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Original Article

Statistical Prediction of the Peak Point (Time) Required for Release of Maximum Number of Sporocysts after Eimeria Tenella Oocyst Excystation

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ABSTRACT

The excystation of sporozoites from the oocyst of genus *Eimeria* is essential for conducting successful in vitro assays on the parasite. We tried to find the most efficient excystation conditions using glass beads for the in vitro excystation of *E. tenella* oocysts. The oocyst suspension was mixed with glass beads in a ratio of 1:1, and vortexed by various combinations of vortexing times and speeds in a Vortex Mixer. We analyzed the sporocyst-release data by regression analysis, and the peak point (duration) of excystation was predicted after differentiation of the obtained regression equation. The results indicated that the 1-mm glass beads at 2,000 rpm could release the maximum number of sporocysts in 1.64 min. Thus, excystation of *E. tenella* oocysts was considered to be the most effective under these conditions. Our data presented the best conditions for efficient excystation of *E. tenella* oocysts.

INTRODUCTION

Apicomplexan parasites belonging to the genus *Eimeria*, the causal agents of coccidiosis, have huge impact on livestock, particularly on the poultry industry (Marugan-Hernandez *et al.*, 2017; Shirley *et al.*, 2004). Coccidiosis is the most common parasitic disease that affects the broiler industry, and the lack of prevention is estimated to cause worldwide losses of about $ 3 billion per year (Williams, 1999; Noack *et al.*, 2019). Seven species of *Eimeria* have been identified as the causal agents of coccidiosis in chickens (Shirley *et al.*, 2007); these seven species develop in specific parts of the chicken's intestines, and their pathogenicity is different for each species (Williams, 1999; Shirley *et al.*, 2013). *E. acervulina*, *E. maxima*, and *E. tenella* are the most commonly found species in intensively reared chickens, and *E. tenella* is considered to be the predominant species that causes serious damage to chickens (Shirley *et al.*, 2013; Macdonald *et al.*, 2017).

Because coccidiosis is one of the most dangerous and economically burdening poultry diseases worldwide, over the past few decades extensive research on *Eimeria* has been carried out for various purposes (Shirley *et al.*, 2013; Blake *et al.*, 2011; Lee *et al.*, 2011). For the successful in vitro study of the parasite, excystation of sporozoites from *Eimeria* oocyst constitutes the first step. Therefore, many researchers have been conducting mechanical disruption of *Eimeria* oocysts.

However, *Eimeria* oocyst wall has a robust biological structure, which makes the release of sporocysts and sporozoites difficult (Belli *et al.*, 2006; Stotish *et al.*, 1978). Usually Teflon-coated tissue homogenizers (Krücken *et al.*, 2008; Chai *et al.*, 1989) or glass beads (Blake *et al.*, 2011; Haug *et al.*, 2007; Tomley 1997; Velkers *et al.*, 2010) are used to crush the oocyst wall. Glass beads are an effective tool for oocyst excystation. In the case of E. acervulina, excystation is done by vortexing glass beads, irrespective of their size, at 3,000 rpm for 5 min (Cha *et al.*,

2014). The excystation of *E. tenella* is the most effective upon vortexing with 1-mm glass beads for 30 s to 1 min (You, 2014). Thus, for optimal oocyst excystation, it is necessary to optimize the size of glass beads, vortexing durations, and speed for each species of *Eimeria*. In this study, we describe the efficacy of different sizes of glass beads with various vortexing speeds and durations on the excystation of *E. tenella* oocysts.

MATERIALS AND METHODS

Parasite

Pure line *E. tenella* oocysts (GenBank: FJ447468.1) were amplified and sporulated in our laboratory according to standard procedures (Holdsworth *et al.*, 2004). Only the normal forms of sporulated oocysts were selected. The oocysts were counted using a hematocytometer, which was adjusted to contain 1.5×10^6 oocysts in 300 µL, and they were transferred to a 2 mL microcentrifuge tube. A total of 250 sample tubes were prepared for this experiment. The sporulated oocysts in each tube were physically excysted by varying the size of glass beads, vortexing speed, and vortexing durations (Table 1).

