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Immune effects of a precocious line of *Eimeria necatrix* with different doses and at different immunization times

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Abstract. This study was designed to assess the immune protective effects of the vaccine strain of a precocious line of *Eimeria necatrix* with different doses and at different immunization times. The immunizations had a negative effect on weight gains of chickens to a certain degree but could be compensated during the "compensatory growth period" after immunity was established in the chickens. The number of oocysts excreted was positively correlated with the immunization dose. All the immunized chickens, whether they were immunized once or twice or immunized with different doses of sporulated oocysts, were able to resist attack from $1x10^5$ virulent sporulated oocysts of *E. necatrix*. The lesion scoring showed that no significant difference existed in the chicken groups immunized with different doses (300 and 600) of sporulated oocysts. However, a difference existed in the immune homogeneity established in the different immunized groups, and two artificial immunizations were superior to one artificial immunization, indicating that two could extend the duration of oocyst excretion and allow more chances for the immunized chickens to become repeatedly infected.

INTRODUCTION

Avian coccidiosis, is a protozoan disease characterized by diarrhea and bloody stools that is caused by one or more species of *Eimeria* that parasitize the intestinal epithelium of chickens. It is one of the most serious diseases and pose great economic impact on the poultry industry worldwide (Yin *et al.*, 2011; Shirley *et al.*, 2012; Wu *et al.*, 2014). The continuous addition of preventive anticoccidial drugs into the feed is a method commonly used in the poultry industry in China and plays an important role in the control of avian coccidiosis; however, this method also causes relatively serious problems, such as drug residues and the rapid emergence of drug-resistant parasites (Suo *et al.*, 2006; Weng, 2009; Wu *et al.*, 2014). Hence, the search for nondrug or limited-drug strategies against avian coccidiosis have intensified, and immunization is the most important method that can be used as a substitute for or in combination with chemical drugs (Chapman *et al.*, 2002; McDonald *et al.*, 2009; Shirley *et al.*, 2012). In China, although imported avian coccidiosis vaccines such as Coccivac-B and Coccivac-D are available, they have not been widely used, due perhaps to the high cost and the limitations on transportation and preservation (Suo *et al.*, 2006; Wu *et al.*, 2014). Currently, the coccidiosis trivalent and coccidiosis quadrivalent vaccines manufactured by Foshan Standard Biotechnology Co., Ltd., China, are the main avian coccidiosis

vaccines used in chicken farms in China. In 2013, more than 1 billion doses of avian coccidiosis vaccines were sold.

Among the seven internationally recognized species of chicken *Eimeria*, *E. necatrix* has a relatively high pathogenicity and primarily damages the middle section of the small intestine. The parasite causes acute intestinal coccidiosis, affects nutrient uptake and causes secondary necrotic enteritis, leading to huge economic losses (McDougald *et al.*, 1990; Mattiello *et al.*, 2000; Liu *et al.*, 2014). *E. necatrix* has a relatively special life cycle. The schizogony of *E. necatrix* occurs in the middle section of the small intestine, and the gametogony of *E. necatrix* occurs in the cecum (Liu *et al.*, 2014). In addition, the production of the *E. necatrix* oocysts is relatively low. Therefore, it is not easy for a chicken group to become homogeneously and repeatedly infected in practical production. One of the key steps of coccidiosis immunization is the smooth realization of "second immunization and third immunizations," which are extremely important to the establishment of strong immunity in a group of chickens (Weng, 2009).

The objectives of the present study were to evaluated the immune effect of a precocious line of *E. necatrix* at with different doses and at different immunization times. The excretion pattern of *E. necatrix* oocysts was monitored after immunization of chickens, and parasite challenge tests were conducted to evaluate the immune protective effects on the immunized chicken groups.

