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 (Su 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 $1 \times 10^5$ 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
cnecatrix (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)

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

*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

<table>
<thead>
<tr>
<th>Group*</th>
<th>Fecal score</th>
<th>Lesion scores of each chicken</th>
<th>Average lesion score</th>
<th>Survival percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Group 2</td>
<td>3.0</td>
<td>3.0 3.0 3.0 3.0 3.0 3.0 3.0 3.0 3.0 3.0 4.0 3.0</td>
<td>3.2±0.42a</td>
<td>90</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.5</td>
<td>2.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0</td>
<td>1.2±0.42b</td>
<td>100</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.5</td>
<td>1.0 2.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0 1.0</td>
<td>1.2±0.57b</td>
<td>100</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.5</td>
<td>1.0 0.0 1.0 2.0 0.0 1.0 0.0 1.0 1.0 1.0 1.0 1.0</td>
<td>0.7±0.67c</td>
<td>100</td>
</tr>
</tbody>
</table>

*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 chickens were autopsied at 7 d after the parasite challenge for lesion scoring. As shown in Table 3, the jejunal 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 ≥ 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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