RECOVERY AND SPORULATION OF BOVINE *EIMERIA* OOCYSTS AFTER EXPOSURE TO SUB-ZERO TEMPERATURE

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Abstract. The persistence of bovine *Eimeria* oocysts under natural conditions between being shed in faeces and infecting new animals has not been studied in detail. Knowledge on how sub-zero temperatures and microbes affect the existence of *Eimeria* is needed to fully understand the parasite's ecology. This study addressed the topic experimentally by focusing on the effects of storage medium and temperature on the numbers and sporulation ability of the oocysts. Tubes containing either unsporulated or sporulated oocysts were kept at either 22 or -18 °C for one month in either a non-sterile medium (faecal suspension) or oxidizing sporulation medium (2% $K_2Cr_2O_7$). After the incubation period, the numbers of oocysts were counted using quantitative flotation. Freezing reduced the number of oocysts within 75.9%–91.5%, indicating the oocysts can handle extreme thermal stress. Fewer unsporulated oocysts weres counted when frozen in an oxidizing medium compared with those stored in a faecal suspension. At room temperature more oocysts were found in the oxidizing medium than in the faecal suspension, indicating a significant effect of microbes on the persistence of oocysts. However, sporulation did not affect the oocysts ability to tolerate freezing. A second batch of unsporulated oocysts was stored at the two temperatures for a month in the faecal solution and then sporulated at room temperature for a month. *E. alabamensis*, *E. ellipsoidalis*, and *E. zuernii* were capable of sporulate after freezing, suggesting that these species handle the thermal stress better than other species, such as *E. bovis*.

Keywords: *Eimeria*, bovine, ecology, freezing, sporulation, coccidian.

Introduction

Unsporulated and noninfective *Eimeria* oocysts are released into the intestinal lumen of the hosts in the final phase of their life cycle and exit the animal with faeces. Outside the host, the oocysts become infective once sporulated. In severe infections of cattle, clinical signs such as diarrhoea commonly occur and cause health problems and occasional mortalities in calves (Daugschies and Najdrowski 2005). In the environment, the parasite is exposed to different natural conditions, which can be harsh and naturally limit the concentration of the parasite. During winters in the Northern hemisphere, thermal stress is considered a major factor for survival of protozoan parasites (Jansson 1990; Svensson 1995; Robertson and Gjerde 2004). Under snow cover, the surface temperature of the soil does not drop far below zero, but in the absence of snow it may reach below -15 °C (Sharratt et al. 1992).

Different *Eimeria* species might survive differently in the environment. It has been suggested that *Eimeria* oocysts from chicken would be more resistant to environmental stresses after sporulation (Horton-Smith and Long 1954), while studies on this in bovine *Eimeria* species are lacking. Sub-zero temperatures seem to impair bovine *Eimeria* oocysts' ability to sporulate (Rind and Brohi, 2001) but it is unknown to what extent these temperatures reduce the number of the parasites in the environment. Oxygen-rich chemicals, such as potassium dichromate ($K_2Cr_2O_7$), are used to shorten sporulation time of oocysts in conditions favouring the development of the parasites (Daugschies and Najdrowski 2005). Potassium dichromate is toxic to microorganisms and plants (European Chemicals Bureau 2005) but the oocyst wall of *Eimeria* seems to provide sufficient protection to the parasite as it is possible to successfully infect animals after sporulation in this media (Bangoura et al. 2007).

In the present study, we tested how the following experiments affected bovine *Eimeria* oocysts after one month incubation at -18 °C compared with 22 °C:

Exp. 1: the effect of faecal suspension or oxidizing sporulation medium ($K_2Cr_2O_7$) on the recovery of unsporulated oocysts.

Exp. 2: the effect of sporulation prior to freezing on the recoverability of oocysts.

Exp. 3: the ability of the oocysts to sporulate after freezing for one month in a faecal suspension.

