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PROTOZOOLOGY - ORIGINAL PAPER

Selection and characterization of a precocious line of *Eimeria media*

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Abstract

Coccidiosis, caused by the infection of *Eimeria* parasites, is one of the most common diseases in domestic rabbits. Live anticoccidial vaccine formulated with attenuated precocious lines of pathogenic eimerian parasites is expected to be valuable for the control of rabbit coccidiosis as a similar strategy to produce anticoccidial vaccines against chicken coccidiosis has being used for several decades. *Eimeria media*, moderate pathogenic, is widespread in China. Therefore, attenuated anticoccidial vaccines against rabbit coccidiosis should contain vaccine strain(s) of *E. media*. In this study, a precocious line of *E. media* (*Empre*) was selected by collecting and propagating the early excreted oocysts with 16 successive generations. The prepatent period of *Empre* reduced from 108 h of its parental strain (*Emwt*) to 70 h. The fecundity of *Empre* was about 1/10 to 1/3 lower than that of *Emwt*. Each sporocyst of *Empre* sporulated oocyst contained only one large refractile body instead of two smaller ones seen in the parental strain. When vaccinated with 1×10^3 or 1×10^4 precocious line oocysts, the rabbits were completely protected against homologous challenge with the parental strain 14 days post challenge by terms of body weight gain and oocyst output counting, indicating the efficacy of *Empre*. Meanwhile, all immunized rabbits showed no clinical sign post immunization, indicating the safety of *Empre*. For co-immunization, 1×10^3 *Empre* oocysts and 5×10^2 oocysts of a precocious line of *E. intestinalis* (*EIP8*) were inoculated to each rabbit in a trial. No diarrhea or mortality was found after vaccination, and the weight gains of the vaccinated group were similar to that of unvaccinated-unchallenged control (UUC) group, while the weight gains of the vaccinated group were similar to that of unvaccinated-unchallenged control (UUC) group ($P > 0.05$), but significantly higher than that of UCC group ($P < 0.01$) after challenge, indicating it is safe and effective when using co-immunization. These results together show that *Empre*, as a precocious line, is a good candidate of precocious line of *E. media* for anticoccidial vaccine development.

Keywords Rabbit · Coccidiosis · *Eimeria media* · Precocious line · Vaccine

Introduction

Coccidiosis, caused by the infection of the genus *Eimeria*, is recognized as one of the major handicaps in rabbit breeding (Cowie-Whitney 1977). It causes considerable economic losses in intensive and semi-intensive production due to the decrease in weight gain, diarrhea, and even death (Drouet-Viard et al. 1997a, 1997b). For a long time, the control of rabbit coccidiosis was based on continuous administration of anticoccidial drugs in feed or in drinking water. The routinely use of anticoccidial drugs resulted in drug-resistant problems in rabbit farming (Coudert 1989; Peeters et al. 1988). As inoculation with live attenuated eimerian parasites could provide sufficient protection against the challenge with the homologous strains (Akpo et al. 2012; Bachene et al. 2018), it provides a practical way to develop the attenuated vaccine against rabbit coccidiosis.

To date, attenuated strains were obtained by precociousness selection of 6 *Eimeria* species, including *Eimeria media* (Licois et al. 1990, 1994; Coudert et al. 1995: Licois et al. 1995; Pakandl 2005; Pakandl and Jelínkova 2006; Akpo et al. 2012; Bachene et al. 2018; Li et al. 2018). Though

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E. media is moderate pathogenic, heavy infection with this parasite can interfere with digestion and absorption and even cause significant reduction of body weight gain (Licois et al. 1994). In addition, *E. media* is widespread in commercial rabbit husbandry (Coudert 1989; Jing et al. 2012). Therefore, the formulation of anticoccidial vaccines against rabbit coccidiosis should contain vaccine strain(s) of *E. media*.

This study describes the selection of a precocious line of *E. media* and the characterization of its reproduction, pathogenicity, and immunogenicity.

Materials and methods

Experimental animals

Coccidia-free rabbits were obtained through the chemotherapeutic method. The breeding rabbits routinely received a coccidiostat in their food (monensin 40 mg/kg or diclazuril 1 mg/kg) (Coudert et al. 1988). Weaned rabbits were housed in HEPA-equipped isolators until 6 to 7 weeks old. Californian rabbits were used for precocious selection, while New Zealand rabbits were used for the tests of fecundity, excretion curve, endogenous development, pathogenicity, and immunogenicity. China Agricultural University Animal Ethics Committee approved all experimental procedures, and due attention was paid to the welfare of the animals (certified by Beijing Laboratory Animal employee, ID: 1114120800096).

