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PROTOZOOLOGY - ORIGINAL PAPER

Selection and identification of a precocious line of *Eimeria intestinalis* with enlarged oocysts and deletion of one generation of schizogony

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Abstract

Rabbit coccidiosis is a common parasitic disease and responsible for enormous economic losses in the rabbit industry. *Eimeria intestinalis*, one of the highly pathogenic and common *Eimeria* species infecting rabbits, is considered as an indispensable species for the development of live oocyst vaccines against rabbit coccidiosis. In this study, we report the successful selection of a precocious line (EIP8) from a wild-type strain of *E. intestinalis* (WT) by successively collecting and propagating the early excreted progeny oocysts. The EIP8 line had a prepatent period of only 132 h compared to 204 h for the WT. Oocysts of EIP8 were notably different from those produced by the WT strain by their significantly larger size (mean length: 29.3 vs 27.6 μm and mean width 20.5 vs 19.8 μm). Examination of tissue sections prepared from EIP8-infected rabbits revealed that this precocious line undergoes only two generations of schizogony before differentiating into gametocytes by 120 h post-infection. In contrast, WT parasites undergo three generations of schizogony and gametocytes are present by 168 h post-infection. EIP8 multiplication capacity reduced by more than 35-fold and a concomitant decrease in pathogenicity was detected. Interestingly, immunization with 10^3 or 10^4 EIP8 oocysts provided sufficient protection against homologous challenge with wild-type parasites, as body weight gain of immunized and challenged rabbits was similar to that of untreated animals, as well as more than 90% reduction of oocyst output was detected in immunized and challenged animals when compared to unimmunized and challenged animals. Together, these results show that the EIP8 precocious line of *E. intestinalis* is an attenuated immunogenic strain and a suitable candidate for the development of a live vaccine against rabbit coccidiosis.

Keywords *Eimeria intestinalis* · Precocious line · Morphology · Endogenous development · Pathogenicity · Immunogenicity

Introduction

Coccidiosis is an important disease of farm animals caused by various *Eimeria* species. The disease is responsible for major economic losses to the rabbit industry. Among the 11 *Eimeria* species that infect rabbits (*Oryctolagus cuniculus*), *E. intestinalis* is one of the most highly pathogenic parasites (Coudert et al. 1995). Previous studies have shown that *E. intestinalis* infection can result in severe clinical signs, including depression, body weight loss, diarrhea, and even death (Coudert et al. 1993).

Licois and colleagues selected a precocious line of *E. intestinalis* with a prepatent period reduced from 8.5 to 5.5 days and a reproduction capacity reduced to less than 1/1000 that of the wild type (WT) (Licois et al. 1990). Electronic microscopy study of this line showed that a large refractile body (RB) existed (in one of the two sporozoites or free in the sporocyst) in two of the four sporocysts while the

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other two sporocysts did not contain RB (Pakandl et al. 2001). This is different from WT strain which has one RB in each sporozoite. Significant changes in the RB morphology were also reported in precocious lines of *E. magna*, *E. media*, and *E. piriformis* (Pakandl et al. 2001; Pakandl and Jelinkova 2006). In the case of the precocious lines of *E. media* and *E. magna*, a very large RB was observed within each sporocyst in an oocyst (Pakandl et al. 2001).

E. intestinalis undergoes four generations of schizogony in the epithelium of the small intestine of infected rabbits, and sexual development initiates following the third or fourth generation (Licois et al. 1992). However, comparison of endogenous development had not been conducted for the precocious line and its parental wild-type strain. Previous studies showed that the second (or third) and fourth generations of schizogony were absent in a precocious line of *E. flavescens*, while the fourth generation of schizogony was absent in a precocious line of *E. piriformis* (Pakandl 2005; Pakandl and Jelinkova 2006).

In the present study, we report the selection of a precocious line of *E. intestinalis* and provide a detailed description of its oocyst morphology, reproductive capacity, pathogenicity, immunogenicity, and the endogenous development. Our results suggest that this precocious line of *E. intestinalis* could be an excellent candidate for the development of a live attenuated vaccine against rabbit coccidiosis.

Materials and methods

Animals

Forty to fifty-day-old coccidia-free New Zealand White rabbits, delivered by breeding rabbits continuously supplied with diclazuril (1 mg/L) or sulphachloropyrazine sodium (2 g/L) alternately in water, were used in this study. After weaning, these rabbits were individually reared in wire cages under coccidia-free conditions and fed with coccidia-free pellets and water ad libitum (pellets and water were pre-heated in 80 °C for 2 h). China Agricultural University Animal Ethics Committee approved all experimental procedures, and due attention was paid to the welfare of the animals.

