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ORIGINAL PAPER

Prokaryotic expression and identification of *3-1E* gene of merozoite surface antigen of *Eimeria acervulina*

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Abstract *Eimeria acervulina* was isolated from chicken at Hebei province, China. The gene of merozoite surface antigen 3-1E was amplified and cloned into pET28a(+) vector and then transformed into *Escherichia coli* BL21 strain. Results showed that 3-1E fusion protein band of about 22 kDa was identified by SDS-PAGE. Western blot analysis indicated that the recombinant protein specifically reacted with *E. acervulina* polyclonal antibody.

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Introduction

Coccidiosis is an ubiquitous avian disease that is recognized as a serious challenge for the poultry industry (Jiang 2000; Dalloul and Lillehoj 2006). The causative agent, *Eimeria* spp., is responsible for the destruction of the enteric epithelium, resulting in malabsorption, reduction of feed conversion, impaired growth rate and body weight gain in broilers, and egg production in layers as well as increased morbidity and mortality (Lillehoj et al. 2004, 2007). Chemoprophylaxis using ionophores and synthetic drugs is the main method for the control of coccidiosis. However, long-term use of these preventative drugs has caused the development of drug resistance in the parasite as well as the high cost of synthesis and test of new anticoccidial agents. Moreover, prophylactic drug usage creates deep anxiety concerned with chemical residues in food products (Min et al. 2004).

Immunoprophylaxis is a reliable alternative to control of coccidiosis in chickens (Han et al. 2007; Qu et al. 2004; Song et al. 2009; Chapman 2009) and to overcome the drug-induced problems. Live attenuated parasite vaccines induce pronounced immune responses (Constantinoiu et al. 2011) and are used to control outbreaks of infection in the field (Chapman et al. 2002). Furthermore, live oocyst vaccines proved to be useful to restore ionophore sensitivity in poultry operations that contain an ionophore-resistant population of *Eimeria* oocysts (Jenkins et al. 2010). However, conventional vaccines including live or attenuated vaccines could cause severe adverse reactions (Vermeulen et al. 2001). Subunit vaccines of *Eimeria* carrying encoding antigen gene fragments induce protective immunity against challenge infection (Garcia et al. 2008; Shah et al. 2010; Song et al. 2010) and are proven to be safe and effective means to prevent the deleterious effects of coccidiosis (Wallach et al. 2008).

3-1E antigen is an immunogenic 20-kDa protein that is located on the surface of *Eimeria* sporozoites and schizonts. This protein is highly conserved among various life cycle stages and species of *Eimeria* (Lillehoj et al. 2000; Song et al. 2000). DNA vaccination based on the *3-1E* gene could induce partial immune protection against coccidiosis (Lillehoj et al. 2000; Song et al. 2000; Min et al. 2001; Ma et al. 2010). In addition, vaccination with the recombinant 3-1E *Eimeria* protein induces protective intestinal immunity against coccidiosis (Min et al. 2001; Ding et al. 2004) and significantly diminish oocyst shedding, reduces intestinal lesion score, improves body weight gain, and increases anticoccidial index (Ma et al. 2010). The present study is designed to develop more simple, productive, and reliable means to express *3-1E* gene. Expression of *3-1E* gene of *Eimeria acervulina* in *Escherichia coli* BL21 strain was developed in order to acquire active protein in a bid to construct anticoccodial DNA vaccine and/or to develop a more sensitive diagnostic kit(s).

Materials and methods

Strain and main reagents

Oocysts of *E. acervulina* Baoding strain isolated from chicken and plasmid PGM-3-1E containing merozoite surface antigen *3-1E* gene of *E. acervulina* were kindly provided by the Laboratory of Parasitology at the College of Animal Science and Technology, Agricultural University of Hebei, China. *E. coli* strain BL21(DE3), expression vector pET28a (+), and PCR reagents were purchased from QIANGEN Beijing Biotech. Co. Ltd. *Eco*RI and *Xho*I restriction enzymes were from TaKaRa Biotech. Co. Ltd (Dalian, China).