Selection for the precocious line of *E. media*

Isolation of the *E. media* and selection for precociousness of this strain was performed following protocol described by Coudert et al. (1995). Briefly, oocysts were isolated from the fecal samples collected from a rabbit farm in Zhangjiakou in 2008. Single-oocyst infection was performed to isolate and propagate the pure strain of wild-type *E. media* (*Emwt*). The precocious line (*Empre*) was obtained by 16 successive collections of early oocysts shed in the feces by the rabbits inoculated with oocysts of the previous generation. The detailed performance of selection is summarized in Table 1. In order to confirm the stability of precocious parasite, the precocious line at passage 16 was consecutively propagated in the absence of selective pressure in rabbits. Three rabbits were individually inoculated with 5×10^3 oocysts of *Empre*, and the daughter oocysts were recovered from feces during days 3 to 10 post inoculation. Sporulated daughter oocysts were inoculated to other three rabbits with the same dose. This propagation without selection was repeated five times. The prepatent time in each generation was measured.

Table 1 Selection of the precocious line of *E. media* (*Empre*)

Oocyst inoculated	Number of oocyst given(10^4)	Time after inoculation when oocysts were collected in feces and caecum content (h)	Prepatent period	Oocyst obtained
P0	Single oocyst	111–117	114	P1
P1	5×10^3	111–115	114	P2
P2	5×10^3	92–98	96	P3
P3	5×10^3	90–94	94	P4
P4	5×10^4	100–104	103	P5
P5	5×10^4	95–99	98	P6
P6	5×10^4	88–93	91	P7
P7	5×10^4	88–93	91	98
P8	5×10^4	90–101	93	P9
P9	5×10^4	87–90	90	P10
P10	1×10^5	88–96	91	P11
P11	1×10^5	87–91	90	P12
P12	1×10^5	86–92	89	P13
P13	2×10^5	76–82	79	P14
P14	2×10^5	76–96	79	P15
P15	2×10^5	67–73	70	P16

Morphological comparison between Emwt and Empre

The photographs of sporulated oocysts and sporocysts were taken using laser confocal microscopy (Leica SP5, Germany). The length and width of 100 oocysts for *Empre* or *Emwt* were individually measured with the Leica LAS AF software. Sporocysts of *Empre* and *Emwt* were released from oocysts by vortexing with 1-mm glass beads (Cha et al. 2014). Sporozoites were collected from excysted sporocysts by purifying with an anion exchange column of DE-52 (Schmatz et al. 1984). Sporozoites were fixed with 2.5% glutaraldehyde and then subjected to transmission electron microscopic photographs (EM-100CXII/S, Japan).

Oocyst output comparison between *Emwt* and *Empre*

Twenty-four rabbits were divided into six groups. Five groups of rabbits were inoculated with *Empre* at doses of 1×10^2, 3×10^2, 5×10^2, 1×10^3, or 5×10^3, respectively. The sixth group of rabbits was inoculated with *Emwt* at dose of 5×10^3. The feces of *Empre*-inoculated rabbits were collected per group between days 3 and 10 post inoculation and subjected to oocyst counting following the method previously described by Licois and Coudert (1980). The feces of *Emwt*-inoculated rabbits were collected between days 4.5 and 12.5 post inoculation and subjected to oocyst counting.

Endogenous development study

Ten rabbits were separately inoculated with oocysts of *Emwt* or *Empre* and euthanized at time points indicated in Table 2. Tissue samples were taken from duodenum, middle jejunum, and ileum, then fixed in 10% formaldehyde solution for 72 h and embedded in paraffin. Tissue sections, approximately 5 μm thick, were cut, stained with hematoxylin and eosin, and studied by light microscopy (Olympus DX71 fluorescence microscope). Due to the lower multiplication rate of the precocious line, the doses of oocysts given for studying the beginning of their life cycle were higher.

Test of pathogenicity and immunogenicity

Thirty-two rabbits were divided into eight groups. Six groups (VC) of rabbits were vaccinated with *Emwt* or *Empre* at doses of 1×10^2, 1×10^3, or 1×10^4, respectively. The other two groups were unvaccinated and unchallenged control (UUC) and unvaccinated and challenged control (UCC). The rabbits in VC groups and UUC group were used to test the pathogenicity of *Empre* and *Emwt*. In order to test the immunogenicity of *Empre*, the rabbits in *Empre*-vaccinated groups and UCC group were challenged with 1×10^4 *Emwt* oocysts on day 14 post vaccination.

