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RESEARCH ARTICLE

Transgenic *Eimeria mitis* Expressing Chicken IL-4 Mediated Decrease in Pathogenicity Compared to Wild Type *Eimeria mitis* Strains in Broiler Chickens

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The present study was designed to establish transgenic *Eimeria mitis* (*tE. mitis*) expressing chicken interleukin-4 (ChIL-4) and further exploitation of *tE. mitis* as a novel vaccine vector to control coccidiosis. For this purpose we compared the clinico-hematological alterations induced by wild type and *tE. mitis* carrying ChIL-4 in broilers birds. Wild type *E. mitis* (*wE. mitis*) oocysts were nucleofected with double cassette vector and transgenic oocysts were propagated for stable transfection. Stability of the transfected *E. mitis* was observed by fluorescent microscopy and PCR analysis revealed the appearance of 411bp band suggestive of presence of IL-4 into the genome of *tE. mitis*. Birds inoculated with transgenic EmiChIL-4 excreted fewer oocysts and fecundity potential of *tE. mitis* turned out to be significantly lower than that of the wild type parent strain. Moderate clinical and behavioral signs such as restlessness, diarrhea, emaciation, decreased feed intake and loss of body weight was observed in birds inoculated with *wE. mitis* oocysts. The erythrocyte counts, Hb conc, PCV and MCHC decreased in birds infected with *wE. mitis* as compared to those infected with *tE. mitis*. The total leukocyte count including heterophils, eosinophil and basophils increased while lymphocytes were decreased in birds inoculated with *wE. mitis* as compared to birds in control group and those infected with *tE.mitis.* It was concluded that *tE. mitis* incorporated with ChIL-4 functions well as an adjuvant, conferring reduced fertility and compromised pathogenicity of *tE. mitis*. This discrimination, may be applied as a potential vaccine vector to control coccidiosis as well as pathogens from which antigens are expressed by *tE. mitis*.

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INTRODUCTION

The poultry production has grown in leaps and bounds from a mere backyard to a mammoth industry throughout the world, so has its problems. Avian coccidiosis takes lead in reducing the poultry industry profit by causing morbidity and mortality in commercial poultry in developing as well as developed countries of the world (Shirley, 2004; Qin *et al*., 2014; Zaman *et al*., 2015). Different pathogenic species of Eimeria (Eucoccidiorida: Eimeriidae), a protozoan, induce coccidiosis in poultry (Suo *et al.*, 2006; Alemayehu *et al*., 2013). The development of poultry coccidiosis depends on various factors including but not limited to: a) age of animals, b) number of oocysts in the host, c) species of Eimeria, d) involvement of concurrent infections and e) husbandry practices (Daugschies and Najdrowsk, 2005; Gussem, 2007). Various control strategies such as prophylaxis with anti-coccidial drugs, selection of disease recalcitrant chicken strains, live and attenuated vaccines

and augmentation of innate immunity are actively applied to control coccidiosis. Each of these methods has advantages and some disadvantages in controlling coccidiosis (Qin *et al.,* 2014). Estimation of pathogenicity, virulence of *Eimeria* spp. and inclusion of different parasites as the vaccinal strains are imperative factors for devising effective control strategies against avian coccidiosis.

Genetic manipulation is potent tool to probe the biology of bacteria, viruses and parasites for devising novel strategies to control infections induced by these microbes (Hoelscher *et al*., 2006). Stable and transient transfection systems are well established in different parasites like: *Plasmodium falciparum* and *Toxoplasma gondii* (Goonewardene *et al*., 1993; Sibley *et al*., 1994). Recently, these strategies have also been successfully developed in *Eimeria* spp. (Yan *et al*., 2009) which resulted in the development of transgenic *Eimeria* spp. through genetic complementation. This encouraged the use of *Eimeria* spp. as novel vaccine delivery vehicles with enhanced yellow fluorescent proteins incorporated as model antigens.

