Attenuation of *Eimeria tenella* by Serial passage in Chicken Embryos

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**ABSTRACT.** A strain of *Eimeria tenella* of Japan origin was serially passaged 51 times in chicken embryos. Any obvious changes of reproductivity and lethality to chicken embryos of the passage strain were not observed. Chickens infected with oocysts passed 41 times in chicken embryos showed less severe cecal lesions and hemorrhage, and higher values of body weight gain, feed intake and lower value of feed requirent, compared with chickens infected with oocysts passed once in chicken embryos. Less severe lesions were also observed in chickens infected with oocysts passed 51 times in chicken embryos than those in chickens with oocysts passed once. It was indicated that pathogenicity of the strain was attenuated after serial passage in chicken embryos.—**KEY WORDS:** attenuation, chicken embryo, *Eimeria tenella.*

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Prophylactic chemotherapy is the major method for controlling coccidiosis in chickens. Although immunological prophylaxis has been shown to be effective against coccidiosis experimentally, vaccination has not been fully established yet. Many authors have attempted to obtain attenuated strains for making vaccines. By serial passage of parasites in chicken embryos, attenuated strains of *E. mivati* [5, 6] *E. tenella* [1, 4], and *E. necatrix* [1, 11] were produced. On the other hand, strains of *E. necatrix* [12] and *E. acervulina* [7] were attenuated by selection of the parasites for characteristics of precociousness.

We have serially passaged a strain of *E. tenella* of Japan origin in chicken embryos. In this paper, we describe the process of the passage and the change of the pathogenicity of the strain.

**MATERIALS AND METHODS**

**Source of Eimeria strain:** *Eimeria tenella* K-2 strain which originated in National Institute of Animal Health, Japan and was maintained in chickens serially at the Department of Animal Science, Faculty of Agriculture, Tohoku University, was used.

**Passage of the parasite in chicken embryos:** Inoculation and harvest of the parasite was performed by the methods described by Nakai et al. [8]. It is briefly mentioned in the following. Sporozoites excysted from sterilized oocysts *in vitro* were inoculated into the allantoic cavity of 10-day-old white leghorn chicken embryos (a commercial strain Iwaya 505, Sendai, Japan) incubated at 37°C. For the passage of the strain, 1×10³ sporozoites were inoculated to each embryo. Infected embryos were incubated at 41°C for 8 days. Oocysts were harvested from the chorioallantoic membrane (CAM) and urate deposits. CAM and deposits were cut to pieces with scissors, and made up to a volume of 10 ml by adding 2.5% potassium bichromate solution. The mixture was homogenized by a whirling blender in an ice bath and placed in a Petri dish at 29°C for 3 days. Sporulated oocysts were washed 4 times in distilled water by centrifugation, and treated by Purelox (5-6% sodium hypochlorite solution) in an ice bath for 10 minutes. Oocyst
suspension was sieved through 100 mesh stainless sieve, and washed by centrifugation with sterile distilled water. These oocysts were used for the preparation of sporozoites.

**Determination of mortality of embryos:** Embryos which died on and after 4th day post inoculation were judged as killed by *E. tenella* infection. The embryos which died within the first 3 days were excluded from the mortality rate calculation.

**Chicken infection:** Sporozoites of the 44th passage strain and those of the non-passage strain were injected into chicken embryos, and cultivated under same condition to harvest resulted oocysts of the 45th passage strain (P45) and the 1st passage strain (P1). After sporulated under same condition, they were inoculated orally to 10-day-old white leghorn male chickens (Iwaya 505) in 7 groups of 10 birds. Each group was reared separately in wire-floored cages. Birds were sacrificed on 7th day of infection. Intestinal and cecal lesions were graded on a scale between 0 and 4 as described by Johnson *et al* [2]. Cecum with its content were homogenized by the whirling blender, and the number of oocysts was counted.

**RESULTS**

Figure 1 shows the oocyst yield and the mortality rate in each passage.

Oocyst yields in the 2nd-4th passage were up to $1 \times 10^4$/embryo. The yields increased as the passage proceeded, resulting in above $1 \times 10^5$/embryo. Similar numbers were shown by the 12th passage. After that, oocyst yields decreased and the numbers of oocysts were not sufficient for the following passage sometimes. In such case the oocysts harvested in several passages before were used for continuing the passage. There was no tendency for the passage strain to reproduce stabilized number of oocysts.

Some of chicken embryos died from hemorrhage caused by infection of *E. tenella*. Death from hemorrhage was observed.
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Table 1. Pathogenicity of E. tenella passaged 45 times in chicken embryos, in 10-day-old chickens

