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Impact of IL-4–Expressing Transgenic *Eimeria mitis* on Reproductive Performance and Parasite Fecundity

Mohammad Farooque Hassan[1], Syeda Saba Sajjad[2], Ahmed Hussain[3], Syed Sairum Hassan[4*], Syeda Tooba Sajjad[5], Syeda Ukasha Mati[6], Syeda Ayesha Mati[7], Jamal Muhammad Khan[8]

[1] Shaheed Benazir Bhutto University of Veterinary & Animal Sciences, Sakrand, Sindh. drfaruqmati72@gmail.com

[2] Liaquat University of Medical & Health Sciences, Jamshoro, Sindh. matee.saba@gmail.com

[3] Liaquat University of Medical & Health Sciences, Jamshoro, Sindh. ahmedsajjad579@gmail.com

[4] Bilawal Medical College, Liaquat University of Medical & Health Sciences, Jamshoro Sindh. sairumhassan@yahoo.com

[5] Azra Naheed Medical & Dental College Lahore. Toobasajjad11@gmail.com

[6] Liaquat Institute of Medical & Health Sciences, Thatta, Sindh. ukasha.farooquematee@gmail.com

[7] Peoples University of Medical & Health Sciences, Nawabshah, Sindh. aishasyeda955@gmail.com

[8] Department of Parasitology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur. jamalmkhan@cuvas.edu.pk

***(Corresponding Author)** Email: sairumhassan@yahoo.com

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Abstract

It is crucial to understand the effects of genetic modification on the reproductive biology of species of Eimeria to determine their utility as vectors of live vaccines. This research involved a transgenic Eimeria mitis parasite that had been engineered to produce the gene chicken interleukin-4 (IL-4) via the assessment of its reproduction ability against the wild-type parasite. After the successful establishment of the IL-4 expressing IL-4 line, patterns of oocyst shedding, the periods of maximum shedding, the total reproductive output, and the sporulation phenotypes were studied under regulated experimental conditions. There was a strong decrease in fecundity in the transgenic E. mitis, relative to the wild-type strain. In several experiments the overall oocyst production by the IL-4 expressing line was less by approximately six-fold, and the maximum shedding time was 22-24 hours later than in birds infected with the wild type parasite. Despite the similarity in general morphology and sporulation capacity between the groups, the transgenic line produced significantly fewer oocysts per shedding interval and had a substantially lower cumulative reproductive output. The results indicate that the expression of IL-4 can alter the reproduction of E. mitis by making it a safer and less fecund parasite, which does not stop its functionality to complete the life cycle, though. This weakened phenotype increases the possibility of IL-4 expressing transgenic E. mitis as a live vaccine carrier, both of limited environmental contamination and specific immunomodulatory effects.

1. Introduction

Eimeria species-induced coccidiosis has continued to be one of the most significant parasitic diseases in poultry production. Coccidia species are more or less pathogenic, and even comparatively low-intensity infections can alter growth performance, feed conversion, and homogeneity in broilers flocks [1]. Eimeria mitis is regarded as one of the least pathogenic among the known species but it nonetheless does not stop at any stage of its life cycle in the host intestine and has an overall contribution to the total coccidial load in the field. Due to this low pathogenicity and stability of development in the host, E. mitis is being investigated as a potential source of genetically engineered live vaccines with growing frequency [2].

With improved technology in transfection, foreign genes have been introduced into a number of Eimeria species, such as Eimeria tenella, Eimeria maxima and more recently E. mitis. It is now possible to investigate new immunomodulatory or antigen-delivery approaches involving live parasites with these breakthroughs [3]. A potential solution is the engineering of Eimeria to produce host cytokines that can have an effect on local immune responses. One of the Th2-related cytokines that is associated with stimulating B-cells differentiation, promoting antibody synthesis, and regulating inflammation is interleukin-4 (IL-4). Delivered into the gut during the development of the parasite, IL-4 can potentially serve as a biologically targeted adjuvant, which is able to stimulate protective immunity without being administered externally [4, 5].

Although the immunological advantages of cytokine expression in transgenic Eimeria have obtained more and more popularity, little is known about the impact of such genetic modifications on the biology of the parasite itself. The key parameters in terms of accessing the viability of genetically modified parasites as a vaccine carrier are reproduction, oocyst output and developmental timing [6]. An effective vaccine strain should have the capacity to achieve its life cycle and induce a desirable immunity and preferably should have a lower fecundity to reduce contamination of the environment and restrict the spread of parasites. Thus, it is the expression of IL-4 that affects the reproductive potential of E. mitis, the study of which defines its suitability to use as a vaccine [3, 7].

