



Ancylostoma caninum: Calibration and comparison of diagnostic accuracy of flotation in tube, McMaster and FLOTAC in faecal samples of dogs

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ABSTRACT

We performed a calibration of flotation in tube, McMaster and FLOTAC to determine the optimal flotation solution (FS) and the influence of faecal preservation for the diagnosis of *Ancylostoma caninum* in dogs, and compared the accuracy of the three copromicroscopic techniques. Among nine different FS, sodium chloride and sodium nitrate performed best for detection and quantification of *A. caninum* eggs. Faecal samples, either fresh or preserved in formalin 5%, resulted in higher *A. caninum* egg counts, compared to frozen samples or preserved in formalin 10% or sodium acetate–acetic acid–formalin. FLOTAC consistently resulted in higher *A. caninum* eggs per gram of faeces (EPG) and lower coefficient of variation (CV) than McMaster and flotation in tube. The best results in terms of mean faecal egg counts (highest value, i.e. 117.0 EPG) and CV (lowest value, i.e. 4.8%) were obtained with FLOTAC using sodium chloride and faecal samples preserved in formalin 5%. Our findings suggest that the FLOTAC technique should be considered for the diagnosis of *A. caninum* in dogs.

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1. Introduction

The hookworm *Ancylostoma caninum* is the most widespread species of the genus *Ancylostoma* (de Silva et al., 2006). It occurs in the tropics and warm temperate areas throughout the world, whereas in Europe, *A. caninum* is found mainly in the southern part (Grandmange et al., 2006). *A. caninum* is one of the most pathogenic gastrointestinal parasites of dogs (Bowman et al., 2003). Typical morbid sequelae include wasting and anaemia, but a sustained chronic infection can lead to death (Heukelbach and Feldmeier, 2008). *A. caninum* also poses a considerable public health risk, because the parasite can infect humans, causing creeping eruptions known as *larva migrans cutanea* (Provic and Croese, 1996).

The most widely used approaches for the diagnosis of hookworm infection in dogs consist of copromicroscopy and post-mortem examination of dogs (Palmer et al., 2006). Copromicroscopic techniques for the diagnosis of *A. caninum* in dogs are usually based on flotation methods and can be either qualitative (e.g. flotation in tube) or quantitative, hence determining faecal egg counts (FECs). The McMaster technique is the most widely used approach

for estimating FECs, and is often employed in anthelmintic drug efficacy studies (Grandmange et al., 2006; Kopp et al., 2007). However, both flotation in tube and McMaster have drawbacks, as both methods lack sensitivity, particularly when infection intensities are low (Mes et al., 2007). FLOTAC is a multivalent sensitive and accurate copromicroscopic method for the diagnosis of helminth infections in animals and humans. This technique utilizes the FLOTAC apparatus, a cylindrical device with two 5 ml flotation chambers, which allows up to 1 g of faeces to be prepared for microscopic analysis (Cringoli, 2006; Cringoli et al., 2010).

With regard to the diagnosis of intestinal helminths in dogs, thus far, the FLOTAC technique has been successfully used for the detection of *Crenosoma vulpis* larvae (Rinaldi et al., 2007a), *Spirocerca lupi* eggs (Traversa et al., 2008) and *Angiostrongylus vasorum* larvae (Cortese et al., 2010). Additionally, besides the growing application of the FLOTAC technique in veterinary parasitology (Rinaldi et al., 2007b; Gaglio et al., 2008; Keiser et al., 2008; Levecke et al., 2009; Duthaler et al., 2010; Rinaldi et al., 2010), it has been used effectively for the diagnosis of hookworm and other helminth species in humans (Utzinger et al., 2008; Knopp et al., 2009a,b; Glinz et al., 2010).

Flotation solutions (FS) and the way faecal samples are preserved have important bearings for the analytic sensitivity, precision and accuracy of any copromicroscopic technique, be they qualitative or quantitative, based on flotation, including the FLO-

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TAC technique (Cringoli et al., 2004, 2010). In view of these considerations, there is a need for a thorough calibration of the FLOTAC technique, in order to determine the optimal FS and faecal preservation method for an accurate detection and quantification of parasitic elements. Here, we present results from the first in-depth calibration of the FLOTAC technique for the diagnosis of *A. caninum* in dog faeces. Subsequently, the diagnostic accuracy of the FLOTAC technique was compared with two widely used methods, flotation in tube and McMaster.

