**Coproscopical diagnosis of patent *Fasciola hepatica* infections in sheep – a comparison between standard sedimentation, FLUKEFINDER® and a combination of both**

Alexandra Kahla, Georg von Samson-Himmelstjernaa, Christina S. Helma, Jane Hodgkinsonb, Diana Williamsb, Wiebke Weiherc, Werner Terhallec, Stephan Steuberc, Jürgen Krückena\*

a *Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin; Robert-von-Ostertag-Str. 7, 13163 Berlin, Germany*

b *Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, L3 5RF, Liverpool, UK*

c *Federal Office of Consumer Protection and Food Safety, Mauerstr. 39-42, 10117 Berlin, Germany*

\* Correspondence to: Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Robert-von-Ostertag-Str. 7, 14163 Berlin, Germany

*E-mail address:* juergen.kruecken@fu-berlin.de (J. Krücken).

**Abstract**

The liver fluke *Fasciola hepatica* is a highly pathogenic and zoonotic trematode with a cosmopolitan distribution. In livestock, infections may lead to significant economic losses if not diagnosed promptly and treated effectively. Particularly for small ruminants, the standard method for the detection of fluke infection is based on coproscopical methods such as the sedimentation method, which detects *F. hepatica* eggs in faecal samples. In this respect a recent innovative coproscopical approach to diagnose patent infections is the FLUKEFINDER® method, which relies on differential sieving before sedimentation. These two methods and a combination of both methods that allows larger amounts of faeces to be processed with the FLUKEFINDER® apparatus were compared, to assess which method is most appropriate to determine the prevalence and intensity of *F. hepatica* egg shedding. The methods were compared for their ability to recover eggs from ovine faecal samples containing different numbers of fluke eggs per gram (EPG) of faeces and diluting the samples further by mixing with faeces from uninfected sheep. To compare the specificity of the test procedures, positive and negative samples with a low EPG were analysed in parallel by an investigator blinded to the nature of the samples. Significant differences concerning the EPG outcome were found: The FLUKEFINDER® method demonstrated the highest EPG values (p<0.001) in the undiluted samples as well as in all mixing levels, followed by the modified FLUKEFINDER® method. The standard sedimentation showed the lowest EPG values and the highest variability between technical replicates. The precision of the FLUKEFINDER® method and the modified FLUKEFINDER® method were significantly higher than the precision of the standard sedimentation as determined by comparison of variability between technical replicates. The highest raw egg counts were detected using the modified FLUKEFINDER® method. The FLUKEFINDER® method and the combined method showed a sensitivity of 100% even at the lowest egg concentrations, whereas the sensitivity of the standard sedimentation was 98.1% for the same set of samples (i.e. one false negative sample). In a separate investigation aiming to estimate the specificity no differences were found between the three methods: all protocols showed 100% specificity and were able to correctly distinguish between truly positive and truly negative samples without any evidence of cross-contamination between positive and negative samples processed in parallel.

**Keywords**: Liver fluke, diagnostics, coproscopy, sedimentation, small ruminant

**1. Introduction**

The common liver fluke *Fasciola hepatica* is a trematode with a worldwide distribution. It is of great importance for sheep farmers and infections may result in acute to chronic disease with for example wasting and significant production losses but also acute mortality (Forbes, 2017; Hayward et al. 2021; Kahl et. al 2020; Williams, 2020; Stuen and Ersdal, 2022). Fasciolosis has a major economic impact leading to considerable financial losses in both small and large ruminants (Charlier et al. 2020). The diagnosis of patent infections is important but often challenging since egg shedding occurs intermittently (Düwel und Reisenleiter, 1990; Sargison and Scott, 2011) and the number of eggs in the faeces is generally low (e.g. considerably lower than the number of eggs shed by major gastrointestinal nematodes) and not necessarily correlated with worm burden or the degree of clinical disease.

