



# Immune protection provided by a precocious line trivalent vaccine against rabbit *Eimeria*

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## ABSTRACT

A pilot study was conducted to evaluate the pathogenicity and immunogenicity of vaccinated rabbits with different doses of oocysts ( $5 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ , and  $5 \times 10^4$ ) of a precocious line, including *Eimeria magna*, *E. intestinalis* or *E. media* following the challenge with their corresponding parent strains. Our results showed that each precocious line had weak pathogenicity but good immunogenicity in terms of clinical symptoms, average daily weight gains (ADGs), and oocyst outputs. Therefore, a precocious line trivalent vaccine, including *E. magna*, *E. intestinalis*, and *E. media* was formulated. A total of sixty 40-day-old coccidia-free rabbits were allocated to ten treatments with a  $2 \times 3$  factorial arrangement that included 2 vaccination doses ( $5 \times 10^2$  or  $1 \times 10^3$  oocysts of the precocious line). Groups I to VIII and Unimmunized Challenged Control group were challenged with mixed oocysts of their corresponding parent strains ( $1 \times 10^4$  oocysts of each parent strain) 14 days after vaccination. No clinical symptoms were observed in the immunized groups after vaccination. Average daily weight gains (ADGs) were similar to those of unimmunized unchallenged controls ( $P > 0.05$ ) after vaccination or after challenge. Oocyst outputs in the vaccinated challenged groups were significantly different from those of unimmunized challenged controls ( $P < 0.01$ ) after challenge. These results indicated that the trivalent vaccine could provide immune protection against coccidiosis and therefore, it could be used as a candidate vaccine.

## 1. Introduction

Rabbit coccidiosis, caused by the genus *Eimeria*, is one of the most harmful diseases in the rabbit industry (Donald W. Duszynski and Couch, 2013). With the emergence of drug resistance and drug residues, vaccine prevention has become an preferred solution against rabbit coccidiosis. Therefore, the design and manufacture of safe and effective rabbit coccidiosis vaccines has become a priority.

Although a number of studies have been conducted on rabbits coccidiosis, attempt to immunize rabbits and protect rabbits from subsequent infection with killed/attenuated coccidia or material derived from these parasites have not been successful. To date, only a few studies have investigated rabbit coccidial precocious vaccines in China and other countries.

Therefore, the aim of this study was to investigate the pathogenicity and immunogenicity of three precocious lines of *E. magna*, *E. intestinalis* and *E. media*. Then the immune protection effect of the precocious line trivalent vaccine was evaluated under laboratory conditions.

## 2. Experimental materials and methods

### 2.1. Parasite lines

In order to constitute a strain with larger variability in genetic background for selection pressure, oocysts of different geographical strains of each rabbit coccidian species (*E. magna*, *E. intestinalis* and *E. media*) were separated from fecal samples, and then the same numbers of oocysts of the different geographical strains were then mixed for

**Abbreviations:** Emag, *Eimeria magna*; Eint, *Eimeria intestinalis*; Emed, *Eimeria media*; PS, parent strain; PL, precocious line; UCC, unimmunized challenged control; UUC, unimmunized unchallenged control; OPG, oocysts per gram; ADG, average daily gain

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precociousness selection (Licois, 1990, Licois et al., 1994; Pakandl and Jelinkova, 2006). The precocious line was obtained by successive collections of early oocysts shed in the feces by the rabbits inoculated with oocysts of the previous generation. (Jeffers, 1975). To test the genetic stability, oocyst propagation without selection pressure was repeated 5 times and the prepatent time in each generation was measured.

## 2.2. *Coccidia-free rabbits*

Self-propagated and self-raised coccidia-free Californian rabbits were used in this study. Rabbits were housed in cages (two rabbits/cage) and fed a concentrate supplemented with diclazuril(1 ppm) *ad libitum* until 40 days of age to prevent potential *Eimeria* infections during the raising period, which leads to an inadequate immune response. The feed was changed to a diclazuril-free feed five days before the experiment.

