PROCEEDINGS OF THE 11th WORLD RABBIT CONGRESS
Qingdao (China) - June 15-18, 2016
ISSN 2308-1910

Session
Pathology and Hygiene

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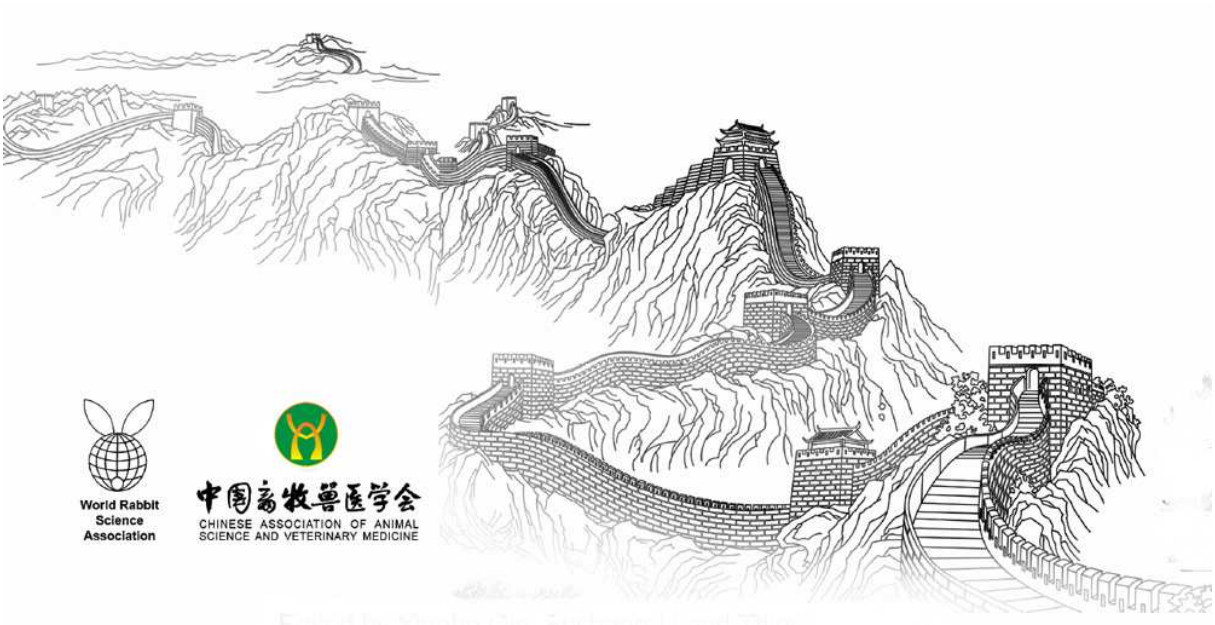
How to cite this paper :
Tao G., Wang Y., Li C., Gu X, Liu X., Suo X., 2016 - Cloning and characterizing *profilin* gene from rabbit coccidia *Eimeria magna*. *Proceedings 11th World Rabbit Congress - June 15-18, 2016 - Qingdao - China, 587-590. + Poster.*



CLONING AND CHARACTERIZING *PROFILIN* GENE FROM RABBIT COCCIDIA *EIMERIA MAGNA*

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ABSTRACT

Coccidiosis caused by parasites of the genus *Eimeria* is one of the most important diseases that affect rabbit industry. Profilin protein is essential in migration and host cell invasion for the apicomplexan parasites. In this study, the coding sequence (CDS) of *Eimeria magna* profilin protein (EmagPRF) was cloned using degenerated primers and genome walking technique. EmagPRF CDS was of 522 bp encoding a polypeptide of 174 amino acids. Successful expression of recombinant EmagPRF was realized in *E. coli.* Western-blot revealed that EmagPRF was continuously expressed in unsporulated oocysts, sporulated oocysts, sporozoites and merozoites. Immunofluorescence analysis for subcellular localization of EmagPRF showed specific staining on the membrane and the posterior end of *E. magna* sporozoites. Our work laid the groundwork for further studies on immunobiology of EmagPRF.

Key words: Rabbit coccidia, *Eimeria magna*, Profilin, CDS.