Parasites

Original oocysts were collected from fecal samples of naturally infected rabbits from Hebei Province, China. The pure strain of *Eimeria intestinalis* was established by single-oocyst isolation (Coudert et al. 1995). Oocysts were propagated and maintained according to standard protocols (Long et al. 1976). The purity of the wild-type strain (WT) was assessed as previously described for *E. perforans* (Coudert et al. 1979). Selection of the precocious line from the WT was conducted as previously reported (Jeffers 1975). Briefly, the oocysts shed during the early time of the patent period were collected and used for propagation in the next round of selection. The 8th generation progeny (EIP8) was obtained (Table 1) and tested for its precociousness. Freshly sporulated oocysts of both WT and EIP8 were used in the follow-up experiments.

Stability test of EIP8

Three rabbits were individually inoculated with 5×10^3 oocysts of EIP8. Progeny oocysts excreted between days 9 and 14 post-inoculation were collected. The recovered oocysts were inoculated to other naïve rabbits with the same dose for the next propagation. This propagation was performed successively five times without selection pressure. The prepatent time in each generation was measured.

Morphological characteristics of the oocysts

Freshly sporulated oocysts of WT or EIP8 were observed under light microscopy (Leica SP5, Germany). The oocyst shape, location of RB, and residual bodies were examined. After oocyst imaging with the built-in Leica LAS AF Software, the length and width of 200 oocysts for each strain were individually measured with the same software.

Endogenous development study

Fifteen rabbits were separately inoculated with different doses of oocysts of WT or EIP8 and euthanized at time points indicated in Table 2. Tissue samples were taken from duodenum, jejunum, ileum, and subjected to section preparation and then HE- staining.

Fecundity, pathogenicity, and immunogenicity test

Eight groups of 32 rabbits were used for the study. Group 1 was an uninfected and unchallenged control (UUC); group 2 was uninfected but challenged with WT (UC); groups 3 to 5 were inoculated with 1×10^2, 1×10^3, and 1×10^4 WT oocysts; and groups 6–8 were inoculated with 1×10^2, 1×10^3, and 1×10^4 EIP8 oocysts, respectively. Fourteen days post-infection, animals except for UUC were each challenged with 1×10^5 WT oocysts. The clinical condition of animals was monitored throughout the duration of the study and rabbits were weighed each week. Daily oocyst output per rabbit in each group was detected, respectively, from days 5 to 14 post-immunization. The total oocyst output per rabbit was measured from days 9 to 14 post-challenge.

Table 1 Selection of the precocious line of *E. int* (EIP8)

Date	Strain inoculated	Rabbits	Prepatent time in feces(h)	Dose	Oocysts output	Strain obtained
2013.9.7	P0	2	204	5×10^4	6.8×10^7	P1
2013.11.7	P1	2	199	7×10^4	6118	P2
2014.1.7	P2	2	205	7×10^4	1.5×10^6	P3
2014.3.5	P3	3	197.5	1×10^5	9.2×10^5	P4
2014.3.18	P4	2	190	1×10^5	2.8×10^5	P5
2014.5.6	P5	3	174	1×10^5	4.0×10^6	P6
2014.6.9	P6	3	154	1.5×10^5	4.0×10^5	P7
2014.6.21	P7	3	132(−72)	1×10^5	1.5×10^5	P8(EIP8)

Statistical analyses

Statistical analyses were performed using *t* test of Excel, one-way ANOVA of SPSS 19.0 Software. Duncan's multiple range test was used. Differences between groups were considered statistically significant when p value < 0.05.

Results

Selection of an *E. intestinalis* precocious line

In order to obtain a precocious line of *E. intestinalis*, we performed a selection by collecting oocysts that emerge early after a series of 8 passages. By the 4th passage, the prepatent period of the selected lines was reduced to 197.5 h, whereas by the 6th, it was reduced to 174 h, and by the 8th passage, this was further shortened to 132 h (Table 1). As a result, the prepatent period of EIP8 line was 72 h shorter than the WT (132 h for EIP8 vs 204 h for the WT). This shorter prepatent period of EIP8 was maintained after 5 generations of successive passages without selection pressure.