The housing environment and cage utensils (rabbit cages, feed boxes and drinking water tanks) were sterilized by dipping them in 70% ethanol, and then carefully drying them over a flame. Feed and drinking water were heated in an 80 °C oven for more than two hours and then cooled down to room temperature before feeding. Experimental rabbits were subjected to a bivalent vaccine against rabbit hemorrhagic disease virus (RHDV) and *Pasterella multocida* at 37 and 62 days of age

## 2.3. *Oocyst proliferation*

The preserved oocysts of the parent strains and precocious lines of *E. magna*, *E. intestinalis* and *E. media* were washed to remove potassium dichromate and used to inoculate coccidia-free rabbits. According to the prepatent periods of the parent strains and precocious lines of the different species, the oocysts were collected separately by floatation, incubated until sporulation and stored in a refrigerator at 4 °C prior to use (Table 1).

## 2.4. *Experimental design*

### 2.4.1. *Pathogenicity and immunogenicity of the three precocious lines of rabbit coccidia*

A pilot study was conducted to determine a suitable dose of oocysts of the precocious lines that can protect against their respective parent strains. Forty eight coccidia-free 40-day-old rabbits were divided into six groups with eight rabbits per group for each precocious line. The immunized groups I-IV were inoculated orally with sporulated oocysts of the precocious line of *E. magna* at doses of  $5 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$  and  $5 \times 10^4$ , respectively. The other two groups were Unimmunized Unchallenged Control group(UUC) and Unimmunized Challenged Control group(UCC). After 14 days, each group of rabbits except UUC was challenged with  $1 \times 10^4$  sporulated oocysts of the parent *E. magna* strain. The same experimental design was used for *E. intestinalis* (V-VIII, UUC, UCC)and *E. media* (IX-XII, UUC, UCC). The details are shown in Table 2.

Rabbits were observed for clinical manifestations and weighed daily after inoculation. The average daily gain (ADG) was calculated for each

rabbit in each group both after inoculation or after challenge. Feces were collected and weighed starting from the oocyst discharge day for one week. The number of oocysts per gram of feces (OPG) and total oocyst outputs were calculated for each group of rabbits.

### 2.4.2. *Immune effects of the rabbit coccidia precocious line trivalent vaccine*

According to the pathogenicity and immunogenicity of each precocious line, two doses ( $5 \times 10^2$  and  $1 \times 10^3$  oocysts) were selected for all three precocious lines of *E. magna*, *E. intestinalis* and *E. media* ( $5 \times 10^2$  and  $1 \times 10^3$  oocysts). The doses were arranged in eight mix ratios and subjected to laboratory studies of a precocious line trivalent vaccine for rabbit coccidia. Sixty coccidia-free 40-day-old rabbits were divided into 10 groups (I-VIII, UUC, UCC). Each group of rabbits from the immunized groups was orally immunized with different combinations of mixed oocysts. After 14 days, all groups except UUC were challenged with mixed oocysts of the parent strains of *E. magna*, *E. intestinalis* and *E. media* ( $1 \times 10^4$  of each). Specific experimental groups are shown in Table 3.

After inoculation, rabbits were observed daily for their animal behavior, drinking, appetite, fecal quality. In addition, rabbits were weighed, and the ADG of each rabbit in each group was calculated for 14 days after immunization and for 0–7 days and 7–14 days after challenge. Feces were collected starting from the oocyst shedding for one week after challenge to calculate OPG and total oocyst output for each group of rabbits.

## 2.5. *Data processing*

Descriptive was summarized in Excel 2010 spreadsheets and analyzed with SPSS 20.0 statistical software. All data were presented as means  $\pm$  standard deviations (SD). Statistical analysis was performed by one-way ANOVA of SPSS software (Version 20.0). Before the one-way ANOVA analyses, One-sample Kolmogorov-Smirnov tests were made to checked the datas for normal distribution ( $p > 0.05$ ).

