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**RESEARCH ARTICLE**

# Immunological Evaluation of Two Local Isolates of *Eimeria tenella* Gametocytes against Coccidiosis in Poultry

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**ABSTRACT**

Two local isolates of *Eimeria tenella* gametocytes against coccidiosis were immunologically evaluated in chickens. Cell mediated immune response was detected by modified splenic cell migration inhibition assay (MSCMIA) and data were expressed in terms of per cent migration index. No significant difference in per cent migration index was detected for the chickens immunized either with Vaccine-I (local isolate-I) or with Vaccine-II (local isolate-II); however per cent migration index was comparatively lower in chickens immunized with Vaccine-II as compared to Vaccine-I; indicating a somewhat higher cell mediated immune (CMI) response. Humoral immune response was monitored by ELISA in vaccinated and control chickens. Significantly elevated ($P<0.05$) antibody titer (IgG) in chickens immunized with Vaccine-II as compared to Vaccine-I was detected. Significantly higher protection (67%) in chickens immunized with Vaccine-II followed by vaccine-I (49%) was recorded. Further, oocyst count was significantly lower ($P<0.05$) in chickens immunized with Vaccine-II as compared to those immunized with Vaccine-I. It was concluded that vaccinal strain (Vaccine-II) contained additional protein of high molecular weight (49.23 kDa) in its gametocytes provided cross protection and can be used to prepare commercial vaccine against coccidiosis in poultry.

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## INTRODUCTION

Coccidiosis remains one of the major menaces for poultry industry throughout the world (Hafez, 2011). The disease is traditionally controlled by the use of chemoprophylactic measures including anti-coccidials in feed that inhibit the developmental stages of *Eimeria* (Calnek *et al*., 1997). No doubt, the continuous use of anti-coccidials has proved to be quite helpful in controlling the disease in commercial poultry production systems (Allen and Fetterer, 2002). But there could be several shortcomings related to this strategy. These include cost, variable withdrawal times, emergence of drugs resistant strains (Dalloul and Lillehoj, 2005) and the possible constraints by the regulatory authorities on medication of feed in animals (McEvoy, 2001) that are used for human consumption. Thus, vaccination of birds seems to be a safer and promising tool to control avian coccidiosis.

Vaccines available in market such as Immucox, Coccivac, Livacox, Paracox and Viracox (Chapman *et al*., 2002; Williams, 2002) contained live virulent or attenuated lines of Eimerian parasites (Bedrnik *et al*., 1995) except CoxAbic that contained native gametocytes (Wallach, 2002). Many of the commercial vaccines are being used in different parts of the world with variable results due to the fact that coccidial strains from different geographical regions exhibit different antigenicity (Martin *et al*., 1997). Laboratory and field trials conducted previously by using gametocyte vaccines from local isolate of *Eimeria tenella* were found to be effective in mixed spp. coccidial infection in chickens (Hafeez *et al*., 2006; Anwar *et al*., 2008). Present study reports the

immunological evaluation of the two isolates of *Eimeria* against coccidiosis in chickens.

## MATERIALS AND METHODS

**Parasite Stages:** Sporulated oocysts of 2 local isolates of *E. tenella* maintained in Immunoparasitology Laboratory, Department of Parasitology, University of Agriculture, Faisalabad-Pakistan were processed for excystation to get sporozoites following the methodology of Speer *et al*. (1973). The sporozoites were washed twice with phosphate buffered saline (PBS) and their concentration was adjusted to $1.8x10^3$- $2x10^3$ per 100 µL and stored at 4°C until use.

Nine days old chicken embryos (2 batches of 200 each) were purchased from a hatchery at Faisalabad, Pakistan and maintained in an incubator at 39°C with 70% humidity. The viability of the embryos was confirmed by candling. On day $12^{th}$ of age, 100 µl from sporozoites suspensions of each isolate, containing penicillin (2000 IU) and streptomycin (0.05mg), was inoculated into the embryos through chorio-allantoic membranes (2 batches). The embryos were maintained for 5-7 days at 39°C temperatures and 70% relative humidity. On day $5^{th}$-$7^{th}$ post-inoculation, gametocytes were harvested and washed according to the methodology described by Hafeez *et al.* (2006).

**Vaccine formulation**: Gametocyte antigens of both the isolates were prepared separately following the methodology described previously (Hafeez *et al*., 2006). In brief, gametocytes were homogenized by sonication (1x3 minutes; Nissei, Model US 330, Japan) at 4-8°C. Supernatant obtained after centrifugation (9500 g/15 minutes) was used as antigen and their protein contents measured by using the methodology of Bradford (1976) were adjusted to 500µg/0.2ml with PBS. Each vaccine dose contained 500 µg proteins. Both the vaccines were designated as Vaccine-I and Vaccine-II, respectively.

**Experiment:** A total of 180 chicks (Hubbard) procured from a hatchery at Faisalabad, Pakistan were reared under standard management conditions (specific pathogen free environment) at Experimental shed, Department of Parasitology, University of Agriculture, Faisalabad-Pakistan. On day $5^{th}$ of age, chickens were separated into three equal groups A, B and C (n= 60 in each group). On day $10^{th}$ of age, chickens in groups A and B were administered with experimental gametocyte vaccines (Vaccine-I and Vaccine- II, respectively) with the help of an oral gavage; whereas group C served as control was administered with PBS. Chickens were confirmed for their coccidia free status by coprological examination.

