



Comparative Efficacies of *Eimeria tenella* Apical Membrane Antigen-1 (*Etama*-1) DNA and Live Attenuated Oocysts Vaccines against Experimental Infection with Field Isolate of *Eimeria tenella* in Broiler Chickens

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SUMMARY

This study evaluated the efficacy of vaccination of broiler chickens against coccidiosis using *Eimeria tenella* (*EtAMA*-1) DNA vaccine in comparison to vaccination using live polyvalent oocyst vaccine (Livacox[®]). Forty-day-old broiler chicks were randomly assigned into 4 groups; group 1 birds were unvaccinated uninfected control, group 2 were unvaccinated infected, group 3 were vaccinated with 0.1ml containing 100µg *EtAMA*-1 vaccine plasmid on the thigh muscle at days 14 and a booster at days 21 of age, while at 10 days old, group 4 birds were administered 0.2ml of Livacox[®]. At 28 days of age, all chickens except group 1 were inoculated with 1×10^4 sporulated oocysts of the *E. tenella* and evaluated for weight gain (WG), oocysts production (OP), lesion scoring (LS) and anti coccidial index (ACI). Higher WG (116±43.89 g) were recorded in group 4 compared to group 3 (110±29.15 g), although, statistically insignificant but was significant ($p < 0.05$) when compared with group 2 (40±12.25 g). OP was significantly ($p < 0.05$) higher in group 2 ($1.3 \times 10^5 \pm 11.00$) compared to groups 3 and 4 ($0.58 \times 10^5 \pm 18.00$ and $0.44 \times 10^5 \pm 42.36$) respectively. Both vaccines significantly ($p < 0.05$) reduce the effects of *E. tenella* on LS (1.4 ± 0.6 and 1.0 ± 0.62) for groups 3 and 4 respectively compared to the unvaccinated group 2 (4.0 ± 0.00). Higher ACI was observed in groups 3 and 4 (177 and 180) respectively compared to group 2 (170). Conclusively, *EtAMA*-1 DNA vaccination ameliorated the effects of *E. tenella* infection in broiler chickens comparable to Livacox[®].

Key words: *Eimeria tenella*, Vaccination, Apical Membrane Antigen-1 DNA, Livacox[®], Broiler chickens.

INTRODUCTION

Coccidiosis in chickens is caused by apicomplexan parasites belonging to the genus *Eimeria* of which at least seven species are incriminated in the cause of the disease (Conway and Elizabeth, 2007; McDougald and Fitz-Coy, 2008). Currently, vaccination against coccidiosis using live oocysts vaccine is the most effective way of control and prevention of the disease (Williams, 2006). However, production limitation which includes requirement for *in vivo* passage has limited the production capacity and elevated the cost of live vaccines (Williams, 2006).

Apical membrane antigen-1 (AMA-1) is a micronemal protein of apicomplexan parasites that appears to be essential during the entry into the host's cells by the invasive stages of the parasites (Santos *et al.*, 2011; Lamarque *et al.*, 2011; Curtidor *et al.*, 2011; Jiang *et al.*, 2012). Preliminary reports have established that recombinant protein vaccines derived from AMA-1 of *E. brunetti* (EbrAMA-1), *E. maxima* (EmaAMA-1) and *E. tenella* (EtAMA-1) have conferred significant immune protection against experimental challenge by the respective *Eimeria* parasites (Blake *et al.*, 2011; Jiang *et al.*, 2012). There is an established knowledge of the existence of antigenic variation among strains of *Eimeria* parasites from different geographical locations of the world (Fitz-Coy, 1992; Martin *et al.*, 1997; Danforth, 1998). This has temper confidence on whether the recombinant AMA-1 vaccine derived from foreign strains of these parasites will protect against challenge by local isolates in Nigeria.

However, recent report on low level of coding polymorphism for AMA1 across Nigeria and spatially diverse *E. tenella* samples, and the cross-protection detected between genetically distinct isolates, supports the cogency of this antigen to confer universal protection against field strains of *Eimeria* species (Jatau, 2014; Blake *et al.*, 2015). There is paucity of information describing the

efficacy of the homologue of EtAMA-1 DNA vaccine derived from the reference laboratory strain of *E. tenella* against regional field strain in Nigeria. The present study evaluated the comparative efficacies of *E. tenella* EtAMA-1DNA and live oocyst vaccines against experimental challenge with field isolate of *E. tenella* in terms of weight gain, oocyst production and lesion score in broiler chickens.