Eimeria mitis, one of the seven species of Eimeria causing chicken coccidiosis, is considered as less pathogenic. Previously, genetic complementation of other Eimeria species like *E. maxima*, *E. tenella*, *E. acervulina* and *E. praecox* have been successfully accomplished; nevertheless, there is room for the *E. mitis* as a potential Eimerian pathogen to be manipulated as a vaccine vector to develop fresh immunity conferring vaccines. Biological characteristics of *Eimeria* spp. such as: prepatent period, intestinal localization and severity of necropsy lesions may also have huge implications in the probable potential of Eimeria spp. as vaccine vectors (Qin *et al.,* 2014). Cytokines have remarkable effects on the immunogenicity of antigens and play a pivotal role in shaping the nature of immune responses. Interleukin-4 (IL-4), for instance, importantly activates peripheral CD4+ T cells and stimulates antibody production against gastrointestinal parasites (Shirley *et al*., 2005). Therefore, keeping in view the above mentioned factors, we have presented *E. mitis* as an alternative vaccine vector to express the foreign elements. After determining the stable transfection, a prerequisite for the development of vaccine vector, we have transfected a double-cassette vector expressing enhanced yellow fluorescent protein (EYFP) and chicken IL-4. To estimate the level of aberrations in clinico-haematological indexes, and measure the oocyst output potential of the *tE. mitis* we have compared the parental *wE. mitis* with the *tE. mitis* strain.

MATERIALS AND METHODS

Ethical code: The study got approval from the Provincial Administrative Committee of Laboratory Animals, Beijing, China and study was in accordance with the China Agricultural University, Institution of Animal Care and Use Committee guidelines.

Parasites and experimental chickens: Coccidia-free, 2-5 week-old Arbor Acre (AA) broiler chickens were used for passaging and maintaining the *wE. mitis* (Zhuozhou strain). For this purpose, one-day-old broiler chickens were procured from a local hatchery. The birds were fed on pathogen free feed. Feed and water was provided *ad libitum* during the whole experimental study. Standard measures were adopted regarding collection, purification and sporulation of *E. mitis* (Zhuozhou strain) oocysts as reported earlier (Huang *et al*., 2011; Yin *et al*., 2011).

Construction of Plasmid and transfection of *E. mitis:* Based on the double expression-cassette plasmid pMic-EYFP/ACT-RFP, we constructed double expression-cassette plasmid His4-DHFR-EYFP/ACT-chIL-4-ACT (pHDEAAssIL4A) as previously (Yin *et al*., 2011). The red fluorescent protein RFP and EYFP genes were replaced with chicken IL-4 and reporter genes, dihydrofolate reductase DHFR and EYFP, respectively (Fig.1). The constructed plasmid was linearized by using restriction enzyme SnaBI that released the two expression cassettes from the backbone of plasmid (Fig. 1). Freshly extracted sporozoites of *E. mitis* were purified with the help of diethylaminoethy l-52 cellulose column and re-suspended in a cytomix buffer containing 5 mM glutathione and 2 mM ATP (Huang *et al*., 2011). The Amaxa Nucleofector system and restriction enzyme mediated (REMI) strategy was followed for transfection of wild type *E. mitis* sporozoites. For the *in vivo* studies two million nucleofected sporozoites were inoculated into the seven day-old chicken via the cloacal route according to the previously described protocol (Yin *et al*., 2011). Collection and purification of oocysts from fecal material was done at 8 days post-inoculation. After the provision of standard sporulation time and temperature, oocysts were observed using fluorescence microscope (Leica, Germany). The transfected oocysts exhibiting DHFR-EYFP were observed and sorted from the progeny population by the MoFlo Cell Sorter (Dako-Cytomation, Fort Collins, CO) on the single cell mode and further inoculated into coccidia-free chicks for the propagation of next generation (Yan *et al*., 2009). This process of sorting and propagation was carried out 10 times to procure enough number of transgenic oocysts for the second phase of experiment. Finally the purified transgenic oocysts were re-suspended in 2.5% $K_2Cr_2O_7$ and stored at 4°C for further studies.

