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RESEARCH ARTICLE
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Assessment of humoral immunity to *Eimeria tenella* sporozoites in chickens by ELISA

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Abstract

Aim : To assess the humoral immune response of *Eimeria tenella* sporozoites in broiler chickens by a developed enzyme linked immunosorbent assay (ELISA) and the efficacy in terms of bodyweight, lesion score and oocysts excretion in immunized broilers.

Materials and Methods: Purified live *E. tenella* sporozoites were administered subcutaneously in neck region of broiler chickens in the early life (first week) at different concentrations. The potency of the sporozoite vaccine as assessed by IgG levels and the performance in immunized broilers as assessed by body weight, lesion score and oocysts excretion in faeces after challenge with 10, 000 live *E. tenella* oocysts at 49 days of age were evaluated.

Results: The chickens of group (T4) immunized with 20 µg of antigen on day 6 showed an increase in IgG levels (0.161±0.004) two weeks post immunization (PI) peaking (0.399± 0.016) at 5 weeks PI. The mean weekly weight gain (g) after challenge, at 56 days of age was high in T4 (148±4.751 g) with a low mean lesion score (2.5±0.22) and mean oocyst output (x10^3 oocytes per gram (OPG) in faeces (100.3± 45.72) when compared to unimmunised infected controls.

Conclusion: An early but partial immune response against caecal coccidiosis could be achieved by immunization with *E. tenella* specific sporozoites in chickens of less than a week old. Moreover, the performance of immunized chickens as indicated by weight gain, lesion score and oocyst output was found to be superior to the unimmunized infected controls.

Keywords: bodyweight, broiler chickens, *Eimeria tenella* sporozoites, ELISA, IgG levels, lesion score.

Introduction

Globally, India ranks fifth in chicken meat production with the value of poultry exports being around INR 4410 million during 2008-09 and the demand for chicken meat in next 2-3 decades is expected to increase many-folds very rapidly [1]. However, efficiency of broiler production today seems to be hampered by many diseases. Coccidiosis is one of those diseases that poses a considerable economic loss to broiler industry, with *Eimeria tenella* being one of the most prevalent species in India causing caecal or bloody type of coccidiosis associated with reduced growth rate, poor performance and mortality in broiler chickens [2]. The disease is responsible for 6–10% of all mortality in broilers [3] and in India, 95.61% (INR 1089.17 million) of the total economic loss occurs due to the disease.

Prophylactic medication has been successfully used to control coccidiosis, however there is an increasing emergence of drug-resistance in commercial industry [4]. Numerous vaccination strategies have been attempted to manage avian coccidiosis [5]. However, the live oocyst oral vaccines currently being used have limited utility in the broiler industry in view of reduced weight gain and recycling of oocysts in the litter resulting in coccidiosis outbreaks [6, 7] thus necessitating alternate immunological approaches to control the disease.

Hence, this study presents the assessment of potency of the sporozoite vaccine of *E. tenella* administered by parenteral route in broiler chickens to block the transmission via litter, by a developed enzyme linked immunosorbent assay (ELISA) and the efficacy in terms of bodyweight, lesion score and oocysts excretion in immunized broilers.

Materials and Methods

Ethical approval: The project proposal of the Ph.D. research programme to conduct this study was duly approved by Institutional Animal Ethics Committee of Veterinary College and Research Institute, Namakkal.

Experimental design: In this experimental trial, five groups of day old Cobb 400 broiler chicks were used (n=15 per group) and purified sporozoite antigen was administered subcutaneously @ 0.1 ml per bird in the neck region, to groups T1 to T4 and T5 was kept as control. The groups, T1 and T2 were administered 10 and 20 µg of live sporozoite antigen, respectively on 2nd day of age, and T3 and T4 were administered 10 and 20

