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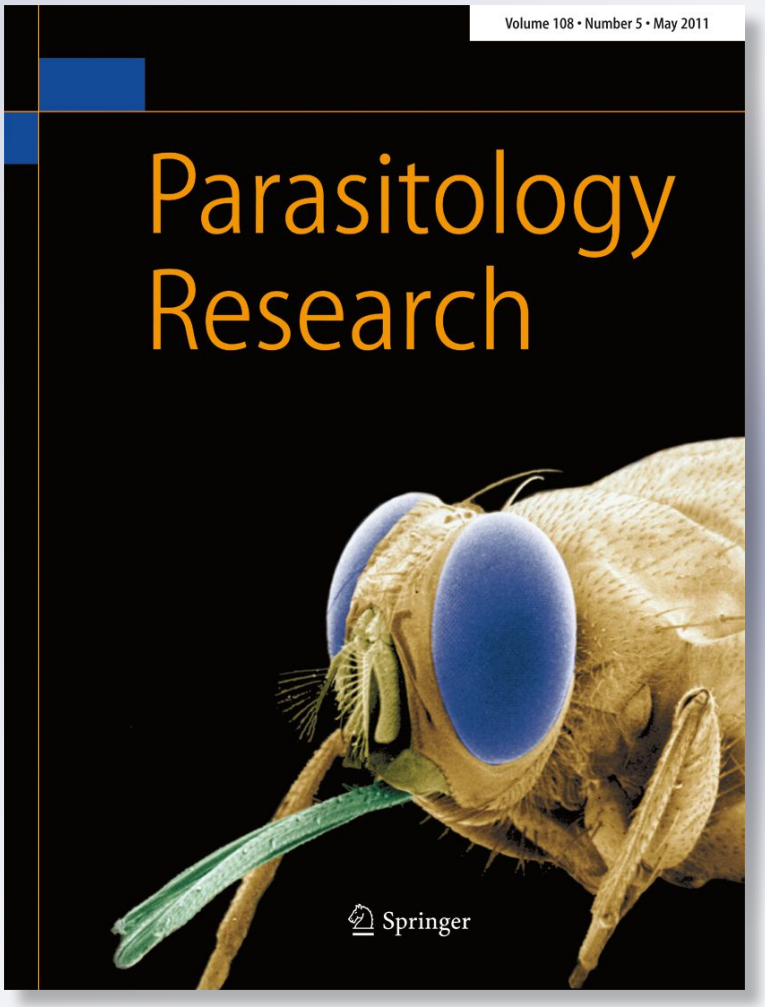

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ORIGINAL PAPER

Which McMaster egg counting technique is the most reliable?

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Abstract This study focuses on the comparison of three selected modifications of the McMaster counting technique, namely the McMaster method modified by Wetzel (W) and Zajíček (Z), as well as the concentration McMaster technique according to Roepstorff and Nansen (R&N). These modifications differ in the weights of faeces examined (W, 2 g/Z, 1 g/R&N, 4 g), flotation solutions (W, NaCl/Z, $MgSO_4$ + $Na_2S_2O_3$/R&N, NaCl + glucose), centrifugation (W, none/Z, 2,000 RPM for 2 min and 2,000 RPM for 1 min/R&N, 1,200 RPM for 5 min), number of McMaster chambers investigated (W, 3/Z, 2/R&N, 2), and multiplication factors used (W, 67/Z, 33/R&N, 20). To investigate the sensitivity and reliability of these methods, nematode eggs (*Teladorsagia circumcincta*) were used. Parasite elements are distributed through negative binomial distribution in naturally infected host faeces, and the number of parasite elements in a given amount of faeces sample is unknown to man. Therefore, we decided to prepare the exact number of eggs which were added to the parasite negative faeces; the faecal sample was then investigated. From this perspective, this is the first time a comparison of the McMaster methods has been so accurately investigated. This approach allows us to evaluate the real sensitivity and reliability of the tested method. As the findings of this study indicate, the highest sensitivity and reliability were obtained using the Roepstorff and Nansen modification. This McMaster modification is able to detect 20 eggs per sample (in 70% of samples). Concentrations of 200 and 500 eggs can be found in almost 100% of samples. Moreover, this method is simple, cheap and fast. For these reasons, we can recommend this method for routine veterinary practice.