MATERIALS AND METHOD

Study area

This research was carried out in the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, Nigeria.

Experimental chickens

Forty-day-old broiler chicks of Mashal breed were purchased from Elim poultry, Kwangila, Zaria and transported in clean brooder cages to an intensive care, animal research unit of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria. They were brooded in a brooder pen for two weeks and fed broiler starter, a product of vital[®], a coccidiostats free commercial feed and clean well water. The feed and water were provided *ad libitum*. Strict biosecurity measures and proper hygiene were ensured during the experimental period to prevent natural infection of the birds. The faeces of the chicks were screened daily to ascertain they are free from *Eimeria* infection prior to vaccination and experimental challenge with the *Eimeria* parasite.

Eimeria tenella isolate

Crude *Eimeria* isolate was obtained from the Parasitology Unit of National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State Nigeria.

From the crude isolate, *E. tenella* was isolated via *in vivo* propagation and isolation as follows: Three 2-weeks old coccidia free broiler chicks were each orally inoculated with 10, 000 sporulated oocysts from the crude *Eimeria* isolate. Progeny oocysts were recovered from caecal tissue and contents of the birds collected during postmortem, 7 days' post infection were sporulated and purified as described by Shirley (1995).

Sources of the *Eimeria* vaccines

The DNA vaccine used in this study was a DNA vaccine construct of *E. tenella* AMA-1 developed from a laboratory strain (Houghton strain) in the Department of Pathobiology and Population Science of the Royal Veterinary College, UK. Live attenuated vaccine (Livacox[®]) a standard polyvalent vaccine comprising *E. tenella*, *E. maxima* and *E. acervulina*. The vaccine was produced by Biopharm and Veterinary drugs, Czech Republic but distributed by PierroDEX, a reputable Veterinary product distributor in Jos, Plateau State Nigeria.

DNA Vaccine constructs extraction from transformed *E. coli* colonies

The AMA-1 construct was extracted from the transformed *E. coli* according to the methods described by Robert (2008) as follows: *E. coli* containing the construct was suspended on brain heart infusion broth, incubated at 37°C for 24 hours. It was then inoculated on eosin methylene blue agar incorporated with ampicillin supplement for 37°C for 24 hours. This was followed by morphological identification and biochemical test for confirmation of the colonies (Mohammed *et al.* 2014), Leuria Bertani (LB) medium was used to grow the *E. coli* for plasmid extraction. Plasmid extraction was performed according to the method of Alkaline Lysis as described by He (2011) and

the resultant plasmids were quantified using a Nanodrop spectrophotometer (Thermo. USA), and stored in the freezer until when needed.

Experimental grouping

At 10 days of age, the chickens were weighed and randomly distributed into 4 groups of 10 chicks each as follows:

Chickens in group 1 (unvaccinated unchallenged control group), chickens in group 2 (unvaccinated challenged positive control group), chickens in group 3 (*Et*AMA-1 DNA vaccinated challenged group) and group 4 (live oocyst vaccinated challenged group).

Immunization of experimental birds

Chickens in groups 1 and 2 were given 0.1 ml sterile PBS as a placebo via intramuscular injection at day 14 and 21 of age using insulin syringe. Chickens in group 3 were vaccinated with 100µg *Et*AMA-1 vaccine plasmid in 0.1 ml phosphate buffered saline (PBS) in the thigh via intramuscular injection at day 14 and a booster dose at 21 days of age. Birds in group 4 were administered single dose of 0.2 ml Livacox[®] vaccine orally in drinking water at day 10 of age following the manufacturers recommendations.

Challenge of experimental birds

At 28 days of age, all chickens except group 1 were inoculated orally with 1×10^4 sporulated oocysts of the *E. tenella* isolate.

Determination of survival rate

Survival rate was estimated by the number of surviving chickens divided by the number of initial chickens per group at the time of challenge (Shah *et al.*, 2011).