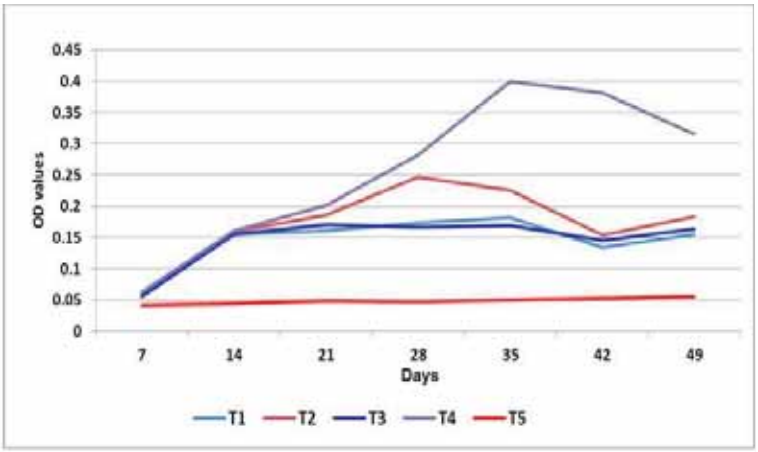

Figure-1: Mean ELISA IgG levels in chickens of experimental trial.

µg of live sporozoite antigen, respectively on 6th day of age. The immune response of coccidian sporozoites vaccinated experimental birds were assessed by ELISA developed as per the recommended method [8] with minor modifications.

Protein estimation: Sporozoites of *E. tenella* (3.6×10^5), extracted and purified by DE-52 column chromatography were washed twice in 0.1M carbonate buffer (pH 9.5), resuspended in 1 ml of carbonate buffer and shaked by vortexing for 5 min with 0.5 mm 50% glass beads. The optimal protein concentration was estimated by Lowrie method using a standard curve derived from bovine serum albumin and purified sporozoites of *E. tenella*.

Positive and negative sera: The *E. tenella* positive serum was obtained from the birds inoculated with 3000 *E. tenella* sporulated oocysts at 3 weeks of age followed by 5000 *E. tenella* sporulated oocysts at 6 weeks of age and after 21 days post inoculation, sera were collected. Negative serum samples were collected from unvaccinated control birds fed with coccidiostats.

ELISA procedure: The optimal dilution of coating antigen, sera and rabbit anti-chicken HRP conjugate were standardized by checker board titration and it was found that 1 µg /well of sporozoite antigen,1:50 dilution of sera and 1:5000 dilution of peroxidase conjugated rabbit anti-chicken IgG (GeNei, Bengaluru) gave optimum results. Each well of a 96 well flat bottom microtitre plate was coated with 1µg of sporozoite antigen in 0.05 M carbonate buffer (pH 9.6) and the plate was kept at 4°C overnight. The plate was washed with 0.05% PBS Tween-20 (PBST) three times and 100µl of 2% skim milk was added to each well to block unreacted sites in the wells. After incubation for 1 h at 37°C, plate was washed three times with PBST and dried by tapping against the filter paper. Test positive and negative control sera were used in 1:50 dilution (in PBST) in triplicate and the plate was incubated for 1 h at 37°C. After washing with PBST three times, rabbit anti-chicken IgG conjugated with peroxidase (Sigma ALdrich ,Bangalore, India) was used at 1: 5000 dilution in 100µl volume per well and each plate had one HRP control well. The plate was incubated for 1 h at 37°C, washed again with PBST three times and substrate tetramethyl benzidine solution was added to all the wells in 100µl volume, with a substrate control. The plate was incubated at room temperature for 30 min and 100µl stopping reagent (sulphuric acid) was added. The plate was then read in multiscan ELISA reader at 450nm and the optical density (OD) value twice and above the negative OD was taken as cut off value (positive).

The potency of the sporozoite vaccine as assessed by IgG levels and efficacy as assessed by bodyweight, lesion score and oocysts excretion in faeces after challenge with 10, 000 live *E. tenella* oocysts at 49 days of age were examined. The relative ratio of body weight gain was calculated from the following formula:

A = Mean weight gain / Mean weight at the end of experiment

B = Mean weight gain of negative control / Mean weight at the end of experiment

Relative ratio of weight gain = A / B x 100

Statistical analysis: Statistical analysis was performed by randomized block design (Snedecor and Cochran) and analysis of variance (ANOVA two-way analysis) with SPSS statistical software (version 10.01).