Materials and Methods

Isolation of oocysts from faeces

Two batches of oocysts were prepared from cattle faeces, from naturally infected calves shedding *Eimeria* oocysts above 80,000 oocysts per gram (OPG) faeces. Batch 1 was used in Exp. 1 and Exp. 2, and batch 2 was used in Exp. 3. The faeces for the two batches were both prepared by mixing with tap water and filtered through double layered gauze. The suspension was spun down at 1209 g for 10 minutes (22 °C), supernatant was discarded, and the pellet resuspended in 100 ml tap water. During constant stirring with a magnetic stirrer, the solution was divided in four tubes.

Species composition

The mixture of species present in the faecal samples was determined by their unsporulated morphology (Levine 1985). Batch 1 contained: *E. ellipsoidalis* (80.5%), *E. zuernii* (15.5%), *E. bovis* (2.0%), *E. auburnensis* (2.0%), *E. wyomingensis* (<1%), *E.*

subspherica (<1%). Batch 2 contained: *E. bovis* (28.9%), *E. ellipsoidalis* (26.8%), *E. zuernii* (24.2%), *E. canadensis* (10.4%), *E. alabamensis* (4.5%), *E. auburnensis* (2.0%), *E. subspherica* (1.8%), and *E. cylindrica* (1.3%). Of all the examined oocysts (N=596) in batch 2, 0.3% of had sporulated at the beginning of the experiment.

Experimental setup

An experimental overview is shown in Fig. 1. The aliquots of samples tested were given codes to describe the meaning of the experimental group: "I" for batch 1 of oocysts, "II" for batch 2 of oocysts, "U" for unsporulated, "S" for sporulated, "N" for oocysts in a natural faecal suspension, or "O" in oxidizing medium, "R" when stored at room temperature (22 °C), "F" when frozen at -18 °C±1.7°C STDV.

At day 0 the aliquots were diluted 7 times in their storage medium: faecal suspension diluted in tap water or sporulation medium (2% w/v $K_2Cr_2O_7$). Final concentration of oocysts (4,900±604 STDV oocysts/ml) was determined by examining 12 x 1 ml samples. Exp. 1: of the original solution [I] 20 x 1 ml samples were stored at 22 °C in either a natural faecal suspension [UNR] or an oxidizing medium [UOR], or stored at -18 °C in either faecal suspension [UNF] or the oxidizing medium [UOF] for 30 days before analysis. Exp. 2: oocysts in the original solution [I] were sporulated at room temperature in sporulation medium for 30 days, before storing 20 x 1 ml samples at 22 °C [SOR], or at -18 °C [SOF] for 30 days before analysis.

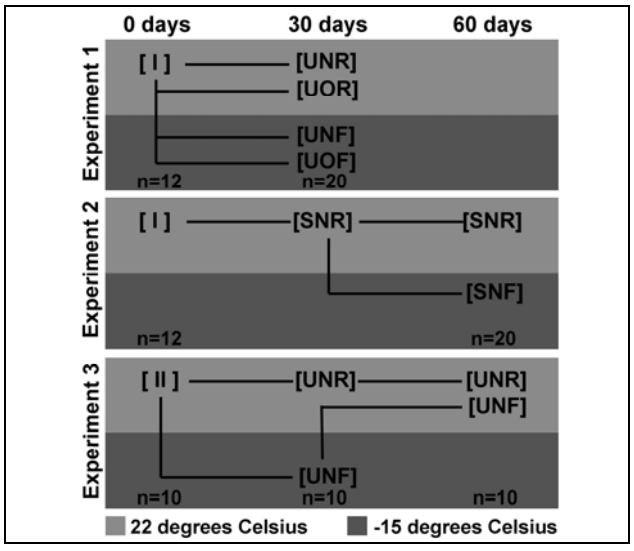

Fig. 1. **Study overview showing the time and number of aliquots analyzed from each group. [I] oocyst batch 1, [II] = oocyst batch 2, [UNR] = unsporulated oocysts in a natural faecal suspension at 22 °C, [UNF] = unsporulated oocysts in faecal suspension at -18 ^{0}C, [UOR] = unsporulated oocysts in an oxidizing medium (2% $K_2Cr_2O_7$) at 22 °C, [UOF] = unsporulated oocysts in an oxidizing medium (2% $K_2Cr_2O_7$) at -18 °C, [SNR] = sporulated oocysts in a natural faecal suspension at 22 °C, [SNF] = sporulated oocysts in a natural faecal suspension at -18 °C.**