2. Materials and methods

2.1. Flotation solutions (FS)

The following nine FS were used according to standardized protocols presented elsewhere (Cringoli et al., 2010): FS1, sucrose and formaldehyde (specific gravity (s.g.) = 1.20); FS2, sodium chloride (s.g. = 1.20); FS3, zinc sulphate (s.g. = 1.20); FS4, sodium nitrate (s.g. = 1.20); FS5, sucrose and mercury II iodide and potassium iodide (s.g. = 1.25); FS6, magnesium sulphate (s.g. = 1.28); FS7, zinc sulphate (s.g. = 1.35); FS8, mercury II iodide and potassium iodide (s.g. = 1.44); and FS9, zinc sulphate and mercury II iodide and potassium iodide (s.g. = 1.45). All FS were prepared on the day of use and the s.g. were checked with a densiometer.

2.2. Composite faecal sample and preservation methods

A fresh faecal composite sample of ~600 g was obtained after pooling five stool samples from different dogs, all being naturally infected with *A. caninum*. The dogs were fed with different diets prior to stool collection. The composite sample was thoroughly homogenized, divided into five aliquots of 120 g each, and then assigned to one of the following five faecal preservation methods: (i) not preserved (i.e. fresh), (ii) formalin 5%, (iii) formalin 10%, (iv) sodium acetate–acetic acid–formalin (SAF), and (v) kept at a temperature of –30 °C for 10 days (for analysis, this sample was unfrozen and processed at room temperature). For three preservation methods, (ii), (iii) and (iv), each fixative was added to the composite faecal sample using a dilution ratio of 1:4 (i.e. one part of faeces plus three parts of fixative).

2.3. FLOTAC calibration

Each aliquot was suspended in community tap water at a dilution ratio of 1:10. The suspension was thoroughly homogenized using a hand-held blender and filtered through a wire mesh (aperture = 250 µm) and the debris discarded. The filtered suspension was divided into 162 aliquots of 6 ml each to have six replicates for each of the nine FS for the three diagnostic techniques to be compared. Each aliquot was placed in a 15 ml Falcon tube. Tubes were centrifuged for 3 min at 170 g, the supernatant was poured off and discarded, leaving only a pellet in the bottom of the tube. Subsequently, tubes were randomly assigned to one of the three techniques, i.e. flotation in tube (MAFF, 1986), special modification of the McMaster technique (MAFF, 1986) and FLOTAC double technique (Cringoli et al., 2010), and one of the nine FS.

2.4. Flotation in tube, McMaster and FLOTAC

For flotation in tube, each tube was filled with FS until a convex meniscus was formed and then covered with an 18 × 18 mm coverslip. After 15 min, the coverslip was transferred on a slide and examined under a microscope. The whole slide was read (analytic sensitivity = 1 egg per gram of faeces; EPG).

With regard to the special modification of the McMaster technique, tubes were filled with the FS to the previous 6 ml level and slowly agitated. The resulting suspension was taken up by a pipette to load the two chambers of the McMaster slide (Weber Scientific International; Teddington, UK; volume = 1.0 ml) and left for 10 min. The whole slide was read under a microscope (analytic sensitivity = 10 EPG).

For the FLOTAC technique, tubes were filled with the respective FS to the previous 6 ml level and slowly agitated. The resulting suspension was taken up by a pipette to load one of the two chambers of the FLOTAC-100 apparatus (volume of each chamber = 5 ml). Thus, a single flotation chamber of the FLOTAC-100 was utilized for each replicate (analytic sensitivity = 2 EPG). The apparatus was centrifuged at 120 g for 5 min and cut transversally. For each diagnostic technique, *A. caninum* eggs were counted for all replicates using a light microscope at a ×10 magnification. Overall, a total of 162 counts were performed; six replications for each of nine FS for each of the three diagnostic techniques employed.