## 3. *Results*

### 3.1. *Pathogenicity and immunogenicity of precocious lines of rabbit coccidia*

#### 3.1.1. *Pathogenicity and immunogenicity of the E. Magna precocious line*

After immunization with the *E. magna* precocious line, the animal behavior, appetite and fecal excretion of the rabbits in the immunized groups were significantly elevated. The ADGs of rabbits in groups I and II were not significantly different from those of UUC ( $P > 0.05$ ), whereas the ADGs of rabbits in groups III and IV were significantly lower than those of UUC ( $P < 0.05$ ) (Fig. 1A).

After challenge, 50% of the rabbits in UUC developed diarrhea, decreased appetite and activity. The ADGs of rabbits in UCC were extremely significantly different from those of UUC and the immunized groups ( $P < 0.01$ ). However, the ADGs of the immunized groups were not substantially different from those of UUC ( $P > 0.05$ ) (Fig. 1B).

Significant differences were detected between oocyst outputs of the

**Table 1**  
Experimental parasite lines.

Type	Origin of strains	Prepatent period
<i>E. intestinalis</i> Parent strain Eint-PS	Jiangsu suining strain, Shandong Jiaodong strain, Sichuan Tongjiang strain, Jiangsu Rugao strain, Hebei Zhangjiakou strain and Hebei Henshui strain	213 h
<i>E. magna</i> Precocious line Eint-PL	Mixed from the above six geographical strains and obtained by generation breeding.	132 h
<i>E. magna</i> Parent strain Emag-PS	Yunnan Kunming strain, Hebei Wuji strain, Sichuan Leshan strain, Zhejiang Zhoushan strain, Hebei Zhangjiakou Strain and Jiangsu Nanjing Strain	156 h
<i>E. media</i> Precocious line Emag-PL	Mixed from the above six geographical strains and obtained by generation breeding.	96 h
<i>E. media</i> Parent strain Emed-PS	Hebei Zhangjiakou strain, Shandong Jiaonan strain, Jiangsu suining strain and Sichuan Leshan strain	108 h
<i>E. media</i> Precocious line Emed-PL	Mixed from the above four geographical strains and obtained by generation breeding.	70h

**Table 2**

Experimental design of the pathogenicity and immunogenicity analyses for the three precocious lines of rabbit coccidia unit: each.

Group	Immunization line	Immunization dose	Challenge strain	Challenge dose
I	Emag-PL	5×10 <sup>2</sup>	Emag-PS	1×10 <sup>4</sup>
II	Emag-PL	1×10 <sup>3</sup>		
III	Emag-PL	1×10 <sup>4</sup>		
IV	Emag-PL	5×10 <sup>4</sup>		
UCC	—	—		
UUC	—	—	—	—
V	Eint-PL	5×10 <sup>2</sup>	Eint-PS	1×10 <sup>4</sup>
VI	Eint-PL	1×10 <sup>3</sup>		
VII	Eint-PL	1×10 <sup>4</sup>		
VIII	Eint-PL	5×10 <sup>4</sup>		
UCC	—	—		
UUC	—	—	—	—
IX	Emed-PL	—	Emed-PS	1×10 <sup>4</sup>
X	Emed-PL	5×10 <sup>2</sup>		
XI	Emed-PL	1×10 <sup>3</sup>		
XII	Emed-PL	1×10 <sup>4</sup>		
UCC	—	5×10 <sup>4</sup>		
UUC	—	—	—	—