Determination of body weight gain

Body weight gain was determined by subtracting the body weight at the time of challenge in each group from the body weight of the chickens at the end of the experiments day 7 post challenged (Shah *et al.*, 2011).

Oocyst production and decrease ratio

Seven days after challenge, five chickens from each group were re-weighed and humanly sacrificed for postmortem analysis and lesion scorings described by Johnson and Reid (1970). 2 g of caecal content for each sacrificed bird in each group were collected separately by opening the caecum longitudinally placed on a clean Petri dish, oocyst per gram of content (OPG) was determined using the McMaster counting technique (Long *et al.*, 1976). Oocyst decrease ratio was calculated as the (mean number of oocysts from (positive control chickens) – (vaccinated chickens)/ (positive control chickens) × 100% (Shah *et al.*, 2011).

Lesion score

Caecal lesion score of the chickens from each group were determined according to the method of (Johnson and Reid, 1970). Briefly each score 0–4 covers a range of gross lesions which are species specific. Generally, a score of 0 = gross lesions absent; 1 = a few scattered petechiae are found on the caecal wall, caecal contents may appear normal; 2 = Lesions more numerous with noticeable blood in the caecal contents, caecal wall is somewhat thickened, 3 = Large amounts of blood or caecal cores present, caecal walls greatly thickened, little if any fecal contents in the caeca and 4 = Caecal wall greatly distended with blood or large caseous cores, fecal debris lacking or included in cores.

Anti-coccidial index (ACI)

Anti-coccidial index (ACI) is a criterion for assessing the protective effect of a medicine or vaccine against coccidiosis was calculated as follows: (Relative weight gain (%) + % Survival rate) –

(Mean lesion value + Mean oocyst value). Anti coccidial index usually ranges from zero to 200

Graham *et al.* (1978). Anti coccidial index numbers above 175 are indicative of good efficacy.

Data analyses

Data collected such as lesion scores were expressed as median while body weight and oocyst count were expressed as mean ± SD and statistically analyzed by one-way analysis of variance (ANOVA), complimented by post hoc analysis using the Tukey's HSD test. Statistical analyses were processed by the SPSS 20.0 Data Editor software (SPSS Inc., Chicago, IL). The differences between groups were considered to be significant at $p < 0.05$.

Ethical statement: The research was approved by the Ahmadu Bello University Committee on

Animal Use and Care with approval number ABUCAUC/2017/015.

RESULTS

Effects of vaccination on body weight gain

Group 2 chickens presents the lowest weight gain (40.00±12.25 g) compared to (110±29.15 g and 116±43.89 g of group 3 and group 4) respectively. The differences in weight gain between infected non vaccinated and vaccinated groups were statistically significant ($P < 0.05$). Although, the differences in weight gain between DNA and live

oocyst vaccinated groups were not statistically significant ($P > 0.05$), however, there is a significant difference in weight gain between all experimentally challenged groups compared to the negative control group 1 (Table I).

Oocysts production and oocysts decrease ratio.

Mean oocyst per gram of caecal content were significantly higher in the unvaccinated infected group with mean OPG values of $1.3 \times 10^5 \pm 11.00$ when compared to the vaccinated groups with $0.58 \times 10^5 \pm 18.00$ and $0.44 \times 10^5 \pm 42.36$ for DNA and live oocyst vaccines respectively. Similarly, the differences in mean OPG between DNA and live oocyst vaccinated groups were statistically significant ($P < 0.05$) (Table II).

Effects of vaccination on lesions scores of broilers chickens experimentally challenged with *E. tenella*.

The caeca lesion score of birds in group 2 was significantly ($P < 0.05$) higher with mean lesion

score of 4.0 ± 0.0 compared to groups 3 and 4 with mean lesion scores of 1.4 ± 0.6 and 1.0 ± 0.62 , respectively. However, the differences in mean lesion score between DNA and live oocyst vaccinated groups were not significant ($P > 0.05$) (Table III).

Anti-coccidial Index

There was lower anti coccidial index for unvaccinated challenged group (170) compared to 177 and 180 for DNA and live oocyst vaccinated groups respectively (Table IV). Although, vaccinated groups had ACI higher than the unvaccinated infected groups, live oocyst vaccine produced higher ACI compared to the DNA vaccine.