Oocyst excystation and counting

The oocyst suspension was mixed with the glass beads at a ratio of 1:1, and vortexed in a Vortex Mixer with selective mixing modes and variable speed control options (Model-VM 96B, Jeio Tech, South Korea) by adjusting the vortexing time and speed according to the combinations given in Table 1. Oocysts and excysted sporocysts were counted according to the method of Cha *et al.* (2014). The experiments were repeated four times to obtain an average value.

Table 1 – Glass bead size, vortexing speed and duration used in the present experiment.

Glass Bead Size (mm)	Vortexing Speed (rpm)	Vortexing Duration	Replication
0.5 mm 1 mm 2 mm 2.5 mm	1,000 2,000 3,000	30 s 1 min 2 min 3 min 5 min	4

Determination of sporozoite viability

E. tenella oocysts were crushed with glass beads to obtain free sporocysts. The sporocysts were washed once in PBS and then incubated for 90 min at 40°C in a hybridization oven (MS major science, USA) with an enzyme solution [Porcine Trypsin (Sigma) 25 mg and Sodium taurodeoxycholate hydrate (Sigma) 75 mg dissolved in 10 mL PBS]. After excystation, the free sporozoites were centrifuged at 3,000 rpm for 10 min and resuspended with pre-warmed PBS. The viability of the sporozoites was determined by trypan blue dye exclusion method (Holdsworth *et al.*, 2011).

Statistical analyses

Data obtained in this study were analyzed by Scheffe's Test with ANOVA and progressing regression using SAS® ver. 9.2.

Predicted peak point (time) of sporocyst number after excystation

Sporocyst data after excystation were analyzed and a functional equation was developed using regression in SAS. The peak point (vortexing duration time) was calculated by differentiation of the equation for each glass bead size and vortexing speed. The sporocyst number at the peak point was calculated by substituting the calculated peak point (vortexing duration time) in the functional equation (Barnett *et al.*, 2008).

RESULTS

Oocysts crushing with various sized glass beads, vortexing duration and speed

We observed that the rupture of *E. tenella* oocysts increased with increasing vortexing speed and duration, regardless of the size of the glass beads ($p<0.01$). At a vortexing speed of 1,000 rpm, large number of oocysts remained uncrushed, but at vortexing speeds of 2,000 and 3,000 rpm, the number of normal oocysts was significantly reduced with increasing vortexing duration ($p<0.01$). Our results also demonstrated that the oocysts were most effectively crushed when 1-mm glass beads were used (Table 2).

The best results were obtained under the following conditions: 1-mm glass beads at 2,000 rpm vortex speed for 1 min. For glass beads of all sizes, a relatively small number of sporocysts were released at 1,000 rpm compared to the number released by the vortex speeds of 2,000 and 3,000 rpm, regardless of all vortexing duration. We found that 1-mm glass beads released the highest number of intact sporocysts at 2,000 rpm with a vortexing period of 1 to 2 min compared to the number released by other glass beads under the same condition. In addition, in case of 0.5, 2 and 2.5 mm glass beads, a relatively higher number of sporocysts was released at 2,000 rpm compared to other vortex speeds. We found that the vortexing speed of 2,000 rpm was efficient for releasing intact sporocysts with glass beads of all sizes (Table 3).

Table 2 – Changing pattern of oocyst number after crushing with different sized glass beads through specified vortexing duration and speed.

