Evaluation of 4 merozoite antigens as candidate vaccines against *Eimeria tenella* infection

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ABSTRACT Coccidiosis, caused by parasites of the genus *Eimeria*, is one of the most widespread and economically detrimental diseases in the global poultry industry. Because the merozoite stage of *Eimeria tenella* is immunologically vulnerable, motile, and functionally important for the parasites, the proteins expressed in these stages are considered to be potentially immunoprotective antigens, especially the secreted antigens and surface antigens. Here, we detected a previously unidentified MIC2-associated protein (Et-M2AP) from *E. tenella* and determined its localization. An immunofluorescence assay revealed that Et-M2AP was distributed in the apical part of second generation merozoites and sporozoites. In addition, an expression profile analysis revealed that the transcriptional level of Et-M2AP is significantly higher in the merozoite stage. To assess the potential of Et-M2AP protein as a coccidiosis vaccine, we expressed recombinant Et-M2AP (**rEt-M2AP**) and compared the immune protective efficacy of rEt-M2AP with 3 surface antigens that are highly expressed by merozoites (rEt-SAG23, rEt-SAG16, and rEt-SAG2 proteins). The immune protective efficacy of these vaccine candidates was assessed based on survival rate, lesion score, BW gain, relative BW gain, and oocyst output. The results show that the survival rate was 90%, which are significantly higher than those in the challenge control group. The BW gain rate was 42% ($P < 0.001$) in rEt-M2AP–immunized chickens, which are significantly higher than those in the challenge control group and rEt-SAG23, rEt-SAG16, and rEt-SAG2 proteins–immunized chickens. In addition, chickens immunized with rEt-M2AP (88% oocyst output decrease rate, $P < 0.001$) had the least oocyst output, compared with those immunized with rEt-SAG16 (59.2% oocyst output decrease rate, $P < 0.001$), rEt-SAG23 (22% oocyst output decrease rate), and rEt-SAG2 (1.36% oocyst output decrease rate). These results demonstrate that rEt-M2AP provided effective protection against challenge with *E. tenella*, suggesting that rEt-M2AP is a promising candidate antigen gene for development as a coccidiosis vaccine.

Key words: MIC2-associated protein, *Eimeria tenella*, merozoite, immune protection

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INTRODUCTION

Eimeria species, protozoan parasites that can infect chickens, cause a considerable disease burden worldwide (Clark et al., 2017). *Eimeria tenella* is highly pathogenic and is one of the most prevalent species of *Eimeria* infecting chickens (Witcombe and Smith, 2014). *E. tenella* infection can result in severe lesions of the caeca, BW loss, hemorrhagic diarrhea, and death (Witcombe and Smith, 2014; Song et al., 2017). Efforts to control *Eimeria* infection have predominantly relied on anticoccidial drugs. However, the rise of drug resistance and public pressure for restrictions on chemicals used in foodborne animal continues to drive the development of anticoccidial vaccines (Williams, 2002; Blake and Tomley, 2014; Dong et al., 2016). Therefore, there is an urgent need to develop a safe and effective vaccine against avian coccidiosis (Blake et al., 2017; Lin et al., 2017; Venkatas and Adeleke, 2019).

E. tenella is an obligate intracellular apicomplexan parasite, which contains secretory organelles such as rhoptries, micronemes, and dense granules. These secretory organelles produce a large number of secretory proteins that mediate parasite invasion and survival

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(Lebrun et al., 2014). The surface antigens (**SAG**) and secreted proteins from these secretory organelles function in attachment and invasion during host–parasite interaction, so they are recognized as potential vaccine antigens (Zhao et al., 2020). In addition, the extracellular stages of *E. tenella* merozoites are immunologically vulnerable, motile, and functionally important for the parasite life cycle (Rafiqi et al., 2018). Thus, screening antigens that are highly expressed in the merozoite stage (especially surface antigens and secreted antigens) is a reasonable approach for anticoccidial vaccine development (Clark et al., 2016).