TABLE I: Mean (\pm SEM) body weights (g) of broiler chickens vaccinated with DNA and Live oocyst vaccines against experimental challenge with field isolate of *E. tenella*.

Group	Pre Infection	Post Infection	Net weight increase	Relative weight gain
1	$930 \pm 39.91_a$	$1190 \pm 40.0_a$	$260 \pm 40.62_a$	100 %
2	$870 \pm 25.49_a$	$910 \pm 24.49_b$	$40 \pm 12.25_b$	15.4 %
3	$900 \pm 35.36_a$	$1010 \pm 36.66_b$	$110 \pm 29.15_c$	42.3 %
4	$904 \pm 16.31_a$	$1020 \pm 46.37_b$	$116 \pm 43.89_c$	45.2 %

Means in the same column with different superscript are significantly different ($P < 0.05$).

TABLE II: Mean (\pm SEM) lesion score of broiler chicks vaccinated with DNA and live oocyst vaccines against field isolate of *E. tenella*

Group	Mean lesion score (\pm SEM)
1	0.00 \pm 0.00 ^a
2	4 \pm 0.00 ^b
3	1.4 \pm 0.86 ^c
4	1.0 \pm 0.62 ^c

Means in the same column with different superscript are significantly different (P<0.05).

TABLE III: Mean (\pm SEM) of oocyst value of broiler chickens vaccinated with DNA and live oocyst vaccines and challenged with field isolate of *E. tenella*

Group	Oocysts value	Relative oocysts production	Percentage oocysts count
2	1.34 $\times 10^5 \pm 11.00^a$	100.0	100
3	0.58 $\times 10^5 \pm 18.00^d$	45.0	45
4	0.44 $\times 10^5 \pm 42.36^f$	34.0	34

*Group 1 omitted because it was the negative control, no oocyst was produced.

Table IV: Anticoccidial index (ACI) of *Et AMA-I* DNA and live oocyst vaccines in broiler 327 chickens challenged with field isolate of *E. tenella*.

Group	Survival rate(%)	Relative weight gain (%)	Mean lesion score	Mean oocyst score ($\times 10^4$)	ACI
1	100	100	0	0	200
2	100	76	4.0	1.34	170
3	100	84	1.4	5.8	177
4	100	85	1.0	4.4	180

DISCUSSION

In the present study, no mortality was recorded throughout the course of the experiment which could be attributed to the strain of *E. tenella* used in this study. Similar observation was made by Song *et al.* (2009), that no chicken died following challenge with a single *Eimeria* species. The observed increase in body weight gain in birds vaccinated with DNA and live oocyst vaccines compared to unvaccinated group of birds could be attributed to effects of vaccination in ameliorating the damages caused by the parasites. Live oocyst vaccination produced higher weight gain that is close to negative control compared to DNA vaccine, this is as a result of proliferation of parasite within the host producing antigens that stimulates strong immune response (Reid *et al.*, 2014). On the other hand, the decreased weight gain (weight loss) observed in the unvaccinated infected birds could be attributed to the changes in the intestinal villi as a result of damage caused by the invasion and replication of the parasite, thus impairing digestion and absorption of nutrients (Shaban, 2012). Similar observations were recorded by Mersha *et al.* (2009) and Jatau *et al.*, (2014) where they attributed decrease weight in caecal coccidiosis to the shared effects of muscle breakdown and anorexia that take place throughout the acute stage of *Eimeria* infection. Song *et al.* (2009) had similarly shown the ability of recombinant DNA vaccine in improving the weight gain of vaccinated birds.

