

RESEARCH ARTICLE

Open Access



# Specific EtMIC3-binding peptides inhibit *Eimeria tenella* sporozoites entry into host cells

Wenjing Chen<sup>1,2†</sup>, Chunli Ma<sup>3†</sup>, Guanghao Li<sup>1</sup>, Zhipeng Jia<sup>1</sup>, Xuelian Yang<sup>1</sup>, Xinghui Pan<sup>1</sup> and Dexing Ma<sup>1,2\*</sup> 

## Abstract

Avian coccidiosis caused by *Eimeria* leads to huge economic losses on the global poultry industry. In this study, microneme adhesive repeat regions (MARR) bc1 of *E. tenella* microneme protein 3 (EtMIC3-bc1) was used as ligand, and peptides binding to EtMIC3 were screened from a phage display peptide library. The positive phage clones were checked by enzyme-linked immunosorbent assay (ELISA). Competitive ELISA was applied to further verify the binding capability between the positive phages and recombinant EtMIC3-bc1 protein or sporozoites protein. The inhibitory effects of target peptides on sporozoites invasion of MDBK cells were measured in vitro. Chickens were orally administered with target positive phages and the protective effects against homologous challenge were evaluated. The model of three-dimensional (3D) structure for EtMIC3-bc1 was conducted, and molecular docking between target peptides and EtMIC3-bc1 model was analyzed. The results demonstrated that three selected positive phages specifically bind to EtMIC3-bc1 protein. The three peptides A, D and W effectively inhibited invasion of MDBK cells by sporozoites, showing inhibited ratio of 71.8%, 54.6% and 20.8%, respectively. Chickens in the group orally inoculated with phages A displayed more protective efficacies against homologous challenge than other groups. Molecular docking showed that amino acids in three peptides, especially in peptide A, insert into the hydrophobic groove of EtMIC3-bc1 protein, and bind to EtMIC3-bc1 through intermolecular hydrogen bonds. Taken together, the results suggest EtMIC3-binding peptides inhibit sporozoites entry into host cells. This study provides new idea for exploring novel strategies against coccidiosis.

**Keywords:** EtMIC3, sporozoites, invasion, protective effects, molecular docking

## Introduction

The phylum apicomplexa includes several well-known unicellular protozoan parasites such as *Plasmodium*, *Toxoplasma*, *Neospora* and *Eimeria*, all of which infect human or animals. *Eimeria tenella* is one of the seven *Eimeria* species, colonizes caecal epithelium cells and causes avian coccidiosis that leads to enormous economic losses to the global poultry industry [1]. Considering the emergence of drug-resistant parasites [2, 3],

alternative measures against coccidiosis are urgently required. With the aim of developing novel anti-coccidial strategies, researchers gradually focus on the mechanism for *Eimeria* invasion of host cells. *E. tenella* microneme proteins are secreted on the surface of parasites by microneme organelles, and could be recognized by receptors located on the surface of the host cells. Microneme proteins play a crucial role in invasion of host cells by *Eimeria* parasites. Several microneme proteins have already been reported, including EtMIC1 [4], EtMIC2 [5, 6], EtMIC3 [7, 8], EtMIC4 [9, 10], and EtMIC5 [11]. EtMIC3 contains seven tandem microneme adhesive repeat regions (MARR), named EtMIC3-MARa, EtMIC3-MARb, EtMIC3-MARc and EtMIC3-MARd, among which MARc consists of four repeated domains

\*Correspondence: madexing@neau.edu.cn

†Wenjing Chen and Chunli Ma are co-first authors

<sup>1</sup> College of Veterinary Medicine, Northeast Agricultural University, NO. 600 Changjiang Road, Xiangfang District, Harbin, Heilongjiang 150030, China

Full list of author information is available at the end of the article



© The Author(s) 2021. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

(MARc1, MARc2, MARc3 and MARc4). MARb, MARc and MARd were reported to actively bind with host cells [7]. Previous studies demonstrated that EtMIC3 effectively facilitates the invasion of sporozoites into host cells by specifically recognizing sialylated glycans, and is responsible for guiding *E. tenella* sporozoites to the invasion sites in chicken gut [12, 13]. Taking into account that EtMIC3 protein plays key roles in the process of sporozoites invasion of host cells, we postulated that peptides specifically binding to EtMIC3 protein could effectively inhibit invasion of cells by *E. tenella* sporozoites. With the aim to verify the above hypothesis and explore novel strategies against coccidiosis, in the present study, the recombinant MARb and one of the four repeated MARc domains of EtMIC3 protein (EtMIC3-bc1) were selected as ligand, and peptides binding to EtMIC3 were screened from phage display peptide library. Then the effects of target phages or corresponding peptides on inhibiting sporozoites invasion of cells were measured in vivo and in vitro. Furthermore, the model of three-dimensional (3D) structure for EtMIC3-bc1 protein was conducted, and molecular docking between target peptides and EtMIC3-bc1 model was analyzed.

## Materials and methods

### Expression and purification of EtMIC3-bc1 protein

The objective fragment consists of MARb and MARc1 of EtMIC3 (EtMIC3-bc1) (Figure 1) was cut off from pUC-EtMIC3-bc1 plasmid (stored in our lab) by restriction enzyme *Bam*H I and *Xho* I. The 864 bp fragment of EtMIC3-bc1 was subcloned into pET30a vector (Novagen, Madison, WI) to generate positive plasmid pET30a-EtMIC3-bc1. The above plasmid was transformed into *E. coli* BL21 bacteria to produce recombinant positive bacteria. The bacteria culture was induced by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and then sonicated (300 W for 3 s, with 3 s interval). The purification of EtMIC3-bc1 protein was performed by affinity chromatography with Ni-conjugated sepharose as previously described [14]. The concentration of purified EtMIC3-bc1 protein was determined, and then separated by 12% SDS-PAGE. The expected protein bands were visualized using Coomassie brilliant blue stain R-250 (Beyotime, China).

### Preparation of antisera against EtMIC3-bc1 protein

The polyclonal antisera against EtMIC3-bc1 protein was prepared according to previous report with some modifications [15]. Briefly, 7-week-old specific pathogen-free (SPF) New Zealand White rabbit was subcutaneously injected with 2.0 mg of purified EtMIC3-bc1 protein (1 mg/mL) emulsified with the same volume of Freund's complete adjuvant (Sigma, USA) around neck, inguinal and axillary lymph nodes. After two weeks, the immunization was boosted with 1.0 mg of purified EtMIC3-bc1 protein emulsified with the same volume of Freund's incomplete adjuvant (Sigma, USA). Another two booster immunizations were performed at one week interval. On day 7 post the last immunization, blood was sampled via the marginal ear vein, and sera were collected and stored at  $-20^{\circ}\text{C}$  until use. The titers of the prepared polyclonal antisera against EtMIC3-bc1 protein were tested by indirect enzyme-linked immunosorbent assay (ELISA) as previously described [16]. The specificity of the prepared polyclonal antisera against EtMIC3-bc1 protein was detected by western blot. Briefly, *E. tenella* sporozoites were purified and sonicated as previously described [14], and protein samples were separated by SDS-PAGE, then transferred to nitrocellulose membranes. The membranes were incubated with rabbit anti-EtMIC3-bc1 polyclonal antisera for 2 h. After washing with TTBS (0.1% Tween 20, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Sigma, USA). The immune complexes were visualized using ECL chemiluminescence detection kit (Sangon Biotech Co., Ltd, Shanghai, China) according to the provided instructions.

