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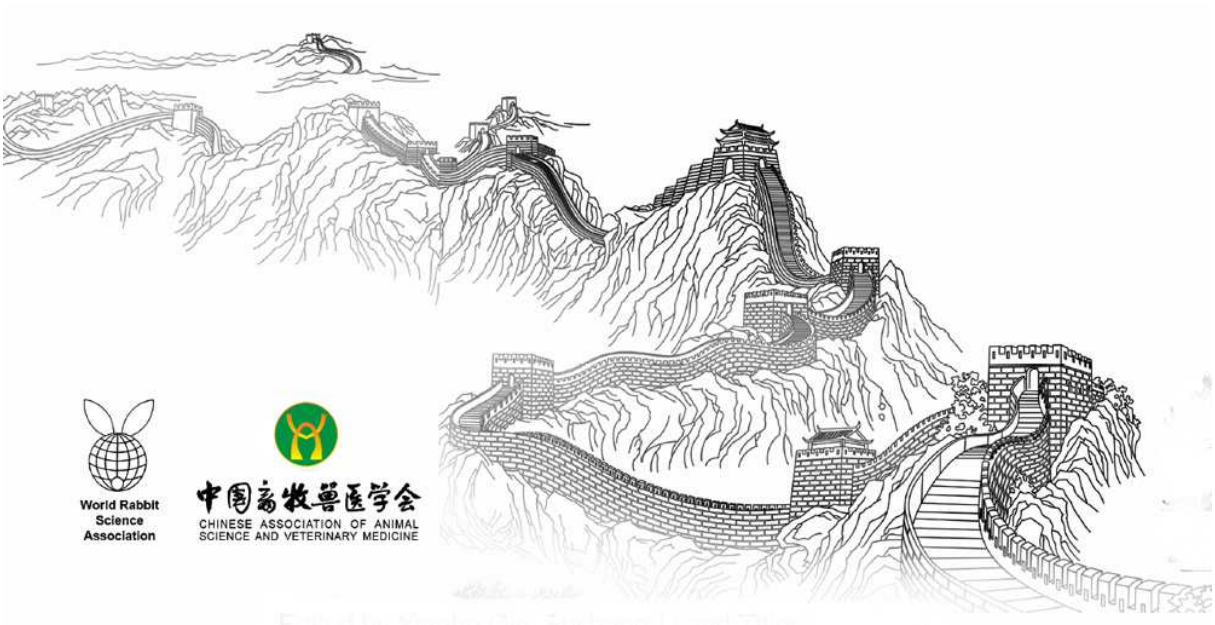

TRANSGENIC *EIMERIA MAGNA* EXPRESSES EYFP THROUGHOUT THE ENTIRE LIFE CYCLE

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ABSTRACT

Rabbit coccidiosis is one of the most prevalent parasitic diseases that causes great economic losses in rabbit farms. Studies on the etiological agents, *Eimeria* spp. focusing on life cycle, pathogenicity and selection of precocious lines were reported. However, little work has done using genetic manipulation for profound knowledge of the parasite. In this study, we constructed a transgenic line of *E. magna* using regulatory sequences of *E. tenella*. Investigation of its life cycle showed that the transgenic parasites expresses EYFP throughout the entire life cycle. This study laid the groundwork for transgenesis of rabbit coccidia.

Key words: Rabbit coccidia, Transgenic, *Eimeria magna*, EYFP, Life cycle.

INTRODUCTION

Eimeria spp. are obligate intracellular parasites that infect gut epithelium of a wide range of hosts. Severe infection of *Eimeria* spp. can result in coccidiosis, which causes huge economic losses both in poultry and rabbit industry. Research on chicken coccidia have been carried on for decades because of the economic importance of the host (Smith et al., 1998). Due to the availability of genetically modified host, studies have been performed on mouse models characterizing detailed knowledge of the host immune response against the parasites, as well (Stange et al., 2012; Schmid et al., 2013). In contrast, little attention was drawn on research of rabbit coccidia in recent years.

