Sci Parasitol 22(1):13-18, February 2021
ISSN 1582-1366

ORIGINAL RESEARCH ARTICLE

Diagnosis of coccidiosis by *Eimeria* spp. in free-range chickens using Mini-FLOTAC and McMaster techniques – preliminary results

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Abstract. Mini-FLOTAC is emerging as a more sensitive and accurate tool to identify gastrointestinal parasites in faecal samples from domestic animals, in comparison with the McMaster method. However, research regarding its specific application in poultry samples, particularly from free-range chickens, is scarce. The current research aimed to test the use of Mini-FLOTAC for the identification of *Eimeria* spp. in free-range chickens and compare its results with McMaster. For this study, 40 faecal samples were collected from free-range chickens in a poultry farm located in North-Western Lisbon (Portugal). Each sample was processed with McMaster and Mini-FLOTAC techniques for the detection and count of coccidian *Eimeria* spp. oocysts. The resulting OPG (oocysts per gram of faeces) data obtained by the two techniques were compared using the Wilcoxon Test and correlated with the Spearman Test, and Mini-FLOTAC's relative sensitivity was assessed, using a significance level of $p<0.05$. The average OPG was higher with Mini-FLOTAC and doubled the one obtained using the McMaster method (2669.3 OPG and 1220 OPG, respectively), although these results were not significant. Mini-FLOTAC's relative sensitivity obtained in this study reached 86% (70.5-95.3%, 95%CI), although this result was not statistically significant. However, correlation of OPG counts between Mini-FLOTAC and McMaster, was significant. These preliminary results suggest the potential interest in the use of Mini-FLOTAC for the diagnosis of coccidiosis by *Eimeria* spp. in poultry, based on its assessment in a free-range poultry production system.

Keywords: Poultry; free-range; McMaster; Mini-FLOTAC; coccidiosis; *Eimeria* spp.

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Diagnosticul coccidiozei produse de către *Eimeria* spp. la puii crescuţi în aer liber, folosind tehnicile Mini-FLOTAC şi McMaster – rezultate preliminare

Rezumat. Mini-FLOTAC apare ca un instrument mai sensibil şi mai precis pentru identificarea paraziţilor gastro-intestinali în probele fecale de la animale domestice, în comparaţie cu metoda McMaster. Cu toate acestea, cercetările privind aplicarea sa specifică în probele de păsări, în special de la puii crescuţi în aer liber, sunt rare. Cercetarea actuală a avut ca scop testarea utilizării Mini-FLOTAC pentru identificarea *Eimeria* spp. la puii crescuţi în aer liber şi compararea rezultatelor obţinute cu McMaster. Pentru acest studiu, 40 de probe de fecale au fost colectate de la puii crescuţi în aer liber, într-o fermă de păsări situată în nord-vestul Lisabonei (Portugalia). Fiecare probă a fost prelucrată prin tehnicile McMaster şi Mini-FLOTAC pentru detectarea şi numărarea oochisturilor de *Eimeria* spp. Datele rezultate privind numărul de OPG (oochisturi per gram de fecale) obţinute prin cele două tehnici au fost comparate folosind testul Wilcoxon şi corelate cu testul Spearman, iar sensibilitatea relativă a Mini-FLOTAC a fost evaluată, utilizând un nivel de semnificaţie $p < 0,05$. OPG-ul mediu a fost mai mare cu Mini-FLOTAC şi s-a dublat faţă de cel obţinut folosind metoda McMaster (2669,3 OPG şi respectiv 1220 OPG), deşi aceste rezultate nu au fost semnificative statistic. Sensibilitatea relativă a Mini-FLOTAC obţinută în acest studiu a atins 86% (70,5-95,3%, 95% CI), deşi acest rezultat nu a fost semnificativ statistic. Cu toate acestea, corelaţia numărului de OPG între Mini-FLOTAC şi McMaster a fost semnificativă statistic. Aceste rezultate preliminare sugerează interesul potenţial în utilizarea Mini-FLOTAC pentru diagnosticul coccidiozei produse de către *Eimeria* spp., pe baza evaluării sale într-un sistem de producţie de păsări, crescute în aer liber ("free-range").