### Screening of phages bind to EtMIC3-bc1

The screening of peptides binding to EtMIC3-bc1 protein was carried out as described in previous reports [14, 17] with some modifications based on phage display peptide library kit (New England Biolabs, USA). Briefly, 96-well plates were coated with 100  $\mu\text{L}$  per well of purified EtMIC3-bc1 protein (100  $\mu\text{g}/\text{mL}$ ), and incubated overnight at  $4^{\circ}\text{C}$ . After blocking with 2% BSA for 2 h, the plates were washed with TBS-T (Tris buffered saline with 0.1% Tween-20, pH 7.4) 6 times. The coated protein in each well was reacted with 100  $\mu\text{L}$  of diluted (1:100)



**Figure 1** Schematic illustration of EtMIC3 protein. *E. tenella* microneme proteins 3 (EtMIC3) contains seven tandem microneme adhesive repeat regions (MARR), and were named EtMIC3-MARa, EtMIC3-MARb, EtMIC3-MARc, and EtMIC3-MARd, respectively. MARc consists of four repeated domains (MARc1, MARc2, MARc3, MARc4).

phage display peptide library ( $1.5 \times 10^{14}$  pfu) (New England Biolabs, USA) for 30 min at room temperature. The plates were firstly washed with 0.1% TBS-T for six times, then washed with 0.2 mol/L glycine-HCL (pH 2.2), and ultimately neutralized with 1.0 mol/L Tris-HCL (pH 9.1). The eluent from plates, called the first round of biopanning phage, was titrated and amplified in *E. coli* strain ER2738 according to the protocol. The amplified target phage was precipitated by 20% PEG8000/NaCl (w/v) (2.5 M NaCl), and titrated again according to the protocol. The next three rounds of biopanning were carried out as described above except that TBS-T (TBS with 0.5% Tween-20, pH 7.4) was applied to wash off the unbound phages. The fourth round of biopanning phages were amplified in *E. coli* strain ER2738 based on the recommended protocol in phage display peptide library Kit (New England Biolabs, USA). The amplified phages were stored at 4 °C until use.

#### Enzyme-linked immunosorbent assay (ELISA)

To further detect the binding capability between the screened phages and EtMIC3-bc1 protein, enzyme-linked immunosorbent assay (ELISA) was performed as previously described [14, 18]. Briefly, the plates coated with 100 µL per well of EtMIC3-b1c protein (100 µg/mL) were incubated overnight, then washed with PBS-T (0.5% Tween-20) and blocked with 5% skim milk. After washing, each well was incubated with 100 µL of target phages ( $1.0 \times 10^{12}$  pfu). The washed plate was incubated with 100 µL per well of diluted anti-M13 rabbit polyclonal antibody (1:1000). After washing, the plate was incubated with 100 µL per well of diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, USA) (1:5000), reacted with substrate solution (0.01%  $H_2O_2$ , 1 mg/mL o-phenylenediamine), and stopped with 2 M sulfuric acid. The optical density at 490 nm (OD490) was recorded by microplate reader (Bio-Rad, USA). Unrelated phages bound to G protein of porcine Vesicular Stomatitis Virus (VSV) (stored in our lab) were used as negative controls. The positive phages that specifically binding to EtAMA1 protein were used as positive control [14]. Each sample was tested in triplicate.

#### Sequence analysis

Phage DNA was extracted according to the provided method in M13 isolation kit (BioTeke Corporation Co., Ltd, China), and was used as template to amplify the fragment that contained target peptide using specific primers pair (New England Biolabs). The above DNA fragment was sequenced, and amino acid sequences were deduced from the sequences of nucleotides. The target peptides were synthesized by GenScript Co., Ltd (Nanjing, China).

#### Binding capability between EtMIC3-bc1 protein and target phages

To verify the binding capability between the selected phages and EtMIC3-bc1 protein, ELISA and competitive ELISA was performed as previously described [14] with some modifications. Briefly, 96-well plates were coated with three selected phages (called A, D and W) that serially diluted from  $1 \times 10^6$  to  $1 \times 10^{12}$  pfu, incubated overnight, blocked with 5% skim milk, washed by 0.5% PBS-T, and then incubated with 100 µL per well of purified EtMIC3-bc1 protein (100 µg/mL). After washing, the plate was incubated with rabbit anti-EtMIC3 polyclonal antisera (60 µg/mL) diluted at 1:1000, reacted with 100 µL of HRP-labeled goat anti-rabbit IgG (1:5000) (Boster, Wuhan, China). After incubating for 1 h at 37 °C, the OD490 values were measured. Each sample was tested in triplicate. The unrelated phages from G protein of VSV (stored in our lab) and the positive phages binding to EtAMA1 protein was used as negative and positive control, respectively.

For competitive ELISA, 50 µL of rabbit anti-EtMIC3 polyclonal antisera diluted at 1:1000, 1:1500, 1:2000 and 1:2500 (with concentrations of 60, 40, 30, 24 and 20 µg/mL, respectively) and 50 µL of EtMIC3-bc1 protein (100 µg/mL) were designed as binding competitors to 100 µL of coated positive phages ( $1.0 \times 10^{12}$  pfu) in each well of the plate. After incubation, the OD490 values were measured, and each sample was tested in triplicate. The negative and positive phages mentioned above were used as controls.

#### Binding capability between sporozoites protein and target phages

*E. tenella* sporozoites were purified by percoll density gradient as described in previous report [14]. Briefly, sporulated *E. tenella* oocysts were broken by shaking with 3 mm diameter glass beads, and the released sporocysts were pooled by centrifugation in 50% Percoll (Sigma-Aldrich). The collected sporocysts were incubated with PBS solution containing 0.25% (w/v) Trypsin, 4% (w/v) taurodeoxycholic acid and 10 mM  $MgCl_2$ . The excysted sporozoites were suspended in 55% Percoll (Sigma-Aldrich), and the sporozoites were harvested after centrifugation. The binding capability between sporozoites protein and target phages were detected by ELISA as described above except that 100 µL sonicated (300 W for 5 s, with 5 s interval) sporozoites ( $1.0 \times 10^6$ /mL) protein (1.2 mg/mL) was added to react with coated target phages. The OD490 values were measured. Each sample was tested in triplicate. The negative and positive phages mentioned above were used as controls.

### In vitro effects of synthesized peptides in inhibiting sporozoites invasion into cells

The maximum non-cytotoxic concentration of synthesized peptides A, D and W to MDBK cells was determined using MTT dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromid as previously described [14]. The effects of synthesized peptides in inhibiting sporozoites invasion into MDBK cells were carried out according to the reported methods [14, 19]. Briefly,  $3 \times 10^5$  sporozoites were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Beyotime, China). The labelled sporozoites were pre-incubated with 100  $\mu$ L target peptides with final concentrations of 25, 50, 75, 100 and 125  $\mu$ g/mL at 37 °C for 2 h. The incubated solutions were centrifuged to collect sporozoites which were then added to monolayer MDBK cells ( $1 \times 10^5$  per well in 24-well plate) for 10 h at 37 °C. After 3 washes, cells were digested with 0.25% (w/v) trypsin, and centrifuged to collect pellets which were resuspended in 500  $\mu$ L PBS (pH 7.2). The MDBK cells invaded by CFDA-SE-labelled sporozoites showing green fluorescent were counted by flow cytometry (Beckman Coulter), and the invasion ratio was calculated. The fluorescently-labelled sporozoites incubating with 100  $\mu$ L of polyclonal antisera against EtMIC3-bc1 protein (300  $\mu$ g/mL) (determined in preliminary test) were designed as positive controls. The fluorescently-labeled sporozoites incubating with MDBK cells were used as negative controls.