Oocyst morphology

To determine whether oocyst morphology was altered following selection, freshly sporulated WT and EIP8 oocysts (200 oocysts each) were examined by light microscopy. Whereas the sporulated oocyst of the EIP8 was similar to that of the WT with a pear-like shape, 1 RB in each of the 2 sporozoites in 1 sporocyst, and 1 residual body in each of the 4 sporocysts (Fig. 1a), a significant difference ($P < 0.05$) in oocyst size between EIP8 and WT was present (Fig. 1b). Oocyst size of EIP8 was (29.3 ± 1.5 μm) × (20.5 ± 0.9 μm), while that of WT was (27.6 ± 2.4 μm) × (19.8 ± 1.2 μm). The length to width ratio of EIP8 was 1.42, which was bigger than that (1.39) of WT ($P < 0.05$). As shown in Fig. 1c, the trendline peaks of both length and width distribution of oocysts suggest enlarged EIP8 oocysts compared to WT oocysts.

Endogenous development

We further observed the endogenous development of EIP8 and compared it to that of the WT following rabbit infection. In all infected animals, schizonts were detected in the duodenum, jejunum, and ileum in both epithelial cells and crypts whereas gametocytes were found primarily in epithelial cells. Whereas three schizogony generations prior to gametogony were observed following infection with WT, only two schizogony generations were observed before gametogony following infection with the EIP8 precocious line (Fig. 2). In both EIP8 and WT-infected rabbits, trophozoites were detected at 24 h post-inoculation (Fig. 2a, b) and schizonts emerged at 48 h post-inoculation (Fig. 2c, d). More schizonts were detected at 72 h (Fig. 2e, f). The peak of the first generation schizogony was reached at 80 h with mature schizonts having an average size of 19.7 × 15 μm for EIP8 and 25.8 × 22.5 μm for WT (Fig. 2g, h). At 96 h post-infection, the peak of the second generation, mature schizonts had an average size of 28.7 × 23.3 μm for EIP8 and 30.3 × 24.7 μm for WT (Fig. 3i, j). At 120 h post-infection with the EIP8 line, both macrogamonts and oocysts could be detected in epithelial cells of infected rabbits (Fig. 3k). In contrast, in WT-infected rabbits, 120 h post-infection corresponded to the third generation schizogony (Fig. 3l) with the peak of schizogony reached at 144 h (34 mature schizonts could be seen in Fig.

Table 2 Intervals of sampling of tissue and inoculation dose in the experiment

Hour (h)		24	48	72	80	96	120	144	168	192
Dose	WT	1×10^7	1×10^7	1×10^7	1×10^7	1×10^6	1×10^6	1×10^6	3×10^5	3×10^5
	EIP8	1×10^7	1×10^7	1×10^7	1×10^7	1×10^6	1×10^6			