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Introduction

There are many parasites that are important for animal and human health. And since some of these organisms can be pathogenic, it is necessary to identify them accurately (Bowman and Lynn 1999; Foreyt 2001). Recently, several diagnostic methodological approaches have been made available, especially in the field of molecular biology, biochemistry and immunology. However, these methods are still expensive and they are not applicable in routine practice (Chiodini 2005). For this reason, the coprological approach, which utilizes faecal samples to determine faecal egg count (FEC) enabling the detection of parasitic elements (e.g. helminths eggs and larvae, protozoa oocysts and cysts), is the most widely used method (Cringoli et al. 2010; Roepstorff and Nansen 1998; Ward et al. 1997).

In many cases, parasitic element detection and determination are not the only important features. We must also take into consideration the quantification of parasites present. FEC plays a crucial role in monitoring helminth burdens in herds and flocks, determining the degree of

pasture contamination, and in epidemiological studies or anthelmintic resistance identification (Coles et al. 1992; Nichols and Obendorf 1994; Ward et al. 1997). All FEC techniques that assess the number of parasitic elements per gram of faeces, e.g. eggs per gram (EPG), larvae per gram, oocysts per gram and cysts per gram, are based on the microscopic examination of an aliquot of suspension from a known volume of a faecal sample. Several methods and subsequent modifications have been reported. However, the McMaster method, developed at the McMaster laboratory of the University of Sydney, is the most universally utilized FEC technique in veterinary parasitology and is advocated by the World Association for the Advancement of Veterinary Parasitology for evaluating the efficacy of anthelmintic drugs in ruminants (Wood et al. 1995) as well as for the detection of anthelmintic resistance (Coles et al. 1992).

In literature, many variations of the McMaster technique can be found, and many scientists continue to introduce new modifications to this method. Its use has been described in detail in many diagnostics, both animal (Coles et al. 1992; Karamon et al. 2008; Morgan et al. 2005; Nichols and Obendorf 1994; Pereckiene et al. 2007; Rinaldi et al. 2007; Ward et al. 1997) and human (Bondarenko et al. 2009; Flohr et al. 2007; Stephenson et al. 1989). Different McMaster method modifications use various weight of faeces examined, volumes and types of flotation solutions, sample dilutions, flotation times, applications of additional centrifugation, durations and speeds of centrifugation, numbers of sections of the McMaster slide counted and different coefficients for interpretation (Cringoli et al. 2004; Karamon et al. 2008; Pereckiene et al. 2007).

The aim of this study was to compare the sensitivity and reliability of three chosen McMaster counting technique modifications, and to conclude which method is the most appropriate for routine veterinary practice examinations. There is no work of literature that evaluates FEC techniques as accurately as this study.

Material and methods

Three modifications of the McMaster method were compared in this study—the McMaster technique modified by Wetzel (1951) and Zajíček (1978) and the concentration McMaster technique according to Roepstorff and Nansen (1998). These modifications differ in the weights of faeces 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. Principal parameters of all the evaluated McMaster egg counting technique modifications are summarized in Table 1.