### In vivo anticoccidial effects provided by target phages

10-day-old specific pathogen-free (SPF) White Leghorn chickens were randomly divided into 5 groups of 15 chicks each. All chickens were individually housed in hanging cages. At 21 days of age, all chickens except in group 1 (non-challenged control group) were orally inoculated with  $1 \times 10^4$  *E. tenella* sporulated oocysts. At days 0, 1 and 2 after challenging, chickens in group 2 were orally fed  $1 \times 10^{12}$  pfu phages A, group 3 with phages D, group 4 with phages W, and group 5 (challenged control group) with PBS (pH 7.2). All animal experiments were complied with the rules of Animal Experiment Ethic Committee of Northeast Agricultural University, China. Chickens in each group were weighed before challenging and at days 7 after challenging to calculate the body weight gain (BWG) [18]. Lesion scores in ceca of chickens ( $n=5$ ) from all the groups were determined on day 7 post challenging as previous described [20]. Fecal samples from each chicken within each group between days 7 and 11 post challenging were gathered to count the oocyst output per gram (OPG), and oocyst reduction ratio was calculated as described by Lillehoj et al. [21]. Briefly, oocyst reduction ratio = (number of oocysts from

challenged control chickens—orally administrated chickens)/ challenged control chickens  $\times 100\%$ .

### Homology modeling and molecular docking

The three-dimensional (3D) structure of EtMIC3-bc1 protein was generated according to the published crystal structures of EtMIC3-MAR1b (PDB code 2LBO) using Swiss-Model (<http://swissmodel.expasy.org>). The software Discovery Studio 2.5 was used to optimize the 3D structure of EtMIC3-bc1, and the model quality was evaluated as previously described [22]. The tertiary structures of target peptides A, D and W were produced by Swiss-Model. The software Autodock 4.2 and Discovery Studio 2.5 were applied to explore the molecular docking between target peptide and constructed 3D structure of EtMIC3-bc1 protein.

### Statistical analysis

Data was subjected to one-way analysis of variance (ANOVA), and was expressed as means  $\pm$  SD. ANOVA Duncan's multiple-comparison procedure was used to compare differences between mean values. Results were considered as significant difference at *P* value less than 0.05.

## Results

### Purification of EtMIC3-bc1 protein

The expression and purification of EtMIC3-bc1 protein was displayed in Additional file 1, showing an expected protein band of 41 kDa.

### Characterization of polyclonal antisera against EtMIC3-bc1 protein

Western blot detection showed that the prepared antisera against EtMIC3-bc1 protein reacted with sporozoites protein and recombinant EtMIC3-bc1 protein, showing protein bands of 41 kDa (Additional file 2). The titer of prepared antisera was  $1:2^{16}$ .

### Screening of phages binding to EtMIC3-bc1

The plaque forming unit per milliliter (pfu/mL) of the positive phages binding to EtMIC3-bc1 protein were identified after four rounds of affinity screening (Table 1),

**Table 1 Titer of phages after four rounds of biopanning and amplification.**

	Rounds of biopanning			
	First	Second	Third	Fourth
Input phage (pfu/mL)	$1.5 \times 10^{11}$	$1.5 \times 10^{11}$	$1.5 \times 10^{11}$	$1.5 \times 10^{11}$
Output phage (pfu/mL)	$6.4 \times 10^4$	$6.3 \times 10^4$	$2.3 \times 10^5$	$8.6 \times 10^5$
Amplified phage (pfu/mL)	$3.0 \times 10^{11}$	$1.3 \times 10^{11}$	$4.5 \times 10^{12}$	$5.7 \times 10^{12}$

demonstrating that the enriched phages bind to EtMIC3-bc1 protein.

#### Characterization of screened phages binding to EtMIC3-bc1

As displayed in Figure 2, among the selected thirty clones, twenty-four phages (except for NO.1, 3, 13, 19, 23 and 28) showed significantly higher binding capability to EtMIC3-bc1 protein than other and control phages ( $P < 0.01$ ).

#### Sequencing of positive clones

After sequencing of 24 positive phages clones, 8 peptides with different amino acid composition were observed. The sequences of AGRLLTPTMSLV (peptide A), DYH-DPSLPTLGK (peptide D), and WKDVHKAWLLEP (peptide W) appeared 8, 6 and 3 times, respectively.

#### Binding assays

All three target phages showed higher binding capability to EtMIC3-bc1 protein than unrelated control phages screened from G protein of VSV ( $P < 0.01$ ). The three phages A, D and W diluted from  $1 \times 10^6$  pfu/100  $\mu$ L to  $1 \times 10^{12}$  pfu/100  $\mu$ L exhibited dose-dependent binding affinity with EtMIC3-bc1 protein (Figure 3A). The results of competitive ELISA showed that antisera against EtMIC3-bc1 protein diluted from 1:1500 to 1:1000 competitively inhibited binding of phages A, D and W to EtMIC3 protein. The irrelevant antisera against hexon protein of fowl adenovirus

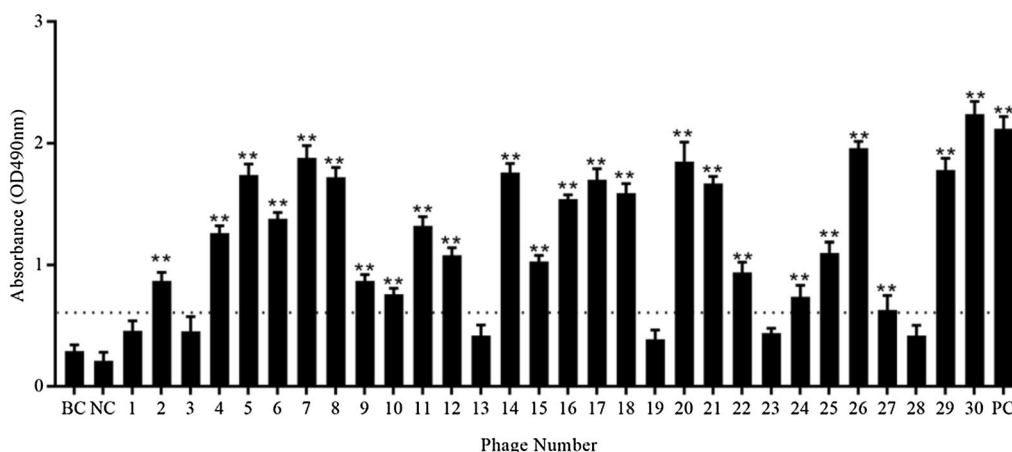
serotype 4 (FADV-4) did not exhibit dose-dependent inhibition (Figure 3B). The three phages A, D and W, especially phages A, showed higher binding capability to sporozoites proteins than negative control phages ( $P < 0.01$ ) (Figure 3C).

#### In vitro inhibition of sporozoites invasion

The maximum non-toxic concentration of the synthesized peptides A, D and W to MDBK cells was 125  $\mu$ g/mL. The three peptides, especially A peptide, showed obvious inhibitory effects on sporozoites invasion of MDBK cells, and the inhibited ratio changed in dose-dependent manners with concentrations of peptides ranging from 25  $\mu$ g/mL to 125  $\mu$ g/mL. The observed highest inhibition ratio of 71.8% was presented by peptide A at concentration of 125  $\mu$ g/mL, and 54.6% and 20.8% was observed for D and W peptide, respectively (Figure 4). The polyclonal antisera against EtMIC3-bc1 protein (300  $\mu$ g/mL) prepared in this study was used as positive inhibitor for sporozoites invasion into cells, showing inhibited ratio of 75.2%.

