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Research Article
Levamisole Hydrochloride as Immunostimulant Drug Synergies the Effect of *Eimeria tenella* Lab-made Vaccine: Experimental Trial

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Abstract

Background and Objective: *Eimeria tenella* is one of the most important parasitic pathogens threaten chickens particularly broilers worldwide. Levamisole hydrochloride is known to be an immunostimulant agent. The present study described the experimental evaluation using Levamisole hydrochloride in combination with a Lab-made vaccine against *E. tenella* infection in broilers. **Materials and Methods:** A lab-made vaccine of *E. tenella* Egyptian strain sonicated sporulated oocysts was prepared. Eighty broiler chickens (one day old) were used; the chicks were divided in to 4 groups. The 1st group (G1) kept as control negative (no vaccine, no challenge). The 2nd group (G2) vaccinated with inactivated Lab-made vaccine. The 3rd group (G3) immunized with inactivated Lab-made vaccine I/M as (G2) then Levamisole administrated orally. The 4th group (G4) kept as control positive. For testing the efficacy and comparison; OPG (oocyst per gram), serum Interleukin 4 (IL4) levels, Immunoglobulin A (IgA) levels in both serum and ceca, as well as histopathological changes in ceca of tested groups were evaluated. **Results:** The results of immunological parameters, parasitological OPG level and histological examination indicated vaccinated groups showed better immunological response than non-vaccinated group, more over vaccinated group that given Levamisole orally showed better response to challenged infection. **Conclusion:** Sonicated *E. tenella* oocysts when used as a vaccine gave good immune response against infection, moreover adding of Levamisole orally may enhance the effect of coccidial vaccine. Further studies on coccidiosis vaccination and its improvements are strongly recommended.

Key words: *Eimeria tenella*, sonicated oocysts, levamisole-HCl, immunostimulant, serum interleukin 4, OPG level

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Eimeria species are unicellular protozoa, infecting wide range of hosts including birds and the disease produced is called coccidiosis. Coccidiosis in poultry industry cause considerable economic losses in the poultry industry[1]. Traditionally, the disease is controlled with chemical feed additives that can inhibit the life cycle stages of the parasite[2]. Several dis advantages related to this strategy including withdrawal periods and development of drug resistance[3]. Vaccines have been used in the poultry industry for more than 50 years, primarily in broiler breeder and replacement layer flocks[4]. Parenteral inoculation of dead antigen is capable of stimulating circulating antibodies against coccidiosis antigen. Vaccination remains the most efficient means of preventing disease and reducing economic losses[5]. Researches dealing with development of effective vaccination against coccidiosis are going on.

Levamisole-hydrochloride (HCl) is a primary anthelmintic drug, with nematocidal properties. Nevertheless, it has been found that Levamisole has also immunostimulant proprieties. Levamisole was found to improve development of immunity to coccidiosis, levamisole given at a dose of 0.25 mg kg^{-1} b.wt., by intraperitoneal route 3 days prior to the coccidiosis vaccine resulted in the greatest potentiation of the immune response, which confirms the ability of Levamisole to potentiate vaccination responses in chickens[6]. So, the current work aimed to test the effect of combining Levamisole-HCl with lab-made vaccine of *E. tenella* and challenge birds with infection.

MATERIALS AND METHODS

Ethical consideration: The experiment was done in Animal Health Research Institute, Tanta Laboratory, Egypt from 1st October-7th November, 2018. All procedures were carried out in accordance to national laws and regulations for the handling of animals to avoid harms and minimize their pain.

Birds and management: One day-old, broiler chicks of the "Avian 48 strain" were purchased from a Fat Hens hatchery. Upon arrival, the chicks were divided, housed in clean, disinfected cages and raised according to the Animal Care Institute recommendations.