Glass bead size (mm)	Duration (min)	**1,000 rpm**	**2,000 rpm**	**3,000 rpm**
		Oocysts number	Oocysts number	Oocysts number
	0.5	294.75± 18.87 ^{bA}	290.50±28.03^{aA}	32.00± 4.69^{aB}
	1	273.25±193.26^{bA}	231.25±35.32^{bB}	12.00± 3.37^{bC}
0.5	2	252.00± 16.41^{bA}	119.00± 8.76^{cB}	1.75± 1.71^{cC}
	3	444.25± 44.49^{aA}	88.00±16.87^{cdB}	0.25± 0.50^{cC}
	5	391.50± 16.54 ^{aA}	43.50± 9.95 ^{dB}	0.00± 0.00^{cC}
	0.5	335.00± 11.37^{A}	129.25±20.76^{aB}	20.75± 3.95^{aC}
1	1	319.00± 30.47^{A}	62.00± 3.92^{bB}	1.50± 0.58^{bC}
	2	313.25± 43.12^{A}	4.50± 1.91^{cB}	0.00± 0.00^{bB}
	3	391.50± 18.36^{A}	3.00± 1.15^{cB}	0.00± 0.00^{bB}
	5	337.25± 51.21^{A}	0.00± 0.00 ^{cB}	0.00± 0.00^{bB}
	0.5	248.00± 34.55ab^{A}	174.50±36.46^{aB}	88.00±18.31^{aC}
2	1	282.75± 15.97a^{A}	100.75±21.36^{bB}	28.00± 7.12^{bC}
	2	228.25± 21.09ab^{A}	50.50± 6.56^{cB}	2.00± 2.45^{cC}
	3	259.75± 19.81a^{A}	16.25± 3.40^{cdB}	1.25± 0.50^{cB}
	5	190.00± 30.30b^{A}	2.00± 1.41^{dB}	0.00± 0.00^{cB}
	0.5	346.50± 25.83^{A}	222.00±30.88^{aB}	133.75±29.43^{aC}
2.5	1	344.75± 63.33^{A}	162.25±26.34^{bB}	63.75± 6.65^{bC}
	2	352.00± 46.21^{A}	45.50± 6.56^{cB}	18.00± 7.79^{cB}
	3	265.75± 19.22^{A}	31.00± 5.23^{cB}	8.00± 4.08^{cC}
	5	279.00± 45.28^{A}	6.75± 2.63^{cB}	0.25± 0.50^{cB}

Mean ± SD.

Small-character: comparison among the vortexing duration (p<0.01).

Large character: comparison among the vortexing speed (p<0.01).

Table 3 – Changing pattern of sporocyst number after crushing with different sized glass beads through specified vortexing duration and speed.

Glass bead size (mm)	Duration (min)	**1,000 rpm**	**2,000 rpm**	**3,000 rpm**
		Sporocysts number	Sporocysts number	Sporocysts number
	0.5	48.25±14.77^{C}	367.75±5.13^{cB}	**664.50±33.51**^{aA}
	1	31.00± 4.55^{B}	408.00±1.14^{bA}	405.00±24.24^{bA}
0.5	2	45.50±17.18^{C}	**507.25±9.36**^{aA}	103.50± 7.55^{cB}
	3	64.00±33.46^{B}	445.50±5.10^{abA}	22.75± 1.26^{dB}
	5	42.25±19.19^{B}	313.25±2.28^{dA}	2.50± 0.58^{dC}
	0.5	84.00± 3.16^{cC}	679.50±8.91^{bB}	**759.25±65.99**^{aA}
1	1	96.25±17.56^{cC}	**805.25±9.43**^{aA}	554.75±50.40^{bB}
	2	103.00±9.42^{cC} 198.00±20.80^{bB} 348.00±21.40^{aA}	785.00±2.10^{aA}	237.25±54.88^{cB}
	3	198.00±20.80^{bB}	662.00±3.38^{bA}	136.25± 7.63^{cdC}
	5	348.00±21.40^{aA}	338.75±4.99^{cA}	34.75± 4.19^{dB}
	0.5	46.75±23.44^{cC}	345.25±1.37^{cB}	497.00±23.12^{aA}
2	1	49.00±15.77^{cB}	571.25±2.37^{aA}	**554.25±75.95**^{aA}
	2	147.50±13.43^{bC}	**630.50±2.12**^{aA}	369.25±33.18^{bB}
	3	168.00±15.56^{abC}	575.25±1.28^{aA}	246.25±15.00^{cB}
	5	207.25±19.52^{aB}	447.75±0.50^{bA}	108.50±14.39^{dC}
	0.5	46.00± 8.37^{dC}	341.75±7.11^{dB}	505.25±42.75^{abA}
2.5	1	80.25±14.95^{bB}	525.75±7.31^{bA}	**546.50±23.46**^{aA}
	2	98.50±18.57^{bC}	630.25±8.14^{aA}	506.75±20.69^{abB}
	3	56.00± 8.76^{cC}	**681.00±6.38**^{aA}	444.75±24.51^{bB}
	5	142.50±13.10^{aC}	483.25±4.25^{bA}	279.75±22.13^{cB}