Genomic DNA analysis of *tE. mitis*: For validation of stable transfection of *E. mitis*, DNA was extracted from sporulated *tE. mitis* by phenol/chloroform extraction method as described (Liu *et al.,* 2013). Briefly, the DNA pellet was dissolved in deionized water with 0.4 mg/ml RNase H to acquire high-quality genomic DNA. PCR verification was performed with primer pairs for chIL-4 (5′-ATGAGCTCCTCACTGCCC -3′ and 5′-TCACTTATTTTTAGCTAGTTGGTGG-3′) to detect the presence of ChIL-4 in *tE. mitis.*

Comparative prolific test of wild type and *tE. mitis:* Broiler birds, separated into two groups, (A and B) aged 2 weeks, were orally inoculated with 3000 *wE. mitis* and *tE. mitis* oocysts, respectively. In the first trial fecal samples from were collected every 24 hours between day 5 and day 11 post inoculation. In the second trial fecal samples were collected at the day five, seven, nine, and day eleven respectively. Finally, the oocyst dejection in droppings was determined using McMaster egg counting techniques (Lee *et al*., 2007; Huang *et al*., 2011).

Clinico-hematological analysis: In order to observe the clinical effects and changes in hematological index we compared *tE.mitis* admission into broiler birds as compared to *wE.mitis*, clinical and hematological alterations were observed meticulously as described earlier by (Hussain *et al*., 2014).

RESULTS

Transfection efficiency*:* The transfection efficiency of transgenic strain of *E. mitis* at different passages in droppings of broiler birds revealed the gradual increase in the appearance of sporulated oocysts population expressing EYFP. Transfection efficiency of transgenic strain of *E. mitis* at different passages in terms of sporulated oocysts in droppings of broiler birds were 0.26, 7.0, 37.0, 55.0, 63.0 and 71.0% in Ist, 2nd, 3rd, 4th, 5th and 6th passage, respectively. After the first *in vivo* propagation and sorting, the rate of fluorescence was estimated by viewing freshly purified oocysts population in different concentration. The appearance of *tE. mitis* sporulated oocysts was 0.26% as compared to 0.8% transgenic unsporulated oocyst with 97.94% sporulated oocyst failed in acquiring EYFP. Only one percent oocysts refused to sporulate and did not acquire EYFP either. Using FACS sorting in the subsequent serial passages we observed gradual increase in the appearance of sporulated oocysts expressing EYFP (Fig. 2), up to 71% at the 6th passage.

Comparative Reproduction Efficiency of *wE. mitis and tE. mitis*: We observed that *tE. mitis* presented a delay of minimum of 22-24hrs in acquiring oocysts dejection peak. As compared to the parental *w*ild type strain, *tE. mitis* oocysts output was reduced by 5-6 fold. The fertility potential of *tE. mitis* turned out to be significantly lower than that of *wE. mitis* ($0.71x10^6$/bird vs $5.1x10^6$/bird).

Validation of the stable transfection of *E. mitis*: The genomic DNA analysis of *tE. mitis* for effective formation of stable transfected EmiChIL-4 revealed the presence of pHDEAAssIL4A containing EYFP and IL-4 gene fragments in the transgenic parasites by PCR detection of genomic DNA (Fig. 3). PCR analysis revealed the appearance of 411bp band which is suggestive of presence of IL-4 into the genome of *tE. mitis*.

Clinico-hematological analysis: The frequency of different clinical and behavioral signs induced by transgenic and wild type strains of *E. mitis* is presented in Table 1. Moderate clinical and behavioral signs like: restlessness, diarrhea, emaciation, decreased feed intake and loss of body weight were observed in *wE. mitis* infection. Very mild to no clinical signs were observed in birds inoculated with *tE. mitis* oocysts. Hematological analysis indicated that total erythrocyte counts, hemoglobin concentration, packed cell volume, mean corpuscular hemoglobin concentration were significantly decreased in birds infected with *wE. mitis* as compared to those infected with *tE. mitis* (Table 2), while mean corpuscular volume significantly increased in birds infected with *wE. mitis*. The total leukocyte counts including heterophil, monocyte, eosinophil and basophil population were significantly increased while that of lymphocyte were decreased in birds inoculated with *wE. mitis* as compared to those in control group and/or infected with *tE. mitis* (Table 3).