Lab-made vaccine preparation: Field strain isolate of *E. tenella* was collected from the cecum of broiler chickens died due to natural outbreak of cecal coccidiosis. Cecal contents were thieved, washed and centrifuged at 2000 rpm for 5 min. A clear *E. tenella* oocysts pellet was concentrated by using saturated salt solution and centrifugation at 4000 rpm for 10 min. The upper third of solution in the tube was collected by rubber pipette. Then washed and kept in sufficient amount of 2.5% potassium dichromate to avoid over growth with fungi and bacteria at 28°C for sporulation[7]. Then after that, Pot. dichromate was removed by washing the pellets 4 times with distilled water followed by centrifugation at 4000 rpm for 10 min. The harvested oocysts were counted using McMaster chamber and aliquoted in Phosphate Buffer Saline (PBS) and stored at 4°C until use.

About 4000 mL^{-1} of *E. tenella* sporulated oocysts stirred continuously on a magnetic stirrer for 12 h at 4-8°C, followed by ultra sonication at 60 kHz for 5 shots of 1 min each with an interval of 30 sec in jacketed vessel at 4-8°C (Ultrasonics Homogenizer 4710 series Cole Parmer instrument Co. Chicago, Illinois 60648), centrifugation at 10.000×g for 30 min at 4°C. Supernatant of sonicated suspension was treated with 0.3% formalin (33% formaldehyde) for 96 h at 37°C and kept in refrigerator until use[7-9].

Levamisole-hydrochloride (10%): The ADWIA Company, 10th of Ramadan city, Egypt. It was given orally at the dose of 0.5 g kg^{-1} b.wt., of chicken.

Experimental design: A total of 80, one day old chicks were divided into four groups (20 chicks each):

Group 1: Non challenged, non-vaccinated and kept as control negative group

Group 2: Immunized with inactivated sonicated vaccine (tested) 0.2 mL by intra-muscular route (I/M) at first day of age and poster dose at 21 day[9] and challenged by 50,000 sporulated oocysts of *Eimeria tenella* at 28 days[10]

Group 3: Immunized and treated group, they immunized with inactivated sonicated vaccine (tested vaccine) I/M as G2 then treated with levamisole orally at 19th-21st day of age (before challenge by 7 days)[11], then challenged by 50,000 sporulated oocysts of *Eimeria tenella* at 28 day

Group 4: Infected by 50,000 sporulated oocysts of *Eimeria tenella* at day 28 of age, but not vaccinated and kept as control positive

Evaluation parameters: The experiment was finished at day 7 after challenge (35 days of age).

This experiment continues for 35 days during which all groups were observed daily and clinical signs were recorded. Blood was collected from wing vein at 16, 21, 28 and 35 day of age. Sera were utilized to evaluate immunity, humeral immunity was estimated by measuring Immunoglobulin A (IgA) level in serum and cecum (ELISA Kit Catalog No: MBS2507630 96T) (MyBiosource), while cellular immunity was performed by Interleukin 4 (IL4) level in serum (ELISA Kit Catalog Number. MBS704068, MyBiosource).

Specimens from cecum were collected and processed for histopathological examination according to method[12] at 5th week (end of experiment). Birds dropping (in each group separately) were collected at zero day of challenge and at days 6 and 7 post-challenge for counting Oocysts per Gram (OPG) using McMaster chamber according to Lillehoj and Ruff[13].

Statistical analysis: Data are represented as mean±SE (standard error). One way Analysis of Variance (ANOVA)-Tukey test was used to compare the mean values of the various groups at significance level of $p \leq 0.05$. Statistical analysis was performed using the method cited in Petrie and Watson[14] and computerized using SPSS 20 statistics software package for Microsoft Windows.

RESULTS

Results of immunological parameters: Regarding the parameters evaluated in the current work; the IL4 level in serum of chicken during the experimental period is shown in Table 1. IgA level in sera is shown in Table 2. While IgA level in ceca is shown in Table 3. The OPG of all groups during experiment is shown in Table 4. The mean significance level observed in various groups are $p \leq 0.05$.