2.5. Statistical analysis

The arithmetic mean EPG, standard deviation (SD), and coefficient of variation (CV) of FECs were calculated for the respective FS, preservation method and diagnostic technique. Differences between FS were analysed using one-way ANOVA with *post hoc* Fisher's least significant difference (LSD). All statistical analyses were carried out using STATA version 10.0 (Stata Corp.; Texas, USA).

In addition, a likelihood ratio test of the equality of the CV of *k* normally distributed populations was performed using software developed by the *Statistical Services at the Forest Products Laboratory* (USA; <http://www1.fpl.fs.fed.us/covtestk.html>).

3. Results

Fig. 1 shows *A. caninum* egg counts in the composite dog faecal sample, stratified by diagnostic method, FS and stool preservation method. The best results in terms of mean FECs (highest value, i.e. 117.0 EPG) and CV (lowest value, i.e. 4.8%) were obtained with the FLOTAC technique using FS2 and faecal samples preserved in formalin 5%. The detailed results from the calibration are summarised below, for each of the three methods separately.

3.1. FLOTAC

The FLOTAC technique performed best in terms of mean FECs when using FS2, followed by FS4 and FS5. Significantly higher *A. caninum* egg counts were obtained with faecal samples preserved in formalin 5% rather than a higher concentration of 10%, or when using fresh or frozen stool (all $P < 0.05$). Of note, fresh, frozen and stool samples preserved in formalin 10% revealed higher FECs, than those obtained with the McMaster and flotation in tube methods. Consistently higher FECs were obtained with the FLOTAC technique using either FS2 or FS4, than the McMaster and the flotation in tube methods (Fig. 1 a–c).

When fresh stool samples were subjected to the FLOTAC technique, all nine FS were capable of bringing *A. caninum* eggs in flotation. However, highly statistically significant differences ($P < 0.001$), both in terms of mean FECs and CV, were found between FS1, FS2, FS3, FS4, FS5 and FS6 on one hand, and FS7, FS8 and FS9 on the other hand. In fresh stool samples, the highest FEC (i.e. 95.0 EPG) and the lowest CV (i.e. 4.9%) were obtained with FS2.

Preservation of dog faecal samples in formalin 5% prior to FLOTAC examination, showed that the use of FS2 and FS4 revealed higher FECs than fresh stool samples using the same two FS. When

