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Molecular Engineering of a Dual-Cassette Transgenic *Eimeria mitis* Expressing Chicken IL-4 and Nuclear-Localized EYFP

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Abstract

Genetic manipulation of *Eimeria* species has emerged as a pivotal strategy for developing next-generation live vaccines capable of delivering immunomodulatory molecules directly to the host intestinal mucosa. In this study, a dual-cassette expression plasmid was constructed to generate a transgenic line of *Eimeria mitis* (*E. mitis*) capable of expressing chicken interleukin-4 (ChIL-4) and enhanced yellow fluorescent protein (EYFP). The ChIL-4 open reading frame was amplified from chicken spleen lymphocyte cDNA, while DHFR-TSm2m3 and EYFP fragments were obtained from previously established constructs to assemble a fused DHFR-EYFP reporter cassette. The final double-cassette plasmid (pHDEAAssIL4A) contained regulatory elements derived from *Eimeria tenella* and a secretion signal to ensure extracellular IL-4 release. Transfection of *E. mitis* sporozoites was performed using nucleofection under REMI conditions with either SnaBI-linearized or circular plasmid DNA. Linear DNA yielded markedly superior transfection efficiency and fluorescence intensity, whereas circular plasmid produced negligible early transgenic oocyst output and weaker fluorescence, consistent with prior observations in *Eimeria* species. Stable EYFP-expressing *E. mitis* populations were enriched through successive *in vivo* passages under pyrimethamine selection and FACS sorting, ultimately reaching over 90% fluorescence-positive sporulated oocysts by the seventh passage for the linear-plasmid group. Molecular verification confirmed successful chromosomal integration of both EYFP and ChIL-4. PCR analysis consistently detected the expected 411 bp IL-4 fragment and 726 bp EYFP fragment in transgenic genomic DNA, whereas these amplicons were absent in wild-type controls. These results validate the fact that a double-cassette vector system can be used to successfully transfect *E. mitis*, and express EYFP and IL-4 across generations in a stable manner. This is because the effective production of this IL-4-expressing line can be a promising step to the use of *E. mitis* as a genetically engineered vaccine carrier and the investigation of the IL-4-dependent suppression of host immunity.

Keywords: Eimeria mitis; transgenic Eimeria; dual-cassette expression system; chicken interleukin-4 (chIL-4); nuclear-localized EYFP

Introduction

Avian coccidiosis is the most economically devastating parasitic illness that impacts the poultry industry globally by causing significant losses through reduced growth, diminished feed ratio, morbidity, and mortality in broilers and layers [1]. Protozoa of the genus *Eimeria* cause the disease and infect the intestinal epithelium, and reproduce in a complex cycle of intracellular development stages [2]. Traditional methods of control anticoccidial drugs, better husbandry, and prophylactic sanitation, have been used extensively, but their effectiveness is short-lived because of the resistance of drugs and the survival of oocysts in the environment [2, 3].

Live vaccination has emerged as an effective alternative in the production of protective immunity against coccidial infections. Attenuated or low-pathogenicity *Eimeria* strains used as vaccines enable the host to carry them to their full life cycle, stimulating species-specific immunity with controlled exposure to oocysts shed following immunization [4]. The recent developments in genetic manipulation of *Eimeria* species such as *E. tenella*, *E. maxima*, *E. acervulina*, and *E. praecox* have provided additional evidence to the fact that transgenic lines can be used as effective delivery vehicles of immunologically relevant antigens, providing the basis of foundation of the development of multivalent recombinant vaccines [5]. *Eimeria mitis* is one of the seven species of *Eimeria* that infect poultry, but it is relatively less pathogenic as it causes mild enteric lesions but can still be able to undergo the developmental cycle and induce protective immunity. *E. mitis* has received interest as a platform on which vaccine vectors can be developed, especially the expression of heterologous immunomodulatory molecules, due to its reduced virulence and good biological properties [6].

Genetic modification of *Eimeria* parasites has also increased significantly, as a result of nucleofection of sporozoites, restriction enzyme-mediated integration (REMI), and cloacal inoculation, which was first developed in *E. tenella* and has now been delivered successfully in *E. mitis* [7]. These procedures have facilitated transient and stable expression of reporter genes like EYFP, and this has allowed researchers to monitor the development of a parasite, confirming the expression of the transgene and also studying host-parasite interactions at the cellular level. It has also been accomplished in stable transgenic lines that are able to test the viability of foreign cytokines or vaccine antigens delivery in the case of infection [8].

Several studies demonstrated that interleukin-2 (IL-2), IL-18, IL-17, and IFN-γ may improve immunity during coccidial vaccination, as plasmids or transgenic *Eimeria* strains. These cytokines serve as adjuvants, as they adjust the T-cell reactions and enhance the proper antigen presentation [6]. The most promising candidate to strengthen the humoral immunity against coccidiosis to enhance the number of B-cells, antibodies, and lymphocyte homing to intestinal tissues is IL-4, which plays a significant role in the activation of B-cells, production of antibodies, and homing of lymphocytes to intestinal tissues [9].

Studies have shown that IL-4 has great potential to improve protective immunity in chickens that are challenged with *Eimeria* spp. Owing to *in ovo* administration of IL-4 plasmids, more weight gain, less shedding of oocysts, more generation of nitric oxide by the macrophages, and also, higher serum levels of anti-coccidia IgG were observed after challenge infection [10]. These immunomodulatory functions were in line with the suggestion that IL-4 expression in the intestinal environment during infection with *E. mitis* could enhance the host immunity and provide enhanced protection [11].