Parasitic elements (eggs) were obtained from parasite naive sheep experimentally infected by *Teladorsagia circumcincta*. For verification of the sensitivity and reliability of the tested methods, low FEC (20 EPG), intermediate FEC (50–200 EPG) and high FEC (500 EPG) egg concentrations were chosen. The 1.5 ml Eppendorf tubes were prepared in 30 repetitions for each of the following egg concentrations: 20, 50, 100, 200 and 500 EPG. The same scale of egg concentrations was prepared for each evaluated method. To minimize statistical error, only one skilled technician prepared all of the Eppendorf tubes with eggs (each concrete number of eggs in 1 ml of tap water) for all methods. This individual always prepared 32 Eppendorf tubes each with a specific number of eggs, and prior to evaluation, the number of eggs in two randomly chosen tubes was determined. Parasite negative faeces were collected from parasite naive sheep. All donor animals were bred for several years at the animal facility of the Czech University of Life Sciences Prague. Faeces were collected into polyethylene bags and stored in a refrigerator at 4°C until examination. Prior to evaluation of the selected method, 1 ml of water with a specific number of eggs was added to the parasite negative faeces (specific amount according to evaluated method), and the faecal sample was mixed for homogenization.

Table 1 Summary of principal parameters of evaluated McMaster egg counting technique modifications

	McMaster technique modifications		
	Wetzel	Zajíček	Roepstorff and Nansen
Amount of faeces (g)	2	1	4
Flotation solution type	NaCl	$MgSO_4 + Na_2S_2O_3$	NaCl + glucose
Solution specific gravity	1.2	1.28	1.3
Centrifugation (RPM)	None	2,000	1,200
Centrifugation (RCF)	None	479	172
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

RMP revolutions per minute, *RCF* relative centrifugal force

Table 2 Comparison of method sensitivity in samples with zero eggs detected

Actual EPG	McMaster modification	Negative samples (%)	Tolerance limit	
			±10%	±20%
20	Wetzel	87	0	0
20	Zajíček	40	0	0
20	Roepstorff and Nansen	27	70	70
50	Wetzel	57	0	0
50	Zajíček	30	0	0
50	Roepstorff and Nansen	0	0	83
100	Wetzel	17	0	0
100	Zajíček	7	40	40
100	Roepstorff and Nansen	0	53	90
200	Wetzel	0	37	37
200	Zajíček	0	30	67
200	Roepstorff and Nansen	0	97	100
500	Wetzel	0	23	43
500	Zajíček	0	33	57
500	Roepstorff and Nansen	0	100	100

The number of negative samples is expressed as percent of the total number of samples investigated

EPG eggs per gram

The sample was then investigated. To minimize the influence of the human factor, possibly resulting in biased results and an incorrect interpretation of the findings, only one qualified technician examined all samples using the three selected methods.

According to the Wetzel method (1951), 2 g of faeces was transferred into a container and 60 ml of saturated sodium chloride was added. The faecal suspension was thoroughly stirred with a glass stick and poured through a tea strainer into the new container. The container with the suspension was closed tightly and carefully inverted several times. One milliliter of suspension was taken up from the centre of the tube and three areas of the McMaster counting chamber were filled. After 2–3 min, the tapeworm eggs were counted and the total number of eggs was multiplied by a coefficient of 67.

According to Zajíček (1978), 1 g of faeces in 5 ml of water was mixed in mortar and the faecal suspension was poured through a tea strainer into a 10 ml centrifuge tube. Centrifugation then followed at 2,000 revolutions per minute (RPM) for 2 min. The supernatant was removed and 10 ml of Breza flotation solution (Breza 1959) (saturated solution of magnesium sulphate, saturated solution of sodium thiosulphate and distilled water at a ratio of 3:3:1, the final density 1.280) was added to the sediment. The sediment and flotation solution were thoroughly stirred and centrifuged for 1 min at 2,000 RPM. The tube was then carefully inverted three times, and 1 ml of suspension was taken up from the centre of the tube and both sides of the McMaster chamber were filled. All eggs observed were multiplied by a coefficient of 33.