Histopathological examination: Briefly; in G1, the intestine of chicken showed normal intestinal glands with its normal small basophilic nuclei (Fig. 1). G2 chickens intestines showed different stages of coccidial cysts in the intestinal glands from schizonts to macrogametes to microgametes associated with infiltration of inflammatory cells between glands (Fig. 2). While, in G3 chickens intestines showed regressed number of coccidial cysts and only little number of schizonts (Fig. 3). Lastly, G4 chickens

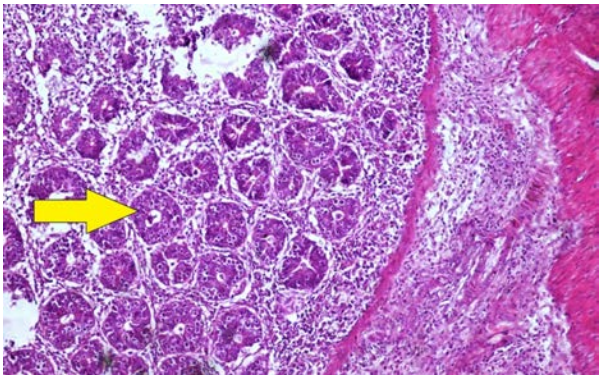

Fig. 1: Histopathological photograph of chickens ceca of G1 (no-vaccine, no-challenge) is showing normal intestinal glands with its normal small basophilic nuclei (Stain H and E, ×100)

Table 1: Interleukin 4 (IL4) level in sera of chickens of different groups as detected during the experiment

	Experimental groups			
Age of chicks/days	1	2	3	4
16	4.90±.32^{a}	5.60±0.36^{a}	5.60±0.36^{a}	4.90±.32^{a}
21	6.03±0.4^{a}	7.56±1.66^{a}	6.43±0.743^{a}	6.03±0.4^{a}
28	6.16±0.61^{b}	13.56±2.57^{a}	9.40±2.14^{ab}	6.17±0.62^{b}
35	5.86±0.35^{c}	9.60±0.98^{ab}	8.66±0.70^{b}	11.70±1.7^{a}

$^{a-b}$Values bearing similar superscript between rows do not differ at $p \leq 0.05$

Table 2: Levels of Immunoglobulin A (IgA) detected in chicken's sera of different groups during the experiment

	Experimental groups			
Age of chicks/days	1	2	3	4
16	0.56±0.031^{a}	0.56±0.082^{a}	0.56±0.082^{a}	0.56±0.030^{a}
21	0.59±0.039^{a}	0.93±0.333^{a}	0.80±0.147^{a}	0.59±0.039^{a}
28	0.56±0.051^{b}	1.86±0.226^{a}	0.80±0.246^{b}	0.56±0.051^{b}
35	0.21±0.055^{c}	0.85±0.042^{a}	0.60±0.120^{ab}	0.46±0.085^{bc}

$^{a-b}$Values bearing similar superscript between rows do not differ at $p \leq 0.05$

Table 3: Levels of Immunoglobulin A (IgA) detected in chicken's ceca of different groups during the experiment

	Experimental groups			
Age of chicks/days	1	2	3	4
16	1.10±0.088^{a}	1.16±0.187^{a}	1.16±0.187^{a}	1.10±0.088^{a}
21	1.32±0.108^{b}	3.84±1.166^{a}	3.66±1.30^{a}	1.32±0.108^{b}
28	1.51±0.189^{b}	3.83±0.706^{a}	2.76±1.00^{ab}	1.51±0.189^{b}
35	1.105±0.08^{c}	2.89±0.390^{a}	2.10±0.35^{ab}	1.88±0.11^{bc}

$^{a-b}$Values bearing similar superscript between rows do not differ at $p \leq 0.05$

Table 4: Oocysts per gram (×10^{4}) detected in chicken's droppings of different groups during the experiment

