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Research Article

Improving The Sporulation of *Eimeria tenella* Oocysts Purified From Chicken Faeces

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ABSTRACT

Coccidiosis is a major and recurrent intestinal disease in the chicken industry caused mainly by *Eimeria tenella* and it is the best-known and most economically significant species in poultry. Oocyst purification is a common and critically important method used for the study of *Eimeria* species. Various methods have been used for the purification of coccidia oocysts. However, no study has outlined the most effective method for increasing the sporulation of *Eimeria tenella* oocysts. Thus, the study compared and validated the applications of the three common oocyst sporulation methods, viz: (i) forced aeration without a shaker, (ii) forced aeration in combination with a shaker, and (iii) magneto base without forced aeration. A total of 9 individual 21-day-old broiler chickens were inoculated individually with a single 1 mL dose containing 2000 *Eimeria tenella* oocysts via oral gavage. Water and food were provided *ad libitum* throughout the experimental period. Pooled group faecal samples were collected on the 7 day post-infection. The average (range) number of sporulated oocysts counts for the forced aeration without shaking was 2250 (1000-2000), forced aeration in combination with shaking was 2000 (1000-1500), and the magneto base without forced aeration was 5500 (2000-3500). The results from the study indicated that the magneto base without forced aeration was the most effective method for enhancing the sporulation of *Eimeria tenella* oocysts.

Key words: *Eimeria tenella*, magneto base method, oocyst, sporulation

INTRODUCTION

Avian coccidiosis, caused by various species of the protozoan parasite of the genus *Eimeria*, presents a significant threat to the global poultry industry (Gugsa Amede *et al*., 2018). This disease causes substantial economic losses due to decreased productivity, increased mortality rates, and the costs associated with preventive and control measures (Madlala *et al*., 2021). Moreover, coccidiosis seriously raises welfare concerns for infected birds and poses challenges towards ensuring food security by impacting poultry meat and egg production.

Effective management of chicken coccidiosis requires a comprehensive understanding of the parasite's biology, the development of robust experimental models for studying disease pathogenesis and evaluating control strategies (Mesa-Pineda *et al*., 2021). Central to these efforts is the acquisition of sporulated oocysts of *Eimeria tenella*, the most pathogenic species of *Eimeria* in chickens (Chengat Prakashbabu *et al*., 2017). Sporulated oocysts serve as the foundation for experimental infection studies, vaccine development, and drug efficacy testing. However, the sporulation process can be complex with varying results, leading to challenges in obtaining consistently high-quality oocysts for research purposes. The incubation (Al-Quraishy *et al*.,

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2009; Elmahallawy *et al*., 2022), orbital shaker without forced aeration,(Abd-ELrahman *et al*., 2022), forced aeration with or without orbital shaker, or magneto base (Hofmann & Raether, 1990; Guimarães Junior *et al*., 2007; Loo *et al*., 2022) and forced aeration by shaking (Hagag *et al*., 2020) methods have been used for oocyst sporulation. The incubation method, which involves keeping *Eimeria tenella* oocysts in a 2.5% (w/v) potassium dichromate solution at 30°C for 48 hr has been widely used in research (Al-Quraishy *et al*., 2009; Nguyen *et al*., 2021). Some researchers have utilized forced aeration to enhance efficiency while maintaining the same temperature and duration of incubation (Guimarães Junior *et al*., 2007).