A dual-cassette plasmid was created to simultaneously express two molecules (1) EYFP, with a nuclear localization signal to enable accurate visualization of parasite growth, and (2) chicken IL-

4, with a secretion signal to facilitate the release of extracellular cytokines. The design took advantage of the regulatory sequences of *E. tenella* to provide reliable transcription in *Eimeria* spp. and applied the method of linearization that increased the effectiveness of genomic integration. Together, this work will create a sophisticated molecular position in designing *E. mitis* as an immunomodulatory vaccine carrier and give initial data on the biological behavior of the IL-4 transgenic coccidia.

Materials And Methods

Parasite and its Maintenance

E. mitis Zhuozhou strain (Zz) was used throughout this study. The Zz strain of *E. mitis* was isolated from a single oocyst, maintained by serial passage through 2 to 5-weeks-old / broiler birds Arbor Acre (AA) and the oocysts were prepared *in vitro* by standard procedures (Zhuoran *et al*., 2015). Susceptible birds were orally given $1x10^3$ to $1x10^4$ sporulated oocysts suspended in 1 ml phosphate-buffered saline (PBS). Sterile droppings pan containing 2.5% potassium dichromate solution (w/v) to cover the bottom of the pan was placed under the cage containing the birds during the collection period (9 days post inoculation). The droppings were collected and washed through stainless steel strainers (80, 100 & 200 μm). The filtrated oocysts were allowed to settle down in the bottom for 2-3 hrs. Supernatant was discarded and oocyst sediment was kept concentrated by centrifugation for 10 mins at 3000 rpm. Oocysts in the faeces pellet were collected using saturated sodium chloride solution. Finally, oocysts were suspended in 2.5% potassium dichromate solution and allowed to sporulate for 48- 72 hr at 30°C using an electric pump. Sporulated oocysts suspended in 2.5% potassium dichromate solution were refrigerated at 2 – 8 °C for further experiments.

Isolation of Sporozoites

Sporozoites were isolated and purified using DE-52 anion-exchange chromatography. DE-52 cellulose was conditioned by sequential washing in distilled water, 0.1 M NaOH, distilled water, 0.1 M HCl, and distilled water at 4°C, followed by equilibration in PBS (pH 7.6). The pH was finally adjusted to 8.0 using glycine buffer. A 20 cm chromatography column was packed with DE-52 suspended in glycine buffer to a bed height of 5–7 cm and equilibrated before use. Surface-sterilized oocysts in PBS were mechanically disrupted until approximately 90% were cracked, releasing sporocysts, which were washed and excysted in PBS containing 10% chicken bile and 0.75% trypsin at 41°C for 30–45 min. Excystation was terminated by centrifugation, and sporozoites were washed in PBS and glycine buffer before loading onto the DE-52 column. Purified sporozoites were eluted with glycine buffer, washed, and counted using a hemocytometer. The total number of sporozoites was calculated using the formula: total amount of sporozoites = (amount in 4 holes/4)x10xdiluted timesxsporozoites solution volume (micro liter). Lastly, the sporozoites were counted and resuspended in 10% DMEM for further experimental trials [12].

Experimental Birds and Husbandry

Coccidia-free Arbor Acres broiler chickens (2–5 weeks old) were used for parasite propagation and transgenic line maintenance. Day-old chicks were sourced from Beijing Arbor Acres Poultry Breeding Co. and housed in isolators under uniform husbandry conditions. Birds received pathogen-free commercial feed (corn–soybean diet; 20% crude protein) and water ad libitum throughout the experiments. Standard procedures for collection, purification, and sporulation of *E. mitis* oocysts were followed as previously described by Huang et al. (2011) and Yin et al. (2013) [13, 14].

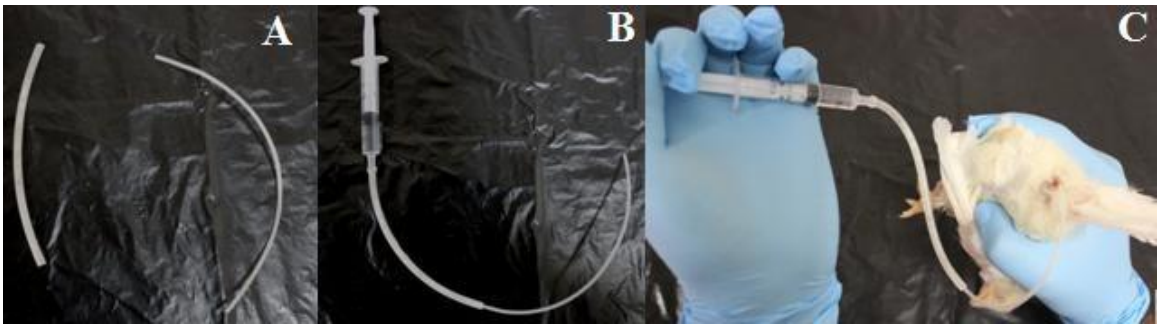

Figure A: Set up for predilection site inoculation of *E. mitis*:

A. Different sizes of clear silicone rubber tubing tube B. Inoculation tool assembly C. Chicken inoculation

Construction of the Dual-Cassette Expression Plasmid

A double-cassette eukaryotic expression vector (pHDEAAssIL4A) was constructed following previously established strategies for *Eimeria* transfection. Total RNA was isolated from splenic lymphocytes of a 3-week-old SPF chicken using TRIzol reagent. cDNA synthesis was performed with random primers and a high-capacity reverse transcription kit. Based on the published *Gallus gallus* IL-4 sequence (GenBank AJ621249.1), the complete ORF of ChIL-4 was amplified with specific primers (ChIL-4-F/ChIL-4-R). To generate the reporter cassette, DHFR-TSm2m3 and EYFP fragments were amplified from plasmids pEtADA and pMIC-EYFP/ACTss-RFP using DHFR-F/DHFR-R and EYFP-F/EYFP-R primers, respectively. These fragments were joined by overlap PCR to create a fused DHFR-EYFP reporter gene. Amplifications were performed using high-fidelity Pfu DNA polymerase to minimize mutation frequency. The parental double-cassette plasmid pHIS-EYFP/ACT-RFP served as the backbone. The EYFP region was replaced by DHFR-EYFP, and the RFP region was substituted with the secretory ChIL-4 gene. A secretion signal sequence derived from *Toxoplasma gondii* GRA8 was incorporated to ensure extracellular release of IL-4. The completed plasmid was linearized with SnaBI to release both expression cassettes, thereby improving recombination efficiency. Commercial reagents used included pEASY-Blunt vectors, PCR supermixes, competent Trans5α and Trans1-T1 cells, DE-52 cellulose, and DMEM medium from suppliers listed in the thesis reagent information section.

Preparation and Excystation of *E. mitis* Sporozoites

Sporulated *E. mitis* oocysts were surface-sterilized and cracked using a mechanical grinder until approximately 90% of oocysts released their sporocysts. Sporocysts were washed twice in PBS (pH 7.6) and incubated in an excystation solution (10% chicken bile + 0.75% trypsin in PBS) at 41°C for 30–45 minutes until most sporozoites were released. The reaction was halted by centrifugation at 2,500 rpm for 5 minutes, and sporozoites were washed twice in PBS and once in glycine buffer to prepare them for purification.

Purification and Enumeration of Sporozoites

Freshly excysted sporozoites were purified using DE-52 anion-exchange chromatography. The DE-52 column was prepared by washing the cellulose with PBS and adjusting its final pH to 8 using glycine buffer. Sporozoites suspended in glycine were layered onto the column and eluted with additional glycine. Eluates were monitored microscopically until pure sporozoite fractions

were obtained. These were washed twice in glycine and once in PBS at 2,500 rpm for 5 minutes. Sporozoite concentration was determined by hemocytometer counting using the manufacturer's formula:
Total sporozoites = (count in 4 large squares / 4) × 10 × dilution factor × sample volume
Purified sporozoites were resuspended in 10% DMEM for transfection experiments.

Nucleofection and Transfection of *E. mitis* Sporozoites
Transfection was performed using the Amaxa Nucleofector II system under REMI conditions, as previously optimized for *Eimeria* spp. Sporozoites were mixed with either circular pHDEAAssIL4A plasmid or SnaBI-linearized plasmid DNA. Transfected sporozoites were inoculated into primary chicken kidney cell monolayers for transient expression studies and injected into broiler hosts for *in vivo* selection. Early transfection success was assessed by fluorescence microscopy at 24 hours post-inoculation, where EYFP-positive sporozoites indicated successful uptake of the plasmid.

Establishment of Stable Transgenic Lines
Following transfection, sporozoites were administered to chickens via cloacal inoculation using the predilection-site apparatus shown in Figure A. Birds received inocula corresponding to each passage number (e.g., 1×10^6 oocysts in the first passage, followed by 5×10^3 oocysts in subsequent passages). Oocysts shed in feces were collected daily from days 5 to 11 post-infection. Selection of transgenic parasites was achieved by supplementing the feed with pyrimethamine and by periodic fluorescence-activated cell sorting (FACS) of sporulated oocysts to enrich the EYFP-positive population. Stable fluorescence exceeding 90% was achieved by the seventh passage in the linear plasmid group.

Table 1. Transfection efficiency of the transgenic strain of *E. mitis* at different passages using circular plasmid in terms of sporulated oocysts in droppings of broiler birds

Reporter	**Generation**	**Inoculate dosage (oocyst per bird)**	**Transfection efficiency (%)**	**Oocyst output**	**Drug selection**
EYFP	1	1×10^6	0.01	1×10^3	Drug
	2	5×10^3	3.4	7.9×10^6	Drug
	3	5×10^3	18	1.5×10^7	Drug
	4	5×10^3	41	1×10^7	Drug
	5	5×10^3	64	1.3×10^7	Drug
	6	5×10^3	70	1.6×10^7	Drug
	7	5×10^3	85	2×10^7	Drug

Monitoring of Oocyst Shedding

Birds were monitored daily for signs of coccidiosis, including diarrhea and changes in fecal quality. Oocyst shedding was quantified using the modified McMaster technique between days 5 and 11 post-infection. Three grams of feces were homogenized in 42 mL saturated sodium chloride solution, and oocysts were counted in McMaster chambers after flotation. Oocysts per gram (OPG) were calculated using established formulae.

Fluorescence Microscopy

Oocysts and intracellular stages (sporozoites, schizonts, gamonts) were visualized using an Olympus IX70 inverted fluorescence microscope. Bright-field, fluorescent, and merged images were acquired at 10× and 40× magnification. Stable nuclear-localized EYFP expression in t*E. mitis* across developmental stages was confirmed with this imaging system.

PCR Confirmation of Transgene Integration

Genomic DNA extracted from sporulated oocysts of the transgenic *E. mitis* line was subjected to PCR to detect inserted transgenes. Successful integration was confirmed by amplification of:

- 411 bp fragment corresponding to IL-4, and
- 726 bp fragment corresponding to EYFP

These amplicons were consistently detected in transgenic samples but were absent in wild-type controls. Plasmid rescue assays further verified stable genomic incorporation, showing characteristic restriction banding patterns after digestion with NdeI or SacII-AgeI, matching expected plasmid-derived fragments.