#### In vivo evaluation of anti-coccidial effects

As shown in Table 2, the weight gain of chickens orally fed phages A, D and W was higher than that in challenged control group ( $P < 0.05$ ). The average cecal lesion scores of chickens in the group orally given phages A or D was significantly lower than that in the group with phages W and challenged control group ( $P < 0.01$ ). The oocyst reduction ratio in the group orally fed phages A,



**Figure 2** Detection of binding capability between selected phages and EtMIC3-bc1 protein. EtMIC3-bc1-coated plate was incubated with thirty selected phages. The washed plate was then successively incubated with rabbit anti-M13 polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Then substrate solution (0.01%  $H_2O_2$  and 1 mg/mL o-phenylenediamine) was added, and the reaction was stopped with 2 M sulfuric acid. The values of optical density (OD) at 490 nm was recorded. The blank control (BC), the negative control (NC) phages from G protein of porcine Vesicular Stomatitis Virus (VSV) (stored in our lab), and the positive control (PC) phages binding to EtAMA1 protein (stored in our lab) were used. Each sample was tested in triplicate. Values represent mean  $\pm$  SD. Twenty-four selected phages clones showed higher binding capability than other clones (with the number of 1, 3, 13, 19, 23, 28), blank control and negative control phages ( $P < 0.01$ ). \*\* $P < 0.01$ .

D and W was 47.42%, 40.78% and 8.52%, respectively. On day 7 PI, chickens from challenged control group displayed severe typical gross pathological changes in ceca, including swelling and thickening, petechial hemorrhage on the serous membranes, and bloody cecal content (Figure 5A). The cecal tissues of chickens in challenged control group presented remarkable histopathological changes, including compromised intestinal structural integrity, damaged intestinal villi, numerous red cells, and infiltration of inflammatory cells, while ceca of chickens in the groups with three phages, especially with phages A or D presented mild gross pathological and histopathological changes (Figure 5B).

### Homology modeling and docking

The secondary and tertiary structure of EtMIC3-bc1 protein was respectively depicted, and an obvious hydrophobic groove was observed in three-dimensional (3D) structure of EtMIC3-bc1 which is responsible for binding target peptides (Figure 6A). The molecular docking between EtMIC3-bc1 protein and target peptides was analyzed, and intermolecular hydrogen-bonds were formed between amino acids in three target peptides and EtMIC3-bc1 protein (Figure 6B). The extent of binding between amino acids in the three peptides and EtMIC3-bc1 protein is different. All 12 amino acids in peptide A (a and d in Figure 6B), partial amino acids in peptide D (b and e in Figure 6B), and only several amino acids in peptide W (c and f in Figure 6B) inserts into the hydrophobic groove of EtMIC3-bc1 protein. The amino acids in peptide A, D and W contributing to the formation of hydrogen-bonds with amino acids in EtMIC3-bc1 protein are shown in Table 3.

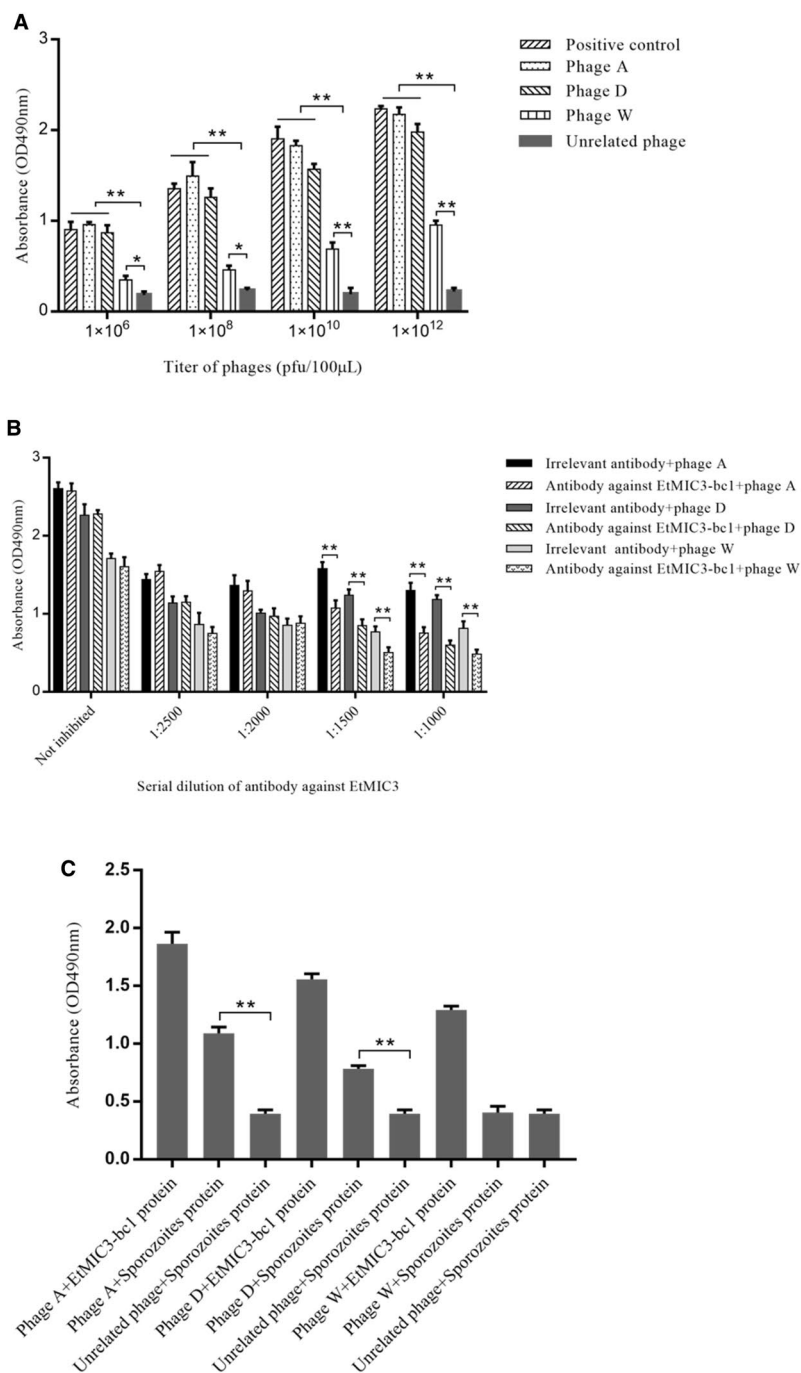
### Discussion

Avian coccidiosis caused by *Eimeria* leads to serious economical losses to the global poultry industry [23]. The development of alternative control strategies would be based on the deep understanding of the mechanism for *Eimeria* invasion of host epithelial cells. The lifecycle for *Eimeria* parasites includes several key developmental stages including sporozoites, merozoites, and gametocytes. Invasion for apicomplexan protozoan parasites was initiated based on the interplay between the apex of parasites and host cells, during which a series of secreted proteins at the parasite-host interface are indispensable for the parasites to penetrate into host cells. Apical membrane antigen 1 (AMA1) is one of the microneme proteins, and previous studies have shown that AMA1 protein secreted by sporozoites is important for invasion of *Toxoplasma gondii* and *E. tenella* into host cells [14, 24]. Besides AMA1 protein, *E. tenella* microneme protein 3 (EtMIC3), one of the important invasion-related proteins