Mean ± SD.

Small character: comparison among the vortexing duration (p<0.01).

Large character: comparison among the vortexing speed (p<0.01).

Characters in bold: higher (average) number of intact sporocysts obtained using the designated RPM.

Predicted peak point of sporocyst number after excystation

At 2,000 rpm, the 1-mm glass bead data were calculated using the following regression equation:

$Y = -39.116x^2 + 128.43x + 665.76$ ($R^2=0.94341$) (Fig. 1). The predicted peak point of vortexing duration for 1-mm glass beads was 1.64 min, and the regression equation predicted that 771 sporocysts would be released at this peak point (Table 4). The predicted peak point was the most efficient vortexing duration for sporocyst excystation using 1-mm glass beads at 2,000rpm.

Viability of released sporozoites

Trypan blue dye exclusion, the method which consists in placing the oocysts with trypan blue solution in hematocytometer and counting the dyed ones, was used to ascertain the viability of the released sporozoites (Cha *et al.*, 2014). The *E. tenella* oocysts were crushed with 1-mm glass beads at 2,000 rpm for 30 s, 1 min, 2 min, 3 min, and 5 min to acquire intact sporocysts. We found that the sporozoites had a viability exceeding 98% at all five time points (i.e., 30 s, 1, 2, 3, and 5 min), and there was no difference (data not shown).

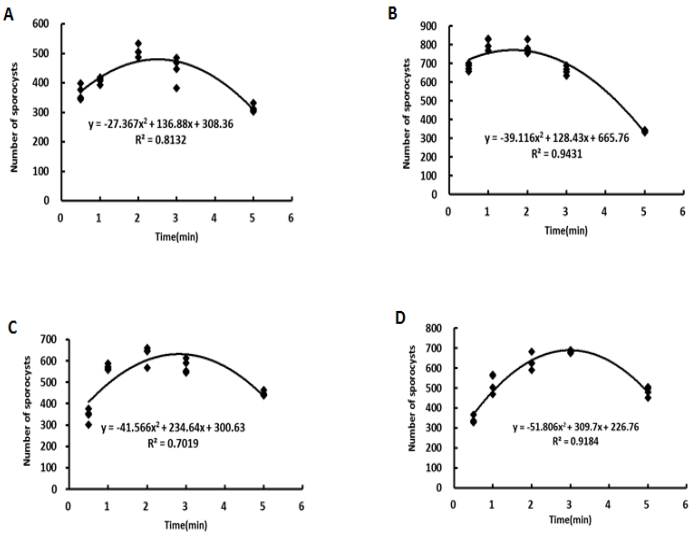

Figure 1 – Regression analysis for each glass bead size at 2,000 rpm vortexing speed. (A: 0.5mm, B: 1mm, C: 2mm and D: 2.5mm).

