



RESEARCH ARTICLE

The Concentration McMaster Technique is Suitable for Quantification of Coccidia Oocysts in Bird Droppings

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ABSTRACT

The objective of this study was to evaluate the suitability of the Concentration McMaster technique in counting coccidia oocysts and to compare it with two others -McMaster method modified by Wetzel and Zajíček. A stock suspension containing *Eimeria* oocysts was prepared, from which five concentrations (20, 250, 500, 1,000 and 1,500) of oocysts were made. Each method was evaluated after the examination of 30 samples in five concentrations. Each sample was presented as a parasite negative fecal dropping into which a known number of oocysts were inoculated. When results of all three methods were compared, statistically significant differences ($P < 0.05$) among them in each tested OPG concentration were found. The most diverse results ($P < 0.01$) were provided by the Wetzel and Zajíček methods. The Wetzel method produced the least accurate results of all the evaluated methods and in all tested oocyst concentrations. This method was found inappropriate for counting coccidia oocysts in fecal droppings. The Zajíček and the Roepstorff and Nansen method produced oocyst counts similar to the true numbers. These two methods were then evaluated using poultry fecal droppings naturally infected by coccidial infection. Differences between these two methods were found in the repeatability of the obtained results rather than in the oocyst counts. Compared to the Zajíček method, the Roepstorff and Nansen method is faster, less laborious, and produces sensitive and reliable results with repeated measurements over time. For all these reasons the Concentration McMaster technique could be recommend for the detection and quantification of coccidia oocysts in bird droppings.

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INTRODUCTION

Accurate detection of parasitic elements (protozoa oocysts and cysts, helminths eggs) as well as the determination of these parasites is of pivotal importance in medical and veterinary parasitology. Nevertheless, a range of immunological and especially molecular biological methods is currently available (Chiodini, 2005), their utilization is still restricted to certain laboratories or specific parasite diagnoses. Therefore microscopic examination of fecal samples for parasitic elements, for its simplicity and specificity, remains the main diagnostic method for many laboratories.

In monitoring parasite burden in the host, determining the degree of environmental contamination and epidemiological studies or efficacy of antiparasitic drugs (Holdsworth *et al.*, 2004), it is necessary to estimate the number of parasitic elements in a certain amount of the host sample. These quantitative techniques, which are based on the microscopic examination of an aliquot of suspension from a known volume of a fecal sample, are expressed as OPG (oocysts per gram) in protozoans (Cringoli *et al.*, 2010; Abbas *et al.*, 2012).

Although several copromicroscopic techniques have been developed, the McMaster technique using special counting chamber, is the most universally used method for

quantification of parasite elements in the fecal sample. This technique has been developed at the McMaster laboratory of the University of Sydney in Australia, primarily for epidemiological studies of gastrointestinal nematodes in sheep. However, the McMaster technique is applicable for other parasitic elements, including protozoa oocysts (Hansen and Perry, 1994; Akhtar *et al.*, 2012), and the World Association for the Advancement of Veterinary Parasitology (WAAVP) advocates this technique for evaluating the efficacy of anticoccidial drugs in chickens and turkeys (Holdsworth *et al.*, 2004). The McMaster counting technique, in some modifications, has also been recently applied in anticoccidial activity testing of different plant extracts (Abbas *et al.*, 2010; Anosa and Okoro, 2011; Awais *et al.*, 2011; Lee *et al.*, 2011; de Almeida *et al.*, 2012; Orengo *et al.*, 2012; Zaman *et al.*, 2012).

The present study verifies the suitability of the three McMaster technique modifications for the detection and subsequent quantification of oocysts in fecal droppings. This study also determines which selected McMaster modification is the most appropriate for this purpose.