MATERIALS AND METHODS

Parasites

Sporulated oocysts of the Hezhou strain of a precocious line of *E. necatrix* (PNHZ) and virulent sporulated oocysts of the Hezhou strain of *E. necatrix* (NHZ) were both provided by Foshan Standard Biological Technology Co., LTD, China. The precocious strain PNHZ was selected from the field strain NHZ by serial passages through chickens of the first oocysts produced during infection.

Animals

One-day-old Chinese Yellow Chickens were obtained from the Animal Husbandry Poultry Farm of the Guangdong Academy of Agricultural Sciences and were housed in an *Eimeria*-free environment with an *ad libitum* supply of filtered water and a specially formulated initial growth feed. The animals were then transferred to wire cages for the experiments.

Feed

Feed was free of anticoccidials and antibiotics and was obtained from Zhongshan Crown Poultry Co., LTD, China.

Immune-protective effects of the vaccine strain of a precocious line of Eimeria necatrix

Four hundred Chinese Yellow Chickens were evenly divided into four groups, with 100 chickens in each group (groups A, B, C and D). Group A was treated as the control. The chickens in groups B and C were immunized orally with 300 and 600 sporulated oocysts of the PNHZ strain, respectively, per bird at 3 d of age. The chickens in group D were immunized twice with 300 sporulated oocysts of PNHZ strain at 3 and 6 d of age. Body weight gains were determined at 0, 13, and 20 d postimmunization. The numbers of oocysts in the cecal feces were determined between 6 and 10 d and between 13 and 18 d postimmunization using a McMaster counting chamber as described (Suo, 1998). Lesion scores were determined between 13 and 16 d postimmunization as described by Johnson and Reid (1970).

At 21 d postimmunization, 20 chickens in group A were divided into a negative group (group 1) and a positive group (group 2). Ten chickens each from groups B, C and D were designated as groups 3, 4 and 5, respectively. The chickens in groups 2, 3, 4 and 5 were challenged with $1x10^5$ virulent sporulated oocysts of the Hezhou strain of *E. necatrix* (NHZ). Body weight gains were determined

at 0 and 7 d postchallenge. Fecal scores were determined between 6 and 7 d postchallenge as described by Suo *et al.* The chickens were observed between 5 and 7 d postchallenge for mortality, and lesion scores were determined at 7 d postchallenge.

Statistical analysis

All data were processed and analyzed using SPSS 18.0 Data Editor (SPSS Inc., Chicago, Illinois, USA). The results from comparisons between groups were considered significantly different if $P < 0.05$.

RESULTS

Effects of the precocious line of Eimeria necatrix *(PNHZ) on body weight gain*

There was no significant difference in the average weight among the experimental groups before immunization. At 13 d postimmunization with a precocious line of *E. necatrix*, the relative weight gain rates of the immunized groups B, C and D were 96.1%, 95.3% and 93.2%, respectively, when compared with the control groups. The data showed that all immunizations, regardless of dose or time, had negative effects on the weight gains of the chickens to a certain degree and that the weight gains of the chickens were negatively correlated with the immunization dose. The relative weight gain rates of immunized groups C and D were lower than that of immunized group B. Table 1 shows that the relative weight gain rates of the immunized groups B, C and D at 20 d postimmunization were all higher than those at 13 d postimmunization, which demonstrated that the negative effects of immunization with PNHZ on the weight gains were transient and could be fully compensated for during the "compensatory growth period" after immunity was established in the chickens.

Determination of the excretion pattern of oocysts of the precocious line of Eimeria necatrix *(PNHZ)*

The numbers of oocysts excreted by the chickens that were immunized with different doses of PNHZ for different times are shown in Fig. 1. The peak oocyst excretion of group B was significantly lower than that of group C. The primary immunization peak period of immunized group D occurred slightly later than those of groups B and C. At 7 and 16 d postimmunization, the number of oocysts excreted by each chicken in groups B and C decreased sharply, whereas the decreasing trend in group D was slower than those in groups B and C. The duration of the oocyst excretion in group D was significantly extended compared to those in groups B and C. Thus, the immunization dose was positively correlated with the number of excreted oocysts, and the application of two artificial immunizations could extend the duration of oocyst excretion.