Mild lesions observed in the vaccinated infected groups shows that the vaccines may have exacted some protective effects in reducing the severity of the lesions in the vaccinated groups. Subramanian *et al.* (2008) recorded a marginal reduction in the lesion score in vaccinated birds while evaluating potential of *E. tenella* micronemal protein as a candidate for sub-unit vaccine for poultry

against coccidiosis. The presence of higher numbers of oocyst in unvaccinated group further buttress the facts that vaccination has limiting effects on the proliferation of the parasites manifested as the reduced oocysts output in the vaccinated groups. Although both DNA and the live oocyst vaccines were able to reduce lesion scores and mean oocyst output in the vaccinated birds, live vaccine exacted the highest percentage reduction. This could be due to the fact that in the live vaccine, the parasite proliferates in the host expressing between 6,000 and 9,000 proteins during the course of its life cycle, this provides a complex portfolio of antigens that stimulates stronger immune protective response (Reid *et al.*, 2014). AMA-1 is just one of such antigens and therefore is expected to produce lesser protective immune response than the one produced by the live oocyst vaccine.

Anti-coccidial index (ACI) is a synthetic criterion for assessing the protective effect of a medicine or vaccine. The fact that broiler chickens vaccinated with the DNA vaccine had relatively close values of ACI to those vaccinated with the live oocyst vaccine strongly suggest that the former could be an effective and cheaper alternative to the later.

CONCLUSION

This study demonstrated that vaccination with DNA and lives oocysts vaccines ameliorated the effects of *E. tenella* on body weight gain, caecal lesions score and reduction in the multiplication and proliferation of the *E. tenella* parasites evident by percentage reduction in oocyst shed and an overall effect on ACI. While this experimental monovalent AMA-1 vaccination was able to induce some level of immune protection against wild strain of *E. tenella*, further studies should be conducted using combinations of two or more antigens (IMP1, MICs, RONS) into a single formulation with the hope of achieving broader

Eimeria species protection that is comparable to live oocysts vaccine.

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REFERENCES

- BLAKE, D. P., CLARK, E.L., MACDONALD, S.E., THENMOZHI, V., KUNDU, K., GARG, R., JATAU, I.D., AYOADE, S., KAWAHARA, F., MOFTAH, A., REID, A.M., ADEBAMBO, A.O., ZAPATA, R.A., ANI S.R., RAO, S., THANGARAJ, K., BANERJEE, P.S., DHINAKAR-RAJ, G., RAMAN, M. and TOMLEY, F.M. (2015). Population, genetic and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development, *Proceedings of the National Academy of Sciences, USA* .www.pnas.org/cgi/doi/10.1073/pnas.
- BLAKE, D.P., BILLINGTON, K. J., COPESTAKE, S. L., OAKES, R. D., QUAIL, M. A., WAN, K. L., SHIRLY, M.W. and SMITH, A. L., (2011). Genetic mapping identifies novel highly protective antigens for an apicomplexan parasite. *PLoS Pathology*, 7:5-9.
- CONWAY, DONALD, P. and ELIZABETH, M. M. (2007). Poultry Coccidiosis Diagnostic and Testing Procedures. Blackwell Publishing, Iowa, USA;1-168.
- CURTIDOR, H., PATINO, L., AREVALO-PINZO, G. and PATARROYO, M. (2011). Identification of the *Plasmodium falciparum* rhoptry neck proteins5 (PfRON5). *Gene*, 474: 22-28.
- DANFORTH, H. D. (1998). Use of live oocyst vaccine in the control of avian coccidiosis. Experimental studies and field trials. *International Journal for Parasitology*, 28:10991100.
- FITZ-COY, S. H. (1992). Antigenic variation among stains of *Eimeria maxima* and *E. tenella* of chicken. *Avian Diseases*, 36: 40-43.
- GRAHAM, W. DONALD, EDWARD, F. ROGERS and WALLACE, T. A. (1978). 5 Deazariboflavin and its derivatives. *Avian Diseases*, 25: 18-20.
- HE, F. (2011). Plasmid DNA Extraction from *E. coli* using alkaline lysis method. *Biology Protocol*, 101:25-30.
- JATAU, I. D. (2014). Prevalence, identification and genetic diversity of *Eimeria* species in commercial poultry farms in Zaria and environs, Kaduna State, Nigeria. *PhD thesis*, Ahmadu Bello University, Zaria, Nigeria.