Amplification of the *3-1E* gene

The complete ORF of *E. acervulina* merozoite surface antigen *3-1E* gene was amplified utilizing forward primer (P1) and reverse primer (P2) designed by Primer 6.0 software, based on the *3-1E* gene sequences of *E. acervulina* (Lu et al. 2009). The primer set was designed to contain restriction enzyme sites *Eco*RI and *Xho*I in forward primer (5′-GGCGAATTCCCCTTA CTCAGTTAAAATG-3′) and reverse primer (5′-CGCTCGAGAAGTCGTTGTTAT GAAAG −3′), respectively. The reaction mixture consisted of 2× PCR Master Mix (12.5 μL), PGM-3-1E template (2 μL), forward primer p1 (1 μL), reverse primer p2 (1 μL), and DEPC-H_2O (up to 25 μL). PCR amplifications were performed by initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 96°C for 1 min, annealing at 47°C for 50 s, and elongation at 72°C for 1 min, and final elongation step at 72°C for 10 min was included. The PCR products were visualized under UV light on ethidium bromide-stained 1% agarose gel after electrophoresis. The PCR products were purified using the PCR Fragment Agarose Gel DNA Purification Kit (QIANGEN) according to manufacturer's instructions and stored −20°C till use.

Construction and identification of recombinant expression vector pET28a-3-1E

The purified PCR products of the *3-1E* gene and pET28a (+) vector were digested by *Eco*RI and *Xho*I, respectively. The reaction mixture contained vector pET28a (+)/PCR products, 7 μL; *Eco*RI, 0.5 μL; *Xho*I, 0.5 μL; and 10× buffer, 2 μL, and sterile ddH_2O of up to 20 μL was mixed by centrifugation and incubation in water bath at 37°C for 4 h. The reaction was terminated by incubation in water bath at 65°C for 10 min.

The digested products were recycled by DNA gel extraction kit and connected with T4 DNA ligase at 16°C for 10 h and transformed into competent *E. coli* BL21(DE3). Transformants were plated on culture medium containing kanamycin, IPTG, and X-Gal overnight at 37°C and subjected for screening and selection. The recombinant expression plasmids pET28a-3-1E were extracted by a plasmid small-scale extraction kit and identified by PCR and *Eco*RI/*Xho*I enzyme digestion.

Induction expression and identification of the positive transformants

The single colonies of transformants were incubated in 5 mL of LB medium containing kanamycin) at 37°C and 180 rpm for 3 h (till OD_{600}=0.5–1.0). The target fusion proteins were expressed after induction by isopropyl-b-D-thiogalactopyranoside (Sigma) to a final concentration of 1 mmol/L. Expressed products were purified by protein A affinity chromatography, identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and specificity was determined by Western blot analysis utilizing polyclonal antibodies against *E. acervulina* merozoites and HRP anti-mouse IgG.

Results

Amplification and cloning of *3-1E* gene

PCR amplification of *3-1E* genes and recombinant plasmids as a template resulted in single band of 603 bp in size

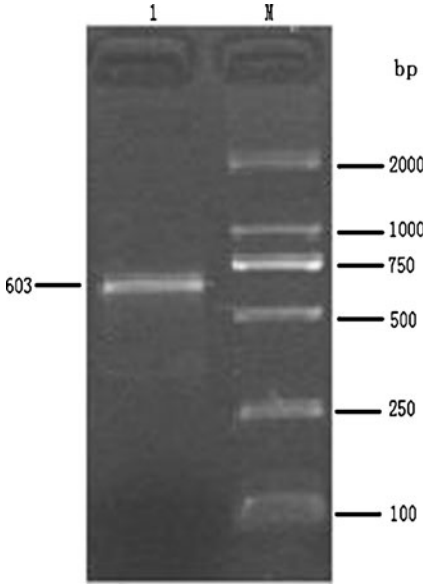

Fig. 1 PCR products of merozoite surface antigen *3-1E* gene of chicken *Eimeria acervulina* Baoding strain by electrophoresis on 1.0% agarose gel. *Line M*, DL2000 DNA marker; *line 1*, *3-1E* gene

(Figs. 1 and 2). The amplified fragment was successfully inserted into the plasmid vector. *Eco*RI/*Xho*I enzyme digestion of the recombinant plasmids resulted in a target band of 603 bp and a band of 5,369 bp in size corresponding pET28a (+) vector genome (Fig. 3).