DISCUSSION

The excystation of sporulated *Eimeria spp*. oocysts is of great importance for molecular, biochemical, immunological and in vitro experiments for studies related to this parasite (Cha *et al.*, 2014; López-Osorio *et al.*, 2020). Therefore, the development of an efficient excystation method for oocysts of *Eimeria spp*. is essential. Various methods have been used to disrupt the walls of the oocysts of *Eimeria spp*., among which glass beads have proven to be the most effective (You, 2014; Zhao *et al.*, 2001).

Previous reports say that the release of sporocysts of *E. tenella* was most efficient when the duration of the treatment was 30 s to 1 min with 1-mm glass beads (You, 2014). In the case of *E. acervulina*, the highest number of intact sporocysts were released upon treatment with 1-mm glass beads at 2,000 rpm for 3 min (Cha *et al.*, 2014). In the current study, we tried to determine the most effective size of the glass beads, and the vortexing speed and duration for the excystation of *E. tenella* oocysts. To achieve a large number of viable sporozoites using glass beads, care and precision is required to avoid cracking the sporocysts while crushing the oocysts. In the present study, we observed that the 1-mm glass beads released the highest number of intact sporocysts with vortexing at 2,000 rpm for 1 min (Table 3, Fig. 1). In order to determine the future outcomes of the different sizes of glass beads on safe release of intact sporocysts, we analyzed the sporocyst-release data by regression analysis to ascertain the peak point (duration time) and calculated the number of sporocysts released at the predicted peak point. We only analyzed the data obtained at the vortexing speed of 2,000 rpm because this speed displayed the highest number of sporocysts (Table 4). The regression analysis revealed that the maximum number of sporocysts could be released in 1.64 min at 2,000 rpm by the 1-mm glass beads.

Our calculation using regression for 3,000 rpm could not provide the peak sporocyst production point for the 0.5, 1, and 2 mm glass beads, as there were

Table 4 – Result of peak point (duration time) and sporocyst number calculated by analysed regression equation at 2,000 rpm each glass bead size.

	Size of glass bead (mm)	Statistical predicted peak point (vortexing duration: min)	Number of calculated sporocysts at predicted peak point	Significant difference for regression equation
A	0.5	2.50	479.5163	P<0.01
B	**1**	**1.64**	**771.1788**	**P<0.01**
C	2	2.82	631.7654	P<0.01
D	2.5	2.99	689.6122	P<0.01

Characters in bold: The peak vortexing duration point with the peak and it's calculated sporocysts number.

continuous decrements in the number of sporocysts from the initial vortexing duration, i.e., 30 s.

Notably, at the vortexing speed of 3,000 rpm with relatively longer vortexing duration (3 and 5 min), the larger glass beads (2 and 2.5 mm) released a small number of sporocysts (Table 3), while crushing most of the oocysts (Table 2). These results signified that this combination is advantageous for extracting DNA, but disadvantageous for obtaining intact sporocysts. For DNA extraction (more crushing is required) all glass beads acted efficiently upon vortexing at 3,000 rpm for 5 min. The four different-sized glass beads provided enough extracted DNA for subsequent PCR amplification and species identification, so they could be used for this purpose at 3,000 rpm for 5 min or more. Based on our results, we recommend 1-mm glass beads, as the 1-mm-sized glass beads were efficient in crushing all oocysts at 3,000 rpm in 2 min (Table 2).

The viability of the *E. tenella* sporozoites released from the sporocysts was also verified. The viability assay was conducted after crushing the oocysts with 1-mm glass beads and releasing the sporozoites from the sporocysts. Trypan blue dye exclusion method revealed that at each time point, the viability of sporozoites was >98%, indicating that the use of 2,000 rpm vortexing speed was safe for obtaining live sporozoites.

CONCLUSION

Conclusively, *E. tenella* oocysts should be mixed 1:1 with 1mm glass beads and vortexed at 2,000rpm for 1.64 min to release the highest number of sporocysts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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