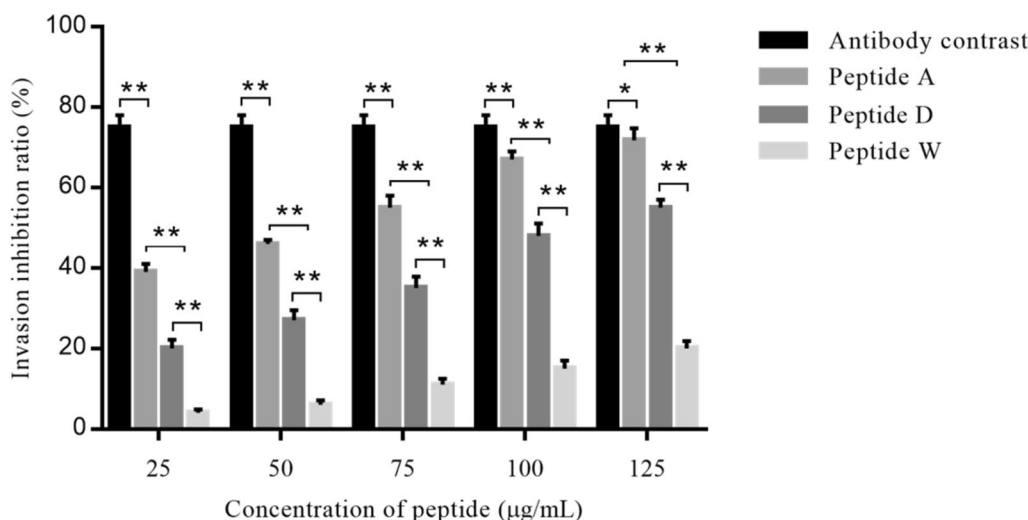
released by secretory organelles existing in sporozoites, plays a crucial role in parasites attachment and subsequent invasion into host cells. It has been shown that EtMIC3 protein consists of seven microneme adhesive repeats (MARs), including four highly conserved internal repeats and three divergent external repeats [7, 25]. EtMIC3 contributes to guiding and binding sporozoites to the invasion site in chicken intestinal tract by recognizing BCL2-associated athanogene 1 (BAG1) and Endonuclease polyU-specific-like (ENDOUL) [26], and sialylated glycans [12, 13]. It was reported that AMA1-binding peptides effectively inhibited *T. gondii*, *P. falciparum* and *E. tenella* invasion into host cells [14, 27, 28]. However, the roles of peptide binding to EtMIC3 protein in inhibiting sporozoites invasion into host cells were not reported until now. In the present study, three phages (named A, D and W) with EtMIC3-binding capability were firstly identified. Analysis of amino acids in the three corresponding peptides revealed that peptide A, D and W appeared 8, 6, and 3 times, respectively, and 2 or 3 consecutive amino acids were similar between peptide A and D, and more hydrophobic amino acids were contained in peptide A, all of which suggesting that peptide A probably exhibited excellent functional characteristics in subsequent test. The ELISA results displayed obvious dose-dependent increase of binding capability between the three phages and EtMIC3-bc1 protein, which were reversely inhibited by polyclonal antisera against EtMIC3-bc1 protein in a dose-dependent manner. The above results demonstrated that the three phages, especially phage A specifically binds to EtMIC3-bc1 protein. Furthermore, the unrelated control phages showed lower binding capability to *E. tenella* sporozoites protein than the three phages, especially phage A and D ( $p < 0.01$ ), further indicating that three target phages were EtMIC3-specific.

To detect the roles of three synthesized linear peptides in inhibiting sporozoites invasion of cells, the non-cytotoxic concentration of target peptides was determined to ensure survival of the labelled sporozoites under cell culture conditions. 125 µg/mL of peptides A, D and W effectively inhibited sporozoites invasion of cells, showing ratio of 71.8, 54.6 and 20.8%, respectively, which are consistent with the above analysis of peptide sequence and ELISA test.

To clarify whether the three phages provided anticcidial effects in vivo, three phages were orally inoculated on day 0, 1, and 2 post homologous challenge. The results suggested that the three phages to some extent offered protection against *E. tenella* challenge. It was documented that the asexual developmental stage for *Eimeria* parasites on day 1 and 2 post infection of *E. tenella* sporulated oocysts was mainly sporozoites. Moreover, our preliminary animal experiment showed that



**Figure 3 Binding detection of three target phages with recombinant EtMIC3-bc1 protein and sporozoites protein.** ELISA (A, C) and competitive ELISA (B) were applied to assay the binding capability of three phages A, D and W with recombinant EtMIC3-bc1 protein and sporozoites protein. The phages A, D and W diluted from  $1.0 \times 10^6$  to  $1.0 \times 10^{12}$  pfu were coated in 96-well plate, respectively, and the three phages showed higher binding capability to EtMIC3-bc1 protein than the unrelated phages from G protein of Vesicular stomatitis virus (VSV) (negative control) ( $p < 0.01$ ) (A). The specific antisera against EtMIC3-bc1 protein significantly inhibited binding of the three phages to EtMIC3-bc1 protein compared with the irrelevant antibody against hexon protein of fowl adenovirus serotype 4 (FADV-4) (negative antibody control) ( $p < 0.01$ ) (B). The phages A, D and W displayed specific binding with sonicated sporozoites protein. The binding of the three phages to recombinant EtMIC3-bc1 protein was used as positive control, and meanwhile the binding of unrelated phages from G protein of VSV to sporozoites was used as negative control to exclude the possibility of binding between any other phages and sporozoites protein (C). Each sample was tested in triplicate. Values represent mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 4** Effect of three target peptides in inhibiting sporozoites invasion of cells *in vitro*. The three target peptides, especially peptide A showed obvious effects in inhibiting sporozoites invasion of MDBK cells. The inhibition ratio showed in dose-dependent manners with concentration of three peptides ranging from 25 to 125 µg/mL, showing inhibition ratio of 71.8% for peptide A, 54.6% for D and 20.8% for W at concentration of 125 µg/mL. 300 µg/mL of prepared polyclonal antisera against EtMIC3-b1c protein was used as positive control, showing inhibition ratio of 75.2%. The values represent mean ± SD (n = 5). \*P < 0.05, \*\*P < 0.01.

**Table 2** *In vivo* evaluation of anti-coccidial effects.

Group	Challenge	Oral inoculation	Body weight gain	Relative body weight gain (%)	Average lesion score in cecum	Oocyst reduction ratio (%)
1	Unchallenged	PBS (pH7.2)	77.75 ± 2.46 <sup>a</sup>	100	/	/
2	Challenged	Phages A	64.33 ± 3.27 <sup>b</sup>	82.74	1.40 ± 0.34 <sup>A</sup>	47.42%
3	Challenged	Phages D	60.08 ± 2.94 <sup>b</sup>	77.27	1.57 ± 0.38 <sup>A</sup>	40.78%
4	Challenged	Phages W	56.27 ± 3.85 <sup>b</sup>	72.36	2.50 ± 0.46 <sup>B</sup>	8.52%
5	Challenged	PBS (pH7.2)	46.04 ± 2.65 <sup>c</sup>	59.21	3.10 ± 0.40 <sup>C</sup>	0

21 day-old chicken was orally challenged with  $1 \times 10^4$  *E. tenella* sporulated oocysts. At days 0, 1 and 2 post challenge, each chicken was orally inoculated with  $1 \times 10^{12}$  pfu target phages. Significant difference between numbers with different capital letters ( $P < 0.01$ ) and small letters ( $P < 0.05$ ).