On day $14^{th}$ post vaccination (PV), blood and spleens were collected from fifteen chickens in each group. On day $15^{th}$ PV, the remaining chickens of groups A and B (n=45) were administered with the booster dose of the respective vaccine and those in group C with PBS. On day $21^{st}$ PV (primary dose), again blood and spleen samples were collected from fifteen chickens in each group for detection of cellular and humoral immune responses.

Spleens were used immediately just after collection to demonstrate the cell mediated immunity by splenic cell migration inhibition assay (Akhtar *et al*., 1999). On the other hand, to detect the humoral immunity, serum was collected from blood samples and subjected to ELISA (Hafeez *et al.*, 2006).

On day $21^{st}$, the remaining chicken in each group (n=30) were orally challenged with $6.5 X 10^4$ sporulated oocysts of mixed species of *Eimeria* [local isolates; mainly *E. maxima*, *E. tenella* and *E. acervulina* (1:1:1)]. Fecal examination was performed on daily basis up to day $10^{th}$ post challenge and oocysts per gram of droppings (OPG) were calculated by McMaster counting technique. Clinical symptoms and mortality during the experiment in each group was monitored up to 10 days post challenge. Per cent protection and mortality was also calculated in each group post challenge. Dead and survived chickens in all the groups were monitored for lesion scoring upto day $10^{th}$ post challenge (Johnson and Reid, 1970).

## RESULTS AND DISCUSSION

Numerous commercial vaccines are being used in many parts of the world to control coccidiosis in chickens. A generalized limitation regarding their use in broiler and high roster birds is marked reduction in weight gain and feed conversion ratios (FCR) as compared to birds receiving some anticoccidial agents in feed as a prophylactic measures (Shapiro, 2001; Crouch *et al*., 2003). In addition, there is a threat of the introduction of unwanted *Eimeria (E.)* species into the environment due to variant antigenicity of coccidial strains in different constituencies of the world (Martin *et al*., 1997). Further, some strains of *Eimeria* showed immunological variation (Lee, 1993) and such strains could impair the efficacy of vaccines. In such circumstances, vaccines prepared from local isolates of parasite have been reported to give promising results (Hafeez *et al*., 2006; Anwar *et al.*, 2008). This study reports the immunological evaluation of the two isolates of *Eimeria* in terms of cellular, humoral and challenge responses.

In the present study, CMI in vaccinated and control chicken, was demonstrated by MSCMIA; and the results were expressed in terms of per cent migration index. In vaccinated chickens, sensitized splenic T-cells become re-sensitized with the test antigen *in vitro* and therefore the migration of T-cells inhibited more with antigen. It can be speculated that these sensitized splenic $T_{dth}$ cells release lymphokines including interleukins (IL-1, IL-2, IL-4) and other cytokines such as migration inhibition factor, which inhibit the migration (Oldham, 2009). This reflects that the test antigen used for vaccination activated the T-cells to initiate the cellular immune response. In majority of the parasitic infections, protection can be obtained experimentally in normal chickens by the transfer of splenocytes, especially the T-cells, from the immunized birds. This is due to the fact that T-cells secrete IL-10, which hinders the release and activity of the INF-γ needed to activate macrophages and rule out the parasitic infection, possibly by the enhanced production of cytotoxic T-cells and NK cells (Roitt *et al*., 1998). There was no significant difference ($p>0.05$) in per cent migration index in the chickens immunized either with Vaccine-I or with Vaccine-II; however it was comparatively lower in chickens immunized with Vaccine-II as compared to Vaccine-I (Table 1); indicated

to somewhat higher CMI response. Moreover, significant difference in CMI response in terms of per cent migration index was recorded on days 14th and 21st post vaccination, irrespective of the vaccine administered. Further, significantly lower (P<0.05) migration index in vaccinated groups as compared to control; indicated the higher CMI response in vaccinated chickens as compared to the control.

**Table 1:** Migration distance with and without antigen on days 14th and 21st post vaccination

| Days Post Vacci-nation | Vaccine | Migration Distance (µm±SE) | | Migration Index (%) |
|---|---|---|---|---|
| | | With Antigen | Without Antigen | |
| 14 | Vaccine-I | 24.9±1.18[b] | 32.5±1.36[b] | 76 |
| | Vaccine-II | 26.7±1.60[b] | 36.2±1.07[b] | 73 |
| | Control | 36.0±1.27[a] | 38.3±1.33[a] | 93 |
| 21 | Vaccine-I | 49.8±2.25[b] | 62.0±1.87[b] | 80 |
| | Vaccine-II | 46.2±1.27[b] | 59.0±2.24[b] | 78 |
| | Control | 70.1±2.87[a] | 74.8±2.80[a] | 93 |

Means having different letters in a column are statistically significant (P<0.05).