The feces of inoculated animals were collected per group between days 3 and 10 post vaccination, and total oocyst output was calculated for both *Emwt* and *Empre*. Oocyst counting was also carried out from days 4 to 11 post challenge.

Clinical signs (depression, diarrhea, death) were observed for all animals during the whole assay. Body weight was measured at days 0, 14, and 28 post vaccination.

Co-immunization assay

To test the effect of co-immunization of precocious lines of coccidia on rabbits, we carried out a trial using *Empre* and *EIP8*, a precocious line of *E. intestinalis* recently selected (Li et al. 2018). Total of 12 rabbits were divided into three groups: vaccinated and challenged (VC), unvaccinated-unchallenged control (UUC), and unvaccinated-challenged control (UCC). Rabbits in VC group were inoculated with 1×10^3 *Empre* sporulated oocysts and 5×10^2 *EIP8* sporulated oocysts. At the 14th day post inoculation, the rabbits except those in UUC group were challenged with 1×10^4 *Emwt* oocysts and 1×10^4 oocysts of wild type of *E. intestinalis* (*Eiwt*). Body weight was measured at days 0, 7, 14, 21, and 28 post immunization. Total oocyst output was measured for two groups of challenged animals from days 4 to 14 after challenge. Then, the oocyst output of *Emwt* and *Eiwt* was calculated based on the percentage of the two parasites, which are morphologically different as oocysts of *Emwt* were ellipsoid or ovoid while oocysts of *Eiwt* were piriform (Pakandl 2009).

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

Hour (h)		24	42	60	70	96	108
Dose	*Emwt*	1×10^7	1×10^7	5×10^6	1×10^6	1×10^6	1×10^6
	Empre	2×10^7	1×10^7	1×10^7	5×10^6		

Statistical analysis

Statistical analysis was performed by one-way ANOVA of SPSS software (Version 17.0). Data were expressed as mean ± standard deviation; * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

Results

Selection of the precocious line of *Eimeria media*

After 16 successive propagations under selection pressure, the prepatent period of the precocious line *Empre* reduced from 114 to 70 h. The prepatent period was distinctly decreased during the passages of P3, P7, P14, and P16 (Table 1). This shortened prepatent period of *Empre* was maintained after five generations of successive passages without selection pressure.

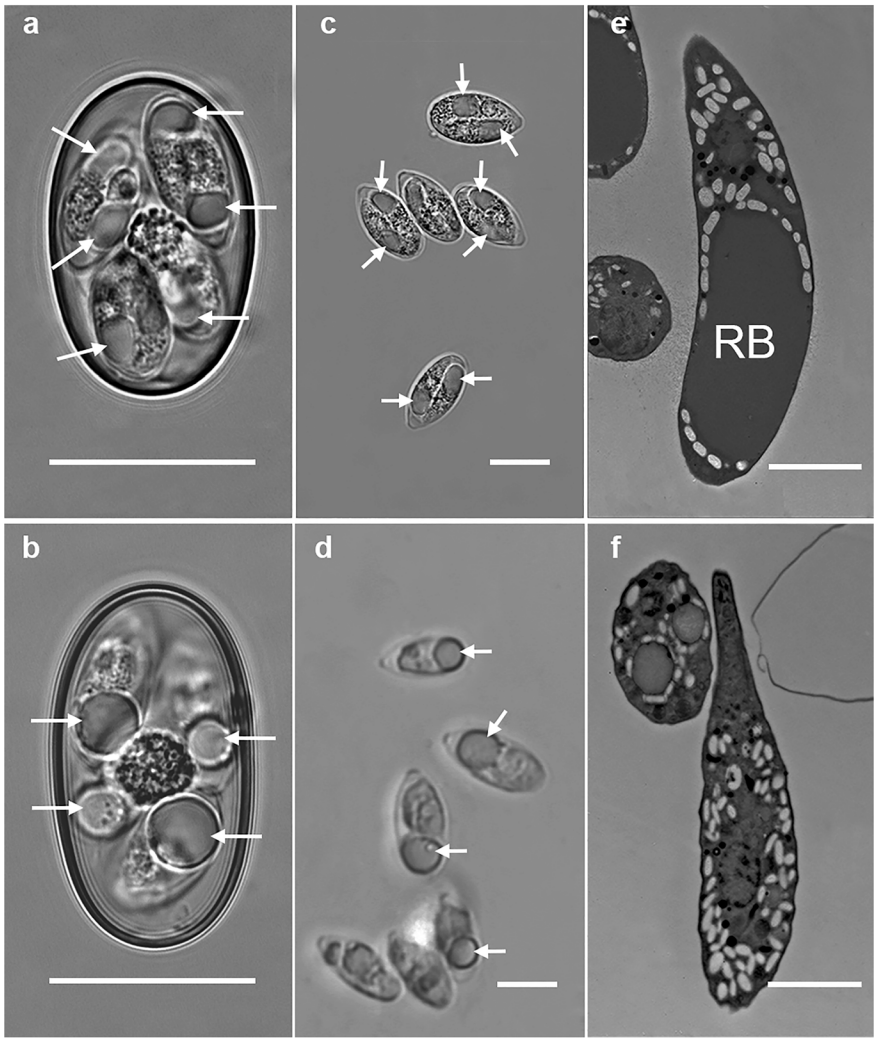
Comparison of oocyst morphology, oocyst production, and endogenous development between *Emwt* and *Empre*