Expression results of 3-1E fragments in *E. coli*

Expression product of pET28a-3-1E in HB121 was detected by SDS-PAGE and Western blot analysis. The expected molecular weight of approximately 22 kDa

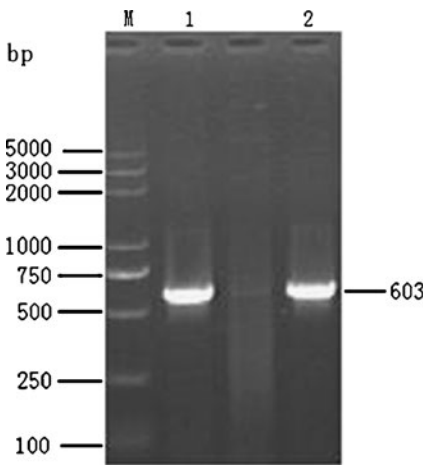

Fig. 2 PCR amplification of the recombinant plasmid. *Line M*, DL5000 DNA marker; *lines 1–2*, *3-1E* gene

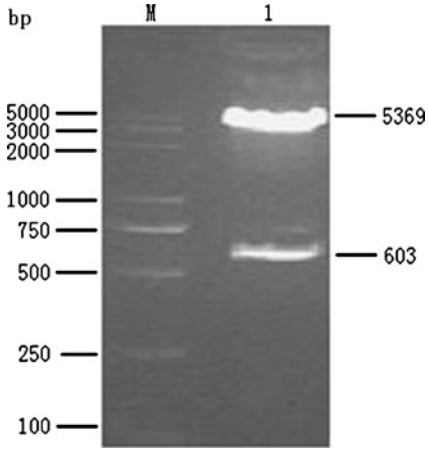

Fig. 3 Restriction enzyme digestion analysis of recombinant plasmid. *Line M*, DL5000 DNA marker; *line 1*, *Eco*RI/*Xho*I enzyme-digested products

specific protein band in HB121 strain was confirmed in SDS-PAGE (Figs. 4 and 5). In confirmation, Western blot analysis showed the band of 22 kDa in size (Fig. 6) and a strong reactivity of antibodies against *E. acervulina* merozoites.

Discussion

Recombinant antigens derived from *Eimeria* spp. could produce definite protective immunity against coccidiosis in chicken (Danforth et al. 1989; Crane et al. 1991; Mark and Marc 1991; Jenkins et al. 1988; Kim et al. 1989). 3-1E protein is a sporozoites and merozoite surface antigen protein that plays a significant role in the interaction

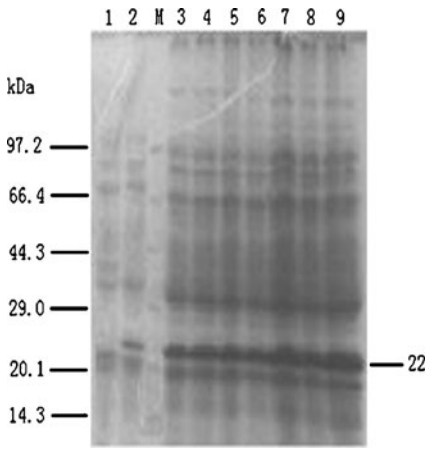

Fig. 4 SDS-PAGE of expression of pET28a-3-1E. *Line M*, low MW protein marker; *line 1*, negative control; *lines 2–9*, induced products at 2–9 h, respectively

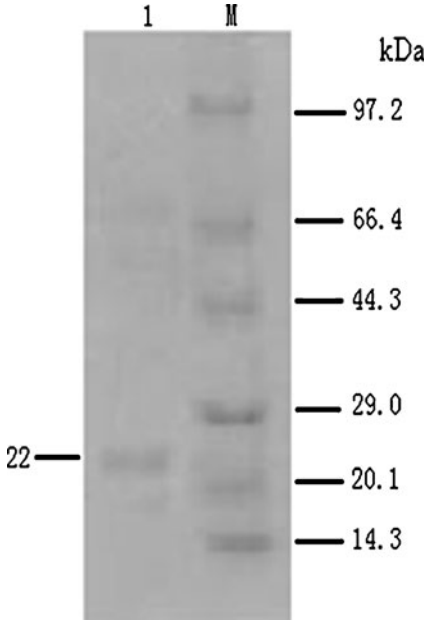

Fig. 5 SDS-PAGE of purified products 3-1E protein. *Line M*, low MW protein marker; *line 1*, purified 3-1E protein