Several recent studies have investigated the potential of merozoite proteins for development into a coccidiosis subunit vaccine. Profilin is a conserved surface antigen of both merozoites and sporozoites that can induce cell-mediated immunity against live *Eimeria* stages (Tang et al., 2018). Profilin also induced protective immunity against *E. tenella* in chickens (Lillehoj et al., 2017), both when used as an anticoccidial vaccine candidate and when applied as an adjuvant (Gowen et al., 2006; Lee et al., 2011; Lillehoj et al., 2017; Bussière et al., 2018). EtSAG4 is specifically expressed in the second-generation merozoite stage of *E. tenella* (Tabarés et al., 2004), and it was able to protect chickens against an *E. tenella* challenge; thus, EtSAG4 might be a candidate gene for use in a vaccine against coccidiosis (Tabarés et al., 2004; Zhao et al., 2020). In the past few decades, numerous microneme proteins such as MIC1, MIC2, and MIC4 have also been evaluated; however, all of these proteins only partially protected against coccidiosis (Du et al., 2005; Subramanian et al., 2008; Chen et al., 2018; Yan et al., 2018).

Here, we expressed a total of 4 *E. tenella* antigens, 1 secreted antigen and 3 surface antigens, all of which are highly expressed during the merozoite stage of this parasite. The protective efficacy of each antigen was evaluated via subsequent challenge with *E. tenella*.

MATERIALS AND METHODS

Ethics Statement

Animal experiments in this study was approved by the Beijing Administration Committee of Laboratory Animals and performed in accordance with the China Agricultural University Institutional Animal Care and Use Committee guidelines (approval number: AW05(7) 069102-2).

Parasites and Animals

E. tenella was maintained and propagated in the Key Laboratory of Animal Parasitology (Beijing City, China) using 2-wk-old specific pathogen free chickens. The oocysts were collected and purified as previously described (Long et al., 1976). Sporozoites and merozoites were also purified as previously described (Hu et al., 2018). The chickens and BALB/c mice were purchased from Merial Animal Health Co., Ltd. (Beijing, China), raised in a coccidia-free environment, and provided with sterilized food and clean water ad libitum.

Bioinformatic Analysis

The nucleotide sequences and amino acid sequences of Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 were downloaded from ToxoDB (ETH_00006930, ETH_00008670, ETH_00013140, and ETH_00034890, respectively) (https://toxodb.org/toxo/). The amino acid sequence alignment was performed using the Basic Local Alignment Search Tool in National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Transcriptional level data for Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 were obtained from the ToxoDB uploaded by Walker et al (Walker et al., 2015). The signal peptide was analyzed using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/).

Cloning, Expression, and Purification *of Et-M2AP*

The full coding sequence of Et-M2AP without the signal peptide sequence was amplified from merozoites cDNA. The plasmid pET28a (+) was linearized by PCR. The two fragments were ligated using seamless cloning (Vazyme Biotech, Co., Ltd., Nanjing) and transformed into *Escherichia coli Transetta* (DE3) cells (TransGen, Beijing). The recombinant Et-M2AP protein (**rEt-M2AP**) was expressed and purified using a Ni^{2+} affinity column following the manufacturer's protocol. The amplification, expression, and purification procedures used for rEt-SAG23, rEt-SAG2, and rEt-SAG16 were the same as those previously used for rEt-M2AP.

Sera

Negative sera were obtained from coccidia-free specific pathogen free chickens. Positive sera against *E. tenella* were collected from SFP chickens that were artificially infected with *E. tenella*. Briefly, each chicken was immunized with 2000 *E. tenella* oocysts orally and immunized again 2 wk later. Polyclonal antibody against rEt-M2AP was produced as previously described (Li et al., 2016). Briefly, BALB/c mice were immunized subcutaneously with rEt-M2AP emulsified in Freund's complete adjuvant (Sigma, St Louis, MO) with 3 boosters. The final vaccination was used Freund's incomplete adjuvant (Sigma, St Louis, MO). After final vaccination, the titers of polyclonal antibodies were examined by ELISA using rEt-M2AP as the antigen and purified using HiTrap Protein A affinity chromatography (Bio-Rad).