<table>
<thead>
<tr>
<th>Number of passages in embryos</th>
<th>Inoculum dose</th>
<th>Mean body weight gain (g)</th>
<th>Feed intake (g)</th>
<th>Feed requirement</th>
<th>Mean packed cell volume (%)</th>
<th>Mean caecal lesion score</th>
<th>Mean number of oocysts recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1×10^3</td>
<td>73</td>
<td>125</td>
<td>1.72</td>
<td>36</td>
<td>2.0</td>
<td>10^6.6</td>
</tr>
<tr>
<td>1</td>
<td>1×10^3</td>
<td>70</td>
<td>122</td>
<td>1.75</td>
<td>37</td>
<td>2.7</td>
<td>10^6.6</td>
</tr>
<tr>
<td>45</td>
<td>5×10^3</td>
<td>69***</td>
<td>123</td>
<td>1.79</td>
<td>34*</td>
<td>3.0</td>
<td>10^6.2</td>
</tr>
<tr>
<td>1</td>
<td>5×10^3</td>
<td>54**</td>
<td>111</td>
<td>2.04</td>
<td>25*</td>
<td>3.3</td>
<td>10^6.1</td>
</tr>
<tr>
<td>45</td>
<td>2×10^4</td>
<td>65*</td>
<td>118</td>
<td>1.82</td>
<td>30</td>
<td>3.1</td>
<td>10^6.5</td>
</tr>
<tr>
<td>1</td>
<td>2×10^4</td>
<td>41*</td>
<td>103</td>
<td>2.50</td>
<td>27</td>
<td>3.7</td>
<td>10^6.7</td>
</tr>
<tr>
<td>−</td>
<td>0</td>
<td>81</td>
<td>128</td>
<td>1.59</td>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Significant difference in the group of the same dose: *, P<0.05; **, P<0.01.

Table 2. Cecal lesion scores of chickens given E. tenella passaged 51 times in chicken embryos

<table>
<thead>
<tr>
<th>Number of passages in embryos</th>
<th>Inoculum dose of oocysts</th>
<th>Number of chickens</th>
<th>Cecal lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>51</td>
<td>1×10^3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1×10^3</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Number of chickens showing the lesion score.
b) Average lesion score of the group.
c) Standard deviation.

also in the 51st passage. Since LD_{50} of the 49th passage strain in chicken embryos was 3×10^3 which was similar to the value of the non-passage strain (4×10^3), there may be no difference in lethality to chicken embryos between the passage strain and the non-passage strain.

Table 1 shows reproduction and pathogenicity of the parasites in chickens. When 5×10^3 or 2×10^4 oocysts were inoculated, the weight gain of chickens inoculated with oocysts of the strain passaged in embryos 45 times (P45) was significantly higher than that with oocysts passaged in embryos once (P1). Both feed intake and feed requirement of chickens with P45 tended to be better than those with P1. The lesion scores of chickens infected with P45 showed lower value than those with P1 in all inoculum doses. Almost constant number of oocysts were recovered from cecum of chickens with P45 and with P1 at 7th day of infection. This fact suggests that there was so-called "crowding effect" and that P45 reproduced sufficiently. On the other hand, the weight gain of chickens with P45 was significantly lower than that of uninfected chickens. From these results it was clear that the effect of the infection with P45 could not be ignored but it was less severe than that with P1.

When the similar experiment was done with P51 (Table 2), chickens infected with P51 had less severe lesions than those infected with P1.

As the results, it was indicated that the pathogenicity to chicken embryos of the passage strain has not been altered, but that its pathogenicity to chickens was attenuated.
DISCUSSION

Passaged strain did not produce a stable number of oocysts (Fig. 1). Many factors are thought to affect oocyst production. The storage period of oocysts may be one of the factors. However, embryos inoculated with the oocysts stored for 82 days yielded higher number of oocysts than those inoculated with oocysts stored for 28 days in the 39th passage. Factors of chicken embryos rather than those of the parasites might affect oocyst yields in some cases.

Maternal transfer of antibodies to chicken embryos was described about *E. tenella* [10] and *E. maxima* [9]. There was a possibility that transferred maternal antibodies in chicken embryos suppressed the development of the parasites in some passages.

Some anti-coccidial drugs inoculated to chickens were shown to transfer to chicken embryos with resulting control of *E. tenella* infection in chicken embryos [3]. As the farm medicated anti-coccidials occasionally, they might affect the infection of the parasites in chicken embryos.

On the other hand, wide genetic variation in the commercial strain of the chickens should affect the oocyst production. Therefore, oocyst productivity of the passage strain have to be investigated furthermore by using genetically controlled SPF chicken embryos.

Death from hemorrhage was observed in chicken embryos receiving oocysts of the passage strain, and LD₅₀ of P49 was similar to that of the non-passaged strain. The lethality of the passaged strain did not attenuate to chicken embryos. Although Long and his colleague described that pathogenicity of *E. tenella* strains passaged in chicken embryos 40 times [4] or 33 times [1] attenuated to chicken embryos, they also mentioned that another strain did not alter their pathogenicity after passaged 29 times in chicken embryos [1]. The tendency of alteration of the pathogenicity to chicken embryos in the course of passage in chicken embryos may be different in each strain. Gene level investigation on the mechanisms of the attenuation of the pathogenicity will give us answers of the question.

Chickens inoculated with P45 showed less severe cecal lesion and hemorrhage and higher values of body weight gain, feed intake and lower value of feed requirement than those inoculated with P1. Less severe cecal lesions were observed in chickens inoculated with P51 than P1. These data indicate that pathogenicity of the strain passaged in chicken embryos attenuated to chickens. Since the passage strain made apparent lesions in chickens, further work must be done in order to make the strain a vaccin.

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REFERENCES


要約

鶏胚継代による *Eimeria tenella* 病原性の減弱：中井 裕・足元敬司*1) （茨城大学農学部家畜衛生学教室、*2) 東北大学農学部家畜衛生学教室）——*Eimeria tenella* の国内分離株を鶏胚で51代継代培養しても鶏胚での増殖率や致死作用に変化はみられなかった。鶏胚45代あるいは51代継代株をニワトリひなに接種すると鶏胚通過初代株に比べて盲腸病変および血便は軽く、増体量、および飼料効率は高かった。