According to the concentration McMaster method (Roepstorff and Nansen 1998), 4 g of faeces was transferred to a container, 56 ml of tap water was added, and the material was mixed thoroughly with a stirring device. The faecal suspension was left for 30 min to rest at laboratory temperature (22°C) and then mixed again. The suspension was poured through a tea strainer into a clean new container, and a 10-ml tube was filled to capacity with the filtered suspension. The tube was centrifuged for 5 min at 1,200 RPM and the supernatant was removed. Shortly before commencing the egg count, 4 ml of flotation fluid (saturated NaCl with 500 g glucose per litre of water) was added to a centrifuge tube. The sediment was then carefully resuspended and both sides of the McMaster counting chamber were filled. The filled McMaster chamber was left for 3–5 min to rest before counting. The numbers of eggs in both fields were then counted. The EPG was calculated by multiplying the total number of eggs by a coefficient of 20.

For microscopic examination of all faecal samples (all investigated methods), the McMaster counting chamber modified from MAFF (1986) (L.E.T. Optomechanika Praha) was used. All samples were investigated using an Olympus BX51 microscope at a total magnification of ×100.

Statistical analysis

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 version 9 (StatSoft, Inc. 2009) was used for statistical analysis.

For comparison of accuracy and possible method evaluation, we quantified the ratio of samples which produced a certain number of eggs within a tolerance limit of ±10% and ±20%, respectively. We used this ratio as usability criteria.

Results

In total, 450 faeces samples were examined using three McMaster method modifications. The sensitivity and reliability of the evaluated methods are summarized in Table 2 and Figs. 1, 2. In the case of concentrations between 20 and 100 EPG, all tested methods provided a certain number of negative findings. At higher concentrations, only the Roepstorff and Nansen (1998) method detected any negative samples. The sensitivity of compared methods ranged in both tolerance limits (±10% and ±20%) from 0% at a 20 egg concentration to 100% at the highest concentration.

The Roepstorff and Nansen (1998) method, at the limited level, detected 20 eggs in 70% of samples in both tolerance limits. The Wetzel (1951) and Zajíček (1978) methods detected 87% and 40% negative samples, respectively and all their results were beyond the selected tolerance limits. The coefficient of variation was over 100% in both latter methods. The Wetzel method (1951) differed significantly from the other methods at this concentration.

Fifty-seven percent of samples examined by the Wetzel method (1951) were negative, and all of the results were within the tolerance limit of ±10% at a concentration of 50 eggs. The Zajíček method (1978), at the same concentration and tolerance limit, provided results similar to the Wetzel method (1951). There was no statistic significant difference among individually tested methods at this concentration. Only 17% of results examined by the Roepstorff and Nansen (1998) method went beyond the tolerance limit of ±20% at a concentration of 50 eggs.

One half of the results produced at a 100 egg concentration by the Roepstorff and Nansen (1998) method were within the tolerance limit of ±10%, and 90% of the results were within the ±20% limit. The Wetzel method (1951), at this concentration, still produced results which went beyond both detection limits, and the Zajíček method (1978) started providing results with a coefficient of variation with values under 30%.

None of the compared methods detected negative samples at concentrations of 200 and 500 eggs. At these levels, almost 100% of the samples examined using the Roepstorff and Nansen (1998) method were within the selected tolerance limits. The Zajíček (1978) and Wetzel (1951) methods were comparable in recovering 200 eggs within a tolerance of ±10%, although the results of the Zajíček method (1978) improved twice when the tolerance limit was increased to ±20%.

At the highest concentration, only 3% of investigated values fell beyond the selected concentration using the Roepstorff and Nansen (1998) method. When compared at a concentration of 500 eggs, the Zajíček method (1978) recovered more eggs than the Wetzel method (1951); these two methods showed significant statistical differences.