Lesion scoring from immunization reactions

During the immunization reaction period (13 to 16 d postimmunization), a small

Table 1. Weight gains of the experimental chickens at 20 days postimmunization

Group*	Number of chickens	Average weight at 13 days postimmunization (g/chicken)	Average weight at 20 days postimmunization (g/chicken)	Average weight gain (g)	Relative weight gain rate (%)
Group A	100	265.8	430.8	165.0	100
Group B	100	257.2	417.6	160.4	97.2
Group C	100	255.5	414.2	158.7	96.2
Group D	100	250.9	408.5	157.6	95.5

*Group A: Treated as the control group; Group B: Each chicken was inoculated orally with 300 sporulated oocysts of a precocious line of *E. necatrix (PNHZ)* at 3 days of age; Group C: Each chicken was inoculated orally with 600 sporulated oocysts of PNHZ at 3 days of age; Group D: Each chicken was inoculated orally with 300 sporulated oocysts of PNHZ at 3 days of age and another 300 sporulated oocysts at 6 days of age.

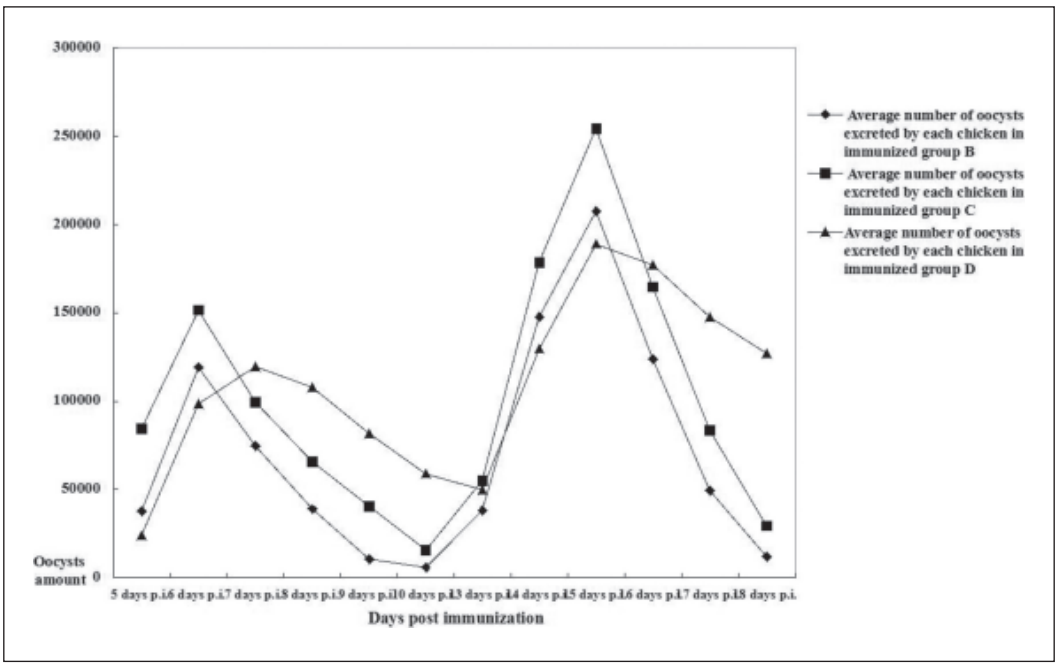

Figure 1. Numbers of oocysts excreted by chickens that were immunized with different doses and at different times.

amount of feed-like feces appeared in each immunized group, and a small amount of tomato-like feces appeared in group C. All of the immunized groups exhibited good mental condition and ate and drank normally, and no chickens died from avian coccidiosis. The autopsy results demonstrated that there were mild intestinal lesions in all of the immunized groups; the lesions appearing in the chickens in groups B and D were slightly milder compared to those in the chickens in group C.