INTRODUCTION

Rabbit coccidiosis is an important infectious disease caused by parasites of the genus *Eimeria*. Although treatment with prophylactic anticoccidial drugs is the primary approach in most rabbit farms, high infection rate was still found (Jing et al., 2012). Vaccination is the most attracting option for coccidiosis control. For the control of chicken coccidiosis, numerous efforts were made to identify parasite antigens as components in subunit vaccines to be developed (Shirley et al., 2005). For instance, the most successful one, CoxAbic®, a subunit vaccine prepared from the affinity purified gametocyte antigen (APGAs) of *E. maxima* has been successfully commercialized (Wallach et al., 2008). However, due to the lack of genomic and proteomic information of rabbit *Eimeria*, little work had been done referring relevant research.

Profilins are well known for their function in actin filament polymerization across eukaryotes. For the Apicomplexan parasites, it is primarily engaged in migration and host cell invasion (Denkers et al., 2008). Profilins derived from several eimerian parasites have been tested as vaccine candidate and adjuvant (Song et al., 2000; Ding et al., 2004; Jang et al., 2010). Profilin protein of *Toxoplasma gondii* is also involved in inducing host innate immune response as a ligand of Toll-like receptors (Yarovinsky et al., 2005; Plattner et al., 2008). Therefore, we aim to identify the profilin protein from *E. magna*, a pathogenic species in rabbits, for the study of its immunogenic property as subunit vaccine of rabbit coccidiosis.

MATERIALS AND MATHODS

Parasites and animals

E. magna Zhangjiakou strain was used in this study. Oocysts were propagated, collected, sporulated and purified as described previously (Huang et al., 2011). Coccidia- free New Zealand White rabbits were provided by Xinglong Laboratory Animal Breeding Center.

RNA extraction, cDNA synthesis and amplification of the CDS

Profilin protein CDS of *E. tenella*, *E. acervulina*, *T. gondii*, and *Neospora caninum* were aligned and 3 pairs of degenerate primers (P1, P2, P3) were designed according to the conserved sequences (Table 1). EmagPRF

partial CDS was amplified using cDNA template derived from total RNA extracted from sporozoites and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) with each pair of the primers. PCR products with the expected size of about 500 bps were retrieved and cloned into pEASY-Blunt cloning vector (TransGen, China). DNA sequence was analyzed and based on which, genome walking primers were designed (Table 1) in order to obtain full-length CDS. Genome walking was conducted following the manufacturer's instruction (TaKaRa, China). The deduced amino acid sequence of obtained EmagPRF was evaluated for domain architecture analysis by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequences alignment was generated by DNAMAN V6.0.

Table 1 Primers used in cloning the full-length EmagPRF

Names of primers	Forward primer 5'-3'	Reverse primer 5'-3'
	Degenerate primers	
P1	ATGGGTGAAGAGGCTGATACTCAGGCGT	TTAGWASCCRSMCTGGTRMAGRTACTCRGCR
P2	ATGTCCGACTGGGACCCTGTTGTCAAGGART	TTAGWASCCRSMCTGGTRMAGRTACTCRGCR
P3	AAGGARTGGCTYGTKGAYACRGG	YTCYTTYTCYTCRTCRTANAR
	Genome walking Primers	
5'-SP-1	Random primer in kit	TACAAGCCTAGGTCAAGAGGGCATTGG
5'-SP-2	Random primer in kit	AGAAGCATCTTTCTTGGGCTTTAATGAT
5'-SP-3	Random primer in kit	AACCTTGCCAGAGTCCACCAGCCAC
3'-SP-1	CAAATGTATTTTGATTTATCCCCCG	Random primer in kit
3'-SP-2	CCCTACGACATCGCGCTTCTGACA	Random primer in kit
3'-SP-3	ACAAAGTAGATGCATTCAACACAGC	Random primer in kit

Preparation of recombinant EmagPRF (*r*EmagPRF) and polyclonal antibody

The EmagPRF CDS was subcloned into pET-28a (+) and expressed in *E. coli*. The soluble *r*EmagPRF expressed in the bacteria was purified from bacterial lysate using the HiTrap™ chelating HP column (GE Healthcare, USA). Polyclonal antibody (pAb) was prepared by 3 times of subcutaneous immunization of BALB/c mice with 100 μg *r*EmagPRF at 2- week intervals.