For Exp. 3 the final concentration (15,893 oocysts/ml±219 STDV) of the sample in a faecal suspension diluted in tap water was established by counting 10 samples [II]. Oocysts in faecal suspension from [II] were stored in 20 x 1 ml aliquots either at 22 °C [UNR] or -18 °C [UNF] for 30 days before analysing 10 samples of each, and sporulating the remaining samples at 22 °C for an additional 30 days before analysing them. All aliquots were stored in 5 ml plastic tubes and sealed with a plastic cap.

Freezing and thawing

Samples to be stored at -18 °C±0.9 °C 95% confidence interval (CI) in a freezer were kept 4 hours at 5 °C before being frozen. For thawing, the samples were kept 24 hours at 5 °C before being prepared and read.

Sporulation of oocysts

In Exp. 2, one 25 ml aliquot of batch 1 was stored at room temperature in the sporulation medium (2% $K_2Cr_2O_7$), aerated three times a week and mixed on the tipping table. After one month, 76% [69-82 95% CI] of the oocysts were counted to have sporulated by examining oocysts in 12 sub-samples.

In Exp. 3, following incubation of one month at either 22 or -18 °C in a faecal suspension, the aliquots were kept at 22 °C and aerated with a pipette three times a week for a month before analysing for sporulation by species.

Reading of samples

Each sample (1 ml) was mixed with 3 ml sugar-salt solution (specific gravity = 1.24) and transferred to a McMaster chamber (Kruuse, Langeskov, Denmark). After 5 minutes of flotation, the number of oocysts was quantified by counting them in both chambers at x200 magnification and multiplying the obtained count with 13.3 (4-times diluted sample / 0.3 ml). Only intact oocysts were counted. In Exp. 1 and Exp. 2, only the number of oocysts was counted. In Exp3, the number of sporulated and unsporulated oocysts for each species was determined.

Statistical analyses

Data analysis was performed using R v. 2.8.0 (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Vienna, Austria). Unpaired t-test was used for comparing different groups and proportions of sporulated oocyst species. Two samples from the group [UNR] and three from [UOR] were removed from the dataset because of the evaporation of liquid due to the tubes having faulty seals.

Results

Exp. 1: Effect of oxidizing medium on unsporulated oocysts handling freezing

In both [UNF] and [UOF] significantly fewer oocysts were detected following exposure to -18 °C after 1 month ($P<0.001$) compared to aliquots kept at room temperature (Fig. 2). The reduction in the number of recovered oocysts from samples kept at room temperature compared with the sub-zero temperature was 75.5±4.1% 95% CI in the faecal suspension and 91.5±2.1% 95% CI in the oxidizing medium. Following freezing, more oocysts were recovered in [UNF] than [UOF] ($P<0.001$), whereas

the opposite was observed for samples, [UNR] and [UOR], stored at room temperature (P=0.001).

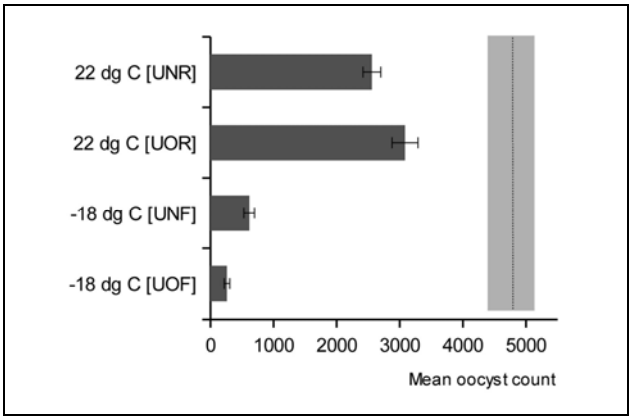

Fig. 2. **Results of experiment 1 showing mean recovered oocyst counts of samples stored in a natural faecal suspension at either -18 [UNF] or 22 °C [UNR] or in oxidizing medium (2% $K_2Cr_2O_7$) after 30 days at either -18 [UOF] or 22 °C [UOR]. Original oocyst count of the batch used is shown as the dotted line and confidence interval in light grey. Error bars are 95% confidence intervals.**