Oxygenation is vital for the viability of oocysts, prompting the use of forced aeration in sporulation procedures. This technique has seen refinements over time, including the integration of orbital shaking or the use of a magneto base (Guimarães Junior *et al*., 2007), and also the combination of the magneto base method with forced aeration (Choi *et al*., 2023). However, there are limited comparative studies on these sporulation methods, which makes it difficult to decide the most efficient technique for enhancing the sporulation of *Eimeria* oocyst. Furthermore, limited research has been conducted on the combination of forced aeration with traditional agitation using an orbital shaker in sporulation. Additionally, the potential of the magneto base method in improving the sporulation of *Eimeria tenella* oocysts remains largely unexplored. The primary concern with these proposed methods is the separation of *Eimeria* oocysts from extraneous debris in faecal material. To obtain highly purified oocysts, we utilized a combination of established agitation techniques and forced aeration. This approach allowed us to harvest parasite oocysts with the desired level of purity and viability on a large scale, whilst minimizing physical effort, time, chemical use as well as glassware requirements. Therefore, this study addressed the existing knowledge gap in sporulation studies by identifying the most effective method for enhancing the sporulation of *Eimeria tenella* oocysts. In this study, we attempted to compare the efficiencies of forced aeration without a shaker, forced aeration in combination with a shaker, and magneto base operation without forced aeration methods for oocysts sporulation.

MATERIALS AND METHODS

Experimental animals

A total of 9 individual 21-day-old broilers were used for this experiment. The chickens were housed at the poultry experimental unit of the Putra Agriculture Centre, Universiti Putra Malaysia Campus Bintulu Sarawak (3°12'38.2"N 113°05'32.7"E). The birds were kept in a pen equipped with a pan feeder and drinker. The chickens were fed *ad libitum* with a formulated diet that was free from coccidiostats. The average optimum temperature of the pen was maintained at 25 (24-26)°C. Faecal samples were obtained and were subsequently examined on day 9 during acclimatization to assess oocyst shedding, thereby confirming the absence of coccidia infection in the chickens before the experimental infection.

Experimental design

The wild-type strain of *Eimeria tenella* used in this experiment was originally isolated from birds with clinical signs of coccidiosis during a pilot study. The oocysts were suspended in normal saline, and the saline suspension was used to individually inoculate the chickens on day 21 via oral gavage. All the chickens were individually inoculated with a 2 mL dose of saline suspension containing 2000 oocysts. The faecal samples were collected on day 7 post-infection for oocysts recovery and sporulation procedure.

Sample collection and parasitology technique

The faecal samples were collected on the 7 day post-infection. Approximately, 10 g of faecal material from each pen was collected and transferred into a Falcon tube (50 mL), followed by the addition of 50 mL of deionized water to facilitate softening of the faecal contents. After 1 hr period, the contents were homogenised with a hand blender until thoroughly mixed (Rentería-Solís *et al*., 2020). Subsequently, the samples were stored at 4°C at the Parasitology Laboratory, UPMKB, until further processing. The homogenates were filtered with a wide-mesh wire sieve (1.8 mm pore size) followed by a narrow-mesh wire sieve (1.0 mm pore size). The filtered contents were then transferred into a 50 mL Falcon tube and centrifuged at 750 x g (2560 rpm) for 10 min (Loo *et al*., 2022). This centrifugation step was repeated three more times and the supernatant was discarded and replaced with a saturated salt solution by using a modified flotation technique utilizing a specific gravity of 1.2 (up to the 50 mL mark) to remove the faecal debris (Hagag *et al*., 2020), followed by rinsing twice with deionized water (Loo *et al*., 2022).

Sporulation method

Forced aeration without shaker method

In the first experiment (Figure 1), a modified forced aeration without a shaker method was used for oocyst sporulation. In this method, the *Eimeria tenella* oocysts recovered from the faeces were incubated in a 125 mL Erlenmeyer flask containing 100 mL of 2.5% (w/v) potassium dichromate pumped via airline tubing by an air pump at room temperature of 30°C for 48 hr before enumerating the sporulated oocysts (Hofmann & Raether 1990; Guimarães Junior *et al*., 2007; Hagag *et al*. 2020; Loo *et al*. 2022).