To determine whether oocyst size was altered following selection, freshly sporulated *Empre* and *Emwt* oocysts (100 oocysts each) were examined by light microscopy. Oocyst size of *Empre* was (30.6 ± 2.2 μm) × (18.5 ± 1.4 μm), while that of *Emwt* was (29.9 ± 3.4 μm) × (18.2 ± 1.0 μm). The length-to-width ratios of *Empre* and *Emwt* were 1.66. There was not a significant difference ($P > 0.05$) in oocyst size between *Empre* and *Emwt*. In *Emwt* sporulated oocysts, a small refractile body (RB) could be seen in each sporozoite (Fig. 1a–e); while only a large RB could be observed within each sporocyst of *Empre* sporulated oocysts and the RB was free in the sporocyst (Fig. 1b–f).

The peak of oocyst shedding occurred on day 5 for *Empre*, whereas it was on day 6.5 for *Emwt* (Fig. 1b). The oocyst output for rabbits inoculated with 5×10^3 *Empre* oocysts was 6.6×10^7; it was 2.0×10^8 for those inoculated with 5×10^3 *Emwt* oocysts (Fig. 1b). The total oocyst output of *Empre* was 1/3 that of the *Emwt*.

In both *Empre*- and *Emwt*-infected rabbits, schizonts emerged at 24 h post inoculation (Fig. 2a and b). The second

a


a
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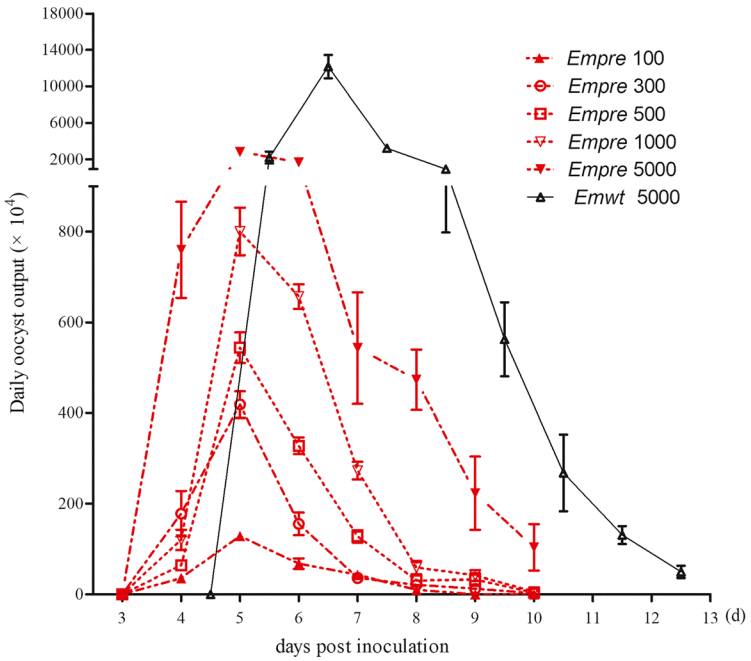
b


18000
14000
10000
6000
2000
800
600
400
200
0
Daily oocyst output (× 10^4)
3
4
5
6
7
8
9
10
11
12
13 (d)
days post inoculation
Empre 100
Empre 300
Empre 500
Empre 1000
Empre 5000
Emwt 5000