Expression analysis of EmagPRF

Indirect immunofluorescent assay (IFA) for subcellular localization was performed on *E. magna* sporozoites using anti- EmagPRF pAb (1: 2k) and cy3-conjugated goat anti-mouse IgG (Proteintech, China) (1:500) as primary and secondary antibodies, respectively. Images were recorded under a confocal microscope (Leica SP5, Germany). To determine whether EmagPRF was continuously expressed in all developmental stages of the parasite, total parasite proteins of unsporulated oocysts, sporulated oocysts, sporozoites and merozoites were obtained either by grinding pellet of parasites in liquid nitrogen or by freezing and thawing method according to a previous study (Fetterer et al, 2003). Protein concentrations were determined by a BCA Protein Assay Kit (Cwbiotech, China), and the same amount were subjected to 15% SDS-PAGE followed by Western-blot. Anti-EmagPRF pAb (1:2k) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) pAb (1:1k) were used.

RESULTS AND DISCUSSION

Cloning and molecular characterization of EmagPRF

A single ~500 bp band was obtained by using the 3rd degenerate primers (Fig. 1A). Sequencing results revealed partial CDS of EmagPRF. Following genome walking analysis revealed the full length CDS of EmagPRF which was of 522 bp encoding a polypeptide of 173 amino acids. The predicted domain of deduced amino acids belongs to the profilin protein family (from 9 to173 amino acid) (Fig. 1B). The identity of deduced amino acid sequences among four other apicomplexan parasites was from 48% to 71.2% (Fig. 1C). Moreover, successful periplasmic expression of *r*EmagPRF in *E. coli* was acquired for subsequent experiments (Fig. 1D).

Expression of EmagPRF in different life stages

Detection of EmagPRF in unsporulated oocysts, sporulated oocysts, sporozoites and merozoites was

performed by western-blot using pAb obtained from mice serum immunized with *r*EmagPRF. Whole parasite protein probed with anti-EmagPRF antibodies showed the same ~20 kDa bands, indicating that EmagPRF was constitutively expressed in the four stages aforementioned (Fig. 2A).

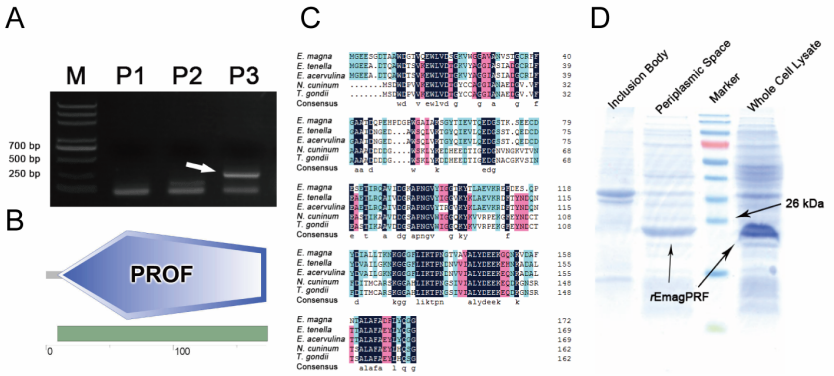

Fig. 1 Cloning and identification of EmagPRF. A. Electrophoresis of PCR products of EmagPRF using 3 degenerated primer pairs (P1, P2 and P3). **B**. Simple domain architecture analysis was performed by SMART. **C**. Deduced amino acid sequence alignment of profilin protein of the five species by DNAMAN V6.0. **D.** Recombinant *r*EmagPRF expression was realized and coomassie blue- staining indicated that protein was expressed in the periplasmic space of *E. coli*.

Subcellular localization of EmagPRF in sporozoites

Profilin is expected to be aggregated in the cytoplasm within areas of highly dynamic actin filaments (Schlüter et al., 1997). Indirect immunofluorescent assay showed that sporozoites were stained primarily in the posterior cytoplasm and parasite membrane (Fig. 2B). The current results are consistent with the identification of profilin proteins of *E. tenella* and *E. acervulina* (Laurent et al., 1994; Fetterer et al., 2004).

