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Attenuation of *Eimeria tenella* with Immersion Various Concentration of Formaldehyde in Inducing Protective Immunity after Challenge Test by Featuring Macroscopic and Microscopic Caecum

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ABSTRACT

Background: *Eimeria tenella* parasite is one of the most pathogenic coccidia that can infect chickens. The strategy for prevention and control of coccidiosis is done by administering anticoccidia drugs and live oocyst vaccines. However, anticoccidia can cause resistance to coccidiosis. So it is necessary to make vaccines (attenuation) using formalin or formaldehyde.

Aim: The purpose of this study was to determine whether the attenuation of the pathogenicity *E. tenella* with immersion various concentrations of formaldehyde can induce protective immunity and the concentration of formaldehyde that is most effective in inducing protective immunity for attenuation of *E. tenella* in featuring cecal macroscopic and microscopic (lesion score).

Method: Twenty-five chickens at three weeks old were divided randomly into five groups. Challenge test did after the first infection. The first infection was inoculated *tenella* by divided the first group (P0) is chicken group was inoculated with 0% formaldehyde soaked *E. tenella* at 1 x 10⁴ doses as control, the 2nd, 3rd and 4th groups (P1; P2, P3 and P4) were inoculated 0.15%, 0.3%, 0.6% and 1.2% of formaldehyde soaked *E. tenella* at the same doses, respectively. On challenge test performed two weeks after the first infection by inoculated 15x10³ infective oocysts of *E. tenella*.

Result: The results showed that the attenuation of *E. tenella* with immersion various concentrations of formaldehyde can induce protective immunity by featuring cecal macroscopic and microscopic (lesion score).

Conclusion: The most effective concentration of formaldehyde in inducing protective immunity of the attenuation pathogenicity *E. tenella* was 1.2%.

Keywords: attenuation, *E. tenella*, formaldehyde, protective immunity

Introduction

Poultry coccidiosis is an intestinal disease caused by Genus *Eimeria* parasitic protozoa¹. *Eimeria* multiplies in the digestive tract and causes damage to the intestinal mucous tissue². *Eimeria* causes damage to the intestine

thereby reducing the efficiency of feed use, body weight, decreased endurance, and decreased egg production³. Coccidiosis in chickens is located in two places, namely the caecal (cecal coccidiosis) caused by *Eimeria tenella*⁴. *Eimeria tenella* parasites develop in cecum cells, which are two dead end sacs near the back end of the small intestine. *Eimeria tenella* parasite is one of the most pathogenic coccidia to infect chickens. Acute infections often occur in young chickens. Infection can be characterized by blood in the stool and with high morbidity and mortality⁵. In addition, the immune response for *Eimeria* involves many aspects of both the cellular and humoral immune mechanisms^{6,7}.

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So far, strategies to prevent and control coccidiosis are carried out by anticoccidials and live oocyst vaccine^{8,9}. However, anticoccidials can cause resistance to coccidiosis¹. The development of increasing anticoccidial drug resistance has stimulated the search for biological control methods by vaccination. Some vaccines have been tried in the form of whole attenuated oocysts^{10,11}. One potential explored vaccine material is a live vaccine from oocysts that can be developed, produced and applied in the field¹². The development of live vaccines using low virulence lines from *Eimeria* oocysts can be used as an alternative for more efficient and effective protection compared to other coccidiosis vaccines, such as vaccines containing switched off microorganisms and subunit vaccines. Vaccine making can be done by activating or weakening the organism (attenuation)¹³. A simple way of attenuation can expose the organism to active chemicals to the limits of sublethal concentrations such as the use of formaldehyde or formalin.

Giving vaccines can stimulate the body to form antibodies to the *Eimeria* antigen so that the chicken is able to deal with new infections (challenge tests). The use of separated *Eimeria* types in vaccines makes it easy for users to apply vaccines according to the prevention of the desired disease. Based on this, the purpose of this study was to determine whether the attenuation of the pathogenicity *E. tenella* with immersion various concentrations of formaldehyde can induce protective immunity and the concentration of formaldehyde that is most effective in inducing protective immunity for attenuation of *E. tenella* in featuring cecal macroscopic and microscopic (lesion score).