Results

Construction of the Dual-Cassette IL-4/EYFP Plasmid

The double-cassette plasmid pHDEAAssIL4A was successfully constructed. Replacement of the EYFP cassette in pHIS-EYFP/ACT-RFP with the fused DHFR-EYFP reporter gene, and substitution of RFP with the ssChIL-4 gene, produced a functional dual-expression construct. Incorporation of the *Toxoplasma gondii* GRA8 secretion signal ensured extracellular targeting of IL-4. The plasmid was linearized with SnaBI, releasing both expression cassettes as expected (Fig. 1 and 2). All PCR products, including ChIL-4 (amplified from spleen lymphocyte cDNA), DHFR-TSm2m3, and EYFP, were obtained with high specificity using high-fidelity Pfu polymerase.

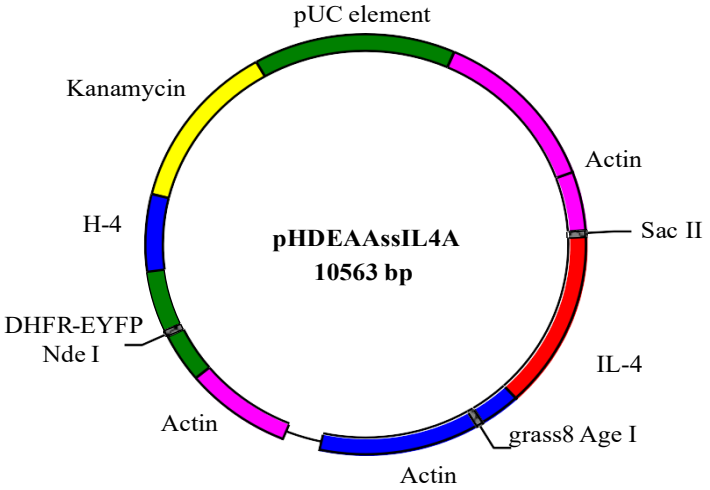

Figure 1. Schematic representation of circular IL-4 expressing plasmid

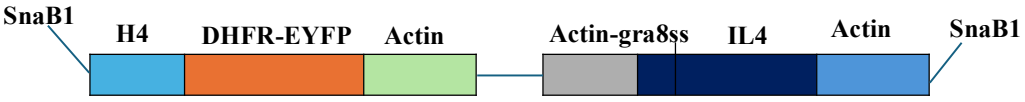

Figure 2. Schematic representation of linear IL-4 expressing plasmid

In Vitro Transient Expression of EYFP

At 24 hours post-inoculation, numerous transfected sporozoites showed detectable EYFP fluorescence when observed in primary chicken kidney cells (Fig. 3). The transient transfection ratio ranged between 1×10^{-5} to 4×10^{-5}, indicating successful uptake of the plasmid by a small but consistent proportion of sporozoites. Fluorescent signals were predominantly localized within the parasite nucleus, confirming the functional activity of the Histone-4 promoter and nuclear localization sequence. Bright-field, fluorescent, and merged imaging clearly differentiated EYFP-positive and EYFP-negative cells (Fig. 3)

Fluorescent **Bright** **Merged**

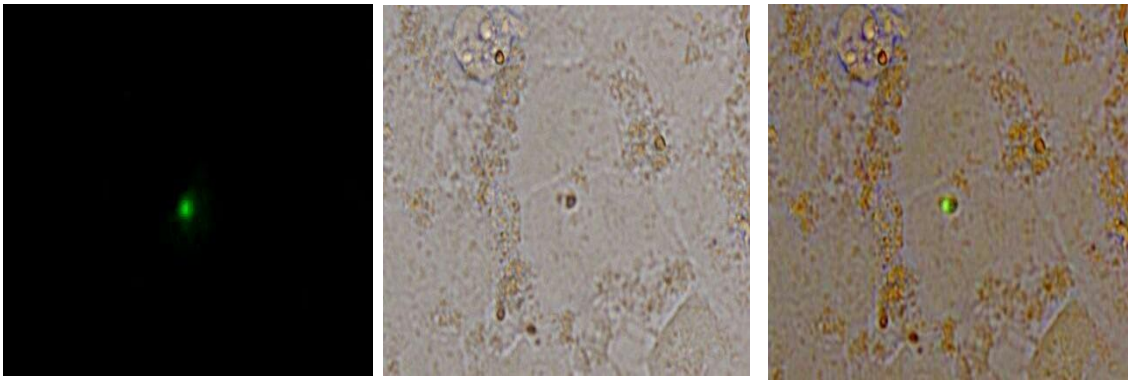

Figure 3: Sporozoites transfected with pHDEAAssIL4A in chicken primary kidney cell. Bar 20 µm.

Establishment of Stable Transgenic *E. mitis* Lines

Linear vs Circular Plasmid Transfection Efficiency

Markable differences in transfection efficiency were observed between linear and circular plasmid DNA forms.

A. Linear Plasmid Group

Table 2: Across seven sequential *in vivo* passages, the proportion of EYFP-positive sporulated oocysts increased consistently:

Passage	EYFP-positive (%)	Oocyst Output
1	1.5%	1×10^{3}
2	16%	9.8×10^{6}
3	37%	1.5×10^{7}
4	51.8%	1.8×10^{7}
5	82%	1.3×10^{7}
6	92.61%	1.6×10^{7}
7	93.16%	2.9×10^{7}

By the 7th passage, a stable population (>93%) of fluorescent oocysts was established, and fluorescence intensity was strong and uniform (Fig. 4).