Cuvinte cheie: Păsări; "Free-range"; McMaster; Mini-FLOTAC; Coccidioză; *Eimeria* spp.

Received 24.07.2020. Accepted 02.12.2020.

Introduction

Coccidiosis is one of the most pathogenic parasitic diseases in worldwide aviculture industry, being responsible for heavy economic losses, and it is caused mainly by protozoa belonging to the genus *Eimeria* (Zajac and Conboy, 2012; Kaboudi *et al.*, 2016; Prakashbabu *et al*., 2017).

Quantitative detection of gastrointestinal parasites in poultry faeces is usually performed using the McMaster method, which has been the reference technique for most parasitological surveys in poultry farms (Bortoluzzi *et al*., 2018).

Over the past seven years, the diagnosis of gastrointestinal parasites in several animal hosts has been also performed using an emerging technique, called Mini-FLOTAC, which is commonly considered a good alternative to the McMaster method, since it allows simultaneously the identification of helminth eggs and oocysts of protozoa with relatively higher sensitivity, accuracy and precision (Cringoli *et al*., 2017; Maurelli *et al*., 2020).

Several authors have been applying this novel technique in faecal and urine samples from a wide range of domestic and wild animals, namely herbivores, monkeys, dogs and cats, and birds, allowing to compare its sensitivity and precision to the traditional quantitative coprological techniques. The sensitivity of Mini-FLOTAC in the diagnosis of poultry gastrointestinal parasites can reach values up to 100%, depending on the type of flotation solution, threshold of detection, groups of parasites under analysis and their burdens, as well as the use of technical replicates (Cringoli, 2006; Cringoli *et al.*, 2010; Maurelli *et al.*, 2014;

Alvarado-Villalobos *et al*., 2017; Bortoluzzi *et al*., 2018; Capasso *et al*., 2019; Daş *et al.,* 2020).

The current preliminary study aimed to identify *Eimeria* spp. oocysts with both Mini-FLOTAC and McMaster techniques, as well as to compare their results, in terms of average oocyst shedding and sensitivity rates obtained.

Materials and methods

Sampling

This brief cross-sectional study was performed in the scope of the first author's Master Thesis program, and therefore the sampling period was defined according to the first author and farm manager's agenda. For this study, a poultry farm located in north-western Lisbon district (39°13'59.5"N, 9°17'15.2"W) was selected, in which chickens are produced in a typical free-range system and therefore are exposed to the outdoor environment in more than 50% of the production cycle, which lasts 3 months.

In March 2019, a total of 40 faecal samples were randomly collected in the fattening park, with a flock size of 100 chickens, and then transported in a cooling bag to the Laboratory of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Lisbon, and stored at 4-5 ºC before analyses. Since animals were scavenging freely in the fattening park and the farm manager did not allow the direct contact with the flock, samples were just collected from soil, and therefore no technical replicates were used for *Eimeria* spp. identification.

McMaster method

Each faecal sample was briefly homogenised, before being separated for each counting technique. Samples were initially analysed by the McMaster method for the quantification of oocysts per gram of faeces (OPG). McMaster protocol was adapted and modified from Madeira de Carvalho (2002), Vadlejch *et al.* (2013) and Zajac and Conboy (2012). For each sample, 2 g of faeces were mixed with 28 ml of saturated solution of sucrose (specific gravity 1.2) and the filtered suspension was transferred to a McMaster slide and visualized under a light microscope (total magnification 100x), to calculate the *Eimeria* spp. prevalence with a threshold of 50 OPG.

Mini-FLOTAC

The Mini-FLOTAC technique was applied using the protocol for exotic animals optimized in the Parasitology and Parasitic Diseases Unit of the University of Naples Federico II (Mini-FLOTAC – Exotic Animals, 2019), due to the current absence of a specific protocol for poultry faecal samples.