There are 11 species of rabbit coccidia, among which *E. magna* is one of the most prevalent one in rabbit farms. Detailed information describing the endogenous stages and selection of precocious line were given in previous studies (Licois et al., 1995; Pakandl et al., 1996). *E. magna* owns moderate pathogenicity and high immunogenicity which makes it an excellent model for research on rabbit coccidia (Niilo, 1967). In this study, we constructed a transgenic line of *E. magna* expressing double marker genes, enhanced yellow fluorescent protein (EYFP) and red fluorescent protein (RFP) (*E. magna* E-R) using regulatory sequences of *E. tenella* and *Toxoplasma gondii*, which helped to fill in the gap of research on transgenic rabbit coccidia.

MATERIALS AND METHODS

Parasites and animals

The wild type of *E. magna* was originally isolated in Hebei, China. For propagation of both wild type and transgenic parasite, 500 sporulated oocysts were given per rabbit. Oocysts were collected from feces excreted 7~9 days post inoculation.

All animals used in this study were obtained from Hebei Zhuozhou rabbit breeding farm. 3-week-old New Zealand White rabbits were weaned and reared by bottle-feeding of powdered milk under coccidia-free condition away from their mothers till 4 weeks to completely avoid coccidial

contamination. 4-week-old rabbits for parasite propagation or other assays were kept in isolators and fed with coccidia-free pellet and water *ad libitum* .

Transfection and selection of transgenic *E. magna*.
We adapted a single expression cassette in which DHFR-Ts2m3m, a pyrimethamine resistance gene from *T. gondii*, EYFP and RFP were inserted between *E. tenella* histone 4 promoter (His4) and actin 3' untranslated region. A porcine teschovirus-1 2A peptide which was proved to be able to cleave two contiguous proteins (Kim et al., 2011) was added between EYFP and RFP-His. Meanwhile, dense granule protein 8 (GRA8) from *T. gondii*, a regulatory sequence was ligated with RFP (Figure 1 A).

Transfection of *E. magna* sporozoites was conducted according to an established protocol (Liu et al., 2008; Yan et al., 2009). Briefly, 2×10^7 purified sporozoites were electroporated using a nucleofector (Program U-033, AMAXA, Switzerland). Sporozoites were resuspended in DMEM and added in the medium of MDBK culture for observation of a transient transfection, or injected into a 4- week old rabbit duodenum in a simple laparotomy for *in vivo* transfection. Screening of the transgenic oocysts were conducted by fluorescence- activated cell sorting (MoFlo Cell Sorter, Dako-Cytomation, Fort Collins, Co) and addition of 150 mg/kg pyrimethamine in the rabbit pellet as a drug selection.

Genomic and expression analysis of exogenous genes
Integration site and expression status of the exogenous DNA were analyzed by genome walking and western blot. First, *E. magna* E-R genome was isolated by phenol/chloroform extraction. Specific primers were obtained and flanking sequences were identified as previously described (Qin et al., 2014). Second, we conducted western blot to identify whether RFP was cleaved from EYFP by P2A peptide. Parasite soluble extracts were prepared as previously described (Fetterer et al., 2003). The lysis were subjected to SDS-PAGE and western blot in the same volume. Monoclonal antibody against 6×His(ABclonal) and *E. tenella* GAPDH polyclonal antibody were applied.

Observation on whole life cycle of *E. magna* E-R.
Total seven 4-week-old rabbits were inoculated with different doses of *E. magna* E-R oocysts and euthanasia was performed 24, 48, 72, 86, 120, 144, 152 h.p.i as described before (Pakandl et al., 1996). Jejunum and ileum were washed with cold HBSS, and smears were made by scraping the mucosa of the intestine. Fresh smears and sporulation process of newly collected oocysts were visualized under a confocal laser scanning microscopy (SP5, Leica, Germany) for detection of EYFP-expressing parasites.

RESULTS AND DISCUSSION

Sucessful transfection of *E. magna*
For *in vitro* transfection, fluorescent sporozoites in MDBK cell culture were observed 24 h after nucleofection (Figure 1 B). As the foreign genes were initiated by his4 promoter, EYFP was mainly expressed in the nuclei of the sporozoites and RFP was observed in the nuclei and the cytoplasm indicating that regulatory sequences from *E. tenella* can be utilized by transgenic *E. magna*. After several trials on *in vivo* transfection, finally we obtained a transgenic oocyst population at a transfection efficiency of 0.01‰ (data not shown). Transgenic oocysts were propagated in coccidia-free rabbits, and grew up about 40% of the whole population after 5 successive passages. Unfortunately, higher fluorescent rate was not realized in subsequent passages. The very low transfection rate, both *in vivo* and *in vitro* puts a serious threshold for further study of transgenic rabbit coccidia.