Materials and Method

This research was conducted at the Laboratory of Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, from August to September 2016. The experimental animals used in this study were 25 broiler chickens strain CP 707 produced by Charoen Pokphand. The chickens were two weeks old and breed in a battery cage in Besah Village, Kasiman District, Bojonegoro Regency, Indonesia. *Eimeria tenella* inoculation was performed on 3 weeks old chickens after being adapted for 1 week by ad libitum feeding and drinking. Feed which given, it does not contain koksidiostat.

Calculation of Dosage: Calculation of dosages of *E. tenella* oocysts was carried out using micro pipets measuring 1-10 µl and white chips. The suspension of *E. tenella* oocysts was diluted using distilled water then the diluted liquid was vortexed so that both materials are homogeneous. The oocyst calculation was carried out by dripping 1 mL oocyst into a glass object with 5 replications and then calculating the number of oocysts found in all replications with a 100x ratio and looking for an average. From the results of calculations in the average can be 15,000 oocysts/ml.

Formula: This research was an experimental laboratory research with the Completely Randomize Design (CRD). The chicken grouping is based on the treatment given, namely:

Treatment 0: As a control, oocysts were not soaked in formalin

Treatment I: *E. tenella* was soaked in formalin with a concentration of 0.15%

Treatment II: *E. tenella* was soaked in formalin with a concentration of 0.3%

Treatment III: *E. tenella* was soaked in formalin with a concentration of 0.6%

Treatment IV: *E. tenella* was soaked in formalin with a concentration of 1.2%

After *E. tenella* was soaked, then chicken were inoculated as many as 10,000 oocysts orally. After 14 days, a challenging test was carried out by re-inoculating *E. tenella* without immersing 15,000 formaldehyde as a formalin.

Chicken and *Eimeria tenella* infections: Twenty-five 21-day old chickens were inoculated with 10,000 *E. tenella* oocysts that were patented using formalin with various levels of concentration (0.15%, 0.3%, 0.6% and 1.2%) for 96 hours. Chickens were inoculated using *E. tenella* orally which had been washed using distilled water and centrifuged at a speed of 1500 rpm for 10 minutes at five times. After two weeks, twenty-five 34-day-old chickens were re-inoculated with 15,000 *E. tenella* oocysts without formaldehyde immersion. Chicken is inoculated with *E. tenella* orally.

Cecum Lesion Scoring: Scoring of cecal lesions was carried out on the fifth day after inoculation,

macroscopically. The cecal abnormalities were recorded and the degree of damage to the mucosal surface of the chicken cecum was calculated based on a score of 0-4¹⁴.

Histopathology observation: Histopathology observation was carried out at the Pathology Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. Microscopic examination was carried out based on the histopathological changes that occurred in the cecum which had previously been made histopathological preparations, assessed by scoring seen based on the Goodwin method¹⁵.

Challenge Test: The challenge test was carried out 14 days after the first *E. tenella* inoculation, *E. tenella* which was soaked with various formalin concentrations. The challenge test was carried out by re-inoculating *E. tenella* without immersing as much as 15,000 oocysts formally. The indicator that shows the success of protective immunity is the reduced scoring of cecum lesions observed macroscopically and microscopically.

Data Analysis: Data to be obtained was macroscopic lesion scoring and microscopic lesion scoring. Data were arranged in table form using Kruskal Wallis analysis, if there are differences data, then proceed with the Mann Whitney test.

Results

Macroscopic Scores of Chicken Fecal Lesions

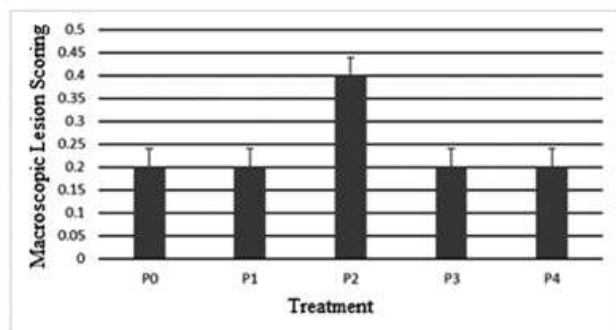


Figure 1: Macroscopic score of Chicken Fecal Lesions

The figure above is a comparison of the scores of chicken cecal lesions macroscopically after being challenged with *E. tenella* infective in each group of chickens with a dose of 15 x 10³ oocyst *E. tenella*. Each shows mean ± SD (N = 5). NS, not significantly different; *, p < 0, 05.