between coccidia and host (McDonald and Shirley 2009). Recombinant or DNA-based vaccines of 3-1E boosted the immune response of recipients and provided a pronounced levels of protection (Lillehoj and Ruff 1987; Lillehoj and Trout 1993; Choi and Lillehoj 2000; Lee et al. 2010a, b). In spite of the recorded discrepancies, accumulated evidence indicated that 3-1E protein is highly conserved among various life cycle stages and different species of *Eimeria* (Laurent et al. 1994; Lillehoj et al. 2000; Song et al. 2000). The assumption that the conserved nature may be a good qualifier for 3-1E protein as a candidate to the work concerning different *Eimeria* spp., on the terms of diagnosis and vaccination, needs further studies.

In the present study, *3-1E* gene of *E. acervulina* merozoite surface antigen was cloned and expressed in *E. coli* BL21. The results reported herein indicated that ORF of *3-1E* gene of *E. acervulina* merozoite surface antigen is approximately 603 bp, which is obviously different from the one (510 bp) reported by Ma et al. (2010). Interestingly, the molecular weight of the expressed product of *3-1E* gene for *E. acervulina* merozoites, whether reported in the present study (22 kD) or (23 kD) by Lillehoj et al. (2000), was considerably different from that reported for *Eimeria tenella* sporozoites by Kim et al. (1989). These discrepancies may be attributed to the differences in the expression systems, complexity of chicken coccidia life history and hugeness of genomes, difference of immunogenicities and antigen formation in different development stages of chicken coccidia, and difference of immunogenicities in different kinds of coccidian. Construction of recombinant plasmid pET28a-3-1E and expression and purification of the 3-1E protein may facilitate the means to develop novel vaccine.

High expression of foreign gene, in the terms of yield and bioactivities, is affected by many factors, such as DNA secondary structure, 5′ end and 3′ end sequence stability, selections of expression vector and expression cell, constructive form of expression, induced expression ways and treatment of expression product, etc. (Gu et al. 2008) . *E. coli* expression system has become a first choice system for expression of foreign gene/s because of its clear genetic background, high expression of target gene, convenient culture, simple operation, and low cost. In this experiment, the target gene could be expressed successfully in *E. coli* BL21 with considerably high yield pET28a (+) vector selected in this experiment is encoded by gene labeled with 6×His (Gu et al. 2008). Therefore, expression target protein were isolated and purified with nickel column affinity chromatography. N-terminal protein expressed by pET32a (+) has 6×His label and can bind with Ni-NTA column whereas other undesired protein cannot. The Ni-NTA binding proteins were eluted, and the target protein was purified and characterized by SDS-PAGE and Western blot analysis. Western blot analysis utilizing polyclonal antibodies against *E. acervulina* merozoites and HRP anti-mouse IgG indicated that the *3-1E* gene of chicken *E. acervulina* could be expressed in *E. coli* BL21, the expression product showed reactivity with antibodies against *E. acervulina* merozoites, and can be used as a candidate gene for chicken coccidiosis recombinant DNA vaccine.

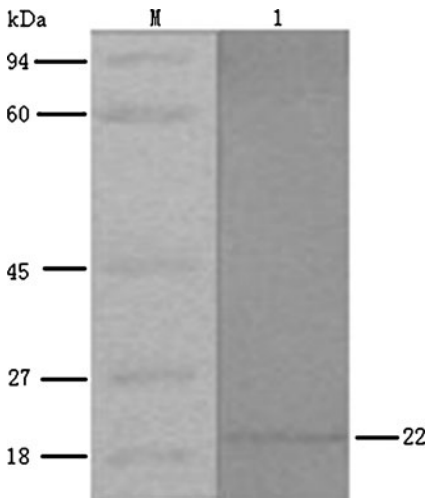

Fig. 6 Western blot analysis of expressed 3-1E protein. *Line M*, low MW protein marker; *line 1*, to 3-1E protein

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