All transgenic oocysts express EYFP in the nuclei, and RFP both in the nuclei and cytoplasm (Figure 1C). To identify the integration site, genome walking analysis was conducted (Figure 1E). Recovered DNA fragments were sequenced, and identified (Figure 1F). To further identify P2A peptide for its self-cleaving function in transgenic *E. magna*, western-blot using a mAb against His tag was performed. A 25 kDa (RFP- 6×His) band was detected demonstrating that P2A worked well in *E.*

magna (Figure 1D). This result favored future research on transgenesis when expressing more than one gene is required in *E. magna*.

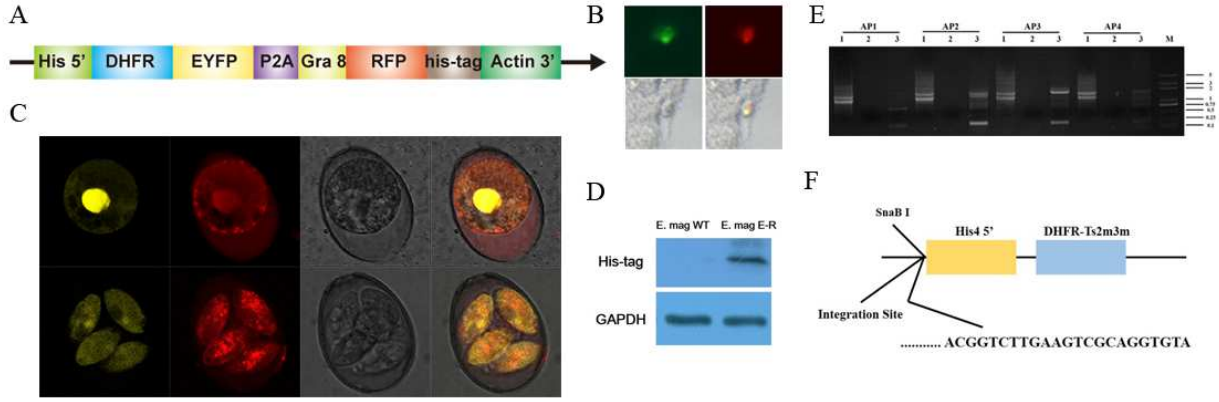

Figure 1: Construction of transgenic *E. magna* expressing double marker genes. **A.** Plasmid *p*HDEp2aRA used in transfection. **B.** Transient transfection of *E. magna* sporozoites in MDBK culture. **C**. Unsporulated and sporulated oocysts of *E. magna* E-R. **D.** Western blot identifying P2A function. **E & F.** Genome walking analysis revealed that exogenous genes were inserted into the parasite genome.

***E. mag* E-R expresses EYFP throughout the life cycle.**

Taking advantage of the fluorescent protein expressed by the trasgenic parasites, all developmental stages of the entire life cycle were easily distinguished (Figure 2). Invading sporozoites, as well as trophozoites ongoing nucleus division were found at 24 h post inoculation (Figure 2 A&B). Mature meronts of each generation were found at 48, 72, 86, 120 h post inoculation, respectively (Figure 2 C~J). Number of merozoites in each developmental stages were consistent with the wild type as previously described (Pakandl et al., 1996). Interestingly, since EYFP was mainly expressed in the nuclei, both multinucleate and uninucleate merozoites were vividly distinguishable. Gametes and immature oocysts were discovered at 144 h, 152 h post inoculation (Figure 3 K~N). In addition, several typical phases of nuclear division and cytokinesis during sporogony of the parasites were also observed (Figure 2 O~S).