MATERIALS AND METHODS

Preparation of oocysts inocula: *Eimeria tenella* oocysts were obtained from chickens bred at the animal facility of the BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs (Czech Republic), for experimental purposes. For the comparison of the three selected McMaster method modifications, a stock suspension of sporulated oocysts was prepared in a 2.5% (w/v) potassium dichromate solution which prevents bacterial degradation of oocysts. From this suspension containing approximately 10,000 oocysts, five concentrations were obtained from samples diluted with tap water. These concentrations ranged from the minimum detection limit of the Concentration McMaster technique to a value of 1,500 oocysts that commonly occur in field conditions. The Eppendorf tubes with a specific number of oocysts in 1 ml of suspension (potassium dichromate with tap water) were prepared in 32 repetitions. Overall 94 inocula doses were prepared for each oocyst concentration (20, 250, 500, 1,000 and 1,500). Prior to evaluation, the predicted oocyst number in two randomly chosen tubes was confirmed. A sufficient quantity of the fecal droppings from coccidia-free chickens was collected at the BIOPHARM institute and these were stored at 4°C until used. Prepared oocysts inocula doses were stored under the same conditions. Prior to evaluation of the selected method, 1 ml of suspension with a specific number of oocysts was added to the coccidia-free fecal droppings (specific amount according to evaluated method), and the fecal sample was thoroughly mixed for homogenization. The sample was then investigated.

Natural coccidia infection: Fecal droppings of chickens naturally infected by coccidia were obtained at a small farm in southern Bohemia (Czech Republic). Overall 200 g of chicken feces were collected into polyethylene bags and stored in a refrigerator at 4°C until examination. The obtained feces were homogenized before coprological examination and 80 samples were separated (specific

amount according to evaluated method) from this material. Two methods that produced the best results in the first part of this study were used for investigation of naturally infected fecal droppings.

Description of compared McMaster methods: Three modifications of the McMaster counting technique were compared in this study-the McMaster technique modified by Wetzel (1951), Zajiček (1978) and the Concentration McMaster technique (Roepstorff and Nansen, 1998). These modifications differ in the weights of feces examined, dilution factors used, the presence / absence of additional centrifugation, centrifugation times and speeds, different flotation solutions, flotation times, numbers of McMaster counting chambers investigated and multiplication factors used. The principal parameters of all the evaluated McMaster counting technique modifications are summarized in Table 1. For microscopic examination of all fecal samples, the McMaster counting chamber modified from Ministry of Agriculture, Fisheries and Food (Anonymous, 1986) was used. All samples were investigated using an Olympus BX51 microscope at a total magnification of 400x.

Table 1: Summary of principal parameters of evaluated by McMaster counting technique with modifications

| Parameters | McMaster technique modifications | | |
|---------------------------------|----------------------------------|---|-----------------------|
| | Wetzel | Zajiček | Roepstorff and Nansen |
| Amount of feces (g) | 2 | 1 | 4 |
| Flotation solution type | NaCl | MgSO ₄ + Na ₂ S ₂ O ₃ | NaCl + glukose |
| Solution specific gravity | 1.2 | 1.28 | 1.28 |
| Centrifugation (RPM) | none | 2,000 | 1,200 |
| Centrifugation time (min) | none | 2/1 | 5 |
| Flotation time in chamber (min) | 2 – 3 | 5 | 3 – 5 |
| McMaster counting chamber | 3 | 2 | 2 |
| Multiplication factor | 67 | 33 | 20 |

Statistical analyses: Basic descriptive statistics were computed. The normality of the data obtained from each method was tested separately using a Shapiro-Wilk test. Considering the results of the normality test, a nonparametric Kruskal-Wallis test was used for the evaluation of differences among all three methods. Statistica ver. 9 (Anonymous, 2009) was used for statistical analysis.

For the comparison of accuracy and possible method evaluation, we quantified the ratio of samples which produced a certain number of oocysts within a tolerance limit of +10% and +20% respectively. This ratio was then used as usability criteria.