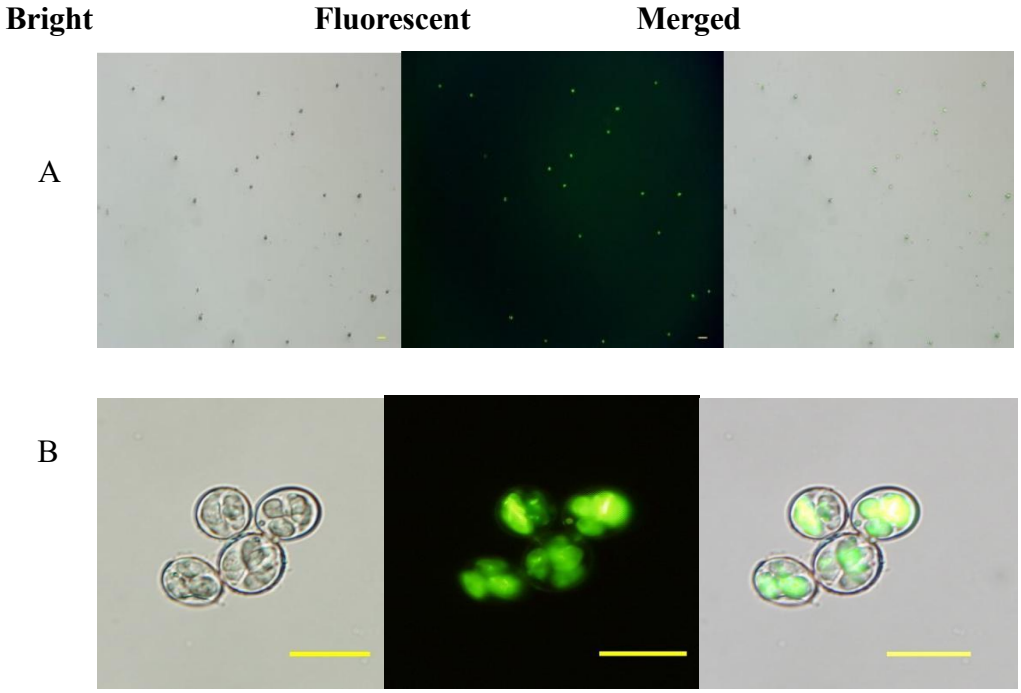

Figure 4: Establishment of stable *tE. mitis* expressing EYFP molecule.

(A) Fluorescent images of the 7th passage of sporulated oocysts of *tE. mitis* viewed under 10x **(B)** Fluorescent images of the *tE. mitis* viewed under 40x. All the pictures were taken as bright, fluorescent and merged images. Bar = 20µm

B. Circular Plasmid Group

Table 3: Circular plasmid transfection produced noticeably lower expression levels:

Passage	EYFP-positive (%)	Oocyst Output
1	0.01%	1×10^{3}
2	3.4%	7.9×10^{6}
3	18%	1.5×10^{7}
4	41%	1×10^{7}
5	64%	1.3×10^{7}
6	70%	1.6×10^{7}
7	85%	2×10^{7}

Linear plasmid consistently achieved higher fluorescence intensity, as demonstrated in Fig. 5:

- **Circular plasmid:** weak and sparse fluorescence.
- **Linear plasmid:** strong, consistent nuclear fluorescence across oocysts and intracellular stages.