◀ **Fig. 1** Comparison of morphology (**a**) and oocyst production (**b**) between *Empre* and *Emwt*. Light arrow: refractile body (RB). **a** *Emwt* sporulated oocyst. **b** *Empre* sporulated oocyst. **c** *Emwt* sporocyst. **d** *Empre* sporocyst. **e** *Emwt* sporozoite. **f** *Empre* sporozoite. Bar = 20 μm (**a**, **b**); Bar = 5 μm (**c**, **d**); Bar = 2 μm (**e**, **f**). B, oocyst output curve of *Empre* and *Emwt*. Each rabbit was inoculated with 1×10^2, 3×10^2, 5×10^2, 1×10^3, 5×10^3 *Empre* oocysts or 5×10^3 *Emwt* oocysts, respectively ($N = 4$). Fecal samples were collected daily from days 3 to day 10 post inoculation for *Empre* or daily from days 4.5 to day 12.5 post inoculation for *Emwt*

generation schizonts were detected at 42 h (Fig. 2c and d). At 60 h post infection with *Empre*, macrogamonts could be detected in epithelial cells (Fig. 2e). At 70 h post infection with *Empre*, oocysts could be detected (Fig. 2g). In contrast, in *Emwt*-infected rabbits, the third generation of schizonts was detected at 70 h post infection (Fig. 2h), while gametocytes and oocysts were detected at 96 h post infection (Fig. 2i). By 108 h post infection, more *Emwt* oocysts could be detected (Fig. 2j).

Pathogenicity and immunogenicity

To evaluate the pathogenicity of *Empre* and *Emwt*, six groups of rabbits were inoculated with *Emwt* or *Empre* at doses of 10^2, 10^3, or 10^4, respectively. No diarrhea or loss of body weight was detected in these *Empre*-immunized groups. In contrast, mild and short-time diarrhea was found in the rabbits inoculated with 10^4 *Emwt* oocysts during days 4 to 6 post inoculation. Compared with UUC group, rabbits infected with 10^3 or 10^4 *Emwt* oocysts lost 41.4% or 51.7% of their weight gain on average (totally 217 g and 270 g) from days 0 to 14 post inoculation. In contrast, the weight gains of rabbits infected with *Empre* oocysts were similar to that of UUC group (totally 524 g) from days 0 to 14 post inoculation (Fig. 3a). Meanwhile, the fecundity of *Empre* was also detected. When inoculated with 10^2 oocysts, a single rabbit excreted 2.8×10^6 *Empre* oocysts on average, whereas 2.8×10^7 *Emwt* oocysts on average. When inoculated with 10^4 oocysts, a single rabbit excreted 9.2×10^7 *Empre* oocysts or 2.5×10^8 *Emwt* oocysts on average. The fecundity of *Empre* was about 1/3 to1/10 that of the *Emwt*. (Fig. 3c).

To further determine the immunogenicity of *Empre*, rabbits inoculated with 10^2, 10^3, or 10^4 *Empre* oocysts were challenged with 10^4 *Emwt* oocysts on day 14 post inoculation and both body weight gain and oocyst production were measured. From 4 to 6 days post challenge, rabbits in UCC group presented mild diarrhea. Interestingly, no diarrhea was found in all *Empre*-immunized rabbits post challenge. Weight gain of rabbits immunized with *Empre* oocysts at each doses (10^2, 10^3, and 10^4) was similar to that of the UUC group animals (35 g per day on average from days 0 to 14 post challenge). On the other hand, rabbits in the UCC group showed a significant decrease of body weight gain from days 0 to 14 post challenge (15 g per day on average from days 0 to 14post challenge). In contrast, rabbits inoculated with 10^3 or 10^4 *Empre* oocysts increased the body weight gain on average (39.3 g per day or 38.5 g per day) from days 0 to 14 post challenge, while rabbits immunized with 10^2 *Empre* gained less weight gain (27.1 g per day) than that of the UUC animals from days 0 to 14 post challenge (Fig. 3b).

In all study groups, when the body weight gain from day 14 post challenge was calculated, there was no significant difference between *Empre*-immunized groups and UUC group (Fig. 3b). After challenge, oocyst production in VC groups immunized with 10^2, 10^3, and 10^4 *Empre* oocyst were 38.7%, 6.2%, and 1.1% of that of the UCC group, respectively (Fig. 3d).

Protection against homologous challenge after co-immunization

After co-immunization or co-challenge with two species, no diarrhea was found in VC group and UUC group, while three of four rabbits in the UCC group presented severe diarrhea. After vaccination, the body weight gain of VC group was similar to that of UUC group on day 7 (23 g per day on average) and day 14 (32 g per day on average). After challenge, the body weight gain of VC group was similar to that of UUC group on day 21 (23 g per day on average) and day 28 (26 g per day on average) post vaccination, but significantly higher than that of the UCC group ($P < 0.01$) (Fig. 4a).