	Experimental groups			
Age of chicks/ days	1	2	3	4
28	0.15±0.278^{a}	0.28±0.177^{a}	0.19±0.204^{a}	0.21±0.292^{a}
34	0.15±0.262^{b}	0.80±0.107^{b}	0.28±0.248^{b}	4.58±0.319^{a}
35	0.15±0.355^{c}	1.29±0.086^{b}	1.41±0.142^{bc}	21.00±0.575^{a}

$^{a-b}$Values bearing similar superscript between rows do not differ at $p \leq 0.05$

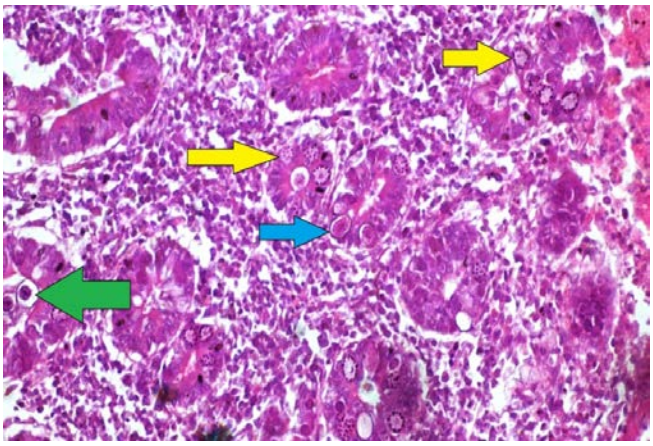

Fig. 2: Histopathological photograph of chickens ceca of G2 (vaccinated alone, challenged), showing different stages of *Eimeria tenella* developmental stages in the cecal tissue from schizonts (yellow arrow) to macrogametes (blue arrow) to microgametes (green arrow) associated with infiltration of inflammatory cells between glands (Stain H and E, ×400)

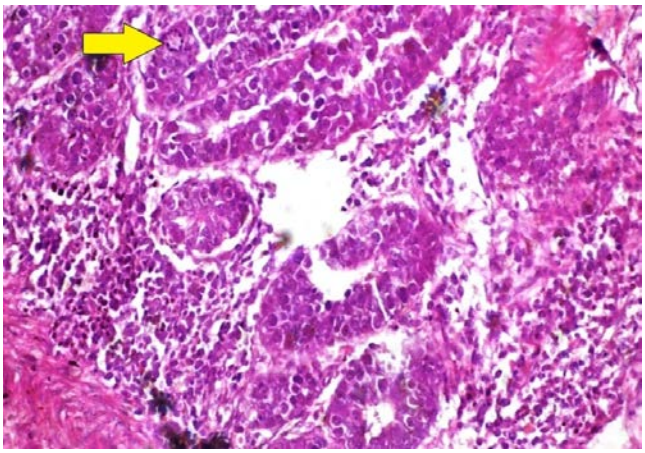

Fig. 3: Histopathological photograph of chickens ceca of G3 (vaccinated plus Levamisole orally, challenged) showing regressed number of coccidial cysts, only few number of schizonts (yellow arrow) (Stain H and E, ×200)

ceca showed highly necropsied degenerated epithelium of intestine of chicken most of degenerated enclosed intestinal glands contain different stages of coccidial developmental stages, highly proliferated intestinal epithelium and infiltration of inflammatory cells between glands (Fig. 4).