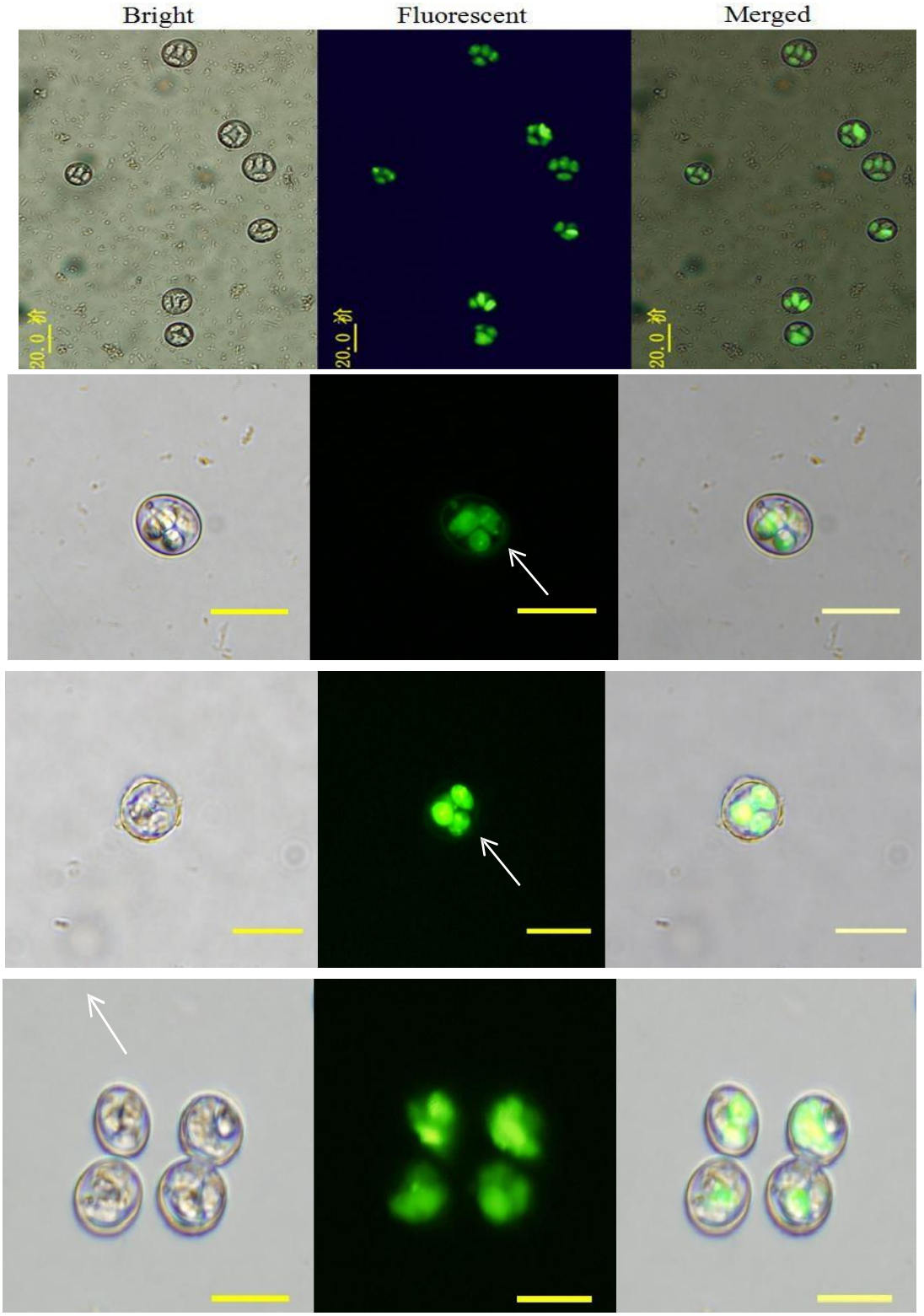

Figure: 5 Optimal transfection efficiency and fluorescence intensity of transgenic yellow fluorescent protein-expressing *E. mitis* using linear plasmid DNA. Arrow pin-pointing the stronger optimal fluorescent intensity Bright, fluorescent and merged images were taken. Bar=20µm

Fluorescent Localization Across the *E. mitis* Life Cycle

In both in vitro and *in vivo* analyses, EYFP expression was consistently observed in:

- sporozoites
- early trophozoites
- schizonts (merogony stages)
- gametocytes
- sporulated oocysts

Fluorescence was predominantly nuclear, aligning with the function of the Histone-4 promoter and nuclear localization tag. Fig. 6 demonstrates this pattern under 10× and 40× magnifications

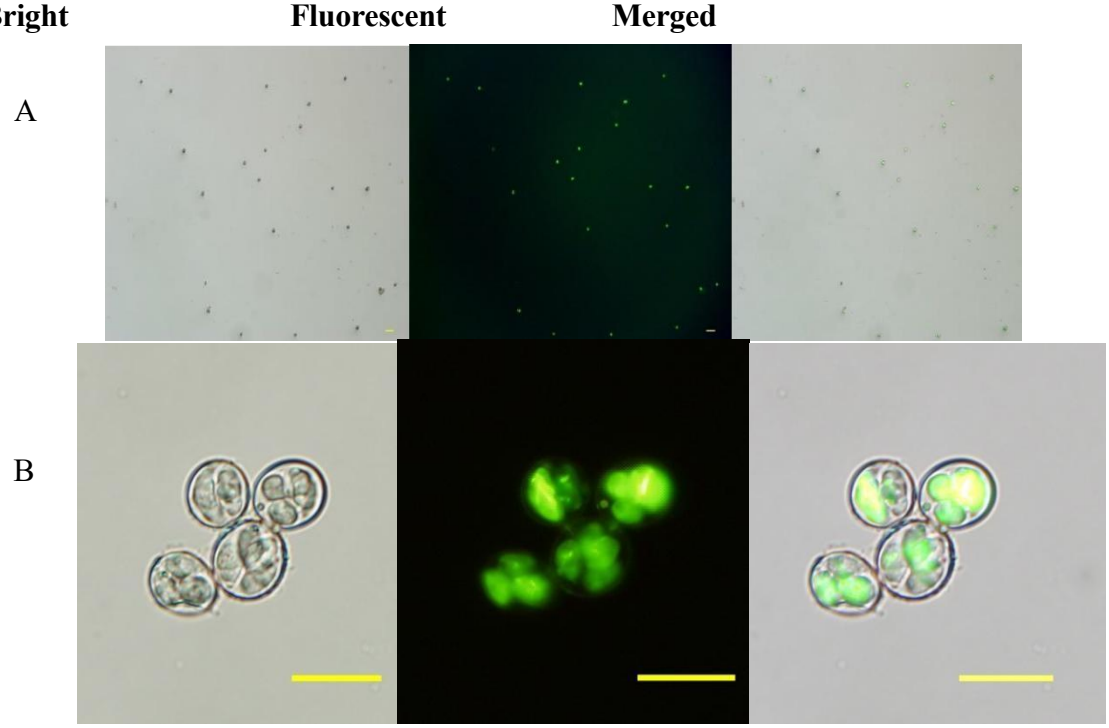

Figure 6: Establishment of stable *tE. mitis* expressing EYFP molecule.