RESULTS

Fecal droppings inoculation: The comparison of three selected McMaster method modifications were carried out on 450 fecal samples. The sensitivity and reliability of the evaluated methods are summarized in Table 2 and Figs 1-2. All tested methods provided a certain number of negative findings at the limited concentration of 20 oocysts. In all other tested concentrations no negative samples were observed using any of the tested methods. The sensitivity of compared methods ranged, in both tolerance limits (+10% and +20%), from 0% at a 20

Table 2: Comparison of method sensitivity. The numbers of negative samples are expressed as a percentage of the total number of samples investigated

| Real OPG | McMaster modification | Negative samples (%) | Tolerance limit | | Multiple comparisons of difference | | |
|----------|-----------------------|----------------------|-----------------|-------|------------------------------------|------|--------|
| | | | ± 10% | ± 20% | W: R&N | W: Z | R&N: Z |
| | | | | | | | |
| 20 | Wetzel | 90 | 0 | 0 | * | ** | NS |
| 20 | Zajíček | 43 | 0 | 0 | | | |
| 20 | Roepstorff & Nansen | 30 | 63 | 63 | | | |
| 250 | Wetzel | 0 | 13 | 47 | n.s. | ** | NS |
| 250 | Zajíček | 0 | 53 | 53 | | | |
| 250 | Roepstorff & Nansen | 0 | 23 | 67 | | | |
| 500 | Wetzel | 0 | 17 | 50 | ** | ** | NS |
| 500 | Zajíček | 0 | 37 | 77 | | | |
| 500 | Roepstorff & Nansen | 0 | 43 | 77 | | | |
| 1,000 | Wetzel | 0 | 0 | 0 | ** | ** | ** |
| 1,000 | Zajíček | 0 | 83 | 100 | | | |
| 1,000 | Roepstorff & Nansen | 0 | 43 | 93 | | | |
| 1,500 | Wetzel | 0 | 0 | 0 | ** | ** | NS |
| 1,500 | Zajíček | 0 | 60 | 97 | | | |
| 1,500 | Roepstorff & Nansen | 0 | 70 | 100 | | | |

Statistically significant differences between two particular methods are marked as "*" (P<0.05) and "**" (P<0.01). Non-significant difference are marked as „NS“ W-Wetzel, Z-Zajíček, R&N-Roepstorff & Nansen method.

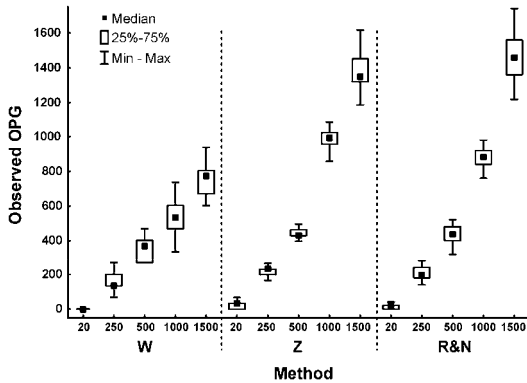


Fig. 1: Box plots showing differences based on number of oocysts obtained by three methods in five different OPG concentrations (20, 250, 500, 1,000 and 1,500). W-Wetzel method, Z-Zajíček method, R&N-Roepstorff and Nansen method

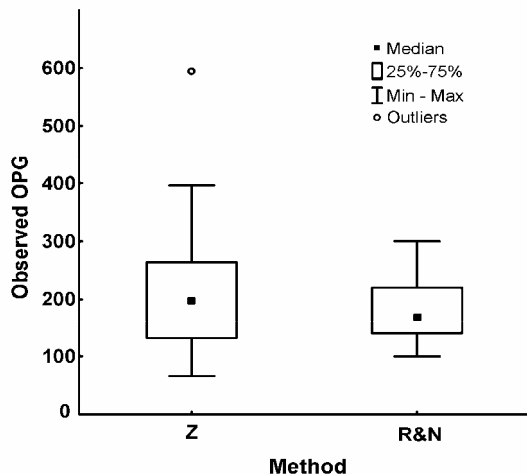


Fig. 2: Box plots presenting the comparison of the results of two methods (Z-Zajíček method, R&N-Roepstorff and Nansen method) evaluated on the examination of naturally infected poultry fecal droppings.

oocysts concentration to 100% at the highest concentration of 1,500 oocysts. Only the Wetzel method provided zero sensitivity at a concentration of 1,000 and 1,500 oocysts.