Exp. 2: Effect of sporulation on oocysts handling freezing

Fig. 3 illustrates that the majority (77.3±2.1% 95% CI) of the sporulated oocysts were lost during the storage in -18 °C (P<0.001). The number of oocysts in the [SNF] samples after freezing was significantly higher (P<0.001) than [UOF] in Exp1, whereas this difference was not seen in [UNF] (P=0.60). By contrast, after incubation in room temperature, more (P=0.006) oocysts in [SNR] than [UNR], but not compared to [UOR] (P=0.08).

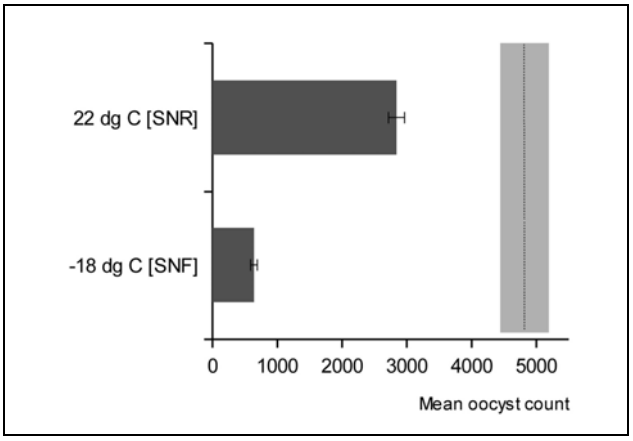

Fig. 3. Results of experiment 2 showing mean recovered oocyst counts of samples stored at -18 [UNF] or 22 °C [UNR] for 30 days in a natural faecal suspension and subsequently left to oxidizing naturally by frequently aerating the solution. Original oocyst count of the batch used is shown as the dotted line and confidence interval in light grey. Error bars are 95% confidence intervals.

Exp. 3: Effect of freezing in faecal suspension on sporulation

After the oocysts from batch 2 were frozen in a faecal solution for a month, thawed, and sporulated, 3.3±1.8% 95% CI of them were able to sporulate. Ten times more (P<0.001) oocysts sporulated (31.5±9.0% 95% CI) in the control group that was left at room temperature for the duration of the experiment. Although all species sporulated at room temperature (Fig. 4A), only *E. alabamensis* (N=3, 10.0±15.1% 95% CI), *E. ellipsoidalis* (N=3, 4.8±5.7% 95% CI), and *E. zuernii* (N=3, 6.5±7.6% 95% CI) sporulated after exposure to sub-zero temperatures in low numbers (Fig. 4B). Results showed significant differences between the proportions of oocysts that sporulated at both room temperature and sub-zero temperature in *E. alabamensis* (P=0.05), *E. ellipsoidalis* (P=0.004), and *E. zuernii* (P=0.005). At room temperature a smaller proportion of *E. auburnensis* sporulated compared to *E. ellipsoidalis* (P=0.04) and *E. zuernii* (P=0.05). Similarly, fewer *E. canadensis* oocysts sporulated compared to *E. ellipsoidalis* (P=0.03) and *E. zuernii* (P=0.04).