Immunoblotting and Immunofluorescence Assay

Purified rEt-M2AP, rEt-SAG23, rEt-SAG16, and rEt-SAG2 proteins were separated via 12% SDS-PAGE and

transferred onto a nitrocellulose membrane (Millipore). The membrane was incubated with chicken serum against *E. tenella* (1:500) and horseradish peroxidase–conjugated goat anti-chicken IgY (Bio-Rad) for 1 h at 37°C. The membrane was then reacted with chemiluminescence reagents (CoWin Biotech Co., Ltd., Beijing) to observe the protein bands. Healthy (preimmune) mouse serum was used as a control.

The immunofluorescence assay was performed as previously described with several improvements (Liu et al., 2018). Briefly, the purified sporozoites and merozoites were adhered to coverslips precoated with poly-lysine, fixed with 4% (w/v) paraformaldehyde, and permeabilized with 0.1% Triton. The coverslips were then blocked with 3% BSA and incubated with primary mouse anti-Et-M2AP (1:200) for 1 h at 37°C. After washing, the coverslips were treated with fluorescein isothiocyanate- or Cy3-conjugated antibodies used for labeling (Sigma) and Hoechst 33,258 (Sigma). The images were obtained using a Leica confocal microscope system (TCS SP52; Leica, Germany).

Evaluation of Protective Efficacy

To evaluate the efficacy of the 4 recombinant proteins (rEt-M2AP, rEt-SAG23, rEt-SAG16, and rEt-SAG2), animal experiments were performed (Figure 1). Seven-day-old chickens were divided indiscriminately into 6 groups, each comprising 10 birds (n = 10). These six groups included 4 recombinant protein (rEt-M2AP, rEt-SAG23, rEt-SAG16, rEt-SAG2)–immunized group, challenged group, and unchallenged group. All chickens were fed with a coccidia-free diet and water in sufficient supply. Different groups of chickens were kept in different cages. The cages were kept at a constant temperature in isolators. Each chicken in the test groups was intramuscularly injected with 50 μg purified recombinant protein, whereas those in the challenged group and the unchallenged group were inoculated with PBS. One week later, a booster immunization was administered via an intramuscular injection into the leg of the same amount of antigen used in the first immunization. Seven d after the booster immunization, the chickens in the test and challenged groups were challenged with 1×10^4 sporulated *E. tenella* oocysts. Those in the unchallenged group were mock-challenged with PBS. The protective effects of each of the 4 recombinant proteins were evaluated based on BW gain, survival rate, lesion score, and oocyst output

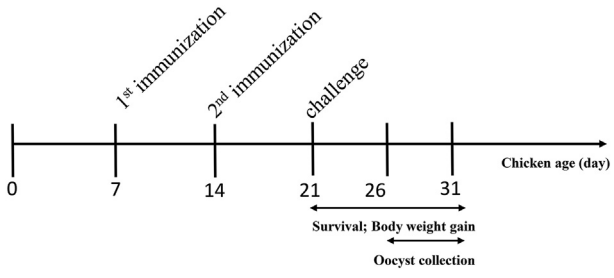

Figure 1. Schematic outline of the experimental design.

decrease rate. The cecum lesion score was assessed as per the method of Johnson and Reid (Johnson and Reid, 1970). The McMaster's counting technique was used to count the oocyst output. Total feces of each group were collected, mixed, and weighed from the sixth to tenth day after challenge. Three samples were randomly selected to calculate the oocysts per gram per sample. The average amount of oocyst output per chicken was calculated. Oocyst output decrease rates were calculated as follows: (oocyst output from positive control chickens - oocyst output from vaccinated chickens) × 100/oocyst output from positive control chickens. BW gain of chickens in each group was determined by the BW of chickens at the end of experiment (day 31) subtracting the BW at the time of challenge (day 21). BW gain of chickens was calculated as follows: weight at the time of slaughter − weight at the time of challenge. The relative BW gain rate was calculated as follows: (weight gain of the experimental group × 100)/weight gain of the unchallenged group. Three independent experiments were performed.