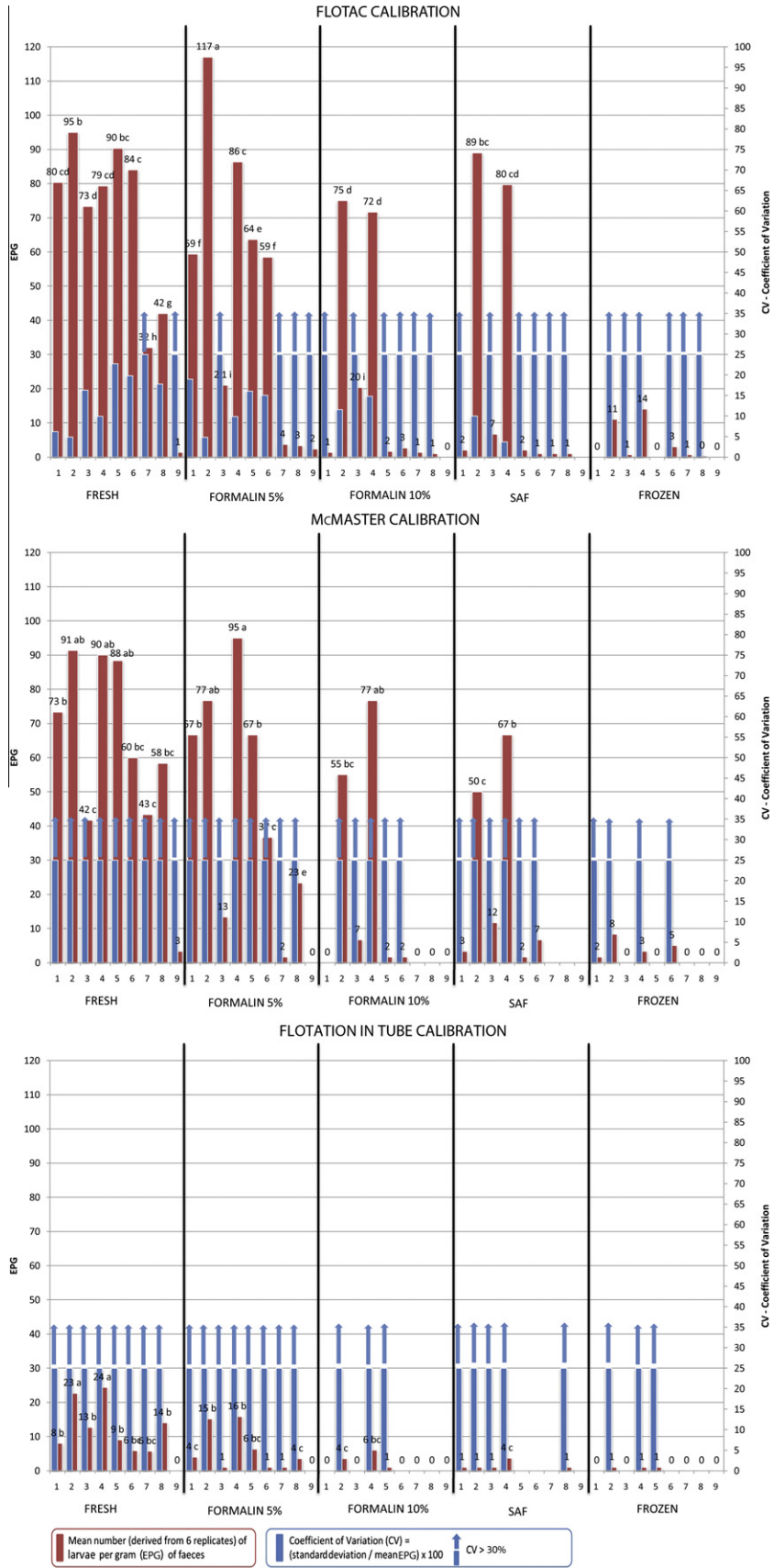


Fig. 1. Calibration of FS and counting techniques (FLOTAC, McMaster and flotation in tube) in fresh and preserved faecal samples. *P < 0.05; significant differences for different letters.

formalin 5% was used as preservation media, FECs obtained with FS2 and FS4 were 117.0 EPG and 86.3 EPG, respectively, and this difference showed statistical significance ($P = 0.019$). The remaining seven FS resulted in lower FECs when comparing stool samples preserved in formalin 5% rather than fresh samples.

When stool samples were preserved in formalin 10%, lower FECs and higher CVs were observed compared to fresh stool samples for all nine FS. As with formalin 5%, FS2 and FS4 in samples preserved in formalin 10% produced the highest FECs (75.0 EPG and 71.7 EPG, respectively) and the lowest CVs (11.5% and 14.8%, respectively). The difference in FECs between FS2 and FS4 was not significant ($P = 0.797$).

Regarding the aliquots preserved in SAF, regardless of the FS used, consistently lower FECs and generally higher CVs were obtained when compared to formalin 5%. FS2 and FS4 produced the best results in terms of FECs (89.0 EPG and 79.7 EPG, respectively) and CVs (10.0% and 3.7%). There was no statistically significant difference in FECs between FS2 and FS4 ($P = 0.472$).

Considerably lower FECs and higher CVs were noted when faecal samples were frozen prior to FLOTAC examination for all the FS employed. The best results were obtained with FS2 (FEC = 11.0 EPG, CV = 42.6%) and FS4 (FEC = 14.0 EPG, CV = 37.3%). The difference in FECs between FS2 and FS4 was not significant ($P = 0.362$).