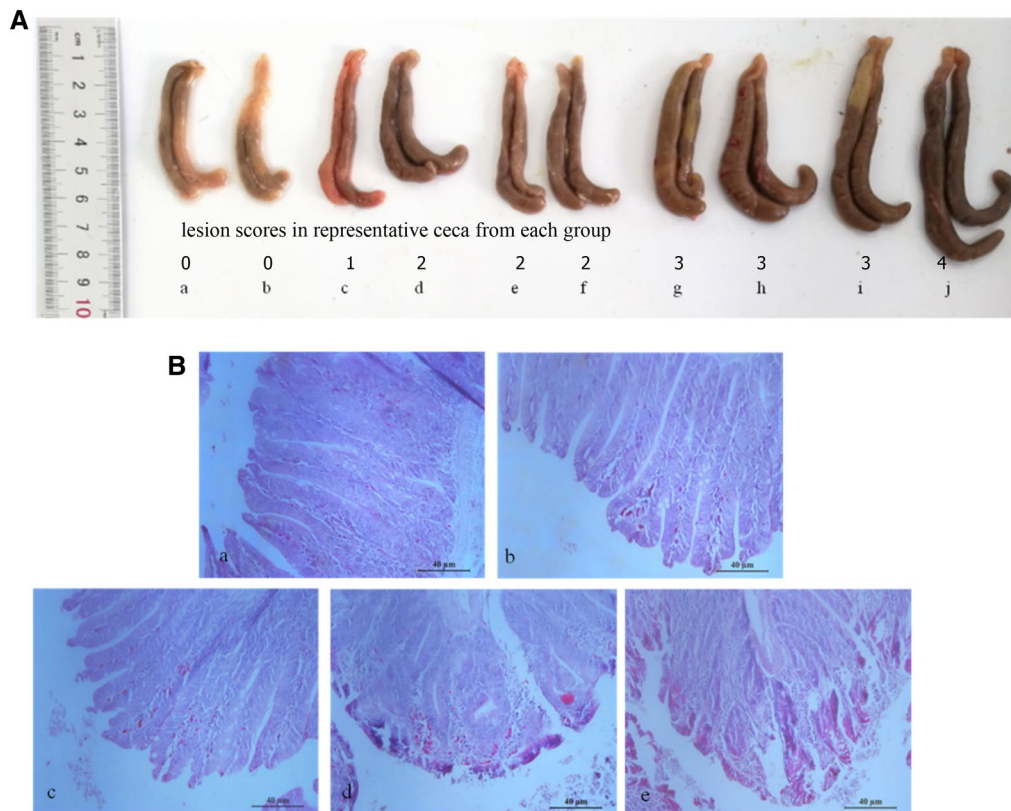
the unrelated control phages did not show any anticoccidial effects. Therefore, the protections against homologous infection could be explained by the fact that the functional phages effectively bound to EtMIC3 protein secreted by *E. tenella* sporozoites in the intestinal tract, and then inhibited sporozoites invasion of cecal epithelial cells. These results and analysis were supported by previous report by Zuercher et al. [29], which showed that the functional phages retained activity in the gastrointestinal environment.

The molecular docking between target peptides and the tertiary structural model of EtMIC3-bc1 protein revealed that binding between the three peptides and EtMIC3-bc1 protein was coordinated by hydrogen-bonds. The three amino acids Q<sup>145</sup>, M<sup>172</sup> and R<sup>175</sup> in EtMIC3-bc1 protein that are responsible for forming nine hydrogen-bonds with peptide D, and the three amino acids V<sup>204</sup>,

T<sup>213</sup> and D<sup>207</sup> in EtMIC3-bc1 protein that are responsible for forming six hydrogen-bonds with peptide W were all contained in the twelve amino acids G<sup>217</sup>, S<sup>216</sup>, C<sup>215</sup>, V<sup>204</sup>, T<sup>213</sup>, R<sup>175</sup>, A<sup>206</sup>, D<sup>207</sup>, Asn<sup>208</sup>, Q<sup>145</sup>, R<sup>229</sup>, M<sup>172</sup> that are responsible for twelve hydrogen-bonds with peptide A. The above analysis provides the probable explanations for the function of 3 peptides *in vitro* and *in vivo*.

Periz et al. [11] and Lai et al. [12] reported that EtMIC3 specifically recognized and bound to α 2,3-linked sialic acid that predominantly distributed in ceca, which to some extent explained the tissue tropism of *E. tenella*. Moreover, Lai et al. [12] reported that competitors of EtMIC3-sialic acid binding inhibited sporozoites invasion of MDBK cells, which is consistent with the results in the present study that sporozoites invasion of MDBK cells was inhibited by EtMIC3-binding peptides. Cowper et al. [13] demonstrated that some amino acids in type I





**Figure 5** Pathological changes in ceca of experimental chickens in each group. On day 7 post infection (PI), chickens from challenged control group displayed severe typical gross pathological changes in ceca, including swelling and thickening, petechial hemorrhage on the serous membranes, and bloody cecal content (i and j, **A**), while chickens in the group orally fed with three phages A (c and d, **A**), D (e and f, **A**) and W (g and h, **A**), especially phages A and D, presented relatively moderate gross pathological changes. Chickens from unchallenged control group showed no gross pathological changes in ceca (a and b, **A**). The histopathological changes in cecal tissues of chickens in challenged control group were obvious, including compromised intestinal structural integrity, damaged intestinal villi, numerous red cells, and infiltration of inflammatory cells (e, **B**) (H.E.  $\times$  200), while chickens orally fed with phages A (b, **B**) (H.E.  $\times$  200), D (c, **B**) (H.E.  $\times$  200) and W (d, **B**) (H.E.  $\times$  200) presented relatively moderate histopathological changes in cecal tissues. Chickens from unchallenged control group showed no histopathological changes in cecal tissues (a, **B**) (H.E.  $\times$  200). Representative images were randomly selected from each group, and lesion scores in ceca were recorded by arabic numerals in (**A**).

and II MAR (microneme adhesive repeat) from *T. gondii* microneme protein 1 (TgMIC1), *T. gondii* microneme protein 13 (TgMIC13), *N. caninum* microneme protein 1 (NcMIC1), and EtMIC3 are conserved. Based on the above reports, in the present study, it is interesting for us to find that five amino acids Q<sup>145</sup>, R<sup>175</sup>, D<sup>207</sup>, C<sup>215</sup> and G<sup>217</sup>, two amino acids Q<sup>145</sup> and R<sup>175</sup>, and two amino acids D<sup>207</sup> and R<sup>175</sup> contained in EtMIC3-c1(MARc1) protein contributed for forming hydrogen-bonds with peptide A, D and W, respectively, and meanwhile all the above amino acids are also conserved among TgMIC1, TgMIC13, NcMIC1 and EtMIC3, suggesting that the three peptides, especially peptides A, probably to some extent displayed inhibition of *T. gondii* and *N. caninum* invasion of host cells.