Statistical Analysis

Graphs were created by GraphPad Prism (San Diego, CA). Graphs present the means, and the error bars show the standard errors of means. All data were analyzed with SPSS statistical package (IBM SPSS Statistics 19) using 1-way ANOVA Duncan test. All *P*-values of <0.05 were considered to be significant.

RESULTS

Selection and Characterization of 4 Merozoite Antigens

Prior research on other vaccines suggests that surface antigens and secreted proteins make good vaccine candidates. Here, we selected 4 antigens, specifically 1 secreted antigen (ETH_00006930) and 3 surface antigens (Et-SAG16, Et-SAG23, and Et-SAG2) to access their potential utility as vaccine candidates. Sequence alignment revealed that ETH_00006930 shared homology with M2AP from *Toxoplasma gondii*, so it was renamed Et-M2AP. Transcriptional level data for Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 were obtained from the ToxoDB and showed highly expressed at the merozoite stage (Figure 2A). Because Et-M2AP had not been previously identified in *E. tenella*, localization studies were performed on it. We applied an immunofluorescence method using mouse anti-rEt-M2AP antibody and found that endogenous Et-M2AP was distributed in the apical parts of *E. tenella* merozoites and sporozoites (Figure 2B).

Cloning and Expression of the 4 Merozoite Proteins

The cDNA encoding the 4 candidate merozoite genes (Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2) were