3.2. McMaster

For all the FS and the preservation method, the McMaster technique resulted in considerably higher CVs compared to the FLOTAC technique. Indeed, the lowest CV (i.e. 26.3%) was obtained using FS5 for faeces preserved in formalin 5%. The single highest FEC (i.e. 95.0 EPG) using the McMaster method was obtained when dog faeces were preserved in formalin 5% and FS4 was employed. The respective CV was 28.0%.

Similar to the FLOTAC technique, McMaster produced the best results in terms of FECs when using either FS2 or FS4 for most of the faecal preservation methods. Fresh and stool samples preserved in formalin 5% produced the best results, regardless of the FS employed.

3.3. Flotation in tube

Regardless of the FS and preservation method, flotation in tube produced considerably higher CVs compared to the FLOTAC technique. The lowest CV (i.e. 19.7%) was obtained when faeces were preserved in formalin 5% and subjected to FS2. The highest FEC (i.e. 24.3 EPG) was observed when fresh stool samples were processed with FS4. The respective CV was 36.1%.

Similar to the FLOTAC and McMaster techniques, flotation in tube produced the best results in terms of FECs when either FS2 or FS4 were employed, particularly when faeces were processed fresh or after preservation in formalin 5%.

4. Discussion

Significant advances have been made in various aspects of the epidemiology and control of hookworm infections in dogs (Balassiano et al., 2009), including the development of new anthelmintic drugs and improved treatment strategies (Kopp et al., 2008; Schimmel et al., 2009). Insight has also been gained regarding enhanced biological control approaches (Carvalho et al., 2009). Moreover, progress has been made with the -omics sciences, including genomics and proteomics (Mulvenna et al., 2009), as well as bioinformatics (Cantacessi et al., 2009), which, in turn, deepened our understanding of ancylostomiasis in dogs, and might foster the

next generation of tools for their control. However, little emphasis has been placed on the development and validation of novel diagnostics and standardization of existing copromicroscopic techniques. Here, we present the first in-depth calibration of two widely used diagnostic methods (i.e. flotation in tube and McMaster) and the more recently developed FLOTAC technique for the diagnosis of *A. caninum* in dog faeces. Particular emphasis was placed on the effect of FS and preservation method. The following points are offered for discussion.

First, regarding the choice of FS, our calibration showed that, among a panel of nine FS investigated, sodium chloride (FS2) and sodium nitrate (FS4), both having the same s.g. of 1.20, performed best for detecting and quantifying *A. caninum* eggs in a composite dog faecal sample regardless of whether flotation in tube, McMaster or FLOTAC was employed. These findings are encouraging because both FS2 and FS4 are widely and effectively used in veterinary clinics for the diagnosis of helminth infections in pet animals. The choice of FS is important – as underscored in our study – but has received insufficient attention in the past (Cringoli et al., 2004). Although FS2 and FS4 have the same s.g., it is not uncommon that significant differences are found for FECs. Such observations therefore challenge the common practice of uniquely reporting the s.g. of a particular FS, as seen in the manuals of diagnostic parasitology and in the peer-reviewed literature. Hence, we recommend that other important aspects of a FS, such as clarity of subsequent reading under a microscope, likelihood of small and large debris to float concurrently, production of air bubbles, among other issues, should be reported (Oge and Oge, 2000; Cringoli et al., 2004).

Second, our investigation showed that both the media and method of stool preservation plays an important role for the accuracy of *A. caninum* egg detection in a composite faecal sample from dogs. For example, fresh faeces and faeces preserved in formalin 5% resulted in higher *A. caninum* egg counts compared to frozen stool or faeces preserved in formalin 10% or SAF. It is noteworthy that adding a fixative to a faecal sample has the advantage of killing infectious agents (e.g. larvae of *Strongyloides* spp.) and allows shipment of samples to designated reference laboratories (Utzinger et al., 2010). Formalin has been widely used in public, private and commercial laboratories for several decades. This preservation medium is considered an all-purpose fixative, preserving not only helminth eggs, but also larvae and intestinal protozoan cysts and oocysts (Pietrzak-Johnston et al., 2000). However, care is indicated with the use of formalin as it is toxic to humans and harmful for the environment (Cringoli et al., 2010). SAF is another widely used preservation medium and has replaced formalin and merthiolate-iodine formalin (MIF) in some laboratories (Marti and Escher, 1990).

