiments.

[†]Table 1.

[‡]TRAP US-CD 154; Mortality expressed as percentage of death/total chickens. Asterisk indicates a p-value of 0.055. NV, NC: Non-vaccinated, non-challenged; V, NC: Vaccinated, non-challenged; NV, C: Non-vaccinated, challenged; V, C: Vaccinated, challenged.

[a,b]Means with different letters within the same column indicate difference ($p<0.05$)

RESULTS

Organ invasion and colonization of vector strains: Liver, spleen and cecal tonsils enriched in Tetrathionate broth overnight and then streaked on BGA NO/NA resulted in typical lactose-negative colonies of *Salmonella* at 3 d post-hatch in ~90% of the immunized chickens in both experiments. Non-immunized chickens remained negative for *Salmonella* through the entire period of study (Table 2).

Immunization study 1: This study evaluated the potential of three TRAP vectors to ameliorate coccidiosis specifically caused by an EM challenge. BW was recorded prior to EM challenge and at 5 d post-challenge to determine total BWG. The treatment groups showed no differences in terms of either final BW or BWG 5 d post-challenge. However, mortality was significantly reduced in chicks vaccinated with the TRAP US and CD 154 vector and was different from the control, challenged group as well as the two other TRAP vector groups ($p<0.05$). Lesion scores demonstrated no differences between the different treatment groups (Table 3).

Immunization study 2: To corroborate the previously observed protective effects of the TRAP US and CD154 vector, a second immunization study was conducted. BW was evaluated prior to challenge and one week post-challenge. All groups started out with uniform body weights on the day of challenge. Beneficial effects on performance was observed with a significant increase in BWG ($p<0.05$) in the immunized, challenged chickens when compared to the control, challenged chickens (Table 3). In addition, mortality was evaluated in the two challenged groups and there was increased mortality in the non- immunized group when compared to the vaccinated group with a p value of 0.055 (Table 3). No differences were observed in lesion scores between individual treatments.

DISCUSSION

Coccidiosis in poultry is common and has global importance in the commercial industry. Vaccination against coccidiosis has become one of the most sought out aspects of modern day poultry research and is being considered as a viable option for disease control. Ideally, the vaccine candidate should be able to stimulate a significant amount of immune response,

one that is capable of offering long-term protection. New and improved vaccine delivery methods are constantly being tested for their efficacy (Konjufca *et al*., 2006).

Attenuated *Salmonella* strains as vaccine delivery vectors have been effectively used in several experimental models. *Salmonella* is known to colonize the cecum in about 12h and liver/spleen in about 24-48h post-immunization (Cheeseman *et al*., 2008). In addition, it is capable of stimulating mucosal, humoral and cell-mediated immunity by effectively colonizing the gut tissues (Ashby *et al*., 2005). The use of attenuated bacteria that can spark a self-limiting infection leading to the development of sustainable immune responses is a viable alternative to conventional live/attenuated vaccines (Spreng *et al*., 2006). Previous studies done in our laboratory and by other groups have successfully incorporated viral epitopes and other *Salmonella spp*., expressing *Eimeria* epitopes as potential vaccine candidates (Layton *et al*., 2009; Konjufca *et al*., 2006; Konjufca *et al*., 2008).

TRAP is known to be homologous and well conserved in almost all apicomplexan members and therefore the potential of this candidate being able to offer protection against other apicomplexan parasites may be considerable (Tomley *et al*., 2001; Witcombe *et al*., 2003; Carruthers and Boothroyd, 2007). Additionally, the use of CD 154 (CD 40L) as an adjuvant bolsters host immune response. CD 154 is an integral membrane protein expressed only on activated T cells. Activation of its receptor CD40 expressed on the surface of macrophages, dendritic cells and B-cells leads to a plethora of activities including clonal expansion of B-cells, cytokine production and enhanced antigen presentation (Quezada *et al*., 2004). Studies carried out in mice indicate that a lack of CD40/CD40L interaction leads to the absence of naïve T-cell priming thereby suggesting a pivotal role in sustenance of host immune responses (Xu *et al*., 2010). Previous work in our laboratories has indicated that CD154 expression on a *Salmonella* vector may improve in quicker vector clearance and immune responsiveness (O' Meara *et al*., 2010).

Acute mortality is one of the hallmark characteristics of coccidiosis. It is important that vaccines decrease the incidence of mortality and also have beneficial effects on feed absorption and weight gain. Therefore, our studies predominantly focused on reducing mortality and benefitting performance. In the first study, broilers immunized with TRAP US demonstrated reduced ($p = 0.03$) mortality when compared to the non-immunized controls. Similarly, in the second study mortality in the vaccinated group was reduced ($p = 0.055$) (Table 3).

BWG was also evaluated with regard to vaccine candidate efficacy. Body weights in all groups were similar on the day of challenge (d21-Exp. 1; d22-Exp. 2, data not shown). In the first experiment, there were no differences between groups in BWG of surviving chickens (Table 3). However, in the second experiment, vaccinated chickens gained significantly more weight during the challenge period ($p<0.05$) as compared to non-immunized controls in both the presence and absence of an *Eimeria* challenge (Table 3). In experiment 1, the challenge-induced mortality was high (21.7% in non-vaccinated controls) within the first 5 days post-challenge. In contrast, the challenge dose in the second experiment caused a mere 5.5% mortality in the non-vaccinated challenged group (Table 3) and the challenge period was extended to 7 days, possibly allowing for more time to result in measurable declines in BWG in the second experiment. Importantly, all groups were co-mingled during the challenge period, potentially allowing for horizontal transmission of oocysts during the last days of this 7-day challenge period in experiment 2 (Table 3). Indeed, coccidial lesions were detected in all groups (including non-challenged groups) by day 29 when lesions were evaluated. Thus, it may not be surprising that vaccination improved BWG even in the vaccinated but non-challenged group since indirect challenge obviously occurred during the challenge period.

While coccidiosis lesions were seen in all groups in both experiments, no differences were observed in the severity of lesions between treatment groups in either experiment (Table 3). In most situations, lesion scores have not been well correlated with the protective effects of vaccines (Williams and Catchpole, 2000). This may be due to immunopathology in the vaccinated broilers causing interference with the ability to accurately determine lesion scores. As a matter of fact, what one sees as lesions in immunized chicks may actually be the process of recovery and tissue regeneration. Therefore, histopathological analysis of tissue samples by differential staining may be a much more accurate method for understanding gross pathology rather than relying on macroscopic lesions. Such evaluations should be included in any further exploration of this potential vaccine vector approach.

In both experiments, a *Salmonella*-vectored *Eimeria* vaccine was able to markedly reduce aspects of disease caused by a EM challenge. Further studies will necessarily evaluate the ability of the vectored vaccine candidate to offer cross protection against other species of *Eimeria*, majorly *E. tenella* and *E. acervulina*, because these two species along with EM have been considered most important in the commercial industry. As the TRAP-US sequence used in these experiments is conserved within known sequences of EM and the phylogenetically disparate *E. tenella*, it is possible that this sequence will show similar activities with other *Eimeria* species. Additionally, the possibility of vectoring these and perhaps other epitopes in completely non-pathogenic bacteria is currently being investigated.

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