## Supplementary Information

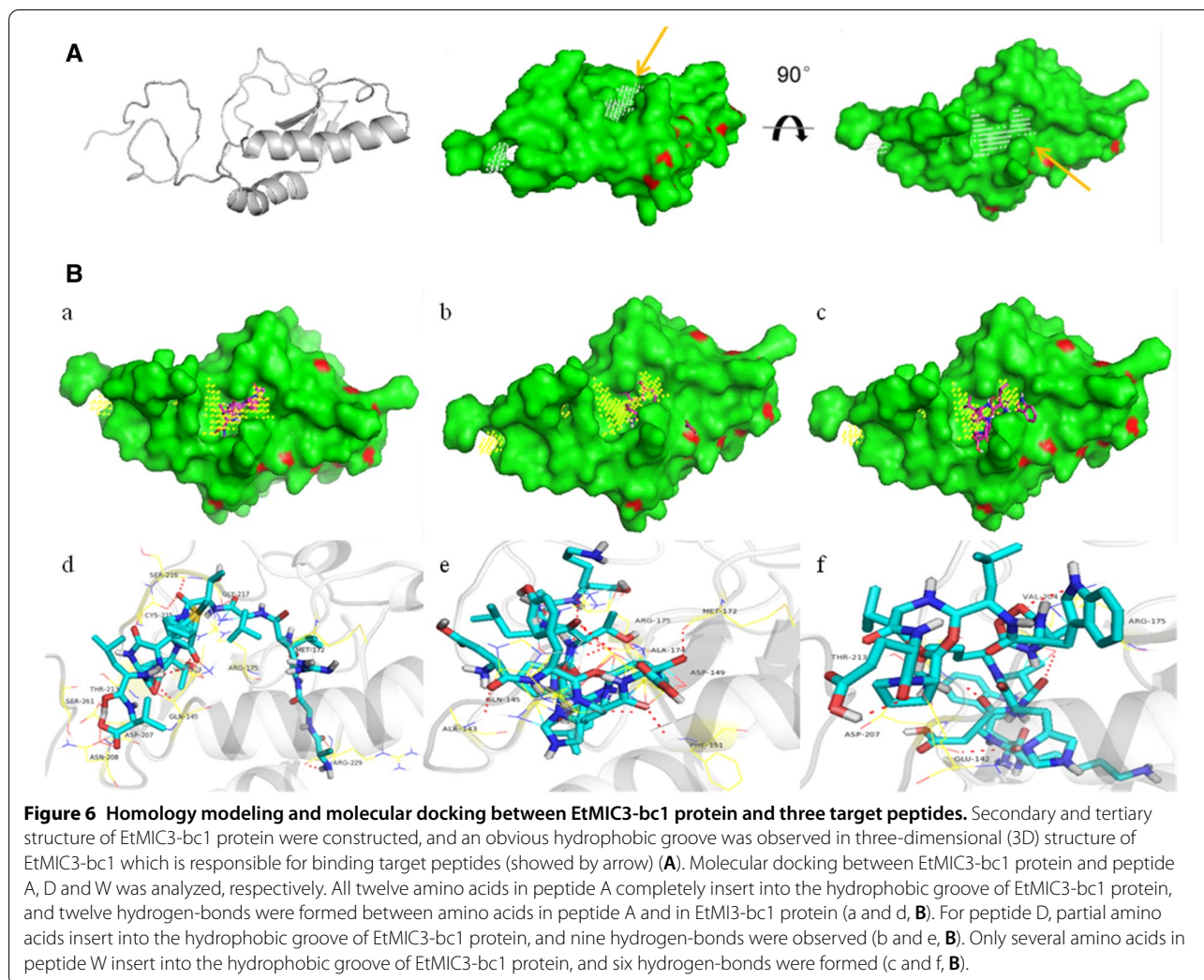
The online version contains supplementary material available at <https://doi.org/10.1186/s13567-020-00873-y>.

### Additional file 1. SDS-PAGE analysis of EtMIC3-bc1 protein expressed in *E. coli* BL21 cells.

A band of 41 kDa corresponding to EtMIC3-bc1 protein was observed. M, Protein molecular weight marker. Lane 1, Recombinant positive bacteria without induction by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (negative control). Lane 2–5, EtMIC3-bc1 protein expressed in *E. coli* BL21 cells induced by IPTG for 0, 1, 2 and 3 h, respectively. Lane 6 EtMIC3-bc1 protein purified by affinity chromatography with Ni-conjugated Sepharose.

### Additional file 2. Detection of prepared anti-EtMIC3-bc1 polyclonal antisera by Western blot.

Sporozoites protein and recombinant EtMIC3-bc1 protein samples were respectively separated by SDS-PAGE, then transferred to nitrocellulose membranes. The prepared rabbit anti-EtMIC3-bc1 polyclonal antisera specifically recognized target proteins, showing band of 41 kDa. Lane M, Protein molecular weight marker. Lane 1, Band of recombinant EtMIC3-bc1 protein expressed in *E. coli* BL21 cells. Lane 2, Band of EtMIC3 protein in sporozoites.



**Table 3 Amino acids in EtMIC3-bc1 protein and in peptide A, D and W that predicted for forming hydrogen-bonds.**

peptide	Hydrophobic amino acids	Amino acids for forming hydrogen-bonds in		Number of hydrogen-bonds	Common amino acids in peptide that contribute for forming hydrogen-bonds between peptides			
		peptides	EtMIC3-bc1 protein		A and D	A and W	D and W	A, D and W
A	A <sup>1</sup> , L <sup>4</sup> , L <sup>5</sup> , M <sup>9</sup> , L <sup>11</sup> , V <sup>12</sup>	A <sup>1</sup> , R <sup>3</sup> , L <sup>5</sup> , T <sup>6</sup> , T <sup>8</sup> , S <sup>10</sup> , L <sup>11</sup> , V <sup>12</sup>	G <sup>217</sup> , S <sup>216</sup> , C <sup>215</sup> , V <sup>204</sup> , T <sup>213</sup> , R <sup>175</sup> , A <sup>206</sup> , D <sup>207</sup> , N <sup>208</sup> , Q <sup>145</sup> , R <sup>229</sup> , M <sup>172</sup>	12	Q <sup>145</sup> , M <sup>172</sup> , R <sup>175</sup>	V <sup>204</sup> , T <sup>213</sup> , D <sup>207</sup>	/	R <sup>175</sup>
D	Y <sup>2</sup> , L <sup>7</sup> , L <sup>10</sup>	D <sup>1</sup> , H <sup>3</sup> , D <sup>4</sup> , Y <sup>2</sup> , T <sup>9</sup>	M <sup>172</sup> , R <sup>175</sup> , A <sup>174</sup> , D <sup>149</sup> , F <sup>151</sup> , Q <sup>145</sup> , A <sup>143</sup>	9	Q <sup>145</sup> , M <sup>172</sup> , R <sup>175</sup>	/		R <sup>175</sup>
W	W <sup>1</sup> , V <sup>4</sup> , A <sup>7</sup> , W <sup>8</sup> , L <sup>9</sup> , L <sup>10</sup>	D <sup>3</sup> , V <sup>4</sup> , H <sup>5</sup> , K <sup>6</sup> , A <sup>7</sup> , E <sup>11</sup>	V <sup>204</sup> , R <sup>175</sup> , T <sup>213</sup> , D <sup>207</sup> , E <sup>142</sup>	6	/	V <sup>204</sup> , T <sup>213</sup> , D <sup>207</sup>	R <sup>175</sup>	R <sup>175</sup>

**Abbreviations**

AMA1: apical membrane antigen 1; CFDA-SE: carboxyfluorescein diacetate succinimidyl ester; ELISA: enzyme-linked immunosorbent assay; EtMIC3: *E. tenella* Microneme protein 3; HRP: horseradish peroxidase; IPTG: isopropyl-β-D-thiogalactopyranoside; MARR: microneme adhesive repeat regions; SPF: specific pathogen-free; 3D: three-dimensional.

**Acknowledgements**

We gratefully acknowledge all the other members working in Molecular Pathological Lab for their kind coordination and support in animal experiments.

**Authors' contributions**

Ma designed the experiments, amended the manuscript. WC, CM, GL, ZJ, XY and XP carried out the experiments. WC and CM finished original draft and statistical analysis. WC and CM prepared figures. All authors read and approved the final manuscript.

**Funding**

This study is funded by grants from National Natural Science Foundation of China (31973003) to Dexing Ma; Postdoctoral Scientific Research Developmental Fund of Heilongjiang Province (LBH-Q14019) and Scientific Research Foundation for the Returned Overseas Chinese Scholars by State Education Ministry to Dexing Ma.