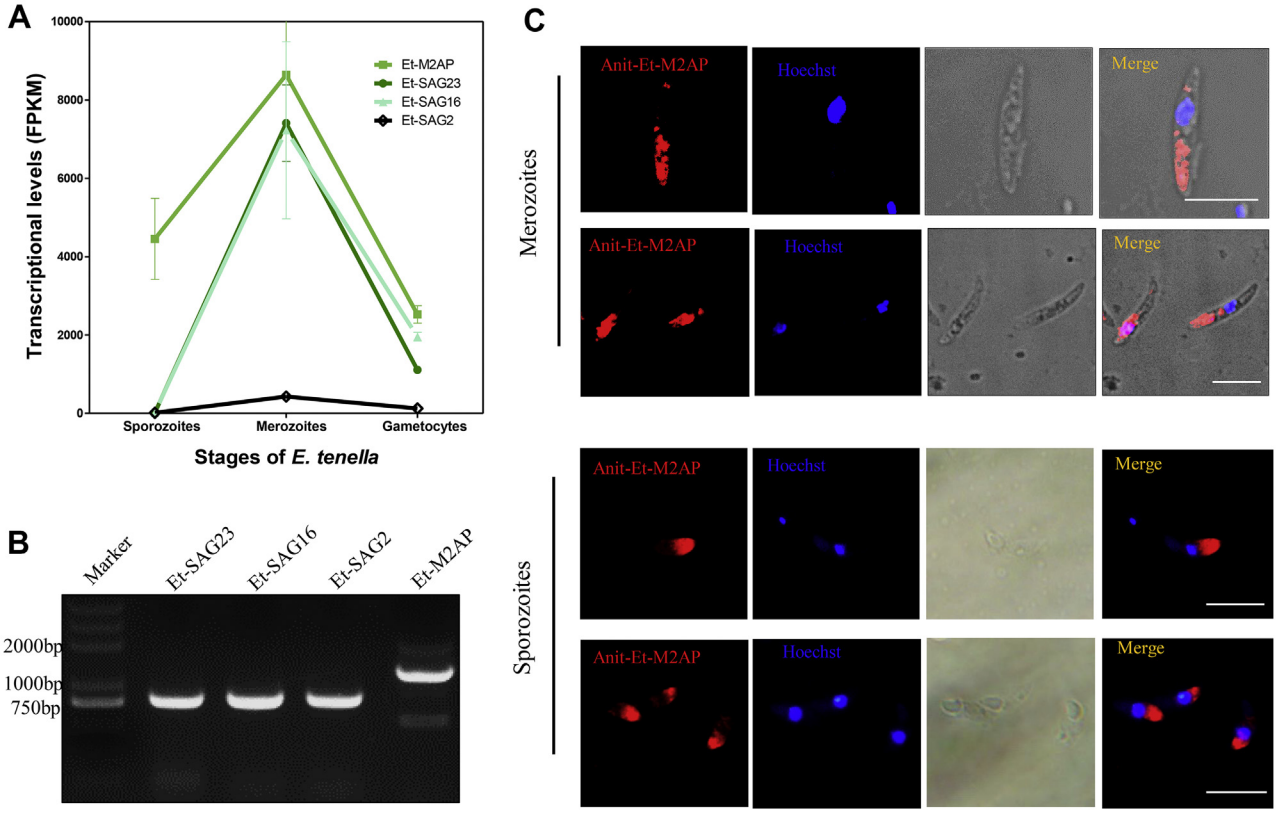

Figure 2. Characterization of 4 merozoiteantigens. (A) The transcription levels of Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 proteins in merozoites and gametocytes as obtained from ToxoDB. (B) PCR amplification of Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 genes. (C) Immunofluorescence using mouse anti–rEt-M2AP serum was performed to investigate the location of Et-M2AP in sporozoites and merozoites. Scale bars: 10 μm. Labeling of Et-SAG and Et-SAG16 (red) served as a parasite surface marker.

1,026 bp, 810 bp, 804 bp, and 759 bp in length (Figure 2C), corresponding to 342, 270, 268, and 253 amino acid residues, respectively. The respective predicted molecular masses of these proteins were ∼38 kDa, ∼30 kDa, ∼30 kDa, and ∼28 kDa. There was a signal peptide cleavage site in the Et-M2AP sequence, which indicates that Et-M2AP is a secretory protein. The predicted molecular weight of Et-M2AP without the signal peptide was ∼34 kDa. The four recombinant proteins (rEt-M2AP, rEt-SAG23, rEt-SAG16, and rEt-SAG2) were expressed in *E. coli*, and the sizes of the resulting products were found to be ∼40 kDa, ∼36 kDa, ∼36 kDa, and ∼34 kDa (including a His-tag), respectively, which are similar to their predicted sizes (Figure 3A).

Western Blot Analysis of the 4 Recombinant Merozoite Proteins

The four recombinant proteins (rEt-M2AP, rEt-SAG23, rEt-SAG16, and rEt-SAG2) were successfully identified by a Western blot performed with anti-*E. tenella* chicken serum, using healthy chicken serum as a control. As shown in Figure 3B, the anti-*E. tenella* chicken serum identified a specific band for each of the 4 recombinant proteins. No specific band was detected by the healthy chicken control serum.

Recombinant Et-M2AP Induced Effective Protection Against E. tenella

To analyze the immune protection provided by rEt-M2AP, rEt-SAG23, rEt-SAG16, and rEt-SAG2 against *E. tenella*, challenge experiments were performed and various markers of disease severity were assessed. The survival rate of the immunized groups were improved compared with the control group (Figure 4A). Vaccination with rEt-M2AP, rEt-SAG23, rEt-SAG16, or rEt-SAG2 resulted in an average BW gain of 25.12 g ($P < 0.001$), 17.6 g ($P < 0.001$), −3.5 g ($P > 0.05$), or 11.9 g ($P < 0.001$), respectively, and a corresponding relative BW gain rate of 42% ($P < 0.001$), 30% ($P < 0.001$), −6% ($P > 0.05$), and 20% ($P < 0.001$) compared with unchallenged chickens. The average BW of the challenged unvaccinated control group decreased (−27 g), and the corresponding relative BW was decreased 45.5%. Therefore, the BW of the chickens immunized with rEt-M2AP, rEt-SAG23, or rEt-SAG2 were significantly higher ($P < 0.001$) than those of the control group chickens (Figures 4B–4C). In addition, compared with the birds in the control challenged group, chickens vaccinated with rEt-SAG16 ($P < 0.05$) had significantly lower cecum lesion scores, whereas those immunized with rEt-M2AP, rEt-SAG23 ($P > 0.05$), or rEt-SAG2 ($P > 0.05$) did not (Figure 4D). The oocyst output was evaluated using McMaster's counting

