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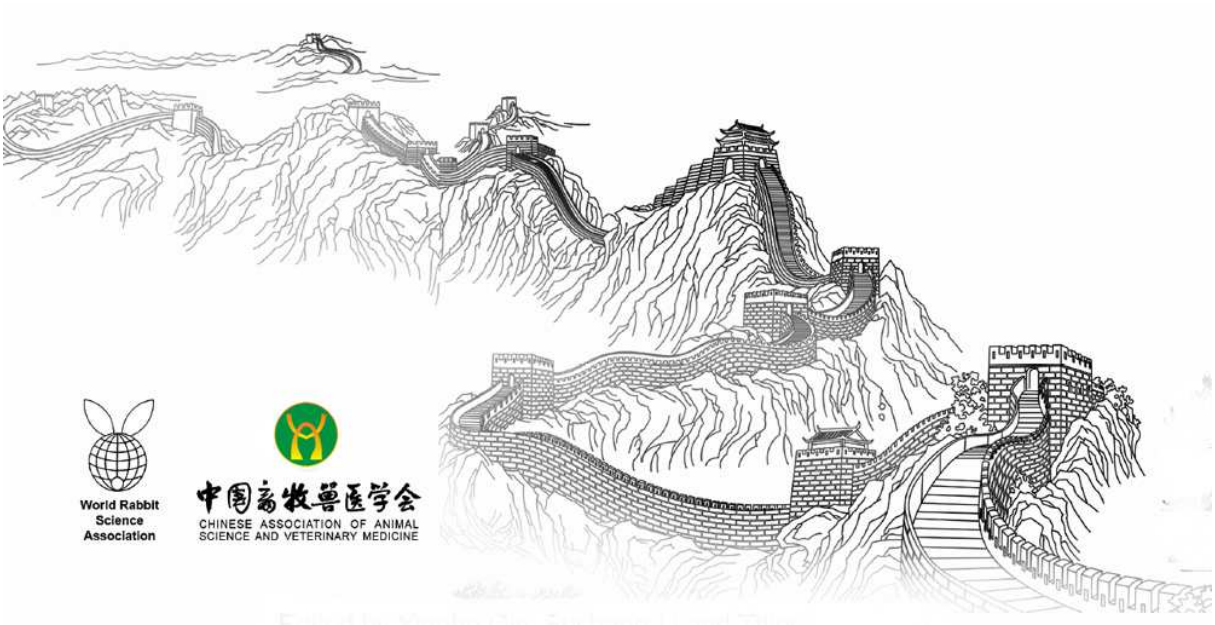

STABLE TRANSFECTION OF *EIMERIA INTESTINALIS* AND INVESTIGATION OF ITS LIFE CYCLE, REPRODUCTION AND IMMUNOGENICITY

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ABSTRACT

Rabbit coccidiosis, which is caused by *Eimeria* spp, is one of the most severe parasitic diseases in rabbit farms. *E. intestinalis* is one of the most pathogenic species in rabbits. Due to the lack of genomic information and unsuccessful *in vitro* cultivation, genetic manipulation of rabbit coccidia lagged behind other apicomplexan parasites. Using regulatory sequences from *E. tenella*, we obtained a transgenic line of *E. intestinalis* expressing yellow fluorescent protein (YFP). YFP was continuously expressed throughout the whole life cycle. Morphological features of *E. intestinalis* in the different developmental stages were dynamically observed with the transgenic line. Some important features in the endogenous development stages were observed as well. In addition, the transgenic parasite was highly immunogenic but less pathogenic than the wild type. The transgenic *E. intestinalis* may be a promising vector for delivering foreign antigens.

Key words: Transgenic, *Eimeria intestinalis*, EYFP, stable transfection.

INTRODUCTION

Coccidiosis, induced by infection of parasites in the genus *Eimeria* is one of the most common and highly contagious diseases in rabbits, causing great economic losses. *Eimeria* parasites are recognized to be potential ideal live vaccine vectors delivering heterologous antigens (Shi et al. 2008; Yan et al., 2009). However research advances on transfection of *Eimeria* are slower than those of *Toxoplasma gondii* and *Plasmodium* spp. due to the difficulty of continuous *in vitro* cultivation of the parasites. Recently, some progresses have been made on the transfection of chicken *Eimeria*. Stable transfection systems have been successfully established in chicken *Eimeria* species including *E. tenella* and *E. mitis* (Yan et al., 2009; Qin et al., 2014), as well as in rat specific species, *E. nieschulzi* (Kurth et al., 2009). Yet, there is no report on transfection of rabbit coccidia. In this study, we established a transgenic system in *E. intestinalis* and explore some of its important biological features.

MATERIALS AND METHODS

Parasite, animals and cell culture

Oocysts of a low virulent *E. intestinalis* strain isolated and identified before was used in the study (Shi et al., 2014). Purified sporozoites used in transfection were gained according to modified method by Schmatz et al. (1984).