Results and Discussion

The ELISA could be an useful technique in the assessment of potency of sporozoite vaccine as it has a high sensitivity and specificity of 100% in the detection of antisporozoite antibodies of *Eimeria* species in vaccination programmes and monitoring of infectivity [9]. In the experimental trial, the antibody levels (IgG) of all vaccinated groups at day 7 were significantly higher ($P<0.05$) than that of control group (Figure-1). The antibody level of T4 at 49 days of age was higher with a mean ELISA OD value of 0.316±0.016 followed by T2 with a mean OD value of 0.183±0.004 when compared to T5 (0.056±0.003) and the antibody level was significantly different ($P<0.01$) from that of other vaccinated groups. However, at 49 days of age, the antibody levels of T1 (mean OD of 0.155±0.011) and T3 (mean OD of 0.164±0.007) administered with 10 µg of antigen to different age groups did not differ

Table-1: Mean O.D. values of ELISA in the *E. tenella* sporozoite immunized broiler chickens

Treatment	OD values (Mean ± SE)						
Groups	7th day	14th day	21st day	28th day	35th day	42nd day	49th day
T1	0.058^{bP} ±0.004	0.156^{BQ} ±0.002	0.161^{bQ} ±0.004	0.173^{BQ} ±0.007	0.182^{BQ} ±0.023	0.134^{BQ} ±0.015	0.155^{BQ} ±0.011
T2	0.058^{bP} ±0.003	0.159^{BQR} ±0.006	0.186^{bcR} ±0.003	0.247^{CS} ±0.011	0.225^{BCS} ±0.013	0.154^{BQ} ±0.007	0.183^{CR} ±0.004
T3	0.055^{bP} ±0.003	0.156^{BQR} ±0.006	0.171^{bcR} ±0.003	0.166^{BQR} ±0.002	0.169^{BQR} ±0.010	0.145^{BQ} ±0.006	0.164^{BCQR} ±0.007
T4	0.062^{bP} ±0.003	0.161^{BQ} ±0.004	0.201^{cR} ±0.016	0.282^{DS} ±0.005	0.399^{CDS} ±0.016	0.382^{CS} ±0.022	0.316^{DS} ±0.010
T5	0.042^{apq} ±0.002	0.044^{Apq} ±0.003	0.049^{Apq} ±0.004	0.047^{Apq} ±0.002	0.050^{Apq} ±0.005	0.053^{Apq} ±0.002	0.056^{Aq} ±0.003

T1 and T2 - administered 10 and 20 µg of live sporozoite antigen, respectively on 2nd day of age, and T3 and T4 were administered 10 and 20 µg of live sporozoite antigen, respectively on 6th day of age.*Row-wise mean (±SE) with different superscript (pqr…) differs significantly (P< 0.05), mean bearing 'upper case' superscript in a row is highly significant (P< 0.01). *Column -wise mean (±SE) with different superscript (abc…) differs significantly (P< 0.05), mean bearing 'upper case' superscript in a column is highly significant (P< 0.01).

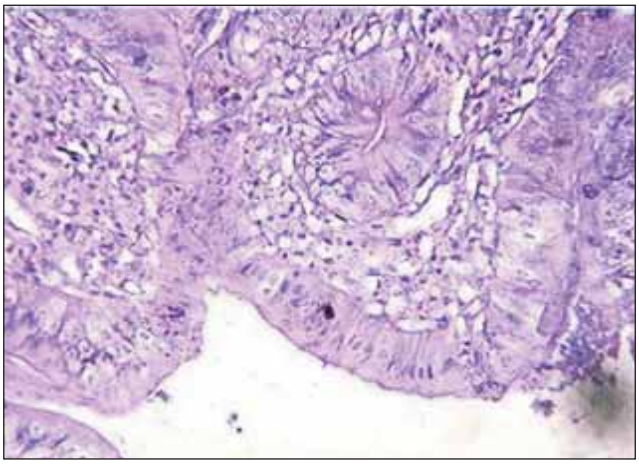

Figure-2: Group T4 showing stunting of villi in the caeca (H & E x400).