At the lowest level, the Roepstorff and Nansen method was the most sensitive. This method detected 20 oocysts in 19 of 30 samples. The other two methods did not find the inoculated oocysts in the fecal samples at any tolerance limits. The Wetzel method detected only 10% positive samples and differed significantly (P<0.01) from the other methods at the 20 oocysts concentration.

The required 250 oocysts, within a tolerance limit of +10%, were found in only 13% of samples examined by the Wetzel method. Seven of the 30 samples examined by the Roepstorff and Nansen method at the same concentration, fell within the tolerance limit of +10%; 67% of samples were within a tolerance limit of +20%. More than half of the results produced by the Zajíček method fell within both the tolerance limits at a concentration of 250 oocysts.

The Wetzel method revealed 500 oocysts in five of 30 samples within the selected tolerance limit. The Zajíček and the Roepstorff and Nansen method provided similar results at the same concentration; a total of 37% and 43% of the samples, examined using these methods was within a tolerance limit of +10%. When the tolerance limit was increased, 23 of 30 samples examined by the Zajíček method as well as by the Roepstorff and Nansen method went beyond this limit.

Surprisingly none of the results provided by the Wetzel method at concentrations of 1,000 and 1,500 oocysts fell within either tolerance limit. All samples examined by the Zajíček method were within the tolerance limit of +20% at a 1,000 oocyst concentration. At the same concentration, 93% of results obtained by the Roepstorff and Nansen method were within the same tolerance limit.

Only 3% of investigated values fell beyond the selected tolerance limits using the Zajíček method at a concentration of 1,500 oocysts. One hundred percent of samples examined by the Roepstorff and Nansen method were found to be at the same level and within the same tolerance limit.

Natural coccidia infection: Eighty fecal samples from naturally infected chickens were investigated using the two selected McMaster technique modifications. The Zajíček and the Roepstorff and Nansen method detected *Eimeria* spp. oocysts in 100% of evaluated samples. Both compared methods revealed mixed coccidial infection (*E. tenella*, *E. maxima*, *E. necatrix*, *E. acervulina* and *E. brunetti*) in all tested fecal samples. Species determination was conducted according to morphology of the sporulated oocysts (Conway and McKenzie, 2007). The intensity of the infection, estimated by OPG, varied using both methods. The OPG value estimated by the Zajíček method was from 66 to 594 while the Roepstorff and Nansen method provided results ranging from 100 to 300 OPG. The mean and median of the OPG values obtained by the two selected methods did not differ significantly (P<0.05), however, the Roepstorff and Nansen method provided results that were more balanced.

DISCUSSION

Although there are many papers dealing with McMaster counting methods for helminth eggs (e.g. Cringoli *et al.*, 2004; Pereckiene *et al.*, 2007; Rinaldi *et al.*, 2011), relatively few works focus on the evaluation of the McMaster method with regards to coccidia oocyst quantification (Haug *et al.*, 2006; Velkers *et al.*, 2010).

In the first part of this study, which deals with the evaluation of the sensitivity and reliability of selected McMaster technique modifications, inocula with a number of oocysts were added to parasite-free fecal droppings. This material was then investigated by the selected methods.

Of all the evaluated methods and tested oocyst concentrations, the Wetzel method produced the poorest results. The failure of this method is probably due to the high dilution ratio (amount of feces / volume of water) and the absence of centrifugation. Some authors (Cringoli *et al.*, 2004; Pereckiene *et al.*, 2007) report attaining the best results through methods which use a dilution factor of 1:10-1:15. The Wetzel method uses a dilution ratio of 1:30, and moreover, the feces are diluted directly in the flotation solution. According to Pereckiene *et al.* (2007), methods that require centrifugation steps produce results that are more precise. The Wetzel method fills the McMaster chamber immediately after mixing the feces with a flotation solution (without further treatment), and flotation takes place directly in the counting chamber. A high amount of fecal debris present in such a sample makes microscopic examination difficult as debris traps some of the oocysts at the bottom of the McMaster chamber. Another problem caused by high amounts of fecal debris is that it creates a viewing obstruction (Haug *et al.*, 2006). This may cause the technician to overlook a number of oocysts, and the probability of error increases. The first evaluated method, the Wetzel method, was found to be inappropriate for the quantification of coccidia oocysts.