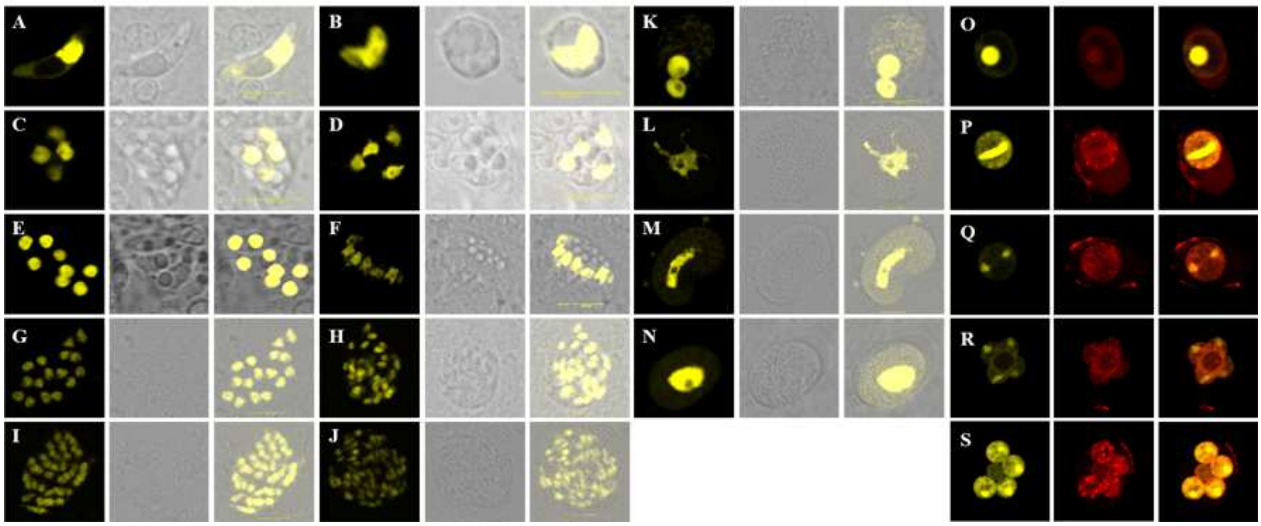

Figure 2 EYFP was expressed throughout the entire life cycle of *E. magna* E-R. **A-I** Different stages of schizogonies of *E. magna* E-R. **K-N** Gametogony of *E. magna* E-R. **O-S** Sporogony of *E. magna* E-R.

CONCLUSIONS

In this study, we constructed a transgenic *E. magna* expressing double marker genes using a plasmid containing regulatory sequences from *E. tenella*. Strategies used for transfection of *E. tenella*, such as restriction enzyme- mediated integration (REMI) and DHFR as drug-resistant gene were helpful for transfection and selection of *E. magna*. However, we did not obtain a transgenic population of a higher fluorescent rate. Thus, investigation of its phenotypes, for example, pathogenicity, immunogenicity was yet to perform. Nevertheless, the constitutive expression of EYFP in transgenic parasites made it convenient to observe and identify the morphology of the parasites throughout the life cycle.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

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Analysis of exogenous genes and observation on whole life cycle of *E. magna* E-R

Integration site and expression status of the exogenous DNA were analyzed by genome walking and western blot.

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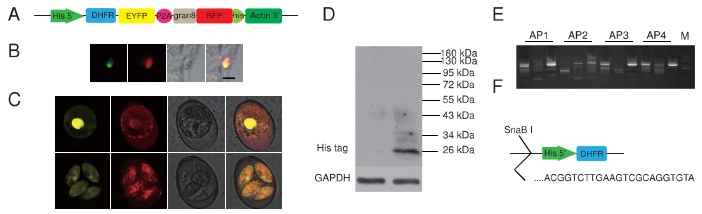

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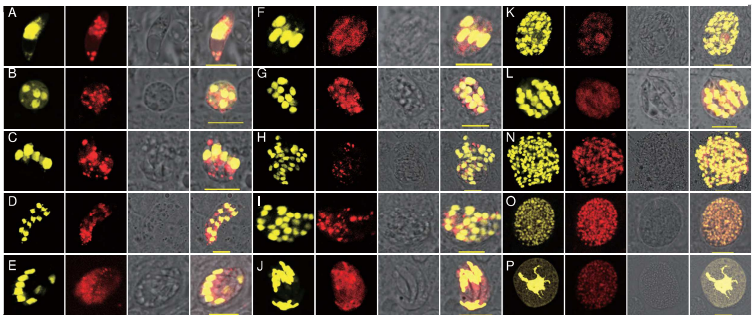

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