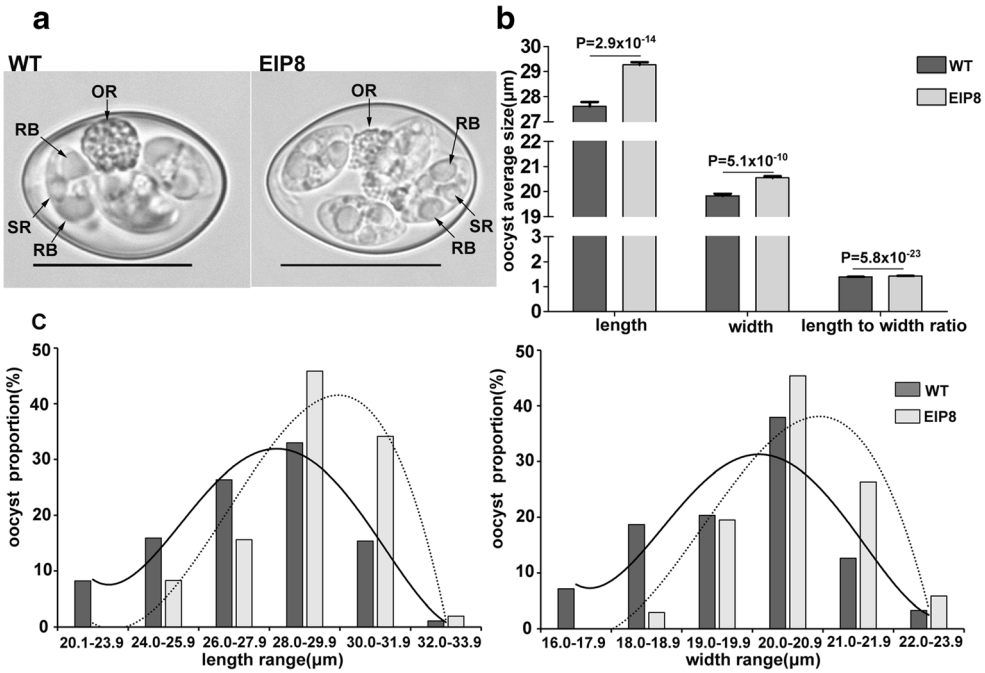

Fig. 1 Morphology comparison between EIP8 and WT. Dark bar: WT. Light bar: EIP8. **a** Photos of WT and EIP8. Bar = 20 μm. OR: oocyst residuum. SR: sporocyst residuum. RB: refractile body. **b** Comparative analysis of length and width of WT and EIP8. **c** Oocyst proportion distribution in different length (left) and width (right) range. The lines were the trend lines of the bar charts. Solid line: WT. Dashed line: EIP8

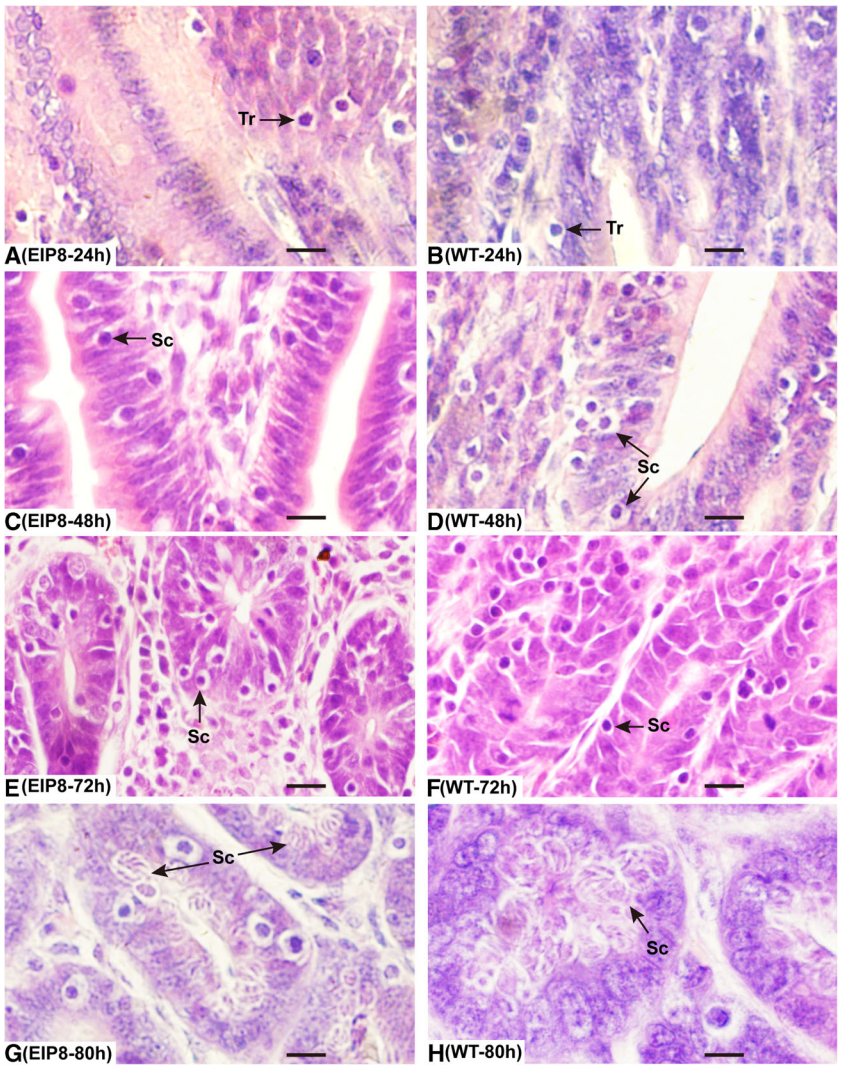

Fig. 2 Observation of endogenous development of EIP8 and WT in ileum. **a**, **b** Trophozoites; **c**–**h** first generation schizonts. Tr: trophozoite; Sc: schizont. Bar = 20 μms