Humoral response in vaccinated and control chickens were monitored through ELISA and results were demonstrated in terms of optical density (OD) values. ELISA results showed a significantly higher (P<0.05) antibody titer (IgG) in chickens immunized with Vaccine-II as compared to Vaccine-I. Further, irrespective of vaccine, the antibody titer was significantly higher (P<0.05) in vaccinated as compared to control chickens (Table 2). The role of antibodies in providing protective immunity against coccidiosis is controversial. It has been assumed that antibodies probably develop in parallel with the cellular immunity (Gilbert *et al*., 1988). In the preset study, vaccines contained gametocyte proteins that are present in the wall forming bodies of the macro-gametes (WFBs) and play a pivotal role in the development of *Eimerian* parasites (Mello *et al*., 2006).

**Table 2:** ELISA results on days 14th and 21st Post Vaccination (PV)

| Vaccine | 14th day pv | | 21st day PV | |
|---|---|---|---|---|
| | OD (Mean) | SD (±) | OD (Mean) | SD (±) |
| Vaccine-I | 0.189[aB] | 0.041 | 0.279[aA] | 0.049 |
| Vaccine-II | 0.302[bB] | 0.037 | 0.424[bA] | 0.044 |
| Control | 0.048[cB] | 0.017 | 0.057[cA] | 0.019 |

Means having different small letters in a column are statistically significant (P<0.05); Mean having different capital letters in rows represents significant difference (P<0.05) within the days.

Results of the challenge experiment on 21 day PV revealed significantly higher protection (67 %) in chickens immunized with Vaccine-II followed by vaccine-I (49%). Significantly lower (P<0.05) oocyst count in group of chickens immunized with Vaccine-II as compared to Vaccine-I was recorded (Figure-I). This reduced oocyst count could be caused by immunity produced that may reduce the development of disease through inhibition of the growth, development and/or fertilization of gametes (Anwar *et al*., 2008). During the course of experiment, vaccinated chickens of both the groups were active and alert with normal feed and water intake contrary to chickens in control group that were dull, depressed with ruffled feathers and decreased feed and water intake.

Survived and dead chickens (during experiment after challenge) in experimental and control groups were monitored for lesion scoring to assess the biological protection. In control group, majority of the chickens (66-70%) demonstrated severe lesions (3.0-4.0) while 25-27 per cent chickens showed moderate lesions (2.0). On the other hand, chickens immunized with Vaccine-I and Vaccine-II developed 74 and 69 per cent mild to moderated lesions (1.0-2.0), respectively.

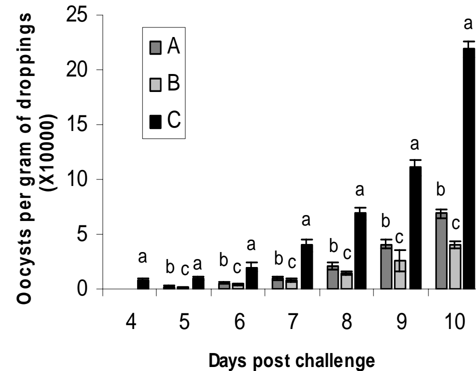


**Fig. 1:** Oocysts per gram of droppings post challenge with *Eimeria* species (local isolates). A = Vaccine-I; B= Vaccine-II; C= Non-vaccinated infected Control. Bars sharing different letters on a particular day are statistically significant (P<0.05).

Gametocytes of the two isolates used in Vaccine-I and Vaccine-II subject to SDS-PAGE contained soluble proteins had molecular weights 27.65, 24.90, 22.75, 13.20 kDa and 49.23, 27.65, 24.90, 22.75, 13.20 kDa, respectively (details not given). Chickens immunized with vaccine-II contained additional gametocyte protein (49.23 kDa) produced high level of antibodies that somehow prevent the formation of dityrosine bonds and their associated protein matrices (Wallach, 2002; Mello *et al*., 2006). It can be speculated that these antibodies might hamper with the oocyst's wall formation (Belli *et al*., 2009). Further, the maternal antibodies produced due to these gametocyte proteins have the ability to protect hatchlings against homologous infection with *Eimeria* species (Hafeez *et al*., 2007; Belli *et al*., 2009).

On the whole, these results demonstrated that vaccinal isolates (Vaccine-II) contained additional gametocyte protein of 49.23 kDa provided maximum protection to chickens in challenge experiment. It can be assumed that the significantly higher cross species protection in our previous (Hafeez *et al*., 2006; Ayaz *et al*., 2008) and present studies may be due to this additional molecular weight gametocyte protein (49.23 kDa), which may contain the major portion of conserved epitopes common to the gametocytes of other *Eimerian species* (Wallach *et al*., 1995). In some other studies, all most similar size gametocyte proteins (56 kDa) purified from *E. maxima* protected the chickens in challenge experiment (Wallach *et al*., 1989). From these results it was concluded that vaccinal strain contained additional gametocyte proteins of high molecular weight provided cross protection against mixed species of genus *Eimeria* (local isolates) and has practical implications to prepare commercial vaccine against avian coccidiosis. Further studies are underway on the role of high molecular weight gametocyte proteins to be used in vaccine preparation against coccidiosis.

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