Preliminary results of previous transgenic experiments indicated that the expression of foreign proteins can change the growth of the parasite or cause a metabolic strain that can lower its replication capacity. A comparative analysis of the transgenic E. mitis strain that expresses IL-4, with a wild-type one was performed in the present study. The study involved controlled experimental infections to determine the daily shedding of oocysts, the periods of maximum shedding, cumulative reproductive output and fecundity in several independent experiments. The main object of the study was to identify whether expression of IL-4 had an effect on the reproductive functioning of E. mitis and to evaluate whether these adaptations produced a weakened but biologically active parasite. It is necessary to understand these effects to not only describe the biological cost of genetic engineering but also to develop transgenic E. mitis as a safe and effective platform to continue the development of vaccines in the future.

2. Materials and Methods

2.1 Parasite Strains and Experimental Design

Two *Eimeria mitis* strains were used in this study:

1. Wild-type *E. mitis*: maintained under laboratory conditions and routinely propagated in coccidia-free chickens.
2. Transgenic IL-4–expressing *E. mitis*: a genetically modified line expressing chicken IL-4 and EYFP, established through nucleofection and sequential in vivo enrichment.

2.2 Experimental Birds and Housing

Healthy, coccidia-free Arbor Acres broiler chickens were used. Birds were reared in isolators under uniform temperature and hygiene conditions. Commercial feed and clean drinking water were provided ad libitum. Birds were screened to ensure the absence of prior coccidial exposure before inclusion in the experiment. Separate isolators were used for each treatment group to prevent cross-contamination.

2.3 Infection Procedures

At the appropriate age, birds were randomly assigned to one of two experimental infection groups:

- Group 1**:** Infected with wild-type *E. mitis*
- Group 2**:** Infected with IL-4–expressing transgenic *E. mitis*

Each bird received a predetermined number of sporulated oocysts via oral inoculation, ensuring equal challenge doses between groups. Infection doses were selected based on pilot trials demonstrating reliable establishment of infection without inducing severe pathology.

2.4 Daily Monitoring and Clinical Observations

Birds were monitored at least twice daily for:

- signs of coccidiosis,
- changes in fecal appearance,
- appetite and behavior, and
- any abnormalities that could influence oocyst shedding.

No anticoccidial drugs or additional treatments were administered during the experiment.

2.5 Collection of Fecal Samples

Fecal samples were collected daily from each group beginning 5 days post-infection and continuing until 11 days post-infection, covering the full expected shedding period.

To ensure accuracy:

- Fresh droppings were collected directly from paper-lined isolator floors.
- Approximately 10 g of feces per group were gathered daily.
- Samples were homogenized thoroughly to produce a uniform mixture.

2.6 Quantification of Oocyst Output

Oocyst shedding was quantified using a modified McMaster counting method.

Preparation of Counting Suspension

1. Three grams of feces were thoroughly mixed in 42 mL of saturated salt solution.
2. Large debris was removed via sieving.
3. The suspension was mixed thoroughly before loading into counting chambers.

Microscopic Counting

- Two chambers were filled per sample.
- Oocysts in defined grid areas were counted under low magnification.
- Counts were converted to oocysts per gram (OPG) using standard calculation formulas.

2.7 Determination of Total Reproductive Output

Cumulative reproduction was calculated by summing daily OPG values across all shedding days. This provided:

- total oocyst output per bird,
- overall reproductive capacity, and
- comparisons of fecundity between transgenic and wild-type strains.

Additionally, the peak shedding day, defined as the day with the highest OPG value, was recorded for each group.

2.8 Determination of Sporulation Rate and Morphological Assessment

To ensure that potential differences in fecundity were not due to altered sporulation:

- A subset of oocysts from both groups was incubated under optimal sporulation conditions.
- Sporulation rate was recorded after the incubation period.
- Oocysts were examined microscopically for morphological abnormalities.

This assessment ensured that any observed reductions in reproduction were biological rather than technical.

2.9 Statistical Analysis

Data from the experiments were analyzed to determine differences between groups. For each parameter:

- means and standard deviations were calculated,
- daily shedding curves were plotted, and
- group differences in peak shedding time and total output were evaluated.

3. Results

3.1 Onset and Pattern of Oocyst Shedding

Both the wild-type and IL-4–expressing transgenic *E. mitis* successfully established infection in chickens, with detectable oocyst shedding beginning on Day 5 post-infection in both groups. However, the magnitude and timing of shedding differed considerably. In the wild-type group, shedding increased rapidly after onset, reaching peak levels within the expected timeframe for *E. mitis*. The transgenic IL-4–expressing group also began shedding on Day 5 but showed a noticeably slower rise in oocyst output (Fig. 3.1 and 3.2).

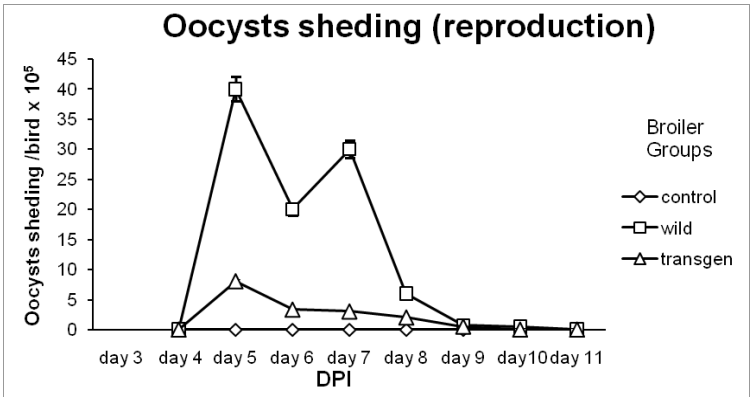
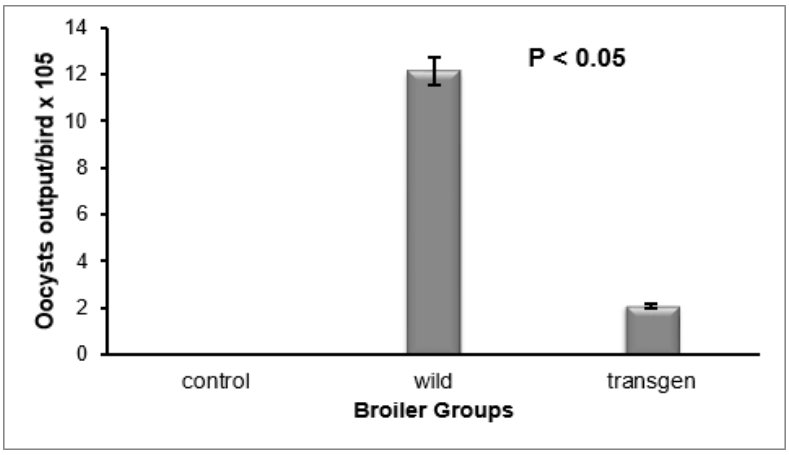


Figure 3.1: Comparison of oocyte shedding pattern. Experiment 1 Group A, Comparison of oocytes shedding pattern of *tE.mitis* with that of the *wE. mitis*. 5 chickens from first group were infected with *tE.mitis*. Oocyst shedding was measured after every 24 hours between day 5 and day 11 post

infection. The data represented one of the three independent experiments with almost similar results and are expressed as the mean ± SD



Mean oocysts output per bird collected from birds treated with EmiChIL-4 and wild type *E. mitis* from 3 to 11 days post-infection.

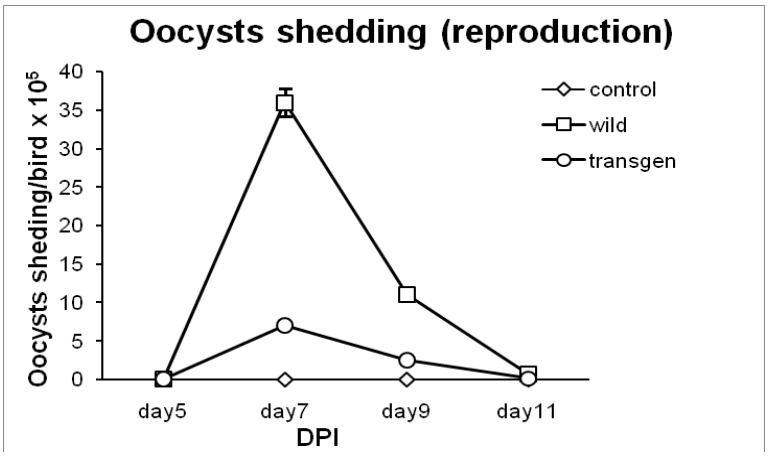
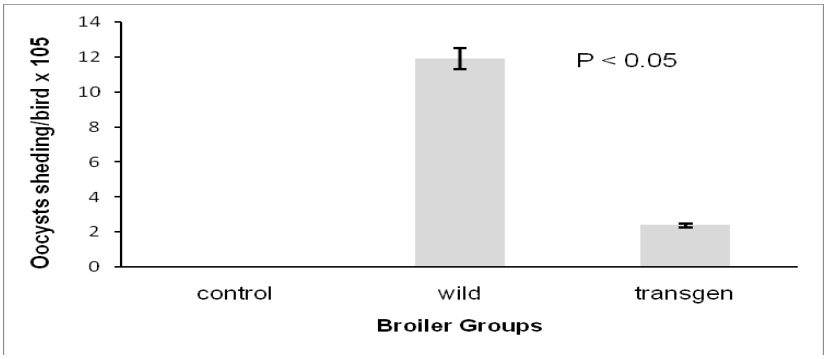


Figure 3.2 Comparison of oocytes shedding pattern

Second Study Group B, Comparison of oocytes shedding pattern of *tE.mitis* with that of the wild type *E.mitis*. Five birds from the first group were infected with *tE.mitis*. Oocytes shedding were measured on day 5, 7, 9 and day11 post infection. The data represented corresponds to three independent experiments with almost similar results and is expressed as the mean ± SD.



Oocyst output per bird of EmiChIL-4 and *wE. mitis*

Mean total oocyst output per bird of EmiChIL-4 and *wE. mitis* on day 5, 7, 9 and day 11 post-infection.

3.2 Delay in Peak Oocyst Shedding in the Transgenic Line

A consistent delay in peak shedding was observed in the IL-4 transgenic parasite line.

- Wild-type peak: typically occurred around Day 6–7 post-infection.
- Transgenic IL-4 peak: occurred approximately 22–24 hours later, generally on Day 7–8.

This delay was reproduced in multiple independent trials, confirming that the shift was linked to the genetic modification rather than experimental variation. The delayed peak suggests that IL-4 expression may alter intracellular development or slow the progression of stages leading to gametogony and oocyst formation (Table 3.1).

Table 3.1. Comparison of Peak Oocyst Shedding Time Between Wild-Type and IL-4 Transgenic *E. mitis*

Parasite Line	Peak Shedding Time (Post-Infection)	Approximate Delay	Observation
Wild-type	Day 6–7	—	Typical sharp peak
IL-4 transgenic	Day 7–8	22–24 hours	Consistent delay across trials

3.3 Marked Reduction in Total Oocyst Output

Across all trials, birds infected with the IL-4–expressing parasite shed significantly fewer oocysts compared with those infected with the wild-type strain (Table 3.2).

Mean cumulative oocyst output:

- Wild-type: approximately 12.1×10^6 oocysts per bird
- IL-4 transgenic: approximately 2.1×10^6 oocysts per bird

This represents a six-fold reduction in reproductive capacity. Daily shedding curves further illustrated this trend:

- The wild-type line produced sharp, high-intensity peaks, followed by gradual decline.
- The IL-4 transgenic line exhibited broader, flatter curves with substantially lower daily oocyst counts.
-

Table 3.2. Characteristics of Daily Oocyst Shedding Curves

Feature	Wild-Type *E. mitis*	IL-4 Transgenic *E. mitis*
Peak intensity	High, sharp peak	Low, flattened peak
Daily oocyst counts	High during peak days	Consistently reduced
Shedding curve shape	Narrow and steep	Broad and flat
Overall fecundity	High	Markedly reduced

3.4 Shedding Intensity and Duration

While both groups shed oocysts across a similar number of days, the intensity of shedding was significantly lower in the IL-4 line (Table 3.3).

Observations included:

- Reduced daily counts throughout the shedding period
- Absence of the sharp spike characteristic of wild-type *E. mitis*
- Slightly prolonged but less intense shedding in the IL-4 group

Despite reduced output, the IL-4–expressing parasite completed its full life cycle, confirming that the genetic modification did not prevent development.

Table 3.3. Shedding Intensity and Duration

Parameter	Wild-Type	IL-4 Transgenic
Duration of shedding	Similar between groups	Similar between groups
Shedding intensity	High	Significantly reduced
Peak spike presence	Present	Absent
Shedding pattern	Short, intense	Slightly prolonged, mild
Life-cycle completion	Complete	Complete

3.5 Sporulation Rate and Oocyst Morphology

Sporulation studies revealed no notable differences between wild-type and transgenic oocysts:

- Both groups achieved normal sporulation under standard incubation conditions.
- Oocyst morphology, including shape, wall appearance, and sporocyst formation, remained typical for *E. mitis*.

This demonstrates that IL-4 expression did not interfere with the later stages of oocyst maturation (Table 3.4).

Table 3.4. Sporulation Rate and Oocyst Morphology

Feature	Wild-Type	IL-4 Transgenic
Sporulation efficiency	Normal	Normal
Oocyst shape	Typical for *E. mitis*	Typical for *E. mitis*
Oocyst wall appearance	Normal	Normal
Sporocyst formation	Normal	Normal
Effect of IL-4 expression	None detected	None detected

3.6 Biological Attenuation of the IL-4–Expressing Line

The combined observations of:

- reduced reproductive output,
- delayed peak shedding,
- diminished shedding intensity, and
- unchanged sporulation quality

This indicates that the IL-4–expressing transgenic *E. mitis* exhibits partial biological attenuation. This attenuation is advantageous for vaccine development because:

- Lower fecundity reduces environmental contamination,
- Delayed and reduced shedding decreases transmission potential,
- Full life-cycle completion ensures adequate antigen exposure for immune stimulation.

Table 3.5. Summary of Biological Attenuation in IL-4–Expressing *E. mitis*

Biological Parameter	Effect of IL-4 Expression	Interpretation
Reproductive output	Reduced	Attenuation
Peak shedding time	Delayed	Attenuation
Shedding intensity	Diminished	Attenuation
Sporulation quality	Unchanged	No impairment
Life-cycle completion	Maintained	Vaccine-relevant
Overall phenotype	Partially attenuated	Favorable for vaccine development

4. Discussion

The wild-type/IL-4-producing transgenic Eimeria mitis comparison can offer valuable information on the mechanism of introducing an immunomodulatory cytokine on parasite biology [8]. The most noticeable fact of this study was the massive reduction of reproductive capacity in the IL-4-expressing parasite line. In all the experiments, the transgenic breed had significantly fewer oocysts and a consistent delay in the peak shedding [9]. These results are great indications that the IL-4 expression poses a biological burden to the parasite and affects its ability to replicate and complete its life cycle with the same efficiency as the wild-type strain [10].

The fact that the shedding of the peak has been delayed by nearly one whole day is especially interesting. The stages of merogony, gametogony, and then oocysts results are indicated by the timing of the peak shedding observed in Eimeria species. A 22-24h shift means that a single or multiple of these developmental stages is implicitly delayed by IL-4 expression. This latency could be evidence of a perturbed cellular response in the infected host cells or high metabolic loads on the parasite during the production and release of IL-4. The expression of those extra proteins also tends to be an energy cost to the parasites, and the witnessed reduced reproduction efficiency is in agreement with this principle [11].

The IL-4–expressing E. mitis line had an ability to pass through its full cycle successfully and generate viable and morphologically normal oocysts despite the fecundity reduction. The rate of sporulation, the appearance of the oocysts, and the features of microscopic analysis were similar to the wild-type strain. This implies that, in spite of the fact that the expression of IL-4 has the power to change the rate and degree of reproduction, it does not affect the parasite developmental transitions occurring in the stage of essential development. These are the essential biological characteristics that any genetically modified parasite should be able to maintain in a vaccine since full development will guarantee that it presents antigens and elicits protective immunity.

The pronounced reduction in the overall oocyst production, which is about 6 times lower than that in the wild-type parasite, is also a good pointer to partial attenuation. This kind of attenuation is useful when it comes to the development of vaccines. Since parasites generate a lower number of oocysts, they lower the chances of environmental contamination, decrease the potential of spreading the disease, and lower the parasite burden in vaccinated flocks. Meanwhile, the weakened parasite is still able to replicate to the extent needed to activate the host's immune system so that the bird is able to acquire a protective immunity without enduring too much intestinal damage.

It is also possible that the results indicate that IL-4 expression could produce local immunogenic responses that disrupt the replication of the parasite. IL-4 is also reported to increase antibody production and control inflammatory reactions at the mucosal surfaces. Even though the immunological consequences are investigated subsequently, it is plausible that the initial expression of IL-4 causes a local environment to change in a manner that constrains the growth of parasites. It will be necessary to find out whether the reproductive reduction is caused mainly by the metabolic cost, immune modulation, or a combination of the two, through further investigation [12].

The study in general illustrates that the expression of IL-4 in E. mitis alters the mating behavior of the parasite in a way that makes it more appropriate as a live vaccine carrier. The resulting effect of decreased fecundity, delayed peak shedding, and maintained developmental ability forms a harmless yet induced immunological response, producing a parasite. Such properties precondition that the IL-4 transgenic E. mitis will prove useful in the future in terms of vaccination application, especially in the direction of antigen delivery and specific immunomodulation.

Conflict of Interest
The authors declare no conflict of interest.

Author`s Contribution
All authors contributed equally.

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