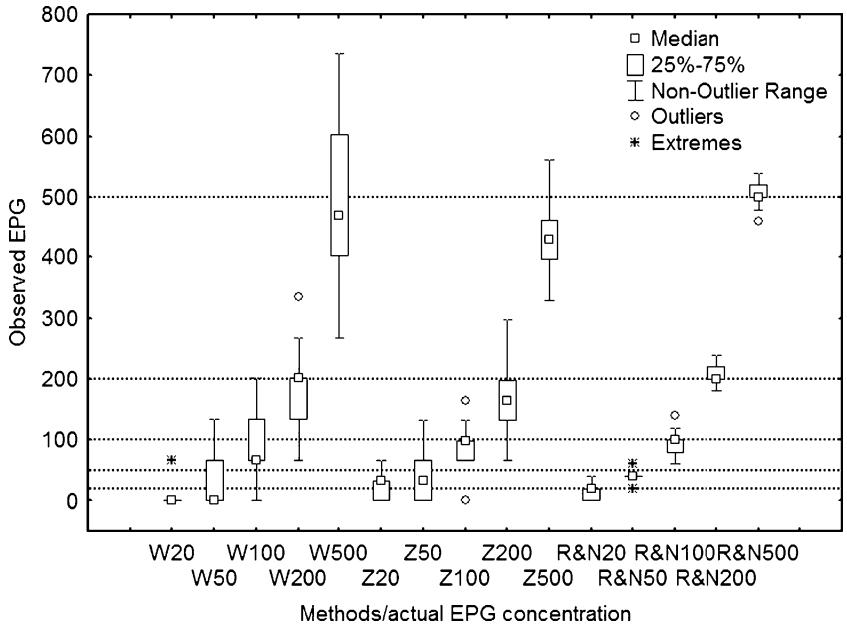

Fig. 1 *Box plots* showing differences based on number of eggs obtained by three methods in five different EPG concentrations (20, 50, 100, 200 and 500). *W* Wetzel method, *Z* Zajíček method, *R&N* Roepstorff and Nansen method

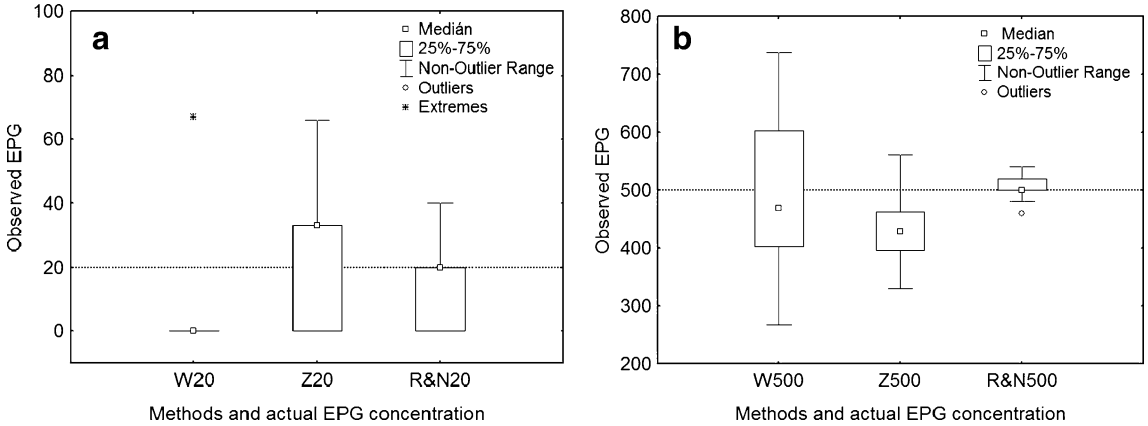

Fig. 2 *Box plots* showing differences among methods in case of lowest (**a**) and highest (**b**) EPG level. *W* Wetzel method, *Z* Zajíček method, *R&N* Roepstorff and Nansen method

Discussion

There are many factors in the methodology that can influence the result of a selected technique. One of the most important factors affecting the reliability of the method is the weight of faeces examined. It is important to effectively analyse maximum amounts of faeces per host to avoid the need for extrapolation in estimating the EPG. When small amounts of faeces are examined, the multiplication factor renders EPG estimates is less precise (Cringoli et al. 2004; Mes et al. 2001).