Immunization effect of the precocious line of Eimeria necatrix *(PNHZ) against challenge with a virulent strain of* E. necatrix *(NHZ)*

The effects of immunization with the precocious line of *E. necatrix* (PNHZ) were evaluated based on body weight gain, fecal scores, intestinal lesion scores and survival percentages. These experimental results are shown in Tables 2 and 3.

As shown in Table 2, the relative weight gain rates of all of the immunized and challenged groups (groups 3, 4 and 5) were slightly lower than that of the negative control group (group 1) but significantly higher than that of the nonimmunized and challenged group (group 2). The weight gains of the chickens in group 2 were affected after these chickens were attacked by a virulent strain of *E. necatrix*. The relative weight gain rates in groups 3, 4 and 5 were 96.6%, 96.1% and 95.6%, respectively, indicating that the chickens were able to establish protection upon challenge with a virulent strain of *E. necatrix*.

At 6 d after the parasite challenge, the chickens in group 2 began to exhibit significantly reduced appetites, became listless, and trembled and their feet became dehydrated and dry. In addition, the visible mucosa exhibited a pale color. The feces of these chickens were shapeless and were covered with large amounts of dark red mucus, and the feces could be scored as a 3. Few tomato-like feces appeared in any of the three immunized and challenged groups. Each immunized and challenged group had a fecal score of 0.5. The chickens in all three immunized and challenged groups showed

Table 2. Immune effect on weight gain in immunized chickens against challenge with a virulent strain of *Eimeria necatrix* (NHZ)

Group*	Average weight before challenge (g/chicken)	Average weight at 7 days postchallenge (g/chicken)	Average weight gain (g/chicken)	Relative weight gain rate (%)
Group 1	421.5	611.8	190.3	100
Group 2	430.8	555.2	124.4	65.4
Group 3	417.6	601.5	183.9	96.6
Group 4	414.2	597.0	182.8	96.1
Group 5	408.5	590.5	182.0	95.6

*Group 1: Negative control; Group 2: Nonimmunized and challenged group; Group 3: Immunized and challenged group; each chicken was inoculated orally with 300 sporulated oocysts of a precocious line of *E. necatrix (PNHZ)* at 3 days of age; Group 4: Immunized and challenged group; each chicken was inoculated orally with 600 sporulated oocysts of a precocious line of *E. necatrix (PNHZ)* at 3 days of age; Group 5: Immunized and challenged group; each chicken was inoculated orally with 300 sporulated oocysts of PNHZ at 3 days of age and another 300 sporulated oocysts at 6 days of age.

Table 3. Immune effects of a precocious line of *E. necatrix (PNHZ)* with different immunization doses and at different immunization times

Group*	Fecal score	Lesion scores of each chicken											Average lesion score#	Survival percentage (%)
Group 1	–	–											–	–
Group 2	3.0	3.0	3.0	4.0	3.0	3.0	3.0	3.0	3.0	4.0	3.0		3.2±0.42[a]	90
Group 3	0.5	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0		1.2±0.42[b]	100
Group 4	0.5	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	0.0		1.2±0.57[b]	100
Group 5	0.5	1.0	0.0	1.0	2.0	0.0	1.0	0.0	1.0	1.0	0.0		0.7±0.67[c]	100

* Group 1: Negative control; Group 2: Nonimmunized and challenged group; Group 3: Immunized and challenged group; each chicken was inoculated orally with 300 sporulated oocysts of a precocious line of *E. necatrix (PNHZ)* at 3 days of age; Group 4: Immunized and challenged group; each chicken was inoculated orally with 600 sporulated oocysts of a precocious line of *E. necatrix (PNHZ)* at 3 days of age; Group 5: Immunized and challenged group; each chicken was inoculated orally with 300 sporulated oocysts of PNHZ at 3 days of age and another 300 sporulated oocysts at 6 days of age.
# The superscript letters indicate the significance of the difference in the lesion score in the experimental groups; the same letters indicate that the difference is insignificant ($P \geq 0.05$), whereas different letters indicate that the difference is significant ($P < 0.05$); n = 10.

good mental conditions, ate and drank normally, and had no noticeable clinical disease symptoms.