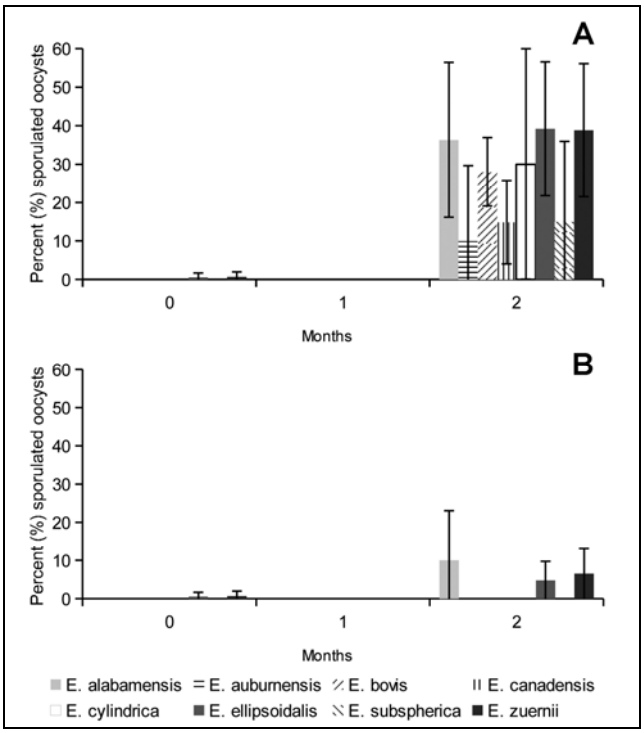

Fig. 4. **Recovered proportion (%) of different species of bovine *Eimeria* oocysts able to sporulate after 1 month at 22 °C (A) or 1 month at -18 °C (B) in a natural faecal solution with no aeration of the oocysts followed by 1 month at 22 °C with frequent aeration. Error bars are 95% confidence intervals.**

Discussion

This study aimed to examine how storage solution and sporulation status affect the recoverability of *Eimeria* oocysts after exposure to sub-zero temperatures, as well as how this temperature affects the sporulation ability of different species. Previous studies of bovine *Eimeria*

traditionally has focused on the quantities of oocysts excreted in faeces (Stewart et al. 2008; Almeida et al. 2011; Dong et al. 2012), or on infectivity of the parasites isolated from faeces (Svensson 1997; Mundt et al. 2005; Bangoura and Daugschies 2007). However, we have limited understanding of the fate of the oocysts once shed to the environment - how different conditions affect the oocysts concentrations under natural and experimental conditions. This knowledge is a needed link between studies reporting shedding of oocysts and studies investigating the survival and infectivity of the parasite.

Our experiment examined the effects at -18 °C on the number of oocysts recovered after one month. Jansson (1990) has presented that -7 °C is representative of the natural conditions that the oocysts are exposed to in Sweden. We chose -18 °C because this temperature reflects the most extreme temperature the oocysts are likely to experience in the top layers of soil under natural conditions in Northern Europe (Sharratt et al. 1992). Thermal stresses of *Eimeria* species was studied by Rind and Brohi (2001) and served as inspiration for the study.

We observed a clear reduction in recoverable oocysts after one month at -18 °C in both oxidizing medium and faecal suspension. However, a considerable amount of unsporulated oocysts remained intact despite freezing, and more than double the amount of oocysts tolerated the thermal stress in the faecal suspension compared to the oxidizing medium. The lower number of intact unsporulated oocysts in the oxidizing and toxic sporulation medium may be explained by the permeability of the outer oocyst wall during sporulation. Tests of different effects on wall permeability of *E. bovis* show that potential toxins such as strong concentrations of urea can go through the wall of unsporulated oocysts (Jensen et al. 1976). In addition, the formation of ice crystals during freezing potentially breaks oocysts, but also likely damages the outer wall of some oocysts, that may further enhance the ability of potassium dichromate to enter the oocyst and act as a toxin. It would be interesting to test the effect of disinfectants on *Eimeria* under similar conditions.

The sporulated and infective oocysts are supposedly more resistant to stress than unsporulated oocysts if observed ecology for *Eimeria* species in chicken can be extrapolated to bovine species (Horton-Smith and Long 1954). Resistance as tolerance to cold was not supported by our findings as originally unsporulated oocysts in the faecal suspension [UNF] handled the sub-zero temperature as well as oocysts that had sporulated [SNF].