Coccidia-free rabbits were reared as follows. The young rabbits were weaned at 18 days, and fed human infant formula in combination with rabbit pellets till 30 days old. The feed and water were sterilized and provided *ad libitum*. Feces from each cage of rabbits (2 rabbits per cage) was checked daily for *Eimeria* oocysts. In addition, primary rabbit intestine cells (PRICs) were used for transient transfection of *E. intestinalis*.

Plasmid, parasite transfection, and screening of stably transfected parasites

The plasmid pEtHEA, carrying a promoter from 5′ flanking sequence of *E. tenella* histone 4 gene, a tandem yellow fluorescent protein genes (YFP-YFP), and a terminator from 3′ untranslated region sequence of *E. tenella* actin gene was used to transfect *E. intestinalis*. Transfection of *E. intestinalis* was carried out according to previous report (Liu et al., 2008).

Observation of the life cycle of transgenic *E. intestinalis*

Total 9 rabbits were inoculated with transgenic *E. intestinalis* oocysts to observe endogenous stages (5 for early stages, 4 for late stages). Euthanasia was performed at 4, 24, 48, 72, 96, 120, 168, 216 and 336 h post infection (p.i.). Also, freshly excreted oocysts were collected at 336 h p.i. from fecal samples, and allowed to sporulate in 2.5% of potassium dichromate solution. The parasites were observed under a fluorescence microscope.

Measurement of reproduction and immunogenicity of the transgenic parasite

Sixteen 34-day-old coccidia-free rabbits were distributed into four groups, with 4 rabbits per group and 2 rabbits per cage. Rabbits in two groups were orally inoculated with 5×10^3 sporulated oocysts of wild-type and transgenic line, respectively. The other two groups were used as non-immunized challenged control and non-immunized non-challenged control. Challenged rabbits were given 1×10^6 sporulated oocysts of the wild-type 16 days pi. Body weights of all rabbits were measured at 0 and 16 days after immunization and at 10 and 19 days after challenge. Feces were collected every day during two periods ranging from 9 to 15 days after the immunization and 9 to 16 days after challenge. Oocysts per gram of feces (OPG) were counted in a McMaster chamber.

Statistical Analysis

Statistical analysis was performed by one-way ANOVA, followed by Duncan's multiple range test using the SPSS 16.0 software. Data were expressed as mean ± standard deviation. Differences between groups were considered statistically significant when *p* values were less than 0.05.

RESULTS AND DISCUSSION

Transient and stable transfection of *E. intestinalis*

In vitro, fluorescent sporozoites and first generation schizonts were observed at 24 h, 48 h after inoculation to PRICs. YFP was mainly located within nucleus of the parasites (Fig. 1A and B). *In vivo* transfection, fluorescent oocysts were observed in rabbit feces on the 9th day after inoculation. The cytometry analysis revealed that only 0.01‰ of the 1st generation expressed YFP. In subsequent passages, fluorescent rate increased to 21.64% in the 2nd generation and 80% in the 3rd generation. The result showed that the regulatory sequences from *E. tenella* were highly functional in *E. intestinalis*.

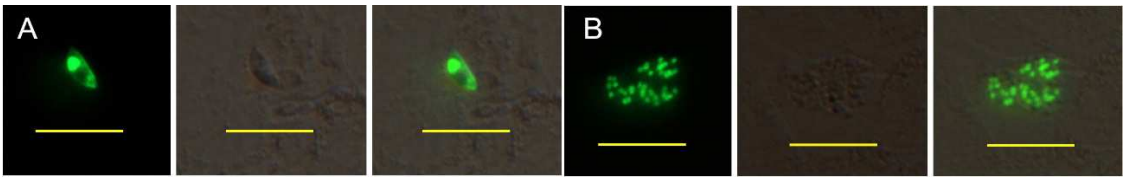

Figure 1: Transient transfection of *E. intestinalis*. Transgenic sporozoite (A) and meront (B) were observed in PRIC culture. Bar=20μm.

Transgenic *E. intestinalis* expresses YFP throughout the entire life cycle

Endogenous development stages of transgenic *E. intestinalis* in intestine of rabbit were observed under a fluorescent microscope between 4 to 366 h p.i. (Fig 2). Particularly, many trophozoites were detected as early as 4 h p.i., which was sooner than 24~48 h pi. reported previously (Licois et al., 1992). Beside lower jejunum and ileum, some parasites of the gametogony were also found in duodenum and vermiform appendix. Sporogony of transgenic oocysts in 2.5% of potassium dichromate solution at 28 °C was also observed using the fluorescence microscopy (Fig.3).