**Table 3**Experimental design for co-immunization of precocious lines of *E. magna*, *E. intestinalis*, and *E. media*.

Group	Immunization line and dose			Challenge strain and dose		
	Emag-PL	Eint-PL	Emed-PL	Emag-PS	Eint-PS	Emed-PS
I	5×10 <sup>2</sup>	5×10 <sup>2</sup>	5×10 <sup>2</sup>	1×10 <sup>4</sup>	1×10 <sup>4</sup>	1×10 <sup>4</sup>
II	5×10 <sup>2</sup>	5×10 <sup>2</sup>	1×10 <sup>3</sup>			
III	5×10 <sup>2</sup>	1×10 <sup>3</sup>	5×10 <sup>2</sup>			
IV	5×10 <sup>2</sup>	1×10 <sup>3</sup>	1×10 <sup>3</sup>			
V	1×10 <sup>3</sup>	5×10 <sup>2</sup>	5×10 <sup>2</sup>			
VI	1×10 <sup>3</sup>	5×10 <sup>2</sup>	1×10 <sup>3</sup>			
VII	1×10 <sup>3</sup>	1×10 <sup>3</sup>	5×10 <sup>2</sup>			
VIII	1×10 <sup>3</sup>	1×10 <sup>3</sup>	1×10 <sup>3</sup>			
UCC	—	—	—	—	—	—
UUC	—	—	—	—	—	—

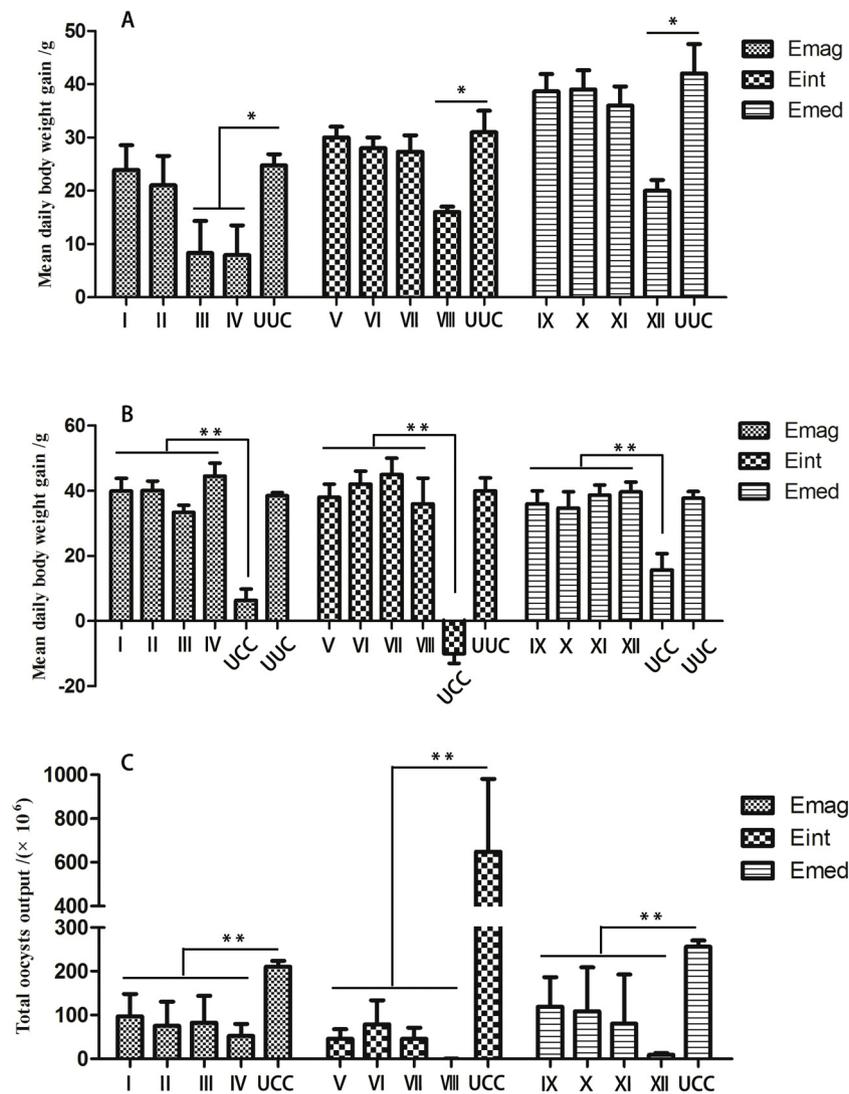
immunized groups and the UCC group ( $P < 0.01$ ) (Fig. 1C).