- JATAU, I. D., ODIKA, A. N., THLAMA, M., TALBA, A. M., BISSALA, M. and MUSA, I. W. (2014). Response of two breeds of broiler chicks to experimental infection with low dose of *Eimeria tenella* sporulated oocysts. *Turkish Journal of Veterinary and Animal Sciences*. 38:398-404.
- JIANG, L., LIN, J., HAN, H., ZHAO, Q., ZHU, S. and HUANG, B. (2012). Identification and characterization of *Eimeria tenella* apical membrane antigen -1(AMA1). *PLOS ONE*, 7(7): 41115.
- JOHNSON, J. and REID, W. M. (1970). Lesion scoring techniques in battery and floor pen experiments with chickens. *Experimental Parasitology*, 28: 30-36.
- LAMARQUE, M., BESTEIRO, S., PAPOIN, J., ROQUES, M., VULLIEZ-LE NORMAND, B., MORLON, GUYOT, J., DUBREMETZ, J. F., FAUQUENOY, S., TOMAVO, S., FABER, B. W., KNOCKEN, C. H., THOMAS, A. W., BOULANGER, M. J., BENTLEY, G. A. and LEBRUM, M. (2011). The RON2- AMA1 interaction is a critical step in moving junction dependent invasion by apicomplexan parasites. *PLoS Pathogen*, 7(2): 34-37.
- LONG, P. L., JOYNER, L. P. MILLARD, B. J. and NORTON, C. C. (1976). A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. 6(3): 201-217.
- MARTIN, A. G., DANFORTH, H. D., BARTA, J. R. and FERNANDO, M. A. (1997). Analysis of immunological cross-protection and sensitivities to anticoccidial drugs among geographical and temporal strains of *Eimeria maxima*. *International Journal for Parasitology*, 27: 527-533.
- MCDUGALD, L.R. and FITZ-COY, S. H. (2008). Protozoal infection. *Diseases of Poultry*; 23: 1068–1080.
- MERSHA, C., TAMIRU, N. and SAMUEL, B. T. (2009). Occurrence of concurrent infectious diseases in broiler chickens is a threat to commercial poultry farms in central Ethiopia. *Tropical Animal Health and Production*, 41: 1309–1317.
- REID, J.S., TREECK, M. and BOOTHROYD, J. C. (2014). Focus on the ringleader: the role of AMA1 in apicomplexan invasion and replication. *Trends Parasitology*, 27(9): 410–420.
- ROBERT CRUICKSHANK (2008). Volume 2 of Medical Microbiology: A Guide to the Laboratory Diagnosis and Control of Infection, pp 58-65.
- SANTOS, J. M., FERGUSON, D. J. P., BLACKMAN, M. J. and SOLDATI-FAVRE, D. (2011). Intramembrane cleavage of AMA1 triggers *Toxoplasma* to switch from an invasive to a replicative mode. *Science*, 331(6016): 473–477.
- SHABAN, K.S. (2012). Exacerbating effect of Newcastle disease virus (NDV) infection on subclinical caecal coccidiosis in broilers vaccinated against Newcastle disease virus (NDV). *Researcher*, 4: 55–59.

- SHAH, A., MUHAMMED, A., XU, L., YAN, R., SONG, X. and LI X. (2011). Construction of DNA vaccines encoding *Eimeria acervulina* cSZ-2 with chicken IL-2 and IFN- γ and their induced immunity against poultry Coccidiosis. *Research Veterinary Science*, 90: 72-77.
- SHIRLEY, M.W. (1995). Maintenance in animal hosts: *Eimeria* species and strains of chickens. In: *Biotechnology guidelines on techniques in coccidiosis research*, Eckert, J., Braun, R., Shirley, M.W., and Coudert, P. (Editors). European Commission, Luxembourg. Pp 1-24.
- SONG, X., XU, L., YAN, R., HUANG, X., SHAH, M. A. A. and LI, X. (2009). The optimal immunization procedure of DNA vaccine pcDNATA4- IL-2 of *Eimeria tenella*, its cross immunity to *Eimeria necatrix* and *Eimeria acervulina*. *Veterinary Parasitology*, 159: 30-36.
- WILLIAMS, R. B. (2006). Tracing the emergence of drug-resistance in coccidian (*Eimeria* spp.) of commercial broiler flock medicated with decoquinate for the first time in the United Kingdom. *Veterinary Parasitology*, 135(1): 1-1