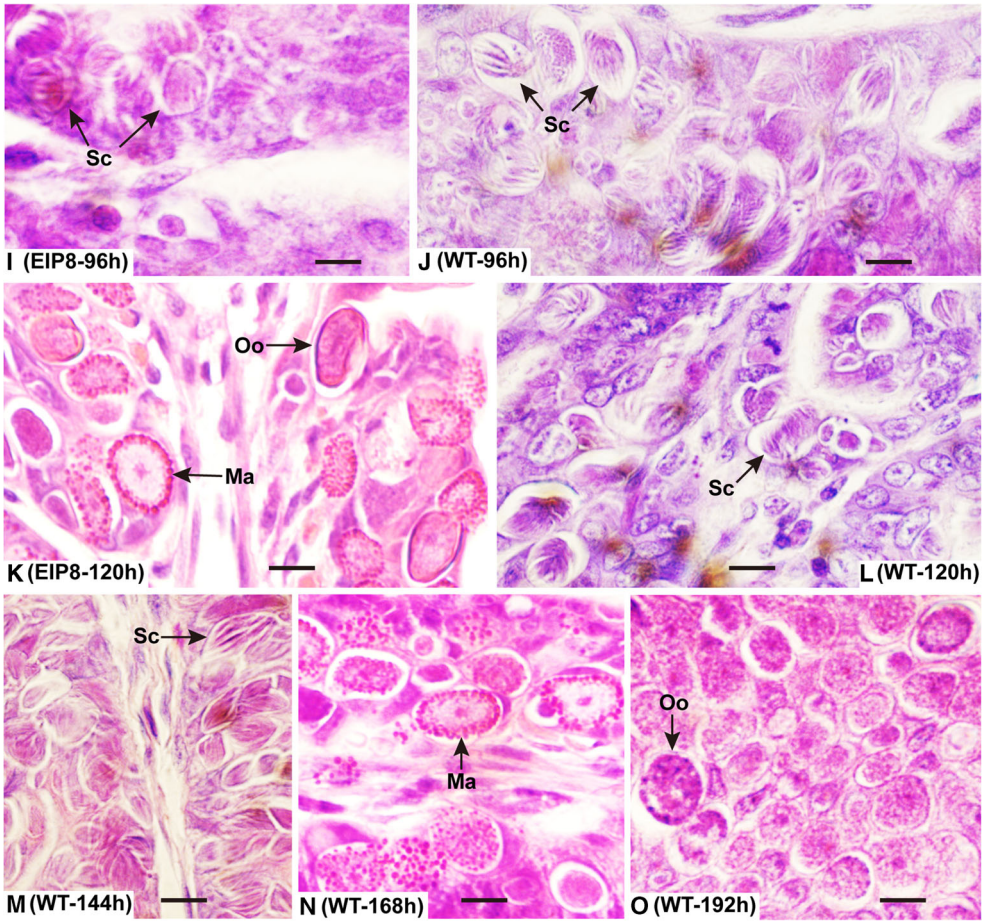

Fig. 3 Observation of endogenous development of EIP8 and WT in ileum. **i**, **j** Second generation schizonts; **k** gametocytes, oocysts, and fourth generation schizonts (related to the fourth generation schizonts of WT) of EIP8; **l**, **m** third generation schizonts of WT; **n** gametocytes and fourth generation schizonts of WT; **o** Oocysts of WT. Tr: trophozoite; Sc: schizont; Ma: macrogametocyte; Oo: oocyst. Bar = 20 μm

3m). At 168 h post-infection with WT oocysts, gametocytes were detected in epithelial cells (Fig. 3n), and by 192 h, oocysts could be detected in epithelial cells (Fig. 3o).

Oocyst production curve and multiplication rate

In order to compare the excretion rates between WT and EIP8, we counted oocyst output following inoculation of rabbits with 10^2, 10^3, and 10^4 oocysts. Daily oocyst output was detected from days 5 to 14 post-inoculation; however, the peak of oocyst shedding occurred on day 6 or 7 for the EIP8 precocious line, whereas that for the WT occurred on day 10 (Fig. 4a). When inoculated with 100 oocysts, a single rabbit infected with EIP8 excreted on average 5.8×10^5, whereas a rabbit infected with WT parasites produces 7.1×10^7 oocysts on average. Our analysis showed that the total oocyst output of EIP8 was only 1/35 to 1/122 that of the WT. Using an infection dose of 1×10^4, the maximal oocyst outputs for EIP8 and WT were 2.4×10^7 and 1.5×10^9, respectively (Fig. 4b).