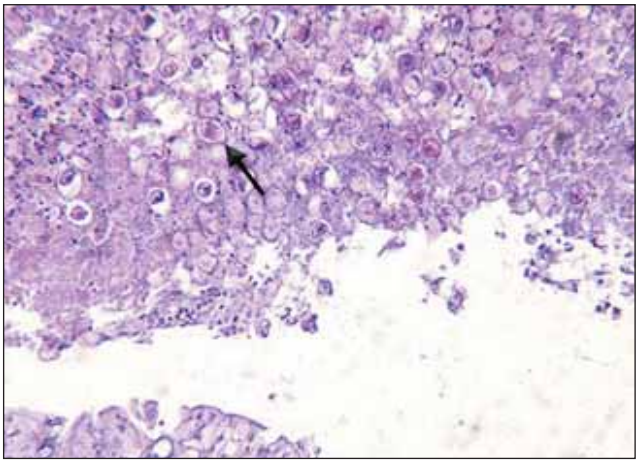

Figure-3: Group T5 showing complete destruction of intestinal villi with large number of megaschizonts (arrow) (H & E x400).

significantly (P>0.05). The antibody levels within all four vaccinated groups up to 49th day of age differed significantly (P<0.01) from the control group (Table-1).

However, the humoral immune response (IgG) observed was high in T4 and T2 which differed significantly (P<0.01) from T1 and T3. Thereafter, the antibody levels of T1, T2 , T3 and T4 rose on day 14 of age, peaked on 35, 28, 21 and 35 days of age, respectively and declined thereafter on 42, 35, 28, and 49 days of age, respectively. These findings are in accordance with Hasbullah *et al.* [10] who observed peak levels of *E. tenella* merozoite antibodies (0.5 to 1.0 OD) on 29 days post inoculation whereas, Constantinoiu *et al.* [8] observed a decline in *E. tenella* sporozoite antibody levels (<2.0) after 42-49 days of age. The peak antibody levels within the groups T4 and T2 were significantly high (P<0.01) when compared to antibody levels after day 7 and antibody levels in T1 and T3 but in groups T1 and T3, the peak antibody levels were not statistically significant (P>0.05) when compared to antibody levels after day 7. Hence, there was a positive dose-response relationship among the vaccinated groups and the group T4 showed higher ELISA IgG levels than the other groups. The findings of the present study are in agreement with Kiani and Farhang [11] who observed increased antibody levels with an OD value of 0.303±0.001 in comparison with the negative controls in chicks vaccinated with live sporozoites vaccine at a dose rate of 20 µg on day 6. However, previous report showed [12] elevated *E. tenella* sporozoite antibody levels with mean OD value of 0.72 to 0.94 in the same regimen in broiler chickens.

Histopathological examination: Histopathological examination (Figure-2 and 3) in chickens sacrificed at 5 days post challenge to assess the severity of infection revealed destruction of caecal epithelium in all groups, however, it was severe in group T3, moderate in group T1 and T2, and mild in T4 whereas the unimmunized infected T5 revealed severe destruction of villi and colonization of large number of megaschizonts [13].

Assessment of bodyweight gain: The mean weekly weight gain (g) after challenge, at 56 days of age was high in T4 followed by T2, T3 and T1. The weight gains of all groups were found to be superior to that of unimmunized infected T5, however, inferior to that of unimmunized uninfected T5. Hence, the relative ratio of weight gain in the vaccinated birds was higher (47.81%) in T4 than in T2, T3 and T1 whereas it was 26.2% in unimmunized infected T5. Similar findings were recorded previously [14] and [15]. However, these weight gains were not significantly high (P>0.05) when compared to unimmunized infected T5 but significantly (P<0.05) low when compared to unimmunized uninfected T5 (Table-2).