The results of our study are in accordance with the opinion expressed by Mes (Mes et al. 2001; Mes 2003) and Pereckiene et al. (2007). The concentrated McMaster method (Roepstorff and Nansen 1998), which examines 4 g of faeces and uses the lowest multiplication factor (20) from all tested methods (33 and 67), was the best technique for estimating EPG in all tested levels. Its assumed detection limit is 20 eggs but only 70% of samples at this limit were detected. At higher concentrations of 200 and 500 eggs, the accuracy of this method was almost 100%.

An important factor is not only the amount of investigated faeces, but also the dilution ratio (grams of faeces/millilitres of water). From the work of certain authors (Cringoli et al. 2004; Dunn and Keymer 1986; Pereckiene et al. 2007), it is evident that methods which use a low dilution factor (optimum 1:10–1:15) produce better results than methods with a ratio of 1:30 or more.

The results of our study are partially inconsistent with those of the authors cited above. In accordance with Cringoli et al. (2004) and Pereckiene et al. (2007), the highest reliability was observed in results obtained by using a dilution ratio of 1:14 (Roepstorff and Nansen 1998). By contrast, the lowest reliability was seen in the Wetzel method (1951), which uses a ratio of 1:30. The Zajíček method (1978) uses the lowest dilution ratio (1:5), and therefore it should be the most sensitive. However, at certain concentration levels, this method provides similar results to those methods with a ratio of 1:30. This low efficiency is evidently due to a higher amount of debris that hinders clear microscopic examination; the technician can overlook a number of eggs, and the probability of error increases.

The next point in the methodology that can affect the accuracy of a selected method is centrifugation. Methods that require centrifugation steps can be considered more complex and labour intensive, and their results are more precise (Pereckiene et al. 2007).

Similar results are evident in our work. The two methods of Roepstorff and Nansen (1998) and Zajíček (1978), which use centrifugation, are more reliable than the Wetzel method (1951), which does not utilize centrifugation steps. It is necessary to point out the possibility of errors caused by failing to strictly adhere to protocol through the incorrect use of rotation speed. In the most recent papers, only RPM as a unit of rotation frequency is used in the methodology. In the past, there were only several types of centrifuges, and their rotors were similar. Therefore, the differences among them were not very evident. Nowadays, there are a number of centrifuges, and they differ in rotor radius and rotation speed. For this reason, it is preferable to cite relative centrifugal force (RCF) or the symbol “g” in the methodology; they refer to the rotor radius of the particular centrifuge.

Some authors (Broussard 2003; Cringoli et al. 2004) write about the influence of flotation solution on the reliability of the McMaster method. According to Cringoli (Cringoli et al. 2004, 2010), flotation solution has a fundamental role in determining the analytic sensitivity, precision and reliability of any analytical method based on flotation.

We agree with the opinion that flotation solution has an effect on the reliability of the flotation method. On the other hand, we believe that when considering all the

methodological factors influencing reliability, flotation solution has the lower effect. Saturated sodium chloride with glucose (density 1.300) is a flotation fluid used in the concentration McMaster technique (Roepstorff and Nansen 1998); this method, which uses this solution, was the most reliable of all the tested modifications. By contrast, the highest number of negative samples was detected using only saturated sodium chloride (Wetzel 1951) with a density of 1.200. This flotation solution is commonly used in many McMaster method modifications (see Pereckiene et al. 2007).

As seen from the results cited above, it is very difficult to individually evaluate any factors influencing the reliability of the analysed method. When a sufficient amount of examined faeces and a low multiplication factor are selected, the results need not be precise when other disproportional factors (high dilution ratio, low RCF or no centrifugation step, unsuitable flotation solution, etc.) are applied. A number of other factors can influence FEC results including parasite biology (fecundity of the species, parasite numbers, prepatent period, arrested development or periparturient rise, etc.), host physiological status (history of prior exposure to the parasite, nutrition, etc.), host management factors (treatments, anthelmintic resistance, herd or flock density) or technician skill level (Eysker and Ploeger 2000; Gasbarre et al. 1996; Gates and Nolan 2009; Pereckiene et al. 2007).