The Zajíček method gave oocysts counts which were in agreement with the true numbers, and at some concentration levels it proved to be the most accurate of all the evaluated methods. When compared with other evaluated methods, the Zajíček method is the most laborious and time-consuming. Two of the parameters which can negatively influence the resulting OPG obtained by this method are amount of examined feces and the choice of the flotation solution. The weight of examined feces is one of the most important factors affecting the reliability of the coprological method. For this reason, it is important to analyse a maximum amount of feces to avoid the need for extrapolation in estimating the parasite elements counts (Mes *et al.*, 2001; Cringoli *et al.*, 2004). The Zajíček method examines only one gram of feces, and this amount could be considered insufficient for an estimation of a sample oocyst count. Another factor which can reduce the number of oocysts revealed by this method is the flotation solution. According to Cringoli (Cringoli *et al.*, 2004; 2010) the type of solution has a fundamental role in determining the analytic sensitivity, precision, and reliability of any analytical method based on flotation. These authors point out that all sucrose-based solutions at densities between 1.200 and 1.350 floated more parasitic elements than the others. The Zajíček

method uses the Breza solution which has a sufficient specific gravity, but is prepared from magnesium sulphate and sodium thiosulphate. The chemical composition of the Breza solution apparently does not create ideal conditions for the flotation of some oocysts. The combination of small amounts of examined samples together with a specific type of flotation solution causes a reduction in the sensitivity of the Zajíček method at some tested concentrations.

The Roepstorff and Nansen method is the most reliable of all the evaluated methods in the majority of concentration levels, including the lowest concentration, wherein two other evaluated methods failed. The concurrence between actual numbers of added oocysts and oocyst counts estimated by this method is attributed to several factors. This method analyses high quantities of fecal sample which is recommended by many authors (Mes *et al.*, 2001; Cringoli *et al.*, 2004) for reliability improvement. The dilution factor is 1:14, which is an optimal ratio for obtaining satisfactory results (Cringoli *et al.*, 2004). The Roepstorff and Nansen method uses an appropriate salt/sugar flotation solution (Cringoli *et al.*, 2004; 2010) which enables the majority of oocysts to float.

The results of the previous part of this study performed on actual oocyst counts of the stock solution indicate that the Zajíček and the Roepstorff and Nansen methods are the most efficient with respect to the quantification of oocysts in poultry fecal droppings. Nevertheless, fecal samples with a known number of oocysts did not necessarily reflect the natural infection intensity. Hence, we evaluated the two most efficient methods from the first part of this study using naturally infected fecal droppings. Each diagnostic method needs to produce results with low variance when samples are investigated by the same person, under the same conditions, and at the same time. Therefore, repeatability is an important aspect of method comparison. In the case of naturally infected fecal droppings, there were differences between two evaluated methods in the repeatability of obtained results rather than in OPG values. The Zajíček method produced an average of 216 OPG and the values ranged from 66 to 594 while the average OPG value obtained by the Roepstorff and Nansen method was 176, and the resulting figures ranged from only 100 to 300 OPG.

Compared to the Wetzel and the Zajíček method, the Concentration McMaster technique (Roepstorff and Nansen, 1998) has some advantages. It is fast, cheap, relatively less laborious, sensitive, and reliable. Moreover, repeated measurements over time produce similar results.

In conclusion, the Concentration McMaster technique, owing to the advantages mentioned above, is a welcome choice for quantifying coccidia oocysts in bird droppings.

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