### 3.1.2. Pathogenicity and immunogenicity of the *E. Intestinalis* precocious line

After immunization with the *E. intestinalis* precocious line, the animal behavior, appetite and fecal excretion of the rabbits in the groups

V-VII increased substantially ; however, the rabbits in the group VIII were less energetic and diarrheic with a decreased appetite.

The ADGs of the rabbits in the groups V-VII were not significantly different from those in UUC ( $P > 0.05$ ). However, the ADGs in the group VIII were significantly different from those in the other groups ( $P < 0.01$ ) (Fig. 1A).



**Fig. 1.** Pathogenicity and immunogenicity tests of the precocious line. (A) The ADGs after immunization with the precocious lines. (B) The ADGs after challenge with the parent strains. (C) The total oocyst outputs after challenge with the parent strains. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ .

After challenge, the rabbits in UCC were less energetic, had decreased appetite and severe diarrhea. ADGs of UCC were significantly lower than that in UUC and the immunized groups ( $P < 0.01$ ), with a negative growth trend. Rabbits in the immunized groups demonstrated no clinical symptoms with a normal appetite, and their ADGs were not significantly different from that in the group VI ( $P > 0.05$ ) (Fig. 1B).

Oocyst outputs of the immunized groups (V-VIII) were significantly different from that of UCC ( $P < 0.01$ ) (Fig. 1C). Differences in oocyst outputs between the immunized groups were not significant ( $P > 0.05$ ), but the oocyst number decreased with the increase in the immunization dose.

### 3.1.3. Pathogenicity and immunogenicity of the *E. Media* precocious line

After immunization with the *E. media* precocious line, rabbits in the groups IX-XI did not show any clinical symptoms; however, the rabbits in the group XII developed a lack of energy, decreased appetite and diarrhea. The ADGs of the group IX-XI were not substantially different from that of UUC ( $P > 0.05$ ), however, the ADGs of group XII rabbits were significantly different from that of the groups IX-XI and UUC ( $P < 0.01$ ) (Fig. 1A). After challenge, rabbits in UCC showed obvious clinical symptoms, with 75% of the rabbits developing severe diarrhea, and the ADGs was significantly different from those of UUC and the

immunized groups ( $P < 0.01$ ). The ADGs of the groups IX-XII were not significantly different from that of UUC ( $P > 0.05$ ) (Fig. 1B). The oocyst outputs of the immunized groups were significantly different from that of UCC ( $P < 0.01$ ). Differences in oocyst outputs between the immunized groups were not significant ( $P > 0.05$ ), and the oocyst numbers decreased when immunization doses were increased (Fig. 1C).

### 3.2. Immune effects of a precocious line trivalent vaccine for rabbit coccidia

After immunization, rabbits in the immunized groups showed no clinical symptoms, and no significant difference were detected between the ADG of the immunized groups and compared with that in the UUC group ( $P > 0.05$ ) (Fig. 2A). Differences in oocyst outputs among the immunized groups were different but not statistically significant ( $P > 0.05$ ) (Fig. 2B).

From days 0–7 after challenge, rabbits in UCC developed clinical symptoms, such as poor behavior, decreased appetite and diarrhea, whereas no clinical manifestations were observed in the immunized groups and UUC group.

No significant differences were detected among the ADGs of the immunized groups or between the ADGs of immunized groups and UUC group ( $P > 0.05$ ). However, ADGs of rabbits in the immunized groups