(A) Fluorescent images of the 7th passage of sporulated oocysts of *tE. mitis* viewed under 10x **(B)** Fluorescent images of the *tE. mitis* viewed under 40x. All the pictures were taken as bright, fluorescent and merged images. Bar = 20μm

(B)

PCR Confirmation of Stable Transgene Integration

PCR analysis confirmed genomic incorporation of both IL-4 and EYFP genes. 1) A 411 bp IL-4 fragment was consistently detected in transgenic lines. 2) A 726 bp EYFP fragment was detectable in all positive samples. 3) Neither band appeared in wild-type *E. mitis* DNA. These results validate the stable insertion of both expression cassettes into the parasite genome (Fig. 7 A &B). Restriction digestion of rescued plasmid fragments produced expected band patterns (NdeI and SacII-AgeI digests), confirming structural integrity following integration.

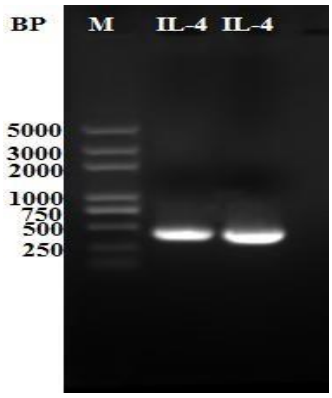

Figure: 7A: Validation of the stable transfection of *E. mitis* by PCR

Lane 1: Trans2K-Plus-DNA-Marker, Lane 2 and 3 represent the genomic DNA extracted from the *t*EmiChIL-4transfected with linear and circular plasmid, respectively by PCR amplification using primers ChIl-4-F and ChIL-4-R, producing a 411bp product verifying the recombination of ChIL-4 into the genome of the parental strain.

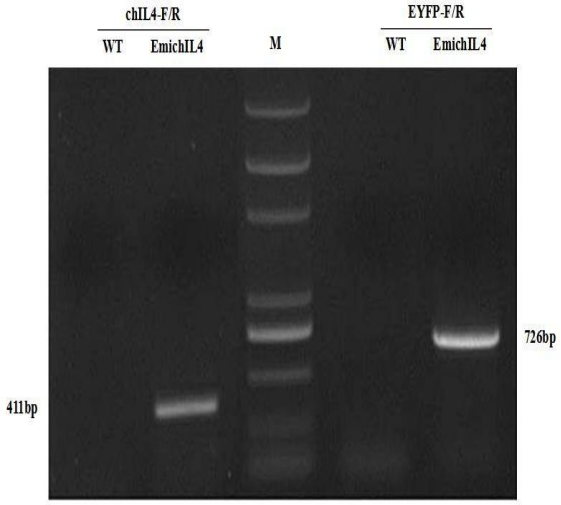

Figure: 7B Validation of the stable transfection of *E. mitis* by PCR

Biological Characteristics of Transgenic *E. mitis*

Although the transgenic line maintained typical morphology and sporulation ability, notable changes in reproductive biology were observed:

- Significant reduction in total oocyst output
- Delay of 22–24 hours in peak oocyst shedding compared to wild-type
- 6-fold reduction in total oocyst production per bird (2.1×10^6 vs 12.1×10^6)
- Lower oocyst shedding in EmiChIL-4 compared to wild type
- Consistent delay in peak oocyst appearance (22–24 hours)
- Markedly reduced reproduction of transgenic parasites

Summary and Discussion

The successful construction of a dual-cassette transgenic *Eimeria mitis* expressing chicken IL-4 and nuclear-localized EYFP represents a substantial step forward in the molecular manipulation of avian coccidia. Earlier genetic studies in *Eimeria* species primarily focused on *E. tenella*, but the extension of these technologies to *E. mitis* is particularly significant because *E. mitis* is

naturally less pathogenic and therefore highly suitable as a live vaccine vector. The engineering strategy in this study demonstrated that this species can reliably express foreign genes without compromising its basic developmental integrity. Li et al. constructed E. mitis expressing chIL-2 and reported enhanced cellular immune responses compared with wild type, supporting the idea that cytokine-expressing Eimeria can modulate host immunity. chIL-2 tends to favor cellular/Th1-type enhancement, whereas chIL-4 biases Th2 responses, so the biological outcomes will differ predictably [15].

One of the most important outcomes of the study was the validation of the double-cassette plasmid design. The engineered vector successfully drove simultaneous expression of EYFP and chicken IL-4, confirming the compatibility of the promoters and signal sequences with the *E. mitis* transcriptional and secretory machinery. The nuclear localization of EYFP observed throughout all examined developmental stages further demonstrated that the Histone-4 promoter remains consistently active and that the nuclear targeting sequence functions effectively in this species. The visualization of fluorescence in sporozoites, schizonts, gametocytes, and sporulated oocysts provided strong evidence of stable and predictable transgene expression across the life cycle. Recent technical advances (CRISPR/Cas9 editing, improved promoters, multiepitope cassettes) have been applied to Eimeria species, meaning future iterations of dual cassette could adopt CRISPR for precise insertion, or stronger/inducible promoters for controlled cytokine delivery [8].

The linear and circular plasmid DNA were compared, where there was a distinct difference in their ability to form stable transgenic lines. The performance of linear DNA was always better than circular DNA and showed greater intensity of fluorescence, earlier emergence of transgenic oocysts, and faster increase in the percentage of occurrence of fluorescent oocysts per passage. These data help to argue that linear DNA forms into the parasite genome more effectively, probably because of more accessibility to recombination processes. The gradual increase in the number of EYFP-positive oocysts and their ultimate prevalence in the population of the parasites suggests that linear DNA is the best choice to produce genetically stable *Eimeria* lines [16].