Macroscopic description of chicken serum



Figure 2: Macroscopic description of chicken cecum

The figure above is a macroscopic picture of chicken cecum which has been challenged by inoculating *E. tenella* infective in each group of chickens with a dose of 15 x 10³ oocyst *E. tenella*. The intensity of petechiae is reduced by *E. tenella* infection which is soaked in formalin with higher concentration. A small circle shows bleeding in the cecal mucosa, the arrow shows thickening of the cecum wall.

Microscopic score of Chicken Fecal Lesions

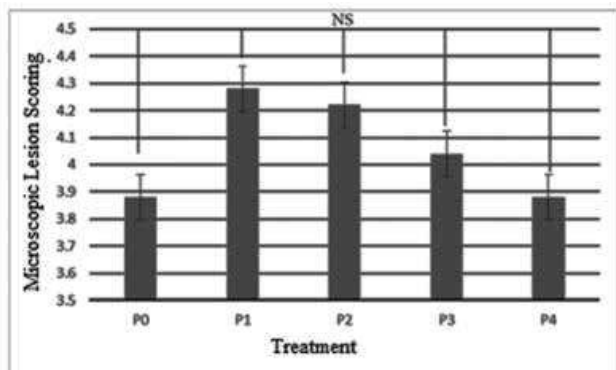


Figure 3: Cecum lesion microscopically in chickens

Figure 3 is a comparison of the scores of chicken cecal lesions macroscopically after being challenged with *E. tenella* infective in each group of chickens with a dose of 15 x 10³ oocyst *E. tenella*. Each shows mean ± SD (N = 5). NS, not significantly different; *, p < 0.05.

Microscopic description of chicken serum

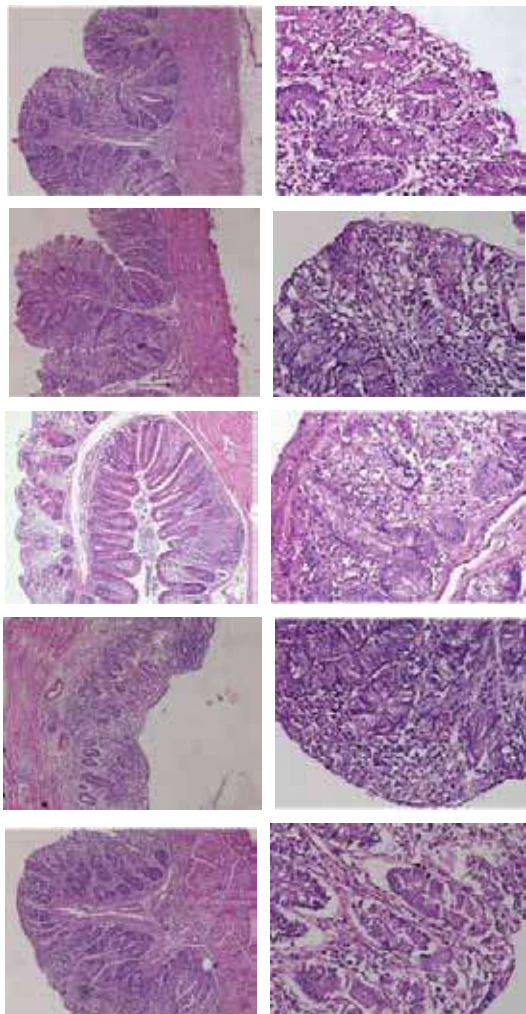


Figure 4: Microscopic description of chicken cecum

Microscopic description of chicken cecum which have been challenged by inoculating *E. tenella* infective in each group of chickens with a dose of 15 x 10³ oocyst *E. tenella* with various magnifications (A (100x) and B (400x)). The small circle shows the *Eimeria* distribution in the cecum and the black line shows the cecum mucosa.

Discussion

Macroscopic description of chicken cecum: From the macroscopic observation of the cecum, data were obtained based on histopathological abnormalities assessed based on a score of 0-4¹⁴. At P0 it shows a normal condition because there is no damage to the caecal lesions macroscopically and there is no thickening of the cecum wall. P1 shows the degree of damage to mild level cecal lesions, characterized by bleeding spots (ptechie) that spread on the surface of the mucosal mucosa with slight changes in the color of the wall or the contents of the digestive tract. P2 shows the degree of damage to moderate cecal lesions, characterized by more bleeding and lesions with a slight thickening of the cecum wall. P3 shows the degree of damage to mild cecal lesions, there are several bleeding spots that spread to the mucosal surface of the cecum with slight changes in the color of the wall or the contents of the digestive tract. P4 shows a normal condition because macroscopically there is no damage to cecum lesions and there is no thickening of the cecum wall¹⁴. These changes are due to the administration of *E. tenella* infective oocysts when the challenge test has no effect on cecal damage macroscopically, which means that the administration of oocysts that have been soaked in formalin before the challenge test can induce protective immunity seen macroscopically from chicken cecum.