Forced aeration with the shaker method

In the second experiment (Figure 2), the modified forced aeration in combination with a shaker method was used for oocyst sporulation (Aljedaie and Al-Malki., 2020; Loo *et al*., 2022). In this method, the *Eimeria tenella* oocysts were incubated in a 125 mL Erlenmeyer flask containing 100 mL of 2.5% (w/v) potassium dichromate. The sporulation was performed by attaching an airline tubing and aeration diffuser via an air pump to the Erlenmeyer flask, which is placed on an orbital shaker at 100 rpm for 48 hr and maintained at a temperature of 30°C (Hagag *et al*., 2020; Abd-ELrahman *et al*., 2022; Loo *et al*., 2022).

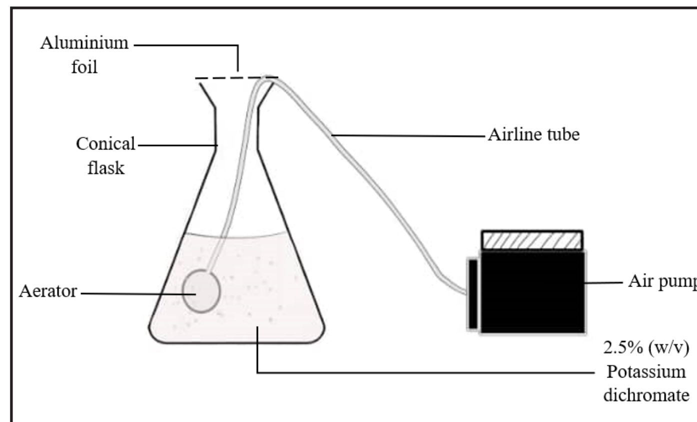

Fig. 1. Forced aeration without shaker method of oocyst sporulation

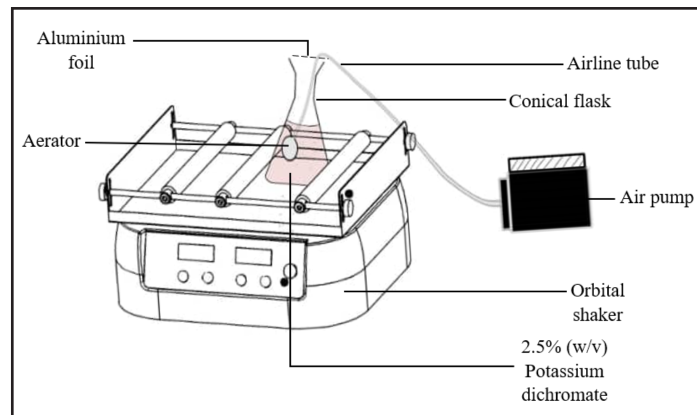

Fig. 2. Forced aeration in combination with the shaking method of oocyst sporulation (Aljedaie and Al-Malki., 2020; Loo *et al*., 2022)

Magneto base without forced aeration

In the third experiment (Figure 3), a magneto base without forced aeration method was used for oocyst sporulation. This method was developed in our laboratory with a slight modification of the method of Loo *et al*. (2022). In this method, *Eimeria tenella* oocyst was placed in 125 mL Erlenmeyer flasks containing 100 mL of 2.5% (w/v) potassium dichromate and placed on the magneto base set at 100 rpm for 48 hr at a temperature of 30°C.

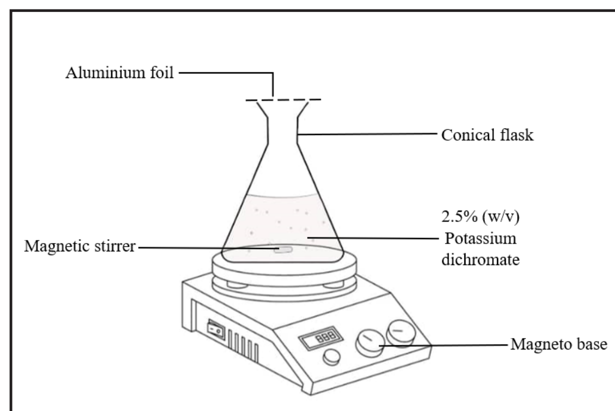

Fig. 3. Magneto base without forced aeration method of oocyst sporulation (Loo *et al*., 2022)