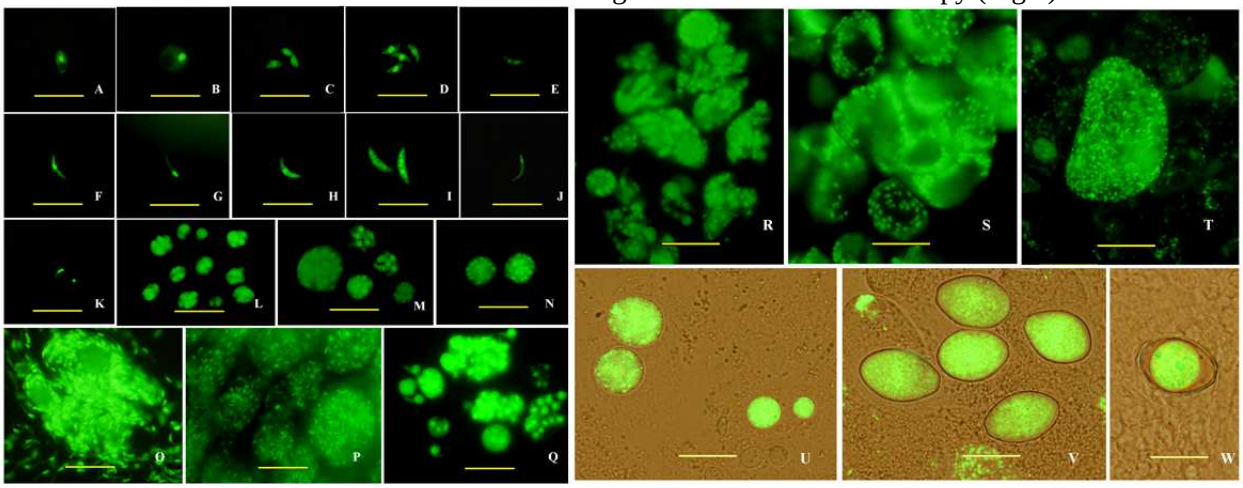

Figure 2 Endogenous developmental stages of the transgenic *E. intestinalis.* Merozoites (A-K) and meronts (L-S) in different schizogonies. T. A microgamont. U. Macrogamonts. V. Zoygotes. W A mature oocyst. Ba r= 20μm.

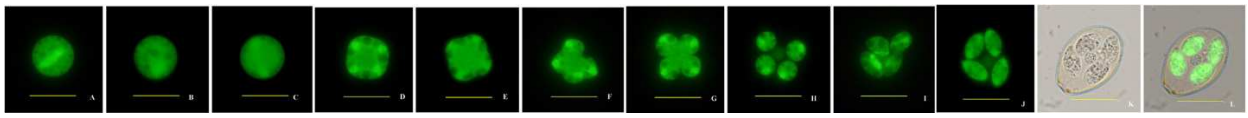

Figure 3 Sporogony of the transgenic *E. intestinalis.* Bar = 20μm.

Transgenic *E. intestinalis* exhibits reduced pathogenicity and preserved high immunogenicity

Mean oocyst output per rabbit immunized with both stains were determined. The result showed that oocyst yield of transgenic *E. intestinalis* was significantly less than that with the wild type (~13%) ($p \leq 0.05$) (Table 1), indicating a weakened reproductivity. In addtion, no case of diarrhea or mortality was seen after the challenge in immunized rabbits, and no significance in mean body weight was found compared with non-immunized non-challenged group ($p > 0.05$)(Fig. 4). Moreover, similar oocyst reduction between the transgenic parasite and the wild type was detected after challenge, indicating that the transgenic *E. intestinalis* possess similar protective efficacy as the wild type (Table 1). Considering the high immunogenicity of *E. intestinalis*, we speculated that transgenic *E. intestinalis* could be a promising eukaryotic vaccine vector.

Table 1. Oocyst output after immunization and challenge.

Group	Wild type Immunized	Transgenic line Immunized	Non-immunized challenge	Non-immunized Non-challenged
Oocysts output per rabbit after immunization	$1.922\times10^{9}\pm2.069\times10^{8}$[b]	$2.569\times10^{8}\pm3.328\times10^{7}$[a]	0	0
Oocysts output per rabbit after challenge	$2.810\times10^{8}\pm2.353\times10^{7}$[a]	$6.785\times10^{7}\pm3.558\times10^{6}$[b]	$1.809\times10^{9}\pm1.815\times10^{8}$[c]	0
Reduction percentage	84.35%±2.23%[a]	62.25%±3.96%[b]	0	—

A, b, c represent statistically significant difference between immunized and control, $a > b > c$ ($p \leqslant 0.05$).

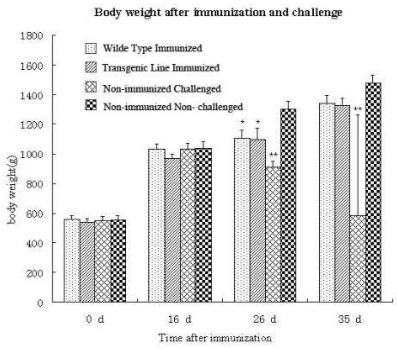

Figure 4 Rabbit body weight after immunization and challenge.

CONCLUSIONS

Here we show a stable transgenic line of *E. intestinalis*. Taking advantages of the fluorescence expressed by transgenic parasite, we identified some important features in the endogenous developmental stages. Moreover, this transgenic line showed reduced pathogenicity while maintained high immunogenicity indicating potential properties as an antigen delivery vehicle in further studies.

ACKNOWLEDGEMENTS

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