For each faecal sample, 2 g were added to the Fill-FLOTAC device and mixed with 38 ml of saturated sucrose solution (specific gravity 1.2). Then, the faecal suspension was transferred to the previously assembled reading chamber and left for 10 minutes resting on the lab bench, before rotating the top piece of the reading chamber. *Eimeria* spp. oocysts were counted under a light microscope (100x), using an analytic sensitivity of 10 OPG.

Relative sensitivity of Mini-FLOTAC was calculated as the percentage of True Positive reads (TP) in the sum of False Negative reads (FN) and TP, assuming the McMaster method as the reference technique, due to its historic (Gordon and Whitlock, 1939) and frequent use in poultry gastrointestinal parasites diagnosis (Bortoluzzi *et al*., 2018).

Statistical analysis

The software GraphPad Prism®, version 8.4.3 for Windows (GraphPad Software, 2020) was used for statistical analysis and chart editing. Data Normality was assessed with the Kolmogorov-Smirnov Test and, for every group included in this study, OPG data failed the Normality Test ($p<0.0001$). These results determined the statistical analysis of the data with the Wilcoxon test for paired samples, to compare the OPG values obtained with the McMaster method and Mini-FLOTAC, as well as the Spearman test was chosen for the correlation between the *Eimeria* OPG obtained with these two techniques. Relative sensitivity analysis of Mini-FLOTAC was also performed

with this software, through a 2x2 contingency table and using the Fisher's exact Test, assuming the McMaster method as the "reference test", being $p<0.05$ the significance level for all tests.

Results

In terms of amplitude of faecal shedding level obtained by these two techniques, the oocysts output ranged from 0 to 38940 OPG, with Mini-FLOTAC, and from 0 to 8850 OPG, with the McMaster method. Both in the Mini-FLOTAC and McMaster techniques, 67.5% of faecal samples averaged 0-500 OPG, 20% were between 500-5000 OPG and the remaining 12.5% were higher than 5000 OPG (figure 1). The average oocysts load was higher and more than doubled with Mini-FLOTAC (2669.3 OPG), in comparison with the McMaster method (1220 OPG). However, the difference was not statistically significant by the Wilcoxon test ($p=0.8445$) and the prevalence obtained with Mini-FLOTAC and McMaster methods were quite similar (87.5% and 90%, respectively).

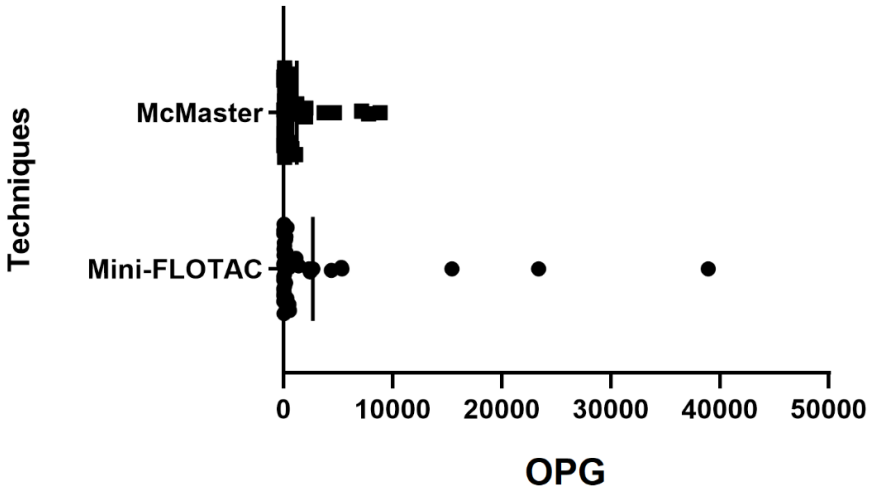

Figure 1. OPG values identified for each sample, per technique; vertical lines correspond to each technique average OPG.

Mini-FLOTAC's relative sensitivity obtained in this study reached 86% (71.34-93.92%, 95%CI), although this value was not statistically significant by the Fisher's exact test ($p>0.9999$).