DISCUSSION

Vaccination induces the most potent immunity against infectious diseases (Liniger *et al.*, 2007; Ullah *et al*., 2014). Live vaccine vehicles, based almost exclusively on recombinant bacteria and viruses expressing defined pathogen-derived antigens, represent powerful vaccine candidates for future developing strategies (Liniger *et al.*, 2007; Medina and Guzman, 2001). There exists a strong possibility to develop Eimeria-based eukaryotic vaccines as transfection manipulation strategy came true to *Eimeria* spp. (Liu *et al*., 2008; Huang *et al*., 2011; Clark *et al*., 2012). Recently, genetic manipulation of *E. tenella*, one of the extremely virulent species of chicken coccidia, using a double-cassette vector proved to be practicable and stable transfection system in this species expressing different fluorescent proteins (Qin *et al*., 2014). This stable establishment of recombinant eimerian parasites with ascertained genetic background, the transgenic lines of *Eimeria* spp is a bold step towards the achievement of great aim of developing potent Eimeria-based eukaryotic vaccines. In this study, we successfully obtained a transgenic line of *E. mitis*, another species of chicken coccidia relatively less virulent, expressing EYFP and chicken IL-4, using a double-cassette expression vector system. In addition to this we estimated the reproductive efficiency and adjuvant efficacy of ChIL-4 in terms of hematological aberrations induced by *tE. mitis* as compared to parental *wE. mitis*. On the top of this we observed the birds for having severe or moderate clinical effects after the admission of transgenic and wild *E. mitis* strains. We observed the successful integration of linear plasmid with regulatory sequences derived from *E. tenella* with successful transfection and expression of EYFP in *tE. mitis*. The integration of linear plasmid as indicated by expression of EYFP confirms the functionality of regulatory sequences derived from *E. tenella*. In our study, PCR analysis revealed the appearance of 411bp band which is suggestive of presence of IL-4 into the genome of *tE. mitis*. These findings are in accordance with a previous report which manifested that *E. tenella* histone 4 promoters could function effectively in the stable transfection of *E. maxima*, *E. acervulina* and *E. praecox* (Zou *et al.*, 2009). In addition, these findings also support the accuracy of the methodologies and authenticity of the results obtained in this study. It has been reported that successful *in*- transfection techniques are valuable tool to study the functional analysis of parasite molecules related to host cell invasion, drug resistance and various aspects of host-parasite interactions (Ding *et al*., 2005).

Strategies which were applied to transfect *E. tenella*, such as nucleofection of sporozoites, REMI transfection and cloaca inoculation (Clark *et al.*, 2008; Yan *et al.*, 2009) proved to be equally useful for transfection of *E. mitis*. Applying the above reported methodologies, we obtained stably transfected *E. mitis* via fluorescent activated cell sorting (FACS), EYFP-expressing

transgenic oocysts through 6 consecutive passages. Ecesis of clonal population of transgenic parasite is a prerequisite, not only for establishment of Eimeria-based vaccines but also for the investigation of their phenotypes like: oocyst output and fecundity owing to the changes in genetic makeup and differences in integration sites. The reproduction and pattern of oocyst output are two considerable characters for Eimerian parasites when comparing a newly isolated population or a precocious line with a parental or reference strain (Yan *et al*., 2009). We observed phenotypic alterations induced by *tE. mitis*. We opine some phenotypic alterations such as oocyst shedding peak days and decreased fecundity could be due to the double cassette plasmid integration in clonal population. In present experimental research different hematological alterations caused by coccidiosis in chickens have also been reported (Adamu, 2013). This integration of linear plasmid in the genome of *tE. mitis* may have played vital role in displaying clinical and hematological alterations, either through expression of foreign genes and/or disruption of host genes. The mechanism involved in giving rise to clinico-hematological alterations needs meticulous mining of the genomic information including the integration locus of the transgene.

Transgenic population of poultry parasites is not necessarily less virulent or less productive (Liu *et al*., 2013). Contrary to our findings, Liu *et al*. (2013) reported an increased fecundity in case of transgenic *E. tenella* expressing M2e-EYFP. In our study, we observed that induced inoculation of one million sporulated transgenic oocysts did not pose adverse impacts on different tissues in chicken as compared to moderate clinical and behavioral changes seen in case of parental wild type inoculation.