Determination of oocysts count (McMaster counting method)

After 48 hr, 1 mL of suspension was mixed with 9 mL of saturated salt solution in a 15 mL falcon tube. Oocysts were counted by filling the two chambers of a McMaster slide with the suspension using a Pasteur pipette and examined under 10x magnification of a compound microscope (Choi *et al*., 2021). The oocysts counts were determined based on the formula: X / 0.15 x V x d**:** where X = oocysts average, 0.15 = the volume/grid of McMaster slide, V = volume of sample + salt solution, and d = dilution factor (= 10) (Loo *et al*., 2022).

Data management

All data collected was recorded in the Microsoft Office Excel spreadsheet program 2019 and used to compute the means

and range of oocyst recovery rates for each sporulation method i. Pair-wise comparison of oocyst counts was conducted using Tukey's HSD multiple comparison test in SPSS. *P* values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The results obtained from this study have shown that the average (range) recovery rate of sporulated oocysts for the magneto base without forced aeration (5500; 2000 - 3500) was significantly (p<0.05) higher than the forced aeration without shaker (2250; 1000 - 2000) or the forced aeration in combination with shaker (2000; 1000 - 1500),

Table 1. Average (range) of oocysts recovery rates according to sporulation methods

Sporulation method	Average (Range) oocysts	Percentage (%)
Forced aeration without a shaker	2250 (1000 - 2000)^{b}	53
Forced aeration in combination with a shaker	2000 (1000 - 1500)^{b}	85
Magneto base without forced aeration	5550 (2000 - 3500)^{a}	100

Means of oocyst counts represented by different superscripts (a, b) were statistically significant.

This study compared the efficiencies of forced aeration without a shaker, forced aeration in combination with a shaker, and magneto base without forced aeration methods for oocysts sporulation. All three sporulation methods used in this study facilitated oxygen provision during the purification process. Oxygen supply is essential for metabolic processes and the development of sporozoites within *Eimeria tenella* oocysts during sporulation (Mesa-Pineda *et al*., 2021). Therefore, the selection of the most appropriate sporulation method is crucial to ensure optimal oocyst recovery rates.

In this current study, the forced aeration without shaker method only achieved a 53% oocyst recovery rate, which is comparatively lower than the 79.44% oocyst recovery recorded by Rentería-Solís *et al*. (2020). Consequently, it can be inferred that the incubation method is more efficient than the forced aeration method. The most common sporulation method used by researchers is the sporulation and incubation of *Eimeria tenella* oocysts in 2.5% potassium dichromate at room temperature (Al-Quiraishy *et al*., 2009; Rentería-Solís *et al*., 2020), which is generally thought to produce a much higher oocyst recovery rate.

In a study conducted by Hofmann and Raether (1990), White Leghorn chickens were exposed to 15,000 *Eimeria tenella* oocysts, and oocyst sporulation from faecal samples was performed using the forced aeration without shaking method. The reported recovery rates ranged from 49.4% to 95.2%, indicating high variability in the efficacy of this sporulation technique. Similarly, Guimarães Junior *et al*. (2007) utilized the forced aeration without shaker method to harvest sporulated oocysts from faecal samples of Hubbard chickens challenged with 116,000 doses of *Eimeria tenella*. Their study documented sporulated oocyst recovery rates ranging from 12.29% to 99.13%, highlighting the variability in oocyst recovery across different experimental conditions. Thus, sporulated oocyst recovery rates from similar methods may be influenced by the source of oocyst and the recovery method, as seen in our study, where a 100% recovery rate was recorded using the novel magneto base method, 85% using the forced aeration in combination with shaker, and 53% using the forced aeration without a shaker. Both previous studies and the current research exhibit a notably high rate of oocyst recovery, with the present study yielding a recovery rate of 53%. However, further comparative studies are warranted to elucidate the most efficient method for oocyst recovery in different experimental contexts.