The presence of transgene integration was also confirmed by molecular techniques, which confirmed the effectiveness of the transformation strategy. The observed IL-4 and EYFP sequences were identified repeatedly in the genomic DNA in multiple passages, and the lack of the same in the wild-type controls indicated the successful integration of the exogenous genes into the genome of the parasites and not as transient products. The integrity of the integrated sequences, which are validated by restriction enzyme digestion of the rescued plasmid fragments, lends credence to the dependability of the system of transformation used [17].

Surprisingly, the wild-type parasite had a very different biological behavior compared to the IL-4-expressing *E. mitis* line. The transgenic line shedding was delayed in time and had many fewer oocysts. These findings indicate some biological attenuation, which may be due to the metabolic cost of the expression of an extra secreted cytokine. Other transgenic strains of *Eimeria* expressing various cytokines have similarly been reported to have similar attenuating effects. This attenuation can be beneficial in vaccine development since it can be better and safer, and still allow appropriate development of the parasite to induce a protective immune response [18].

Overall, the establishment of this IL-4/EYFP-expressing *E. mitis* strain provides a powerful new model for exploring immune interactions during coccidial infection and for developing recombinant vaccine platforms. The consistency of gene expression, the stability of integration, and the favorable safety profile of the transgenic line collectively demonstrate the feasibility of using *E. mitis* as a next-generation live vaccine vector. This foundation enables subsequent studies to evaluate the immunological benefits of IL-4 expression, including its potential to enhance antibody responses, influence inflammatory pathways, and improve protection against coccidiosis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Mohammad Farooque Hassan designed and conducted the experiments, analyzed the data, and wrote the manuscript. Syed Sairum Hassan supervised the study and critically revised the manuscript. Ahmed Hussain assisted with experimental work and data analysis. Syeda Saba Sajjad contributed to data interpretation and manuscript editing. Syeda Tooba Sajjad supported the literature review and preparation of tables and figures. All authors reviewed and approved the final manuscript.

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References

1. Khan, S., G. Naheed, and M.S. Farooq, *Avian health under threat: trends in poultry diseases and their impact on global production.* Pak-Euro Journal of Medical and Life Sciences, 2025. **8**(1): p. 169-186.
2. Ahmad, R., et al., *Management and control of coccidiosis in poultry—A review.* Animal bioscience, 2023. **37**(1): p. 1.
3. Khater, H.F., et al., *Avian coccidiosis: Recent advances in alternative control strategies and vaccine development.* Agrobiol Rec, 2020. **1**: p. 11-25.
4. Tensa, L.R., *Vaccination strategies to control coccidiosis in poultry.* 2018.
5. Liu, Q., et al., *Live attenuated anticoccidial vaccines for chickens.* Trends in Parasitology, 2023. **39**(12): p. 1087-1099.
6. Lee, Y., M. Lu, and H.S. Lillehoj, *Coccidiosis: recent progress in host immunity and alternatives to antibiotic strategies.* Vaccines, 2022. **10**(2): p. 215.
7. Pastor-Fernández, I., et al., *Laboratory growth and genetic manipulation of Eimeria tenella.* Current Protocols in Microbiology, 2019. **53**(1): p. e81.
8. Tang, X., X. Liu, and X. Suo, *Towards innovative design and application of recombinant Eimeria as a vaccine vector.* Infection and Immunity, 2020. **88**(5): p. 10.1128/iai. 00861-19.
9. Tan, F., et al., *Immune synergistic mechanism of recombinant plasmid adjuvant containing chicken IL-4 and IL-2 fusion genes on chicken coccidia live vaccine.* Poultry Science, 2024. **103**(1): p. 103204.
10. Lillehoj, H., et al., *Avian coccidiosis as a prototype intestinal disease–host protective immunity and novel disease control strategies*, in *Intestinal health*. 2015, Wageningen Academic. p. 71-115.
11. Chen, P., et al., *Exploring the interplay between Eimeria spp. infection and the host: understanding the dynamics of gut barrier function.* Veterinary Quarterly, 2025. **45**(1): p. 1-22.
12. Jackson, A., *The isolation of viable coccidial sporozoites.* Parasitology, 1964. **54**(1): p. 87-93.
13. Huang, X., et al., *Transgenic Eimeria tenella expressing enhanced yellow fluorescent protein targeted to different cellular compartments stimulated dichotomic immune responses in chickens.* The Journal of Immunology, 2011. **187**(7): p. 3595-3602.
14. Yin, G., et al., *Expression of Toxoplasma gondii dense granule protein7 (GRA7) in Eimeria tenella.* Parasitology research, 2013. **112**(5): p. 2105-2109.
15. Li, Z., et al., *Transgenic Eimeria mitis expressing chicken interleukin 2 stimulated higher cellular immune response in chickens compared with the wild-type parasites.* Frontiers in microbiology, 2015. **6**: p. 533.

16. Qin, M., et al., *Transfection of Eimeria mitis with yellow fluorescent protein as reporter and the endogenous development of the transgenic parasite.* PLoS One, 2014. **9**(12): p. e114188.
17. Liu, X., et al., *Development of transgenic lines of Eimeria tenella expressing M2e-enhanced yellow fluorescent protein (M2e-EYFP).* Veterinary Parasitology, 2013. **193**(1-3): p. 1-7.
18. Tang, X., et al., *Transgenic Eimeria tenella expressing profilin of Eimeria maxima elicits enhanced protective immunity and alters gut microbiome of chickens.* Infection and immunity, 2018. **86**(9): p. 10.1128/iai. 00888-17.