Finally, a significant correlation by the Spearman test was identified between the OPG obtained with Mini-FLOTAC and McMaster ($p=0.0001$), for thresholds of 10 and 50 OPG, respectively.

Discussion

Comparing the *Eimeria* spp. outputs detected with both methods, it was possible to observe that Mini-FLOTAC averaged a higher oocyst faecal output, for an analytic sensitivity of 10 OPG, in comparison with the McMaster method, using an analytic sensitivity of 50 OPG. These results can be justified by the high enclosed homogenization and accurate filtration process allowed by Fill-FLOTAC apparatus, which minimizes the human-error and allows a better homogenization between faeces and the flotation solution used (Cringoli *et al*., 2017; Scare *et al*., 2017; Bortoluzzi *et al*., 2018). Silva *et al.* (2013) also obtained higher values of OPG for Mini-FLOTAC, in comparison with McMaster, when applying both techniques in goat faecal samples, as well as Bortoluzzi *et al.* (2018) also obtained higher results of OPG for Mini-FLOTAC, in comparison with McMaster, for thresholds of 23 and 25 OPG, respectively, despite their difference not being also statistically significant.

The absence of statistical significance between the average OPG obtained with each technique can be explained by the absence of technical

replicates and the influence of OPG levels in the sensitivity of the tests. Although the relative sensitivity results obtained in this study for Mini-FLOTAC was not significant, it should still be highlighted its relevant value (86%), which overpassed the 68% of sensitivity obtained by Barda *et al.* (2013) for protozoan intestinal parasites in human faecal samples. Also, the results obtained for Mini-FLOTAC relative sensitivity could have been influenced by the oocysts outputs, since the majority of the samples (67.5%) had shedding levels lower than 500 OPG. As recently described by Daş *et al.* (2020), Mini-FLOTAC and McMaster sensitivities depend on the egg per gram (EPG) levels, although their differences being significant only at levels of 50 EPG. Once the shedding levels start increasing, the difference on their sensitivities becomes diluted and without significance.

The high variability of results obtained with Mini-FLOTAC and their concentration in low levels of OPG could have been due to the use of a protocol which has not been optimized for poultry samples. As shown by Bortoluzzi *et al.* (2018) in their research with poultry samples, several experimental factors may influence these results obtained with the Mini-FLOTAC technique, namely the absence of a specific protocol for poultry faecal samples, the selected ratio of saturated solution/grams of faeces, the threshold used for these domestic animals and the incorrect density of the flotation solution. These factors could have been responsible for the high variability of results obtained with this technique.

The significant correlation between the OPG obtained with Mini-FLOTAC and McMaster, for thresholds of 10 and 50 OPG, is in accordance with previous results published by Bortoluzzi *et al.* (2018), whose authors also obtained a significant correlation between *Eimeria* spp. faecal output with Mini-FLOTAC and McMaster, for thresholds of 23 and 25 OPG, respectively.

Despite not using a specific Mini-FLOTAC protocol for poultry samples, the overall results obtained with this method suggest its potential use for the identification of protozoan oocysts in domestic and wild avian hosts, as recently studied for avian nematode eggs by Daş *et al.* (2020).

This was the first study using Mini-FLOTAC for the detection of avian *Eimeria* spp. oocysts, and these results serve as a baseline for further research in this topic, in order to optimize the Mini-FLOTAC technique for poultry samples and include it more frequently in parasitological surveys on poultry farms and other avian facilities, as a diagnostic method that can improve avian production, conservation and its medicine.

Acknowledgements

The authors acknowledge the funding of their research by CIISA (project UIDP/00276/2020, funded by FCT) and CESAM (UIDB/AMB/50017/2020), FCT/MCTES through national funds, and the co-funding by FEDER, within the PT2020 Partnership Agreement and Compete 2020. We would like also to thank the owner of the poultry farm, for allowing the conduction of faecal samplings in his facilities and the completion of our field studies regarding avian parasites.

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