Our data suggested that *tE. mitis* is less virulent than *wE. mitis* as in the latter case, the total leukocyte counts, monocyte population and basophil number increased. In addition to this the therapeutic nature of *tE. mitis* increased as compared to *wE. mitis* as hemoglobin concentration, total erythrocyte count, pack cell volume, mean corpuscular hemoglobin concentrations significantly reduced in birds inoculated with *wE. mitis*. Previously, it has been reported by *Huang et al*. (2011) that transgenic *E. tenella* elicited cellular, humoral and mucosal immunity and maintained immunogenicity with low pathogenicity. Similarly, we observed that *tE. mitis* caused an increased feed uptake and body weight; while, *wE. mitis* led to emaciation and diarrhea. Several others clinical and behavioral indicators suggested that *tE. mitis* might have induced humoral immunity in birds owing to insertion of Chicken IL-4 in linear plasmid; nevertheless, this needs further genetic manipulation and immune trials. However, the success of these initial studies supports extension to further molecular analysis. Based on the findings in this study we may credit insertion of ChIL-4 for the weakened pathogenicity of *tE. Mitis* as compared to *wE. mitis*. The quantity of foreign proteins expressed by the transgenic Eimerian parasite determines the magnitude of protective immunity to pathogen which provides foreign antigens. Relative limitation of expression of foreign proteins in transgenic parasite makes *tE. mitis* an excellent candidate for its usage as promising vaccine vector owing to its transgenic vulnerability; nevertheless, much more study is required to test its biological safety.

Table 1: Clinical Variables observed in different groups of broilers exposed to wild and transgenic *E. mitis.*

Clinical signs	Groups (Intensity of signs)		
	A	B	C
	Control	Wild	Transgenic
Restlessness	----	+++	+
Emaciation	----	+++	----
Diarrhea	----	+++	----
Ruffled feathers	----	+++	+
Decreased feed intake	----	++	----
Decreased body weight	----	++	+
Dullness and depression	----	+++	+

Clinical alteration were categorized on the basis of severity into severe (++++), moderate (+++), mild (++) and very mild (+).

Table 2: Hematological indices in birds exposed to wild and transgenic *E. mitis*

Parameter/	Groups of Broiler Birds		
Days post inoculation	A	B	C
Erythrocyte (10^{12}/l)			
2	3.24±0.03	3.14±0.01	3.2±0.02
4	3.24±0.07	3.13±0.01	3.16±0.01
6	3.29±0.03	2.62±0.03*	3.09±0.01
Hemoglobin (g/dl)			
2	13.84±0.01	12.58±0.2	13.37±0.25
4	13.01±0.24	11.11±0.40*	13.03±0.25
6	13.3±0.17	9.42±0.09*	12.74±0.33
Pack cell volume (%)			
2	34.9±0.53	30.99±0.55	33.56±0.25
4	35.1±0.68	28.41±0.46*	33.29±0.17
6	35.17±0.30	28.14±0.28*	33.6±0.38
Mean corpuscular volume (fL)			
2	126.4±0.37	135.0±0.95	126.4±0.63
4	127.7±0.75	135.2±0.45*	127.5±0.76
6	125.2±0.27	135.5±0.57*	124.8±0.27
Mean corpuscular hemoglobin concentration (g/dL)			
2	35.2±0.28	32.3±0.43	33.4±0.13
4	34.9±0.45	28.5±0.29*	32.9±0.19
6	34.8±0.29	27.9±0.21*	32.7±0.35

Values (mean±SE) in rows bearing asterisk differ significantly (P≤0.05) from control group. The group A served as control while the birds of groups B and C were inoculated with wild and *tE. mitis* respectively.

Table 3: Total and differential leukocyte cunts in broiler birds exposed to wild and *tE. mitis*

Parameter/Days	Groups		
	A	B	C
Leukocyte counts (10^9/l)			
2	12.4±0.13	13.7±0.31	12.3±0.05
4	12.6±0.21	14.3±0.03*	13.7±0.20
6	12.7±0.05	17.5±0.14*	14.3±0.29
Heterophil (%)			
2	51.4±0.59	54.6±0.87	53.2±0.86
4	52.1±0.68	54.8±0.68	53.9±0.83
6	53.3±0.37	56.2±0.56*	56.2±0.61
Lymphocyte (%)			
2	39.1±0.38	35.9±0.28	37.2±0.33
4	38.1±0.57	34.9±0.29*	36.5±0.12
6	38.3±0.22	34.9±0.25*	36.7±0.29
Monocyte (%)			
2	4.93±0.08	4.81±0.02	4.91±0.01
4	4.88±0.03	5.11±0.04*	5.13±0.04
6	4.97±0.05	5.24±0.02*	5.29±0.02
Eosinophil (%)			
2	1.91±0.01	2.01±0.03	1.89±0.00
4	1.92±0.01	2.15±0.01*	1.94±0.01
6	1.95±0.01	2.21±0.02*	1.96±0.00
Basophil (%)			
2	3.79±0.04	3.84±0.02	3.74±0.02
4	3.93±0.01	4.19±0.03*	3.86±0.02
6	3.89±0.02	4.68±0.05*	3.93±0.03