The results of the statistical analysis using the Kruskal Wallis test also did not show significant differences in each treatment. It can be explained that macroscopically, the challenging test by inoculating as many as 15,000 *E. tenella* infective oocysts did not affect the damage to the cecum which had been inoculated with *E. tenella* with soaking various formalin concentrations (0.3%, 0.6%, 0.9 %, 1.2%). So it can be concluded that immersion of *E. tenella* in various concentrations of formaldehyde can induce protective immunity seen from the results of scoring and the results of macroscopic examination statistics after the challenge test which shows the results are not significantly different. The optimal formalin

concentration in patenting *E. tenella* is 1.2% indicating a normal state because macroscopically there is no damage to cecum lesions and there is no thickening of the cecum wall¹⁴.

Microscopic description of chicken cecum:

Histopathological observations on cecum, data obtained based on histopathological abnormalities contained in the preparations were assessed by scores seen based on summations of A and B, where A represents the distribution of *E. tenella* development stages found throughout the caecum, while B represents the severity of damage caused by *E. tenella*¹⁵.

The results of statistical analysis using the Kruskal Wallis test showed no significant differences in microscopic examination. The results obtained by histopathological preparations showed no significant differences in each treatment with the average scale which tended to be the same, namely P0: 3.88, P1: 4.28, P2: 4.22, P3: 4.04 and P4: 3.88. There are three immunity to *E. tenella*, which are totally invulnerable to parasites and do not develop parasites, chicken is immune to a certain degree, where oocysts are able to complete the life cycle but no lesions occur in the intestine, and chickens show no clinical symptoms but lesions occur in intestine¹⁶.

The microscopic scoring value in figure P0 did not experience abnormalities in cecum histopathology. Microscopic observations showed normal intestinal cells, the intestinal epithelium looked compact and did not show any rupture in the cecum villi. The first infected chicken, Schizont will develop properly due to immunity to *E. tenella* infection is still in the process of initiation, causing damage to the cecum.

The results of the P1 scoring data indicate histopathological abnormalities with moderate degrees of damage to the mucosal cecum. The cecal epithelium is compact and there are several villous ruptures. When compared with P0, P1 has a higher cecum damage. This is because previously chickens were inoculated with *E. tenella* which was soaked in formaldehyde with low concentration so it was suspected that schizont could still damage the caecum. Tissue damage to the cecum is caused by the outbreak of the schizont stage which has three generations before entering the gamete stage. P2 shows histopathological abnormalities with mild degrees of damage to the mucosal cecum. The cecal epithelium

appears compact and only a few villi rupture, when it compared with P0, P2 suffers more damage, it suspected that P2 has a low immune system that is lowered by its parent.

At P3 it showed almost no abnormalities in the histopathology of the cecum, the cecal epithelium appeared to be compact and rarely found in ruptured cecum villi. If vaccines are used to better control disease in an animal population and not individually, the concept of group immunity should be considered. If group immunity is carried out by reducing the likelihood of sensitive animals meeting the infected, there will be no spread of disease¹³. In P4 there is no abnormality in the histopathology of cecum. Microscopic observations showed normal intestinal cells, the intestinal epithelium appeared to be compact and did not show any rupture in the cecum villi and rarely found in the development of parasites.

Conclusion

Attenuation of *Eimeria tenella* through immersion in various concentrations of formalin (0.15%; 0.3%; 0.6%; 1.2%) can induce protective immunity in terms of macroscopic and microscopic images of the cecum. The most potential formalin concentration for patenting *Eimeria tenella* in induces protective immunity serum in terms of macroscopic and microscopic images of cecum is 1.2%. Macroscopically there is no damage to cecal lesions and no cecum wall thickening. Whereas microscopically shows the intestinal epithelium looks compact and there is no rupture in the cecum villi.

Ethical Clearance: The research process involves participants in the survey using a questionnaire that was accordant with the ethical research principle based on the regulation of research ethic committee. The present study was carried out in accordance with the research principles. This study implemented the basic principle ethics of respect, beneficence, non-maleficence, and justice.

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