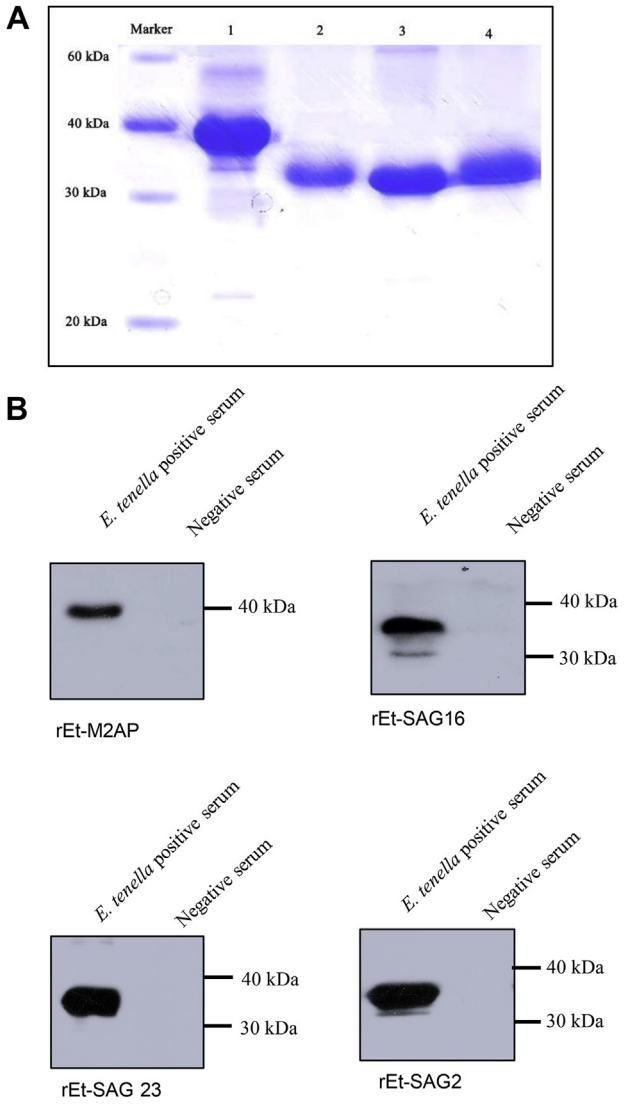

Figure 3. SDS-PAGE and Western blotting analysis. (A) Purification of recombinant proteins. SDS-PAGE gel: Lane 1, purified rEt-M2AP protein; Lane 2, purified rEt-SAG23 protein; Lane 3, purified rEt-SAG2 protein; Lane 4, purified rEt-SAG16 protein. (B) Western blotting analysis of recombinant proteins.

technique. The resulting data show that the oocyst output of the groups immunized with rEt-M2AP (88% oocyst output decrease rate, $P < 0.001$) or rEt-SAG16 (59.2% oocyst output decrease rate, $P < 0.001$) were significantly lower than that of the control challenge group, whereas the oocyst output decrease rates were only 22 and 1.36% in the rEt-SAG23-and rEt-SAG2-immunized groups, respectively (Figures 4E–4F).

DISCUSSION

E. tenella is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa, and it has unique secretory organelles (micronemes, rhoptries, and dense granules) to produce proteins for the invasion process (Carruthers and Boothroyd, 2007; Lebrun et al., 2014). Micronemes are small vesicles that cluster in the apical portion of the parasite and rapidly secrete a large number of proteins when initial contact is made between the parasite and the host cell (Li et al., 2015). The microneme proteins act as major cellular adhesion factors for host cells and participate in parasite recognition, reorientation, and entry; thus, they are reasonable vaccine candidates (Peng et al., 2009; Liu et al., 2010; Li et al., 2015; Pinzan et al., 2015; Zhang et al., 2015). As Et-M2AP was a previously unexplored protein in *E. tenella*, we first performed localization studies on it. Endogenous Et-M2AP was observed to localize in the apical portion of merozoites and sporozoites, which is consistent with the known distribution characteristics of other microneme proteins (Li et al., 2015; Yang et al., 2015).