Pathogenicity

To evaluate the pathogenicity of the EIP8 line, three groups of four rabbits each were infected with either 10^2, 10^3, or 10^4 oocysts and monitored for clinical signs and body weight over a period of 14 days. As a control, two non-infected groups of four rabbits each were included in the analysis. No diarrhea or loss of body weight was detected in these EIP8-infected groups. In contrast, rabbits infected with 10^2, 10^3, or 10^4 WT oocysts showed typical signs of *E. intestinalis* infection including diarrhea and weight loss (Fig. 4c). As shown in Fig. 4c, rabbits infected with 10^4 WT oocysts lost 30.6% of their body weight on average (totally 331.7 g) from days 7 to 14 post-infection. In contrast, rabbits infected with 10^4 EIP8 oocysts increased 25.0% of their body weight on average (totally 288.7 g) from days 7 to 14 post-infection.

Immunogenicity

To further examine the immunogenicity of the EIP8 line, rabbits inoculated with 10^2, 10^3, or 10^4 EIP8 oocysts were challenged with 10^5 WT oocysts on day 14 post-infection and both body weight gain and oocyst production were measured. As control, three groups of rabbits inoculated with 10^2, 10^3, or 10^4 WT oocysts were challenged with 10^5 WT oocysts; one unimmunized but challenged control (UC) group, and unimmunized and unchallenged control (UUC) group were also included. Interestingly, following challenge, the body weight

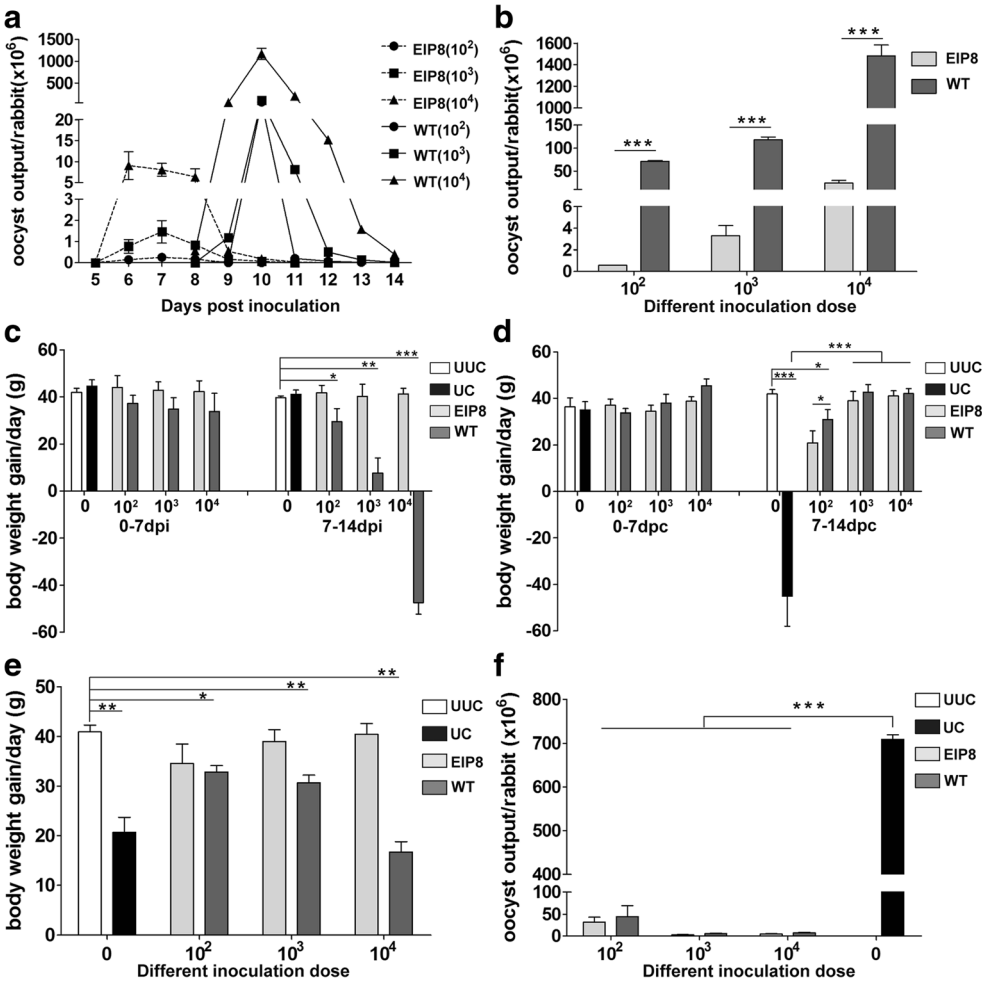

Fig. 4 Productive capacity, pathogenicity, and immunogenicity of EIP8 and WT. Oocyst output (**a**) and total oocyst output (**b**) of EIP8 and WT. Rabbits were inoculated with 1×10^2, 1×10^3, 1×10^4 EIP8 or WT oocysts, respectively. Oocyst output was detected daily from days 5 to day 14 post-inoculation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (the same below). Body weight gain post-immunization (**c**) and post-challenge (**d**) with 1×10^2, 1×10^3, 1×10^4 EIP8 or WT oocysts, respectively. **e** Total body weight gain from day 0 post-immunization to day 14 post-challenge. **f** Total oocyst output post-challenge