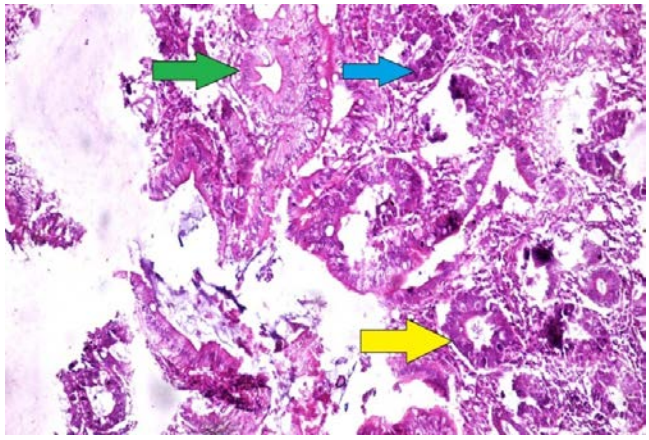

Fig. 4: Histopathological photograph of chickens ceca of G4 (no-vaccine, infected) showing highly necropsied degenerated epithelium of intestine of chicken (yellow arrow) most of degenerated enclosed intestinal glands contain different stages of coccidial cysts (blue arrow) highly proliferated intestinal epithelium plus infiltration of inflammatory cells between glands (Stain H and E, ×100)

DISCUSSION

Avian coccidiosis is the major parasitic disease of poultry particularly broilers all over the world. Vaccinations against coccidiosis on a commercial scale have shown to some extend a limited effectiveness and current disease control remains largely dependent on routine use of anti-coccidial drugs in most countries[3]. The immune response against cecal coccidiosis could be established by immunization with *E. tenella* specific sporulated oocyst (sporozoites) and merozoites as well as other stages in birds less than 4 weeks old[15]. In the present study, an attempt was made to evaluate the use of Lab-made sonicated sporulated oocysts of local isolated strain of *E. tenella* vaccine by intramuscular route of administration. Also to evaluate the use of drug (Levamisole-HCl) as immunostimulant agent with Lab-made vaccine as effective way to combat *E. tenella* infection.

Determination of immune response (both humeral and cellular) was performed. It is well-known that, IL4 regulate macrophage functions in chickens[16]. In the present study, the vaccinated groups (G2 and G3) showed high IL4 values than control groups before challenge. These results agreed with Martinez *et al.*[17] who reported that elevated levels of IL4 are typically associated with tissue injury and TH2 diseases caused by infection with parasites or extracellular pathogens.

At day 28 level of IL4 was decreased in G3 than G2 due to administration of Levamisole which stimulate immunity by indirect way (i.e., non-specifically). These results agreed with Oladele *et al.*[18] who revealed that oral administration of Levamisole resulted in enhancement of cellular immunity due to its ability to prompt initiation of cellular reaction, in addition, the anti-inflammatory effect of Levamisole was evident in both sensitized and un-sensitized subgroups.

At day 7, post-challenge (at day 35) the positive control group (G4) showed the highest level of IL4 this may be due to sever tissue injury of chicken intestine which reported by Hong *et al.*[19] who stated that increased expression of IL4 has been reported in infections caused by avian coccidian parasites. On the other hand, G2 and G3 showed low level due to challenge after vaccination which agreed with Hong *et al.*[19] who detected decreased level of IL4 after primary or secondary infection with *E. tenella* infection. However, these results disagreed with Hoan *et al.*[20] who detected that IL4 levels were higher in vaccinated more than un-vaccinated chickens. Moreover, Tian *et al.*[21] reported that 2 week old chickens were intramuscularly vaccinated with 100 μg (0.5 μg μL^{-1}) of recombinant plasmids, they found that IL4 were significantly increased by the vaccinations.