Reports about emerging resistance of the parasite to different flukicidal agents have increased during recent years (Moll et al. 2000; Alvarez-Sánchez et al. 2006; Mooney et al. 2009; Gordon et al. 2012; Ortiz et al. 2013; Kelley et al. 2016; Novobilský et al. 2016; Kamaludeen et al. 2019). Resistance monitoring requires accurate coproscopical methods with a high sensitivity to identify patent infections reliably and good precision to determine the trematode egg shedding intensity in eggs per gram (EPG) faeces. Precise data and high numbers of eggs observed directly under the microscope in contrast to extrapolated EPGs were shown to be highly relevant to diagnose anthelmintic resistance in parasitic gastrointestinal nematodes using faecal egg count reduction tests (FECRT) (Levecke et al. 2011; Levecke et al. 2012; Torgerson et al. 2012; Torgerson et al. 2014; Levecke et al. 2015; Levecke et al. 2018; Nielsen, 2021).

The most frequently used method for examining faecal samples for the presence of *F. hepatica* eggs is the conventional sedimentation method (“standard sedimentation” in the following), which is a simple and inexpensive method requiring only basic laboratory equipment (Boray, 1969). Up to 10 g of faeces are usually examined with this method. A recently established technique for *F. hepatica* diagnosis is the “FLUKEFINDER®” method (FLUKEFINDER® Diagnostic System, Soda Springs, Idaho, USA), which is based on differential sieving followed by a sedimentation procedure. Two g of faeces can be processed per sample (Zárate-Rendón et al. 2019; Reigate et al. 2021) with a detection limit of one egg per gram of faeces according to the manufacturer. The low egg detection limit is promising for an accurate diagnosis even at low egg concentrations in faecal samples. The first use of the FLUKEFINDER® was promising with every single *F. hepatica* egg visible in the sediment, even without the use of methylene blue, which is often applied in sedimentation protocols to enhance the visibility by counter-staining plant particles in a blue colour. When performing the standard sedimentation, a large amount of coarse faecal components in the sediment frequently impairs the visibility of the eggs and the probability of missing individual eggs during the microscopical examination is high. However, the FLUKEFINDER® method only allows a sample size of 2 g of faeces, whereas up to 10 g of faeces per procedure can be examined using the standard sedimentation. Hence, a combination of both methods was implemented by performing the first steps equal to the standard sedimentation using 10 g of faeces and sieving the resulting sediment through the FLUKEFINDER® device (“Modified FLUKEFINDER®”).

The aim of the present study was to compare the faecal egg counts obtained from repetitive analysis of the same ovine faecal sample using these three different coproscopical methods. Precision was investigated by analysing multiple technical replicates for each biological replicate. Biological replicates contained eggs with various *F. hepatica* EPG values to be able to investigate technical variability between these methods and its dependency on egg counts. Furthermore, a comparison of the sensitivity and specificity of the methods was conducted to investigate whether the methods are able to distinguish between truly positive and truly negative samples or if cross-contamination was likely to occur during parallel handling of both sample types in the laboratory.

**2. Materials and Methods**

*2.1. Collection of faeces*

Faecal samples were collected from patently *F. hepatica*-infected sheep. The first five experimental runs were performed using the faeces of a two-year-old naturally *F. hepatica*-infected dairy sheep (~ 65 kg bodyweight) with moderate egg shedding, collected at different days post infection. The last repetition was conducted using the faeces of a one-year-old experimentally *F. hepatica*-infected crossbred sheep (~ 55 kg bodyweight) with a comparatively high egg shedding intensity. All animal experiments were in agreement with both the European Directive 2010/63/EU and the German Animal Welfare Act (Tierschutzgesetz) and were approved by the Landesamt für Gesundheit und Soziales of the federal state Berlin under the reference number H0337/17.

Since a large amount of defined faecal material was needed for the comparison (286 g of *F. hepatica*-positive faeces per experimental round), faecal samples from infected sheep were collected over several hours using self-made cotton bags placed underneath the sheep's tail (Fig. 1).Since egg shedding may be uneven over time, all samples were thoroughly kneaded for several minutes to obtain homogenous distribution before further analysis. Between collection and analysis, samples were stored at 4°C in the dark. For the mixing procedure to obtain samples with lower *F. hepatica* egg counts than the original sample, *F. hepatica*-negative faeces from non-infected animals were collected in the same way.

In order to evaluate how