Our strategy for vaccines is to block parasite infection by disturbing the invasion and development of *E. tenella* merozoite stage. The secreted proteins and surface antigens are recognized as good candidates based on the general characteristics. Thus, 1 secreted protein (Et-M2AP) and 3 surface antigens (Et-SAG23, Et-SAG16, and Et-SAG2) that are highly expressed at the merozoite stage were selected in our study. Here, we successfully obtained recombinant Et-M2AP, Et-SAG23, Et-SAG16, and Et-SAG2 proteins and assessed the immune protective effect of each when administered as a vaccine against *E. tenella*. Our results show that vaccination with rEt-M2AP improved the survival rate and BW gain of chickens challenged with *E. tenella*. Meanwhile, the oocyst output was significantly lower in birds vaccinated with rEt-M2AP than in control-challenged chickens. Together, these findings suggest that vaccination with rEt-M2AP provided effective protection against *E. tenella*. There was also a significant reduction in the oocyst output of the rEt-SAG16–immunized chickens; however, the BW gain indicators in these birds are not good. Weight loss in *E. tenella*–infected chickens is common because the invasion and reproduction of parasites causes intestinal damage. Therefore, the inability of rEt-SAG16 to prevent infection-induced reductions in weight gain suggests that it could not effectively prevent the invasion and reproduction of *E. tenella* parasites. Otherwise, the protective immunity of rEt-SAG23 and rEt-SAG2 proteins was not satisfactory comparing with rEt-M2AP. The groups immunized with rEt-SAG23 and rEt-SAG2 protein showed significantly increased BW. However, vaccination with rEt-SAG23 and rEt-SAG2 was not effective in oocyst shedding and cecum lesion, which suggests that rEt-SAG23 and rEt-SAG2 did not protect the chicken from *Eimeria* infection, or did it prevent transmission between chickens.

Over the past few decades, numerous microneme proteins such as MIC1, MIC2, and MIC4 have also been evaluated; however, all of them protected only partially against coccidiosis (Du et al., 2005; Subramanian et al., 2008; Chen et al., 2018; Yan et al., 2018). Profilin (3-1E) is a conserved surface antigen of both merozoites and sporozoites, and it can also act as an anticoccidial adjuvant (Lee et al., 2010, 2012; Zhang et al., 2012). Profilin is able to induce cell-mediated protective immunity against *Eimeria* species (Lillehoj et al., 2017; Tang