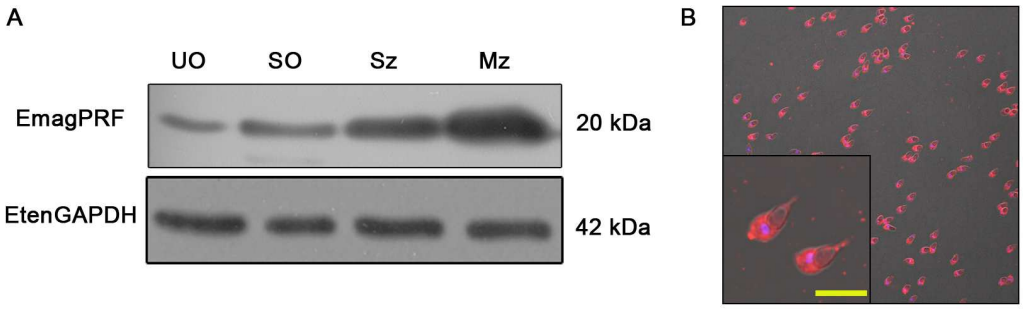

Fig. 2 Expression and subcellular localization of EmagPRF. A. Mice antisera against EmagPRF (1:2k) and EtenGAPDH (1:1k) were used for detection of EmagPRF expression in unsporulated oocysts (UO), sporulated oocysts (SO), sporozoites (Sz) and merozoites (Mz) of *E. magna*. **B.** Freshly purified sporozoites were treated with pAb against EmagPRF (1:2k) and Cy3-conjugated goat anti-mouse IgG (1:500) to confirm the subcellular localization of EmagPRF. DAPI (blue) indicated the parasite nuclei. Bar = 10 μm.

CONCLUSION

In this study, we identified the coding sequence and expression pattern of *E. magna* profilin protein(EmagPRF). Further investigation of the biological and immunological properties of EmagPRF will be needed to determine the potential capability as a subunit vaccine candidate.

ACKNOWLEDGEMENTS

We are grateful for the help from colleagues Jingxia Suo and Xiuling Tian in imaging with confocal laser scanner. We thank colleague Dr. Xinming Tang for revisions that improved the manuscript. This research was

supported by China Agricultural Research System (CARS-44) and the National Natural Science Foundation of China (key project, No. 31330076).

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RNA extraction, cDNA synthesis and amplification of the CDS

Profilin protein CDS of *E. tenella*, *E. acervulina*, *T. gondii*, and *Neospora caninum* were aligned and 3 pairs of degenerate primers (P1, P2, P3) were designed according to the conserved sequences (Table 1). EmagPRF partial CDS was amplified using cDNA template derived from total RNA extracted from sporozoites and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) with each pair of the primers. PCR products with the expected size of about 500 bps were retrieved and cloned into pEASY-Blunt cloning vector (TransGen, China). DNA sequence was analyzed and based on which, genome walking primers were designed (Table 1) in order to obtain full-length CDS. Genome walking was conducted following the manufacturer's instruction (TaKaRa, China). The deduced amino acid sequence of obtained EmagPRF was evaluated for domain architecture analysis by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequences alignment was generated by DNAMAN V6.0.

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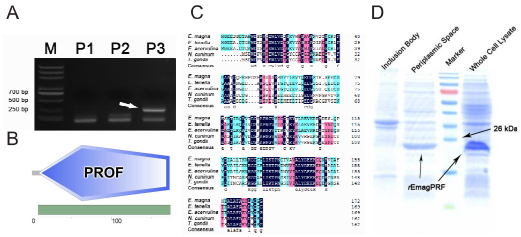

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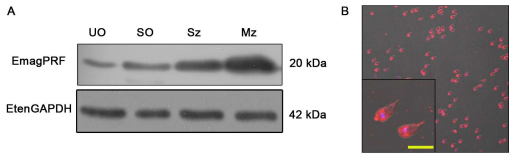

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