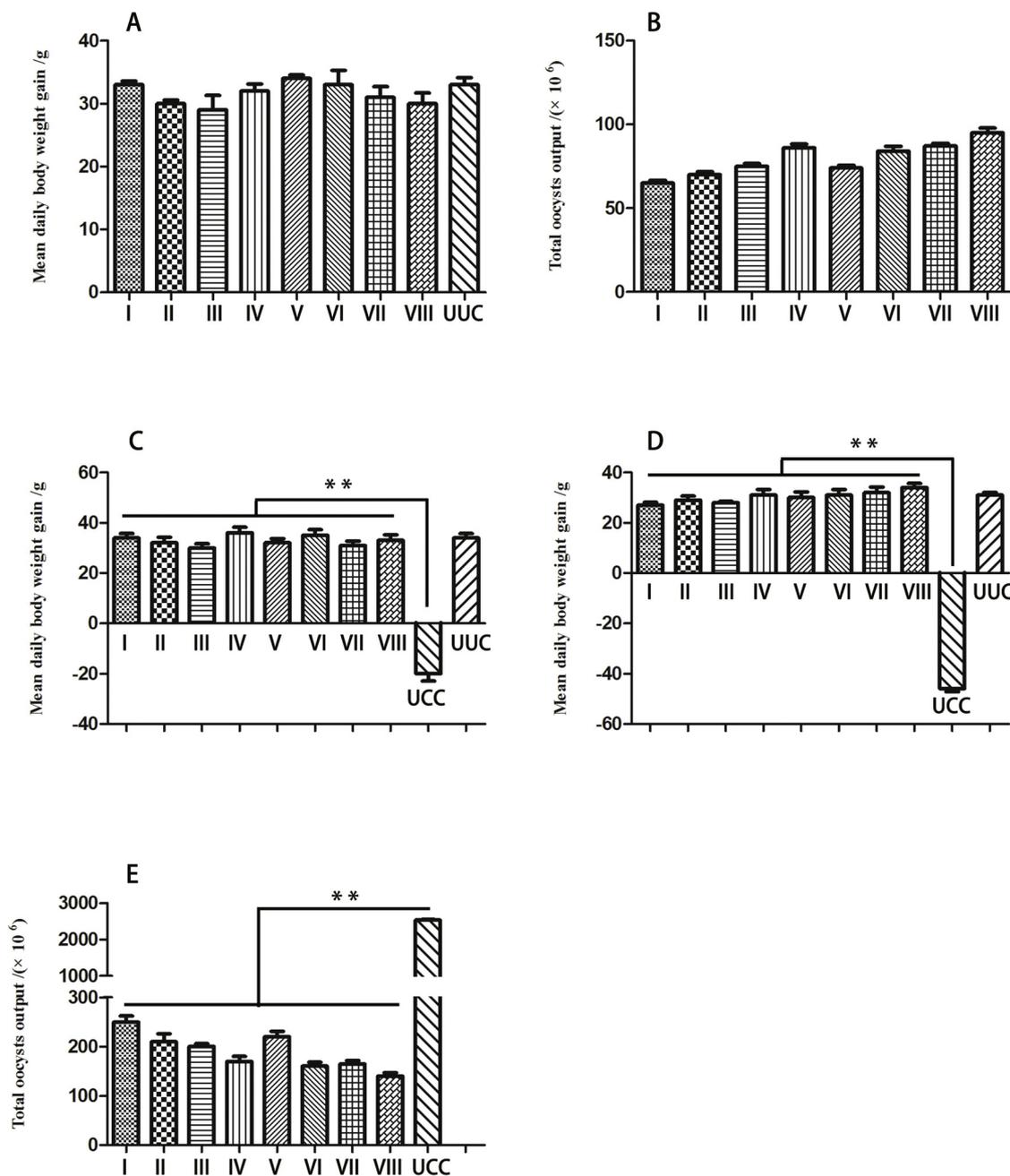


Fig. 2. Immuno-protective effects of co-immunization with three precocious lines. (A) The ADGs after immunization with the precocious lines. (B) The total oocyst outputs after immunization with the precocious lines. (C) The ADGs 0–7 days after challenge with the parent strains. (D) The ADGs 7–14 days after challenge with the parent strains. (E) The total oocyst outputs after challenge with the parent strains. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ .

were significantly different from that of the UCC group ( $P < 0.01$ ) (Fig. 2C).