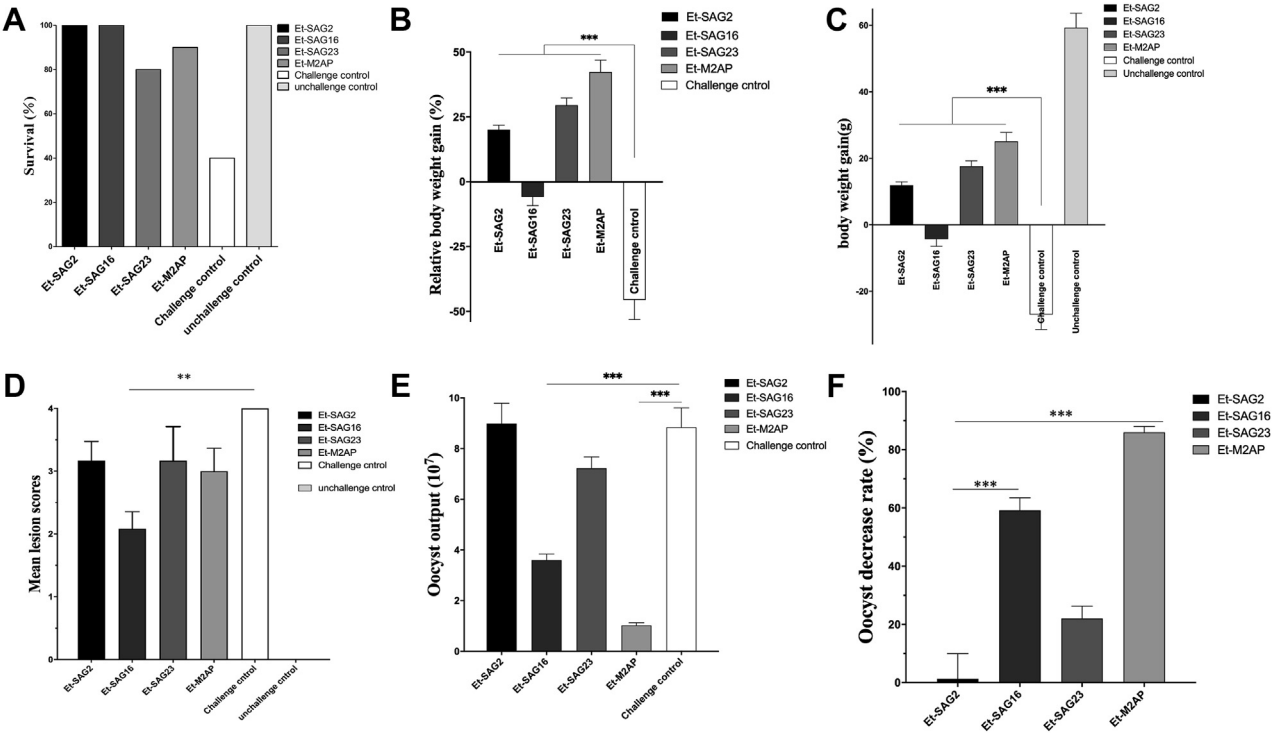

Figure 4. Protective efficacy against *Eimeria tenella* infection of the 4 merozoite antigens. (A–F) The effects of vaccination with 4 merozoite antigens on survival rate (A), cecum lesion score (B), BW gain (C), relative BW gain (D), oocyst output (E), and oocyst decrease rate (F). Three independent experiments were performed. All data were analyzed with ANOVA: $^{**}P < 0.01$, and $^{***}P < 0.001$.

et al., 2018). Compared with profilin, the oocyst output of rEt-M2AP–immunized chickens were lower (Ma et al., 2013). The relative BW gain rates were 79.3 and 64.4% in *Bacillus subtilis* expressing *E. tenella* 3-1E protein (B.S-pBS-H1-3-1E) and *Lactococcus lactis* expressing *E. tenella* 3-1E protein (*L. lactis* pTX8048-3-1E), respectively (Ma et al., 2013; Lin et al., 2015). The relative BW gain rate of rEt-M2AP in the present study was 42%. It is worth noting that different *E. tenella* strains have different levels of virulence; our challenge dose resulted in a weight loss of 46% in the unimmunized control group and a mortality rate of 60%, whereas the previous study using *B. subtilis* and *L. lactis* expressing *E. tenella* 3-1E protein reported weight gain rates of 64.5 and 43.83% in the respective unimmunized control groups. Thus, compared with unimmunized chickens, the rEt-M2AP–immunized chickens in our study had a weight gain of ∼88%, whereas the chickens immunized with B.S-pBS-H1-3-1E and *L. lactis* pTX8048-3-1E in the other study had weight gains of ∼15 and 21%, respectively. EtSAG4 is specifically expressed in the second-generation merozoite stage of *E. tenella* (Tabarés et al., 2004), and it is recognized as a competent vaccine candidate gene against coccidiosis (Tabarés et al., 2004; Zhao et al., 2020). Here, the oocyst reduction rate of chickens vaccinated with the rEtSAG4 protein was 75.5%, which is lower than that of rEt-M2AP–immunized chickens. Similarly, the relative BW gain was also lower in rEtSAG4 protein–immunized chickens as compared with the challenge control (Zhao et al., 2020).

In summary, we identified 4 merozoite proteins, including a microneme antigen and 3 surface antigens, and assessed their immunoprotective effects when administered as vaccines. The resulting data indicate that rEt-M2AP has potential for use as an effective vaccine candidate against *E. tenella* infection, and rEt-SAG23, rEt-SAG16, and rEt-SAG2 proteins were not suitable for vaccine candidate.

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Data Availability Statements: All data sets generated for this study are included in the manuscript/supplementary files.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.12.001.

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