gain of rabbits immunized with EIP8 oocysts at high doses (10^3 and 10^4) was similar to that of the UUC control animals (39 g per day on average from days 0 to 14 post-challenge). Rabbits immunized with WT oocysts at high doses (10^3 and 10^4) gained similar weight as well (Fig. 4d). On the other hand, rabbits in the UC group showed a loss of 18.0% of body weight (315 g) from days 7 to 14 post-challenge. In contrast, rabbits infected with 10^4 EIP8 oocysts increased 16.6% of their body weight on average (totally 286.3 g) in the same period. However, rabbits immunized with 10^2 EIP8 or WT oocysts gained less weight than that of the UUC animals ($P < 0.05$) from days 7 to 14 post-challenge, indicating that immunization with 100 oocysts only provided partial protection following a homologous challenge with 10^5 WT oocysts.

In all study groups, when the body weight gain from day 0 post-immunization to day 14 post-challenge was calculated, there was no significant difference between EIP8-immunized groups and UUC group (Fig. 4e). While the weight gain decreased significantly ($P < 0.05$) in groups immunized with 10^2, 10^3, 10^4 WT oocysts or UC group, analysis of oocyst production in the study groups showed that in rabbits immunized with EIP8 lines, oocyst production post-challenge was 3.1×10^6 to 3.2×10^7, more than 90% reduction compared to UC group, and similar reduction of oocyst production were detected in rabbits immunized with WT oocysts (Fig. 4f).

Discussion

Here, we report the successful selection of a precocious line of *E. intestinalis* with a prepatent time reduced from 204 to 132 h. This precocious line is characterized with large oocyst size, a reduced number of generations of its schizogony, decreased fecundity, low pathogenicity, and retentive immunogenicity. Furthermore, the precocious line was found to provide sufficient protection following subsequent challenge with WT parasites.

As previous studies showed, RB number and morphology in precocious lines of several rabbit *Eimeria* species were different from those of the parental strains which have one RB in each sporozoite. In the precocious lines of *E. media* and *E. magna*, only a large RB was seen in each sporocyst and it was in one sporozoite or free in the sporocyst. Each sporozoite contained no or only one small RB in these precocious lines (Pakandl et al. 2001). In a precocious line of

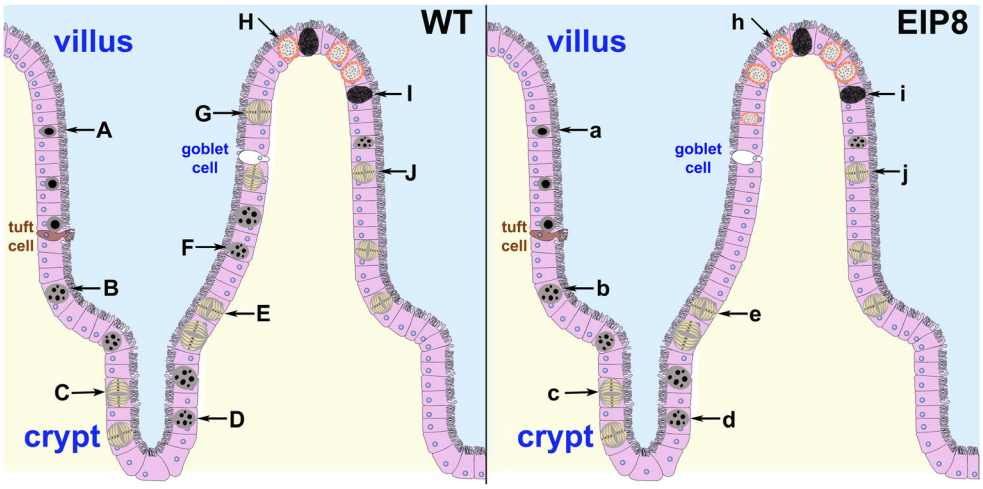

Fig. 5 Model of *Eimeria intestinalis* development in intestinal epithelial cells and crypts. **A/a** trophozoites (epithelial cells and crypts); **B/b**, first generation schizonts in early stage (epithelial cells and crypts); **C/c**, mature schizonts of the first generation schizogony (epithelial cells and crypts); **D/d**, second generation schizonts in early stage (epithelial cells and crypts); **E/e**, mature schizonts of the second generation schizogony (epithelial cells and crypts); **F**, third generation schizonts in early stage (epithelial cells and crypts); **G**, mature schizonts of the third generation schizogony (epithelial cells and crypts); **H/h**, gametocytes (epithelial cells); **I/i**, oocysts (epithelial cells); **J/j**, fourth generation schizonts (epithelial cells)