On the other hand, humoral immune response represented by immunoglobulins including IgG, IgA and IgM play a vital role in the binding of foreign antigens and the presence of these antibody molecules on a microbial or parasitic surface can cause clumping (agglutination) and activation of the complement system[22,23]. The role of humoral immune response in coccidiosis is to some extent can be described as vague and limited[8,20]. In this study there was significance increase in level of serum IgA in vaccinated groups than control ones at day 7 post challenge. These results agreed with results of others[22,24] that maximum numbers of IgG, IgA and IgM were found in chickens vaccinated with sonicated gametocyte formalin inactivated vaccine. Moreover, Del Cacho *et al.*[25] revealed higher numbers of Ag-reactive, IgA -producing cells in jejunal Peyer's patches and spleen were harvested at 6 days post-infection following *in vitro* stimulation with the sporozoite s-antigens compared with the non-immunized/non-infected and non-immunized infected controls. However, our results disagreed with Davis *et al.*[26] who stated that serum IgA levels were similar in control non-infected non-immunized group and immunized group (two sequential infections with an interval of 2 weeks throughout the experiment). There was a significant increase in cecal IgA in vaccinated group G2 than G3 which received Levamisole at day 28 of age, but there was a significant decrease in cecum IgA in control negative groups. These results agreed with Parker *et al.*[27] who suggested that cecal *E. tenella* infection, even if manifested by high lesion score, does not have to decrease the cecal production of IgA. Also, agreed with Davis *et al.*[26] who found that the only immunoglobulin class consistently detected in cecal and intestinal contents was IgA. So, it is clear that further studies are required more to targeting the exact pathway and immuno-response against avian coccidiosis.

The I/M immunized groups showed a great protective immunity against *E. tenella* infection documented by significantly decreased oocysts shedding, compared with non-immunized group. At day 28 (i.e., day zero before challenge), there was no significance differences between groups, at day 34 (i.e., day 6 after challenge) G4 was significantly increased in OPG than other groups. At day 7 (i.e., day 35) G2 and G3 showed significance decrease in OPG and G2 significantly differ than G3 in OPG. Akhtar *et al.*[7] found that the supernatant of sonicated sporulated oocysts vaccine gives the lowest OPG post challenge as compared with the sediment of the same vaccine. Onaga *et al.*[11] stated that Levamisole when administrated with *E. tenella* oocysts as a vaccine for protection against challenged infection; it gave higher protection than those with no Levamisole administration. The results disagreed with Kadhim and Hussien[28] who detected in their study, that no statistical differences were detected in oocysts shedding until *E. tenella* challenge at day 28 after challenge test. But, current results shows the efficacy of Lab-made vaccine alone and in-combination with Levamisole in combating challenge as shown clearly by reduction in OPG output in vaccinated groups. Another evidence of its efficacy is also shown in histopathological findings.

As, histopathological examination of chickens ceca of G2 that received the Lab-made vaccine alone showed limited different stages of *E. tenalla* cyst in the intestinal glands, associated with mild infiltration of inflammatory cells between glands These results were similar to those reported by Kadhim[9] who revealed that *E. tenella* induced-lesions were mild in the immunized group characterized principally by inflammatory cells influxes into the sub-mucosa and thickened mucosa and sub mucosal layer. While, chickens ceca of G3 that received the vaccine plus Levamisole showed regressed number of *E. tenella* developmental stages, only few numbers of schizonts. These results were similar to those reported by Giambrone and Klesius[6] that Levamisole given

prior to the coccidiosis vaccine resulted in the greatest potentiation of the immune response. Ceca of challenged, non-vaccinated control group (G4) showed highly necropsied degenerated epithelium, most of degenerated enclosed intestinal glands contain different stages of *E. tenella*, highly proliferated intestinal epithelium and heavy infiltration of inflammatory cells between glands. These results agreed with Zulpo *et al.*[29].

CONCLUSION

This study comes in-line with other studies on avian coccidiosis vaccination trials and shows that vaccinated chickens against *E. tenella* gave good immunological response to combat infection than non-vaccinated chickens. Moreover, vaccinated group and orally supplied with Levamisole-HCl show some improvement than non-vaccinated supplied group, further studies on coccidiosis vaccination and its improvements are strongly recommended.

SIGNIFICANCE STATEMENT

Coccidiosis is one of the most important parasitic diseases, affect chickens worldwide. Vaccination against coccidiosis is a new challenge with fast steps to achieve the best results. Also, immunostimulant agents that promote more effective immune response are on focus in avian industry to help in combating diseases.

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