Values (mean±SE) in rows bearing asterisk differ significantly (P≤0.05) from control group. The group A served as control while the birds of groups B and C were inoculated with wild and transgenic strains of *E. mitis,* respectively.

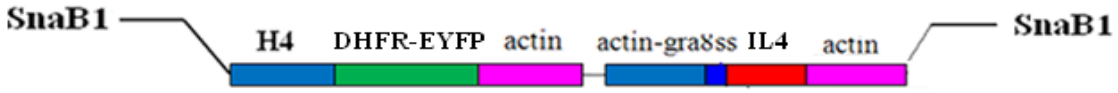

Fig. 1: Plasmid His4-DHFR-EYFP/ACT-chIL-4-ACT pHDEAAssIL4A).

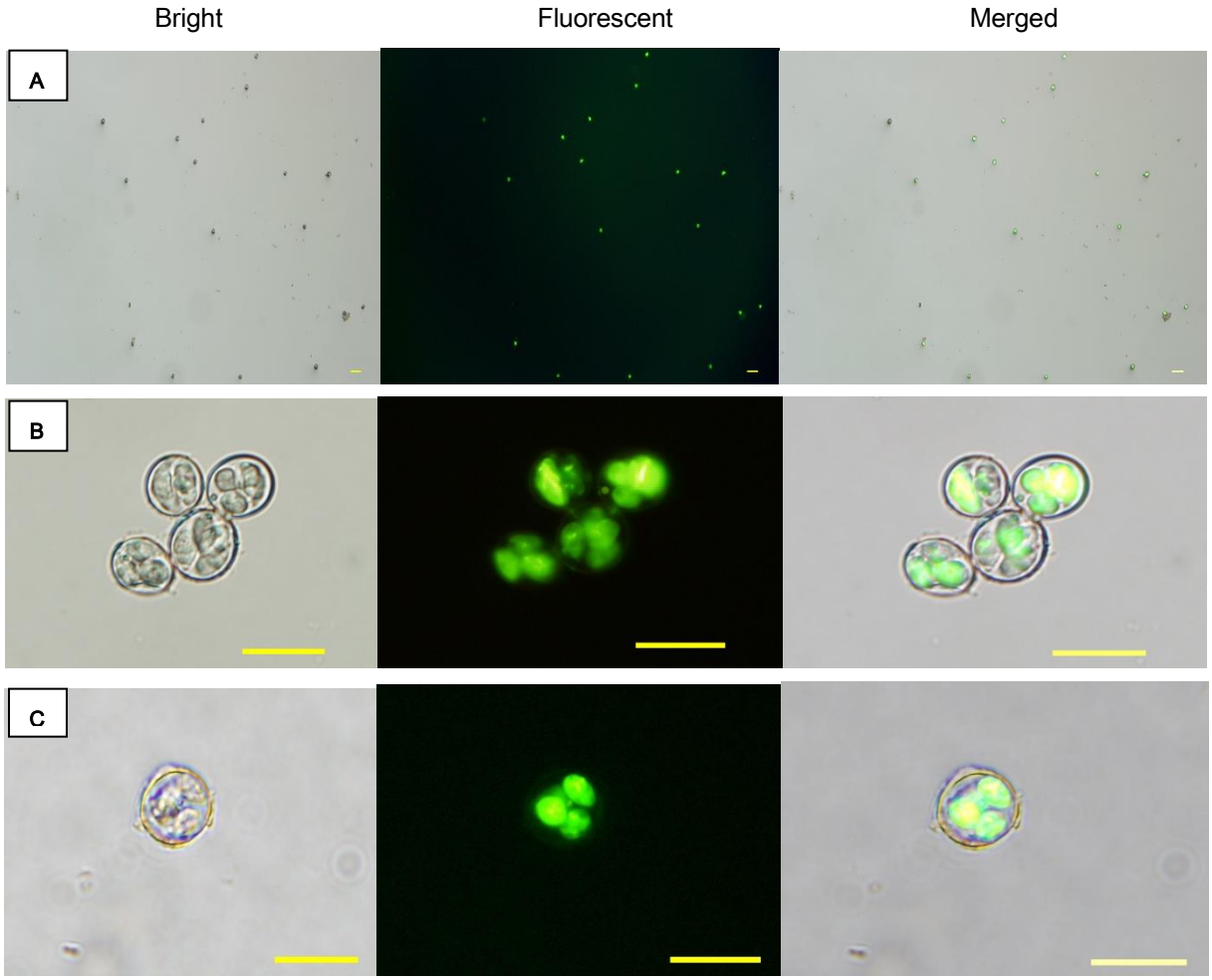

Fig. 2: Establishment of stable *tE. mitis* expressing EYFP molecule. A) Fluorescent images of the 6th passage of sporulated oocyst of *tE. mitis* viewed under 10 ×; B & C) Fluorescent images of the *tE. mitis* viewed under 40×. All the pictures were taken as bright, fluorescent and merged images. Bar=20µm.

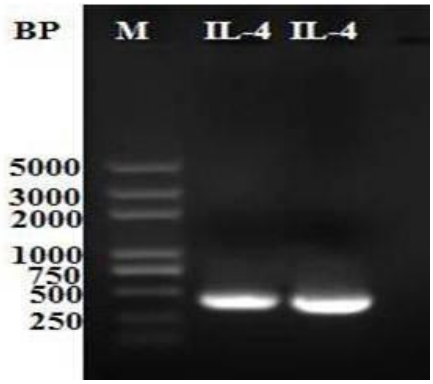

Fig. 3: Trans2K-Plus-DNA-Marker, Lane 2 and 3 represents the genomic DNA extracted from the *t*EmiChIL-4 transfected with linear and circular plasmid respectively by PCR amplification using primers ChIl-4-F and ChIL-4-R producing a 411bp product.

Conclusions: We have successfully developed the *in vivo* transfection of *tE. mitis* and acquired a transgenic population by using double cassette linear plasmid carrying EYFP and IL-4. In addition to this we compared the fecundity potential and hematological aberrations induced by *tE. mitis* and *wE. mitis*. Our results encourage the possible manipulation of *tE. mitis* as a vaccine vector to cope-up with different pathogenic antigens.