From 7 to 14 days after challenge, rabbits in UCC group developed severe diarrhea, and two died were recorded. The ADGs of the UCC group rabbits were significantly different from those of the immunized groups and UUC group ( $P < 0.01$ ). No significant clinical manifestations were observed in rabbits in the immunized groups. The ADGs of the immunized groups were not significantly different from that of the UUC group ( $P > 0.05$ ) (Fig. 2D). After challenge, the total oocyst outputs of the groups I–VIII decreased significantly and were significantly different from that of UCC group ( $P < 0.01$ ). The oocyst output of each immunized group decreased with the increase in the immunization dose (Fig. 2E).

The results showed that when rabbits were challenged on day 14 after immunization, all immunization groups could produce good

immune protective effects and resist infection with 30,000 oocysts of mixed parent strains.

#### 4. Discussion

Coccidial infections are common in almost all rabbit farms, and infections with multiple coccidial species are common. Among them, *E. intestinalis*, *E. flavescens* and *E. magna* are the most dominant (Jing et al., 2012) and highly pathogenic species, *E. media* has moderate pathogenicity and the pathogenicity of *E. stiedai* is dose-dependent.

All four doses of each precocious line ( $5 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$  and  $5 \times 10^4$  oocysts) used in the immunogenicity test induced resistance to infection effectively with  $1 \times 10^4$  parent oocysts. However, the immunogenicity of the *E. flavescens* precocious line was poor (data not shown), which was consistent with the report by Pakandl et al.

(2008a, 2008b). Therefore, three precocious lines (*E. magna*, *E. intestinalis*, and *E. media*) were selected because of their considerable immunogenicity to form a trivalent vaccine. Nevertheless, Akpo et al. (2012) conducted a co-immunization experiment with precocious lines of *E. magna* and *E. media* and proved that these two lines could provide immune protection to rabbits.

Vaccination with 1000 (for each species of interest) precocious oocysts of *E. magna*, *E. intestinalis*, and *E. media* could not only induce clinical resistance to infection with 30,000 mixed parent oocysts but also reduced the oocyst output after challenge.

After all, the precocious lines of other pathogenic coccidia species, including *E. stiedai*, *E. irresidua* and *E. piriformis*, would be selected to develop a complete rabbit coccidiosis vaccine, and a gel delivery system for this trivalent vaccine will be tested to facilitate the vaccination for the commercial rabbit breeders.

As far as the challenge time, rabbits were challenged with the wild-type strains 14 days after vaccination, and the vaccine demonstrated a good immune protection. The time interval between vaccination and challenge was consistent with the report of Licois et al. (1995). Beforehand, rabbits were challenged nine days after vaccination and failed to obtain immune protection. One possible reason could be that the rabbits had not established immune protection 9 days after vaccination and could not resist infection with large doses of the wild-type strain.

Licois et al. (1995) obtained a precocious line of *E. magna*, and decreased ADG was observed by inoculating not less than  $4 \times 10^4$  precocious oocysts of Licois strain.  $5 \times 10^2$  oocysts of *E. magna* precocious line can provide complete protection against the challenge with  $1 \times 10^4$  oocysts of the parent strain of *E. magna*, however the dose of complete protection was  $2.5 \times 10^3$  oocysts for Licois strain. Although the pathogenicity of *E. magna* precocious line was a litter higher than that of Licois strain, it has more excellent immunogenicity, which might be due to the differences in different geographical strains of the same *Eimeria* spp.

China is the largest rabbit-producing country in the world, and rabbit coccidiosis vaccines formulated with the attenuated precocious lines will be widely used in the rabbit industry to avoid the drug resistance problems and drug residues in meat.

## 5. Conclusion

In conclusion, the precocious lines of *Eimeria magna*, *Eimeria*

*intestinalis* and *Eimeria media* had weak pathogenicity but good immunogenicity. The use of dose of 1000 oocysts for each of the three species precocious lines (*E. magna*, *E. intestinalis*, and *E. media*) in the trivalent vaccine reduced significantly the oocyst output after challenge with a healthy growth of rabbits. These results suggest that the precocious line trivalent vaccine could be used as a candidate vaccine.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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