Maquardt et al. (1960) showed that exposing unsporulated *E. zuernii* to temperatures around -7 to -8 °C for two months did not destroy them and that a majority of the oocysts were able to sporulate. Even after 6 and 24 hours exposures to -30 °C some oocysts appeared to tolerate the thermal stress. Rind and Brohi (2001) exposed 10 different bovine *Eimeria* species to -20 °C and reported that only *E. zuernii* and *E. bovis* were able to sporulate after a 24 hour exposure, and none after a longer time. Our study showed a similar proportion of *E. zuernii* oocysts being able to sporulate as the one published by Maquardt et al (1960), despite longer incubation in colder temperature. In addition, we got similar results for *E. alabamensis* and *E. ellipsoidalis* oocysts. The sporulated oocysts are the species commonly found in infecting cattle on pastures, and *E. alabamensis* and *E. zuernii* are known to cause clinical eimeriosis (Daugschies and Najdrowski 2005). Surprisingly, no sporulated *E. bovis* was detected despite it being the dominant species in the original samples. Very low numbers of oocysts completing sporulation could have been missed with our chosen method. However, it seems unlikely as *E. bovis* normally sporulate as fast as *E. zuernii* (Levine 1985; Rind and Brohi 2001). It is thus possible that *E. bovis* is not as temperature tolerant as the other pathogenic species. A smaller proportion of *E. canadensis* and *E. auburnensis* sporulated slower at room temperature than *E. ellipsoidalis* and *E. zuernii*. This may be attributed to species differences in sporulation rates and to lesser extent that *E. canadensis* and *E. auburnensis* were present in lower proportions.

Our results differed from the results of Rind and Brohi (2001) regarding the oocysts ability to sporulate. This may be due to the discussed toxicity of the sporulation medium the oocysts were frozen in and that the sporulation was artificially enhanced at 30 °C for a week before reading in their study. We attempted a more gentle approach that we believe to more closely represent the natural conditions the oocysts may be exposed to, by storing in faecal solution and sporulating for a month using only airing of the solution to aid the process. Further evidence was observed in Exp. 3 where no oocysts sporulated in the sealed containers at room temperature, but one third sporulated following access to oxygen. The natural conditions are probably different from any of the experimental setups, as oocysts either are frozen in a faecal pat, or as an infiltrate in the soil. The amount of moisture likely affects the oocysts chances to remain intact, as previously discussed, and freezing in liquids thus present a more extreme situation than the oocysts would normally experience. Projecting our results to natural pasture conditions, this means that bovine *Eimeria* species appears to handle long periods of extreme sub-zero temperatures, regardless of sporulation status, between grazing seasons. Consequently, the oocysts shed late in the grazing season that sporulate slower due to colder temperatures (Rind and Brohi 2001), could still play an important role in new infections the following year.

In addition to the possible effect of the oxidizing medium during freezing, we also examined whether a significant loss of oocysts would occur in the presence of naturally present microbes. To test this, we compared the toxic sporulation medium, likely not to have active microbes, and a non-sterile medium consisting of the raw filtrate of the suspended faeces containing natural intestinal and airborne microbes. The experiment result was 17% less unsporulated oocysts after 1 month when stored in a non-sterile faecal suspension at room temperature. The amount of unsporulated oocysts in the oxidizing medium was equal to sporulated oocysts in

faecal suspension that were stored an additional month. This suggests that sporulation may provide resistance to microbial breakdown of the oocyst. It is possible that microbes in the faeces as well from soil and air act as important natural predators of the parasite.

Conclusion

Freezing in a potassium dichromate medium negatively affects the recoverability of intact unsporulated bovine *Eimeria* oocysts. Bovine *Eimeria* oocysts can tolerate a month at -18 °C frozen in a liquid, and sporulation did not show any additional resistance to freezing. After a month at -18 °C in a faecal solution, *E. alabamensis*, *E. ellipsoidalis*, and *E. zuernii* are able to sporulate. Oocysts stored in a faecal suspension at room temperature for a month are significantly reduced in recoverable numbers compared to a potassium dichromate medium.

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