For *Empre* or*EIP8*, over 1×10^7 oocysts per animal were obtained in VC group between day 3 and day 10 after co-immunization. After challenge, oocyst output of *Emwt* in VC group was 2.1% of that of UCC group, and oocyst output of *Eiwt* in VC group was 0.9% of that of UCC group (Fig. 4b).

Discussion

In this study, we selected and characterized a precocious line of *E. media*. Compared with its parental strain *Emwt*, the precocious line *Empre* is characterized by a shortened prepatent period (from 108 to 70 h), a single refractile body in each sporocyst of an oocyst, remarkably reduced oocyst production, and highly immunogenic.

Licois et al. (1994) reported a precocious line of *Eimeria media*, which is similar to *Empre* of this study in terms of prepatent time, fecundity, and oocyst morphology. Observation of endogenous development of both *Empre* and *Emwt* showed that oocyst formation occurred around 70 h after inoculation with *Empre*, while meronts of third generation of merogony were majorly found in *Emwt*-infected animals, which may suggest the deletion of third generation of merogony and thus the shortening of the prepatent time in the precocious line *Empre*.

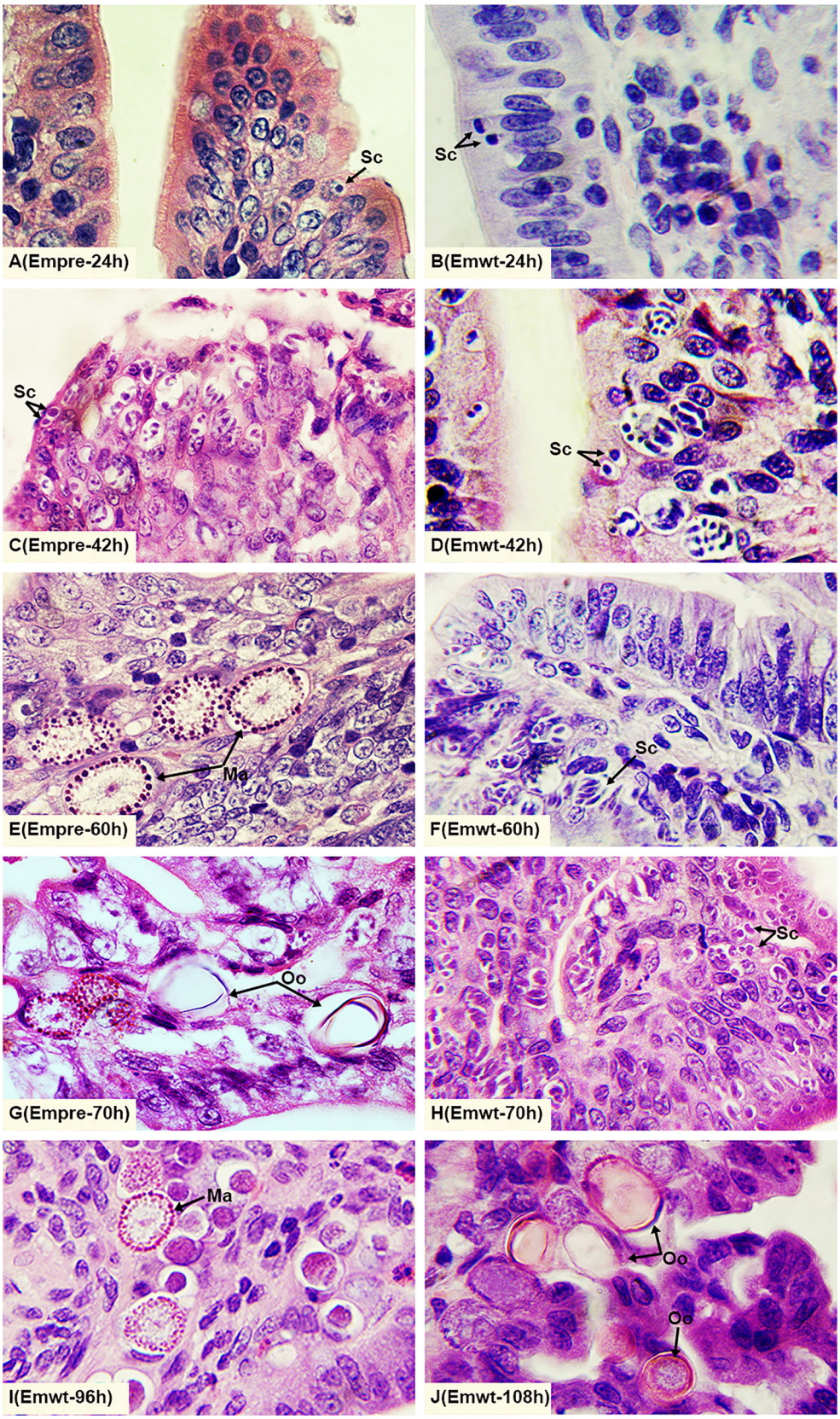


Fig. 2 Observation of endogenous development of *Empre* and *Emwt* in middle jejunum. Arrow(s) indicate the parasite(s) in the panels. **a**, **b** First-generation schizonts. **c** and **d** Second-generation schizonts. **e** Gametocytes of *Empre*. **f** Mature schizonts of *Emwt*. **g** Oocysts of *Empre*. **h** Third-generation schizonts. **i** Gametocytes of *Emwt*. **j** Oocysts of *Emwt*. Bar = 20 μm

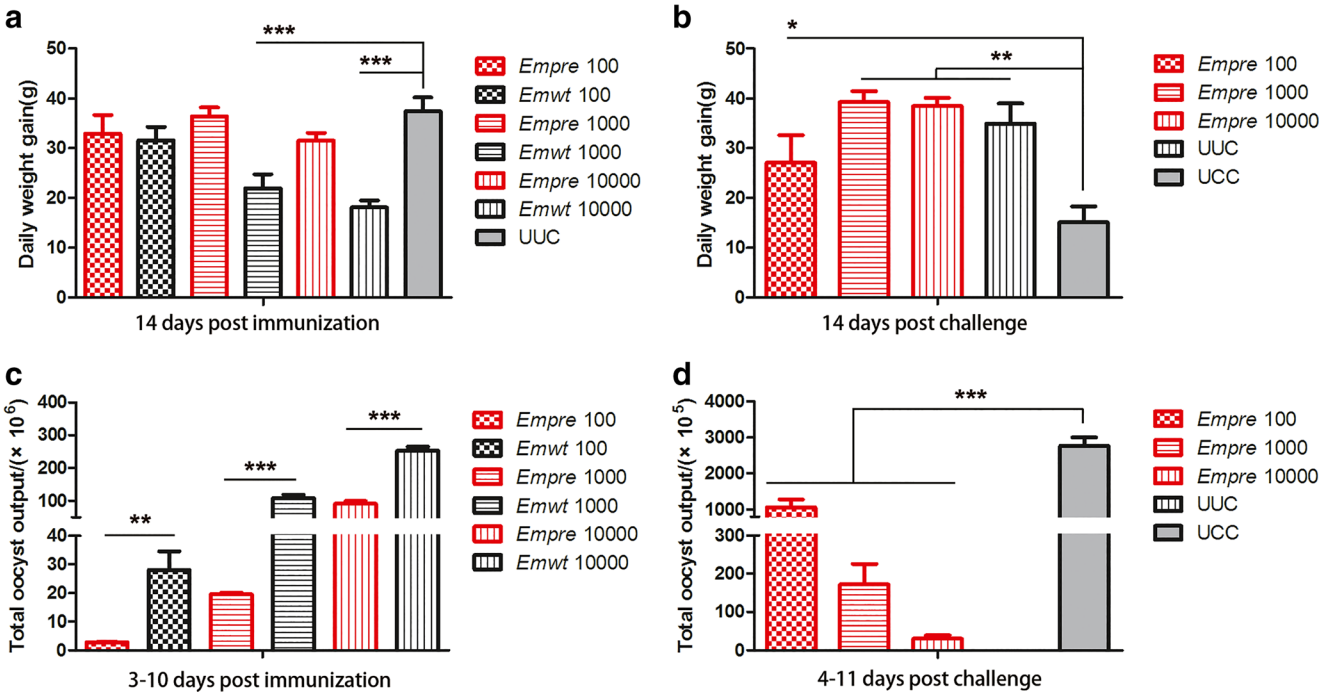


Fig. 3 Pathogenicity and immunogenicity of *Empre*. Rabbits were inoculated with 1×10^2, 1×10^3, and 1×10^4 *Empre* or *Emwt* oocysts, respectively. Challenge infection was performed to rabbits except for those in UUC group and *Emwt*-inoculated group 14 days post immunization. Body weight gain was measured post immunization (**a**) and post challenge (**b**), while total oocyst output was counted days 3 to 10 post immunization (**c**) and days 4 to 11 post challenge (**d**). * $P < 0.05$;** $P < 0.01$;*** $P < 0.001$