When the three selected methods are compared, it is evident that the concentrated McMaster method (Roepstorff and Nansen 1998) is the most sensitive and reliable for the detection of helminth eggs. This method is fast, it has a low detection limit (20 eggs), and is relatively less laborious. Also, due to the centrifugation step, the faecal suspension is sufficiently clear for examination. The modification according to Zajíček (1978) should provide better results due to the low multiplication factor, the two centrifugation steps and the lowest dilution ratio. However, the dilution ratio alone may cause this method to fail. The amount of debris in the examined suspension renders the investigation of the sample more difficult and parasite elements can be overlooked. This method is applicable with accurate results from 100 EPG. The third compared method according to Wetzel (1951) is also the simplest one; however, it produces the worst results. The low sensitivity and reliability of this method is most likely caused by a high multiplication factor and the absence of a centrifugation step. This method provided accurate results from 200 EPG.

There are some articles in literature which deal with the comparison of different McMaster techniques (Pereckiene et al. 2007) or some part of their methodology (Cringoli et al. 2004). However, all these works use only parasite positive faeces from naturally infected animals. These authors are unaware of the real level of infection and therefore the EPG could be under or overestimated. For this reason, we prepared a specific number of tapeworm eggs which we added to the parasite negative faeces; these samples were later investigated. We are convinced that this is the only way to precisely compare McMaster counting techniques. There is no other work in literature that uses a similar approach to the investigation of McMaster methods.

Traditional coprological methods for the estimation of gastrointestinal (GI) nematode infection levels, based on microscopic examination of faeces, are laborious, time-consuming (especially when a large number of samples is examined) and requires an experienced diagnostician. For these reasons, new approaches for parasitological diagnosis are being researched. Advances in molecular technology offer the potential for reliable and sensitive methods, which are fast and reproducible. Some authors (Harmon et al. 2007; Humbert and Cabaret 1995; Learmount et al. 2009; Schnieder et al. 1999; Roeber et al. 2011; Zarlenga et al. 2001) developed different polymerase chain reaction (PCR) tools (multiplex PCR, real-time PCR, RAPD) for discriminating GI nematode eggs from faeces at both the genus and species levels. Yet, although these methods have considerable advantages, they are only semi-quantitative and have problems with extracting DNA directly from faeces resulting in low DNA yield (Bott et al. 2009; Learmount et al. 2009). For this reason, Bott et al. (2009) came up with the idea to combine isolation of nematode eggs from host faeces by conventional flotation procedure with real-time PCR.

Another diagnostic approach is to use immunological methods which detect antigens in faeces. Copro-antigen capture enzyme-linked immunosorbent assays take into account current intestinal infection with adult worms because these methods detect excretory/secretory (ES) antigens released by the metabolic active stages (Agneessens et al. 2001; Deplazes et al. 1992; Roepstorff 1998). Unfortunately, these immunological methods also have their limitations. Parasite ES products are released in the GI tract and these antigens may be digested during its passage (Agneessens et al. 2001). Another problem with these methods is the cross-reactivity with other concurring GI nematodes (Johnson et al. 1996) or other parasites (Agneessens et al. 2001).

An antemortem diagnostic method, which is able to sensitively, reliably and quickly estimate the real number of GI nematodes in the host and can also classify detected parasites up to the species level, has yet to be developed. In the future, molecular assays will become widespread. Combined microscopic–molecular methods could become the optimal tool for GI nematode diagnosis. Therefore, conventional coprological techniques will continue to play an important role in parasitological diagnosis.

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Ethical standards All experiments conducted with laboratory animals comply with the current laws of the country in which they were performed.

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