E. intestinalis, a large RB was seen in one of two sporocysts; however, no RB was seen in sporozoites or sporocyst in the other two sporocysts (Pakandl et al. 2001). In a precocious line of *E. piriformis*, a very large RB was observed free in each sporocyst in addition to the RBs within sporozoites (Pakandl and Jelinkova 2006). In our study, sporulated oocysts of the precocious line EIP8 and WT showed no obvious change in RB morphology and location, which is not in accordance with those in the previous studies. In addition, our finding of enlarged size of sporulated oocysts in this precocious line is, for the first time, reported for rabbit coccidia. However, the mechanisms determine the change of oocyst size and RB morphology and location in these precocious lines remain unknown and need further research work based on genome and transcriptome analysis.

Shortening of the asexual reproduction by one of two generations is common among precocious lines of rabbit coccidia (Pakandl 2005; Pakandl et al. 1996a, 1996b; Pakandl and Jelinkova 2006). One asexual generation (the third) was absent before gametogony in the *E. intestinalis* precocious line we selected. In other species, the fourth meront was absent in both *E. magna*, *E. media*, and *E. piriformis* precocious lines, and two asexual generations, second (or third) and fourth generation, were absent in *E. flavescens* precocious line. An interesting phenomenon was that gametocytes appeared before the last asexual generation in *E. intestinalis*, but they appeared after the last generation in other rabbit coccidia (El-Shahawi et al. 2012; Licois et al. 1992). That means some gametocytes develop from the third generation and later gametocytes develop from the fourth generation merozoites in *E. intestinalis*, whereas all gametocytes develop from the fourth generation merozoites in other species. These findings indicate that the third but not the fourth schizont generation was absent in *E. intestinalis* precocious line.

Our study further characterized the location of the developing parasites in vivo (Fig. 5). We found that trophozoites first appear in epithelial cells and crypts, then they develop to the first and second generation schizonts. Gametocytes and oocysts mainly developed in epithelial cells in EIP8. The fourth generation schizonts appeared in epithelial cells. In WT, the third generation schizonts, gametocytes, and the fourth generation schizonts occurred in crypts and mainly in epithelial cells. WT oocysts developed in epithelial cells. Together, these data demonstrate that EIP8 endogenous development is shorter than WT with only two schizogony generations before gametogony occurs.

Though oocyst production decreased markedly following the deletion of one generation schizogony, the immunogenicity of the precocious lines was retained. Rabbits were totally protected against challenge when immunized with 1×10^3 (this study) or more than 1×10^4 (Licois et al. 1990) *E. intestinalis* precocious line oocysts. In previous studies, rabbits inoculated with 2.5×10^3 or 3.5×10^3 *E. magna* precocious line were found to be totally protected from subsequent challenge with WT parasites (Licois et al. 1995; Drouet-Viard et al. 1997). Similarly, immunization of rabbits with 1×10^3 *E. media* precocious line provides total protection following subsequent challenge with WT parasites when assessed by weight gain (Licois et al. 1994). Together, our findings support the concept that precocious lines of each *Eimeria* species.

In conclusion, by selecting early excreted oocysts through multiple generations, we obtained an attenuated *E. intestinalis* line, which maintained immunogenicity. The prepatent period of this precocious line was shortened by 3 days, the

reproduction capacity decreased markedly, and one asexual generation was lost during the parasite endogenous development. This precocious line represents an excellent candidate for a live attenuated vaccine. Studies aimed at understanding the molecule mechanism that orchestrates the loss of asexual generations are warranted.

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Compliance with ethical standards

Experiments were approved by China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (CAU20160921-2). Animal experiments were carried out in accordance with Chinese National Laboratory Animal Standards (GB 14925-2010/XG1-2011). Enough food and water were provided. Handling of animals was minimal when they were inoculated and weighed. Fecal samplings were performed outside the rabbit cages. Rabbits were euthanized in a humane manner.

Conflict of interest The authors declare that they have no conflict of interest.

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