Assessment of lesion score: The mean lesion score

Table-2: Mean bodyweight gain in broiler chickens

Treatment	Body weight in grams (Mean ± SE)							
	Before challenge				After challenge			
	7th day	14th day	21st day	28th day	35th day	42nd day	49th day	56th day
T1	99.0^{a} ±3.501	180.75^{a} ±3.788	232^{a} ±12.465	314.7^{AB} ±13.149	503.67^{a} ±19.203	509^{AB} ±34.285	300.44^{A} ±16.404	78.89^{a} ±11.6
	Total gain - 2214.67^{a} ±40.426 Relative ratio of weight gain (%) - 26.92							
T2	94.75^{a} ±5.208	187.5^{a} ±6.213	223.25^{a} ±8.079	344.08^{B} ±14.787	458.44^{a} ±15.102	506.22^{AB} ±9.24	326.13^{A} ±14.381	142.5^{a} ±65.568
	Total gain - 2284.50^{a} ±74.407 Relative ratio of weight gain (%) - 47.2							
T3	90.00^{a} ±2.733	191.92^{a} ±3.702	244.5^{a} ±9.865	270.92^{A} ±10.39	502.44^{a} ±12.845	425.00^{A} ±21.016	420.00^{B} ±27.689	101.67^{a} ±63.278
	Total gain - 2241.67^{a} ±67.333 Relative ratio of weight gain (%) - 34.28							
T4	101.83^{a} ±1.934	183.25^{a} ±4.277	254.5^{a} ±12.381	310.25^{AB} ±11.963	449.00^{a} ±36.315	584.63^{B} ±9.809	306.63^{A} ±17.765	148.00^{a} ±4.751
	Total gain - 2342.50^{a} ±42.483 Relative ratio of weight gain (%) - 47.81							
T5 Uninfected								
	100.17^{a} ±6.478	176.33^{a} ±6.627	256.33^{a} ±11.546	363.33^{B} ±16.382	405.50^{a} ±11.337	595.33^{B} ±28.527	289.17^{A} ±29.956	332.67^{b} ±12.521
	Total gain - 2518.83^{b} ±38.704 Relative ratio of weight gain (%) - 100.0							
T5 Infected								
	97.5^{a} ±3.978	177.13^{a} ±6.134	263.50^{a} ±7.023	354.75^{B} ±14.529	412.00^{a} ±14.561	567.50^{B} ±9.839	331.00^{A} ±15.24	78.33^{a} ±15.24
	Total gain - 2278.20^{a} ±85.418 Relative ratio of weight gain (%) - 26.2							

*Row-wise mean (±SE) with different superscript (pqr…) differs significantly (P< 0.05), mean bearing 'upper case' superscript in a row is highly significant (P< 0.01). *Column- wise mean (±SE) with different superscript (abc…) differs significantly (P< 0.05), mean bearing 'upper case' superscript in a column is highly significant (P< 0.01).

was lower in T4 (2.5±0.22) than in T2 (2.83±0.17), T1 (3.33±0.33) and T3 (3.00±0.45) when compared to unimmunised infected T5 (3.33±0.33), indicating a moderate performance and partial protection by the sporozoites. However, there was no significant difference in the mean lesion score of all groups administered with the sporozoites (P>0.05). This finding is in agreement with previous reports [16, 17].

Assessment of oocyst output: The mean oocyst output in faeces (x10^{3} OPG) was low in the group T4 (100.3± 45.72) followed by T2 (128.3±53.25), T3 (139.2 ± 57.99) and T1 (145.3±55.09) in comparison with unimmunised infected T5 (205.5±75.73). The mean OPG between the vaccinated groups was not significant (P>0.05), however, the mean OPG of T4 was significantly lower (P<0.05) than that of unimmunised infected T5. This finding is in agreement with Ziomko *et al.* [16].

Conclusion

It is concluded that, immunization with *E. tenella* specific sporozoites by parenteral administration in broiler chickens of less than a week old resulted in an early but partial protective immune response (IgG) against caecal coccidiosis with a mean bodyweight gain, mean lesion score and faecal oocyst output superior to the unimmunized infected controls.

Authors' contributions

SS and KMP planned and designed the experiment. SS performed the experiment. TJH, PS and GS drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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