Author's contribution: SE, XMT and XS conceived and designed the study. JMK, SHK and MFH executed the experiment. HBL studied hematology. AA, XYL and HW analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

REFERENCES

Adamu M, Chaiwat B, Gongruttananun N and Vongpakorn M, 2013. Hematological, biochemical and histopathological changes caused by coccidiosis in chickens. Kasetsart J, 47: 238-246.

Alemayehu A, Nuru M and Belina T, 2013. Prevalence of bovine coccidia in Kombolcha district of South Wollo, Ethiopia. J Vet Med Anim Health, 5: 41-45.

Clark JD, Billington K, Bumstead JM, Oakes RD and Soon PE, 2008. A toolbox facilitating stable transfection of Eimeria species. Mol Biochem Parasitol, 162: 77-86.

Clark JD, Oakes RD, Redhead K, Crouch CF and Francis MJ, 2012. Eimeria species parasites as novel vaccine delivery vectors: Anti-Campylobacter jejuni protective immunity induced by Eimeria tenella-delivered CjaA. Vaccine, 30: 2683-2688.

Daugschies A and Najdrowski M, 2005. Eimeriosis in cattle: current understanding. J Vet Med, 52: 417-27.

Ding X, Lillehoj HS, Dalloul RA, Min W, Sato T, *et al*., 2005. In ovo vaccination with the *Eimeria tenella* EtMIC2 gene induces protective immunity against coccidiosis. Vaccine, 23: 3733-3740.

Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffy P, *et al*., 1993. Transfection of the malaria parasite and expression of firefly luciferase. Proc Natl Acad Sci, 90: 5234-5236.