The chickens were autopsied at 7 d after the parasite challenge for lesion scoring. As shown in Table 3, the jejunum lesion scores of the chickens in the immunized and challenged groups were significantly lower than those of the chickens in the nonimmunized and challenged group. There was no significant difference between groups 3 and 4 ($P \geq 0.05$), but there was a significant difference between groups 3, 4 and 5 ($P < 0.05$). Therefore, one immunization of a lower or higher dose of PNHZ and two artificial immunizations of PNHZ could all induce the chickens to establish protective immunity against a virulent strain of *E. necatrix*. However, the application of two artificial immunizations was superior to one immunization.

DISCUSSION

A multivalent vaccine containing precocious lines of different *Eimeria* species was able to induce a strong immunity in chickens against virulent strains, but the strength and duration of the immunity stimulated by

single different *Eimeria* species were varied, depending on the different biological characteristics and immunity method (Li *et al.*, 2005; Bian *et al.*, 2006). The immunogenicity of *E. necatrix* is the weakest among the seven chicken *Eimeria* species, and it takes at least three life cycles to gradually establish immunity against virulent *E. necatrix* strains (Nakai *et al.*, 1992; Klinkenberg *et al.*, 2005; Bian *et al.*, 2006). In this study, at 21 d postimmunization with a precocious line of *E. necatrix*, all of the immunized chickens, whether they were immunized once or twice or immunized with different doses of sporulated oocysts, could resist attack from $1x10^5$ sporulated oocysts of the virulent *E. necatrix*. The immunization had a certain temporary negative effect on the weight gain of the immunized chickens, but this effect was compensated for during the "compensatory growth period" after immunity had been established and did not affect the general weight gain (Voeten *et al.*, 1988; Li *et al.*, 2005). Based on these data, the excreted oocysts of the immunized chickens were positively correlated with the dose of vaccinated oocysts, and two inoculations could extend the duration of oocyst excretion.

When the immunization efficacy was compared to the virulent *E. necatrix* challenge, there were no significant differences in lesion scoring between the different immunized groups in which the chickens were immunized with different doses, but two immunizations were superior to one immunization. Furthermore, two immunizations could extend the duration of the oocyst excretion and allow more chances for the chicken group to become repeatedly infected. Therefore, strengthening multiple repeated infections with small doses is effective in inducing immunity against *E. necatrix* to a certain degree.

When the commercial coccidiosis vaccine is used in China, there is usually only one occurrence of artificially planned immunization. The establishment of strong immunity eventually relies on the chicken group becoming repeatedly and naturally infected from vaccine progeny in the litter. In a previous study on Chinese Lingnan Yellow chickens, we found the excretion frequency of cecal droppings was only 2-4 times per day, and the oocysts of *E. necatrix* were mainly distributed in the cecal droppings, which affects the homogeneity of the second and third generations of oocysts ingested by chickens and therefore alters the immune effects during *E. necatrix* immunization (Chen *et al.*, 2016). In this study, the artificial second immunization showed that it could extend the duration of oocyst excretion and increase the immune homogeneity, thus enhancing the immune effect against virulent *E. necatrix*.

In this study, the experimental chickens were immunized with different doses of a precocious line of *E. necatrix* for different artificial immunization times, and the immune effects were compared. The immunized chickens could all resist attack from a virulent strain of *E. necatrix* 21 d postimmunization. Two artificial immunizations were superior to one artificial immunization and could strengthen the immune protection and improve the immunity homogeneity.

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