Previous studies showed that the position, the size, and the number of RB changed when most precocious lines of rabbit coccidia were obtained from their parental strains (Pakandl et al. 2001; Pakandl and Jelínkova 2006). In this study, a large RB was seen in each sporocyst of oocyst in *Empre*, similar to the observation of a previously selected precocious line of *E. media* (Licois et al. 1994). The RB is generally present in the sporozoite stage, fragmented during merogony and incorporated into the forming second-generation merozoites (Lal et al. 2009). During the selection of *Empre*, both types of RB were found in sporocysts of a minor proportion of oocysts for the middle generations (data not shown), which indicates that the alter of RB may precede the reduction of the schizogony generation. Together with the deduction of the deletion of third generation of *merogony* in *Empre*, this observation further prompt a hypothesis that RB may function in maintaining the merogony generations of eimerian parasites.

Two proteins, SO7 and Eimepsin, the most abundant protein identified in *Eimeria* sporozoites, are located at the RB (de Venevelles et al. 2004). Some groups have proposed a role for eimepsin and SO7 in the invasion process and intracellular development (Danforth and Augustine 1989; Augustine 1999,

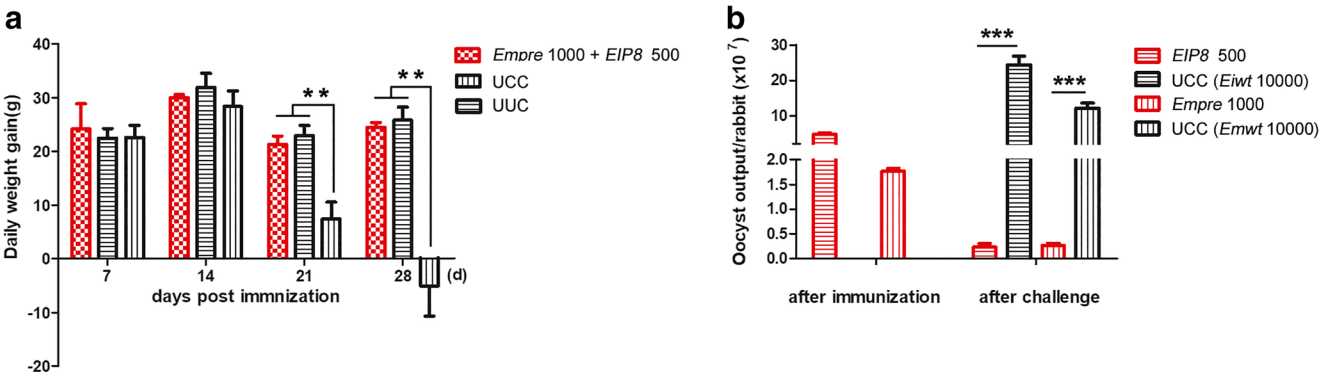


Fig. 4 Protection against homologous challenge after co-immunization with precocious lines of *E. media* and *E. intestinalis*. Rabbits were inoculated with 10^3 *Empre* oocysts and 5×10^2 *EIP8* oocysts, then challenged with 1×10^4 *Emwt* oocysts and 1×10^4 oocysts of wild type of *E. intestinalis* (*Eiwt*) 14th day post inoculation. **a** Body weight gain post co-immunization and post challenge. **b** Total oocyst output post co-immunization and post challenge. Oocyst output was detected daily from days 3 to day 10 post immunization and days 4 to post challenge. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

2001; Jean et al. 2000). The *Eimeria* RB protein eimepsin is similar to the plasmepsins, proteases from Plasmodium that degrade heme in the food vacuole (Liu et al. 2005). Proteomic studies revealed that RB contains proteins such as Eimepsin, SO7, haloacid dehalogenase, hydrolase, subtilase, lactacte dehydrogenase, or ubiquitin family proteins (de Venevelles et al. 2006). The presence of other enzymes, such as lactate dehydrogenase, indicates RB has energetic and metabolic functions in this parasite (Lal et al. 2009; Lemgruber and Lupetti 2012).

In future work, the different proteins of RBs between the parental strain and the precocious line will be identified by a comparative proteomics approach, and the candidate proteins that may function in maintaining the schizogony generations of *Eimeria* will be chosen and validated.

In conclusion, the precocious line of *E. media* selected in this study is immunogenic and thus can be a candidate line for the development of live anticoccidial vaccine for the control of the rabbit coccidiosis.

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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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