We have recently speculated that the use of SAF for preservation of dog faeces might result in some *A. caninum* eggs to be altered or even broken, an issue that warrants further investigation. Indeed, recent studies on hookworm diagnosis in human showed that stool samples preserved in SAF and subjected to the FLOTAC method resulted in significantly lower FECs than the Kato-Katz method (Utzinger et al., 2008; Knopp et al., 2009b).

In the present study, it is important to note that a dramatic reduction in FECs was observed, regardless of the FS and diagnostic technique employed, when faeces were frozen. It is conceivable that *A. caninum* eggs are damaged by the process of freezing and unfreezing pending microscopic examination. There is evidence that freezing indeed has a negative effect on the diagnosis of other gastrointestinal strongyle eggs, as shown for *Haemonchus contortus* in sheep faeces (Van Wyk and Van Wyk, 2002).

Third, with regard to the comparison of the three diagnostic techniques, for any FS and faecal preservation method used, FLOTAC consistently resulted in higher FECs than McMaster and flota-

tion in tube. These findings are in contrast to previous studies for the diagnosis of hookworm infection in humans; FECs based on a single FLOTAC were considerably lower than those obtained after multiple Kato-Katz thick smear readings (Uttinger et al., 2008; Knopp et al., 2009a,b; Glinz et al., 2010). On the other hand, a single FLOTAC showed a higher diagnostic sensitivity than multiple Kato-Katz thick smear readings, and hence it has been speculated that the Kato-Katz method might actually overestimate hookworm and other helminth egg counts. Studies are underway to shed new light on these conflicting results. Given the low sensitivity and the significant underestimation of infection intensity by simple flotation methods, such as flotation in tube and McMaster, we wonder why these techniques serve as the current methods of choice in most veterinary clinics. Similarly to our results, a recent study performed in Pennsylvania showed that passive faecal flotation examinations employed in private practice misses a large fraction of infected dogs (up to 50%), due to inherent limitations to the passive faecal flotation technique or technician errors (Gates and Nolan, 2009).

Fourth, FLOTAC generally resulted in lower CVs than McMaster and flotation in tube, thus indicating a higher precision. This is a key issue, especially for studies on anthelmintic drug efficacy and for the detection and monitoring of anthelmintic drug resistance (Albonico et al., 2004; Knopp et al., 2010). Resistance to different anthelmintics has been reported against *A. caninum* in dogs, and hence it is of pivotal importance to have precise tools at hand (i.e. high sensitivity and high precision) for early detection of anthelmintic drug resistance (Kopp et al., 2008).

The high *A. caninum* infection rates in dogs worldwide seem to indicate that there are failures in individual (use of anthelmintics) and collective (reduction of environmental contamination) preventive measures currently in place for pet animals (Balassiano et al., 2009). Adult dogs must be submitted to faecal examination before treatment, as also suggested in the guidelines from the European Scientific Counsel Companion Animal Parasites (<http://www.escap.org/>). Hence, based on the findings of our study, it is reasonable to suggest that the FLOTAC technique should be considered as a method for the accurate diagnosis of *A. caninum* in dogs, preferably based on stool samples preserved in formalin 5% and subjected to either FS2 or FS4. In addition, since polyparasitism is a common phenomenon in dogs (Grandemange et al., 2006), the availability of a multivalent technique such as FLOTAC for the simultaneous diagnosis of helminths and intestinal protozoa might overcome some of the limits of the classical parasitological methods commonly used for diagnosing canine parasites.

Conflict of interest

The FLOTAC apparatus has been developed and is patented by G. Cringoli, but it is planned that the patent will be handed over to the University of Naples "Federico II". At present, the FLOTAC technique is under detailed validation by several research groups focusing on human and veterinary parasitology. Should these validations continue to be successful, the FLOTAC technique will be provided free of charge to public research centres, including the World Health Organization and universities. The fact that one of the authors is the current patent holder of the FLOTAC apparatus played no role in the present study. All other authors have no competing financial interests.

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