Gussem MD, 2007.Coccidiosis in poultry: review on diagnosis, control, prevention and interaction with overall gut health. 16th European Symposium on Poultry Nutrition pp: 253-261.

Hoelscher MA, Garg S, Bangari DS, Belser JA, Lu X, *et al*., 2006. Development of adenoviral-vectorbased pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet, 367: 475-481.

Huang X, Zou J, Xu H, Ding Y and Yin G, 2011. Transgenic Eimeria tenella expressing enhanced yellow fluorescent protein targeted to different cellular compartments stimulated dichotomic immune responses in chickens. J Immunol, 187: 3595-3602.

Hussain R, Khan A, Mahmood F, Rehan S and Ali F, 2014. Clinico-hematological and tissue changes induced by butachlor in male Japanese quail (*Coturnix japonica*). Pest Bioch Physiol, 109: 58-63

Lee S, Lillehoj H, Dalloul R, Park D and Hong Y, 2007. Influence of Pediococcus-based probiotic on coccidiosis in broiler chickens. Poult Sci, 86: 63-66.

Liniger M, Zunigab A and Naim HY, 2007. Use of viral vectors for the development of vaccines. Review. Expert Review, 6: 255-266.

Liu X, Shi T, Ren H, Su H, Yan W, *et al*., 2008. Restriction enzyme mediated transfection improved transfection efficiency in vitro in Apicomplexan \parasite Eimeria tenella. Mol Biochem Parasitol, 161: 72-75.

Liu X, Zou J, Yin G, Su H and Huang X, 2013. Development of transgenic lines of Eimeria tenella expressing M2e-enhanced yellow fluorescent protein (M2e-EYFP). Vet Parasitol, 193: 1-7.

Medina E and Guzman AC, 2001. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine, 19: 1573-1580.

Qin M, Liu XY, Tang XM, Suo JX and Tao GR, 2014. Transfection of eimeria mitis with yellow fluorescent protein as reporter and the endogenous development of the transgenic parasite. PLoS ONE, 9: e114188.

Raman M, Banu SS, Gomathinayagam S and Raj GD, 2011. Lesion scoring technique for assessing the virulence and pathogenicity of Indian fifi eld isolates of avian Eimeria species. Vet Arhiv, 81: 259-271

Shirley MW, Ivens A, Gruber A, Madeira AM and Wan KL, 2004. The Eimeria genome projects: a sequence of events. Trends Parasitol, 20: 199-201.

Shirley MW, Smith AL and Tomley FM, 2005. The biology of avian Eimeria with an emphasis on their control by vaccination. Adv Parasitol, 60: 285-330.

Sibley LD, Messina M and Niesman IR, 1994. Stable DNA transformation in the obligate intracellular parasite Toxoplasma gondii by complementation of tryptophan auxotrophy. Proc Natl Acad Sci, 91: 5508-5512.

Suo X, Zhang JX, Li ZG, Yang CT, Min QR, *et al*., 2006. The efficacy and economic benefits of Supercox, a live anticoccidial vaccine in a commercial trial in broiler chickens in China. Vet Parasitol, 142: 63–70.

Ullah MI, Akhtar M, Iqbal Z, and Muhammad F, 2014. Immunotherapeutic activities of mushroom derived polysaccharides in chicken. Int J Agri Biol, 16: 269-276.

Yan W, Liu X, Shi T, Hao L, Tomley FM, *et al*., 2009. Stable transfection of Eimeria tenella: constitutive expression of the YFP-YFP molecule throughout the life cycle. Int J Parasitol, 39: 109-117.

Yin G, Liu X, Zou J, Huang X and Suo X, 2011. Co-expression of reporter genes in the widespread pathogen Eimeria tenella using a double-cassette expression vector strategy. Int J Parasitol, 41: 813-816.

Zaman MA, Iqbal Z, Abbas RZ and Ehtisham-ul-Haque S, 2015. In vitro efficacy of herbal extracts against *Eimeria tenella*. Int J Agri Biol, 17: 848-850.

Zou J, Liu X, Shi T, Huang X, Wang H, *et al*., 2009. Transfection of Eimeria and Toxoplasma using heterologous regulatory sequences. Int J Parasitol, 39: 1189-1193.