**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**

This study was approved based on the National Guidelines for Experimental Animal Welfare. Animal experiments were performed according to the regulations of the Ethical Committee for animal sciences in Northeast Agricultural University. The protocol was approved by the Animal Ethics Committee of Northeast Agricultural University NEAU-2018-09-0232-12.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no conflict of interests.

**Author details**

<sup>1</sup> College of Veterinary Medicine, Northeast Agricultural University, NO. 600 Changjiang Road, Xiangfang District, Harbin, Heilongjiang 150030, China. <sup>2</sup> Heilongjiang Key Laboratory for Experimental Animals and Comparative Medicine, Harbin, Heilongjiang 150030, China. <sup>3</sup> College of Food Science, Northeast Agricultural University, Harbin, Heilongjiang 150030, China.

Received: 14 September 2020 Accepted: 2 December 2020

Published online: 17 February 2021

**References**

- Blake DP, Tomley FM (2014) Securing poultry production from the ever present *Eimeria* challenge. *Trends Parasitol* 30:12–19
- Shirley MW, Smith AL, Blake DP (2007) Challenges in the successful control of the avian coccidia. *Vaccine* 25:5540–5547
- Clarke L, Fodey TL, Crooks SR, Moloney M, O'Mahony J, Delahaut P, O'Kennedy R, Danaher M (2014) A review of coccidiostats and the analysis of their residues in meat and other food. *Meat Sci* 97:358–374
- Tomley FM, Clarke LE, Kawazoe U, Dijkema R, Kok JJ (1991) Sequence of the gene encoding an immunodominant microneme protein of *Eimeria tenella*. *Mol Biochem Parasitol* 49:277–288
- Tomley FM, Bumstead JM, Billington KJ, Dunn PP (1996) Molecular cloning and characterization of a novel acidic microneme protein (Etmic-2) from the apicomplexan protozoan parasite, *Eimeria tenella*. *Mol Biochem Parasitol* 79:195–206
- Yan M, Cui X, Zhao Q, Zhu S, Huang B, Wang L, Zhao H, Liu G, Li Z, Han H, Dong H (2018) Molecular characterization and protective efficacy of the microneme 2 protein from *Eimeria tenella*. *Parasite* 25:60
- Labbe M, de Venevelles P, Girard-Misguich F, Bourdieu C, Guillaume A, Pery P (2005) *Eimeria tenella* microneme protein EtMIC3: identification, localisation and role in host cell infection. *Mol Biochem Parasitol* 140:43–53
- Zhao N, Lv J, Lu Y, Jiang Y, Li H, Liu Y, Zhang X, Zhao X (2020) Prolonging and enhancing the protective efficacy of the EtMIC3-C-MAR against *Eimeria tenella* through delivered by attenuated salmonella typhimurium. *Vet Parasitol* 279:109061
- Tomley FM, Billington KJ, Bumstead JM, Clark JD, Monaghan P (2001) EtMIC4: a microneme protein from *Eimeria tenella* that contains tandem

- arrays of epidermal growth factor-like repeats and thrombospondin type-I repeats. *Int J Parasitol* 31:1303–1310
- Periz J, Ryan R, Blake DP, Tomley FM (2009) *Eimeria tenella* microneme protein EtMIC4: capture of the full-length transcribed sequence and comparison with other microneme proteins. *Parasitol Res* 104:717–721
- Periz J, Gill AC, Hunt L, Brown P, Tomley FM (2007) The microneme proteins EtMIC4 and EtMIC5 of *Eimeria tenella* form a novel, ultra-high molecular mass protein complex that binds target host cells. *J Biol Chem* 282:16891–16898
- Lai L, Bumstead J, Liu Y, Garnett J, Campanero-Rhodes MA, Blake DP, Palma AS, Chai W, Ferguson DJ, Simpson P, Feizi T, Tomley FM, Matthews S (2011) The role of sialyl glycan recognition in host tissue tropism of the avian parasite *Eimeria tenella*. *PLoS Pathog* 7:e1002296
- Cowper B, Matthews S, Tomley FM (2012) The molecular basis for the distinct host and tissue tropisms of coccidian parasites. *Mol Biochem Parasitol* 186:1–10
- Ma D, Huang Y, Ma C, Zhang L, Wang J, Wang D, Li J, Dalloul RA (2019) *Eimeria tenella*: specific EtAMA1-binding peptides inhibit sporozoite entry into host cells. *Poult Sci* 98:4480–4491
- Ma D, Ma C, Pan L, Li G, Yang J, Hong J, Cai H, Ren X (2011) Vaccination of chickens with DNA vaccine encoding *Eimeria acervulina* 3–1E and chicken IL-15 offers protection against homologous challenge. *Exp Parasitol* 127:208–214
- Li J, Wang F, Ma C, Huang Y, Wang D, Ma D (2018) Recombinant lactococcus lactis expressing *Eimeria tenella* AMA1 protein and its immunological effects against homologous challenge. *Exp Parasitol* 191:1–8
- Shi LF, Wu Y, Li CM (2015) Identification of high-affinity VEGFR3-binding peptides through a phage-displayed random peptide library. *J Gynecol Oncol* 26:327–335
- Ma C, Zhang L, Gao M, Ma D (2017) Construction of *Lactococcus lactis* expressing secreted and anchored *Eimeria tenella* 3–1E protein and comparison of protective immunity against homologous challenge. *Exp Parasitol* 178:14–20
- Hessenberger S, Schatzmayr G, Teichmann K (2016) In vitro inhibition of *Eimeria tenella* sporozoite invasion into host cells by probiotics. *Vet Parasitol* 229:93–98
- Johnson J, Reid WM (1970) Anticoccidial drug: lesion scoring techniques in battery and floor-pen experiment. *Exp Parasitol* 28:30–36
- Lillehoj HS, Ding X, Dalloul RA, Sato T, Yasuda A, Lillehoj EP (2005) Embryo vaccination against *Eimeria tenella* and *E. acervulina* infections using recombinant proteins and cytokine adjuvants. *J Parasitol* 91:666–673
- Wiederstein M, Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res* 35:W407–W410
- Dalloul RA, Lillehoj HS (2006) Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Rev Vaccines* 5:143–163
- Srinivasan P, Yasgar A, Luci DK, Beatty WL, Hu X, Andersen J, Narum DL, Moch JK, Sun H, Haynes JD, Maloney DJ, Jadhav A, Simeonov A, Miller LH (2013) Disrupting malaria parasite AMA1-RON2 interaction with a small molecule prevents erythrocyte invasion. *Nat Commun* 4:2261
- Tomley FM, Soldati DS (2001) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. *Trends Parasitol* 17:81–88
- Li W, Wang M, Chen Y, Chen C, Liu X, Sun X, Jing C, Xu L, Yan R, Li X, Song X (2020) EtMIC3 and its receptors BAG1 and ENDOUL are essential for site-specific invasion of *Eimeria tenella* in chickens. *Vet Res* 51:90
- Collins CR, Withers-Martinez C, Hackett F, Blackman MJ (2009) An inhibitory antibody blocks interactions between components of the malarial invasion machinery. *PLoS Pathog* 5:e1000273
- Tyler JS, Boothroyd JC (2011) The C-terminus of *Toxoplasma* RON2 provides the crucial link between AMA1 and the host-associated invasion complex. *PLoS Pathog* 7:e1001282
- Zuercher AW, Miescher SM, Vogel M, Rudolf MP, Stadler MB, Stadler BM (2000) Oral anti-IgE immunization with epitope-displaying phage. *Eur J Immunol* 30:128–135

**Publisher's Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.