According to Venkateswara Rao *et al*. (2015), the sporulation of oocysts primarily relies on temperature, humidity, and access to oxygen. This agrees with the current study's results, where a comparison between forced aeration with and without a shaker demonstrates higher oocyst recovery rates when a shaker is employed. Specifically, in this study, the oocyst recovery rate for the forced aeration method combined with a shaker reached 85%, significantly surpassing the recovery rate achieved when using forced aeration alone. The combination of forced aeration with a shaker enhances oxygenation levels, thereby facilitating the sporulation process and resulting in improved oocyst recovery.

The magneto base without forced aeration produces maximum oocyst recovery, which, as is demonstrated in the present study, is the best method among the three evaluated. The magneto base influences oxygen levels during sporulation, creating turbulence and increasing surface area, allowing for better oxygenation of the solution. This method enhances oxygen levels in a solution through mixing and aeration and increases sporulation levels because oxygen is one of the most important factors for oocyst sporulation (Venkateswara Rao *et al*., 2015). Additionally, the magneto base without forced aeration was the most effective method, possibly due to its ability to enhance oxygen levels through turbulence and increased surface area during sporulation. The forced aeration method may have not created sufficient and uniform turbulence in the media whereas the combined agitation and forced aeration may have created too much turbulence. Thus, our results suggest that oxygen supply plays to enhance the sporulation of *Eimeria tenella* oocysts harvested from chicken gut. Other studies have shown that for two species of *Eimeria* from both chickens and turkeys, their site of oocyst development in the gut affects the rate of sporulation (Répérant *et al*., 2021). For *E. meleagrimitis*, which inhabits the gut, studies indicate that oocyst sporulation ability increases as they move through the intestine, influenced by oxygen supply (Répérant *et al*., 2021). Sporulation rates were low (below 20%) for oocysts collected in the duodenum, whereas those collected from the midgut and lower intestine showed much higher sporulation efficiency (around 80%) compared to those from the duodenum at the same time (Répérant *et al*., 2021). A deeper understanding of factors such as oxygen supply, temperature, and moisture, which influence the sporulation process, could lead to new strategies to reduce or prevent sporulation, thereby helping to control these parasites and mitigate their effects on poultry hosts. Other work also strongly suggests that the sporulation of *E. maxima* oocysts was affected by the moisture content of the litter (Waldenstedt *et al*., 2001). Sporulation of *E. maxima* was most efficient under the driest conditions (16% moisture content), and least efficient in samples with the highest moisture levels (62%), suggesting that oxygen availability is reduced in damp faecal material (Waldenstedt *et al*. 2001).

CONCLUSION

The effectiveness of various methods for sporulating *Eimeria tenella* oocysts was studied, including incubation, forced aeration without a shaker, forced aeration with a shaker, and magneto base without forced aeration. Both prior and current research indicate that the incubation method consistently yields a high rate of oocyst recovery, suggesting its efficiency. However, the forced aeration without shaking method also showed promising results, with the present study achieving a notable oocyst recovery rate of 53%. Furthermore, when a shaker was employed in conjunction with forced aeration, the oocyst recovery rate significantly increased to 85%, demonstrating the importance of oxygenation in the sporulation process. Additionally, the magneto base without forced aeration was the most effective method, possibly due to its ability to enhance oxygen levels through turbulence and increased surface area.

In conclusion, while the incubation method remains a reliable choice for oocyst recovery, the forced aeration, when combined with a shaker and the magneto base without forced aeration methods, shows promise for improving oocyst recovery rates. Future research should focus on optimizing these methods to maximize oocyst recovery under various experimental settings.

Through systematic inquiry, this study will not only refine experimental protocols but also contribute to the evolution of poultry research methodologies and deepen our understanding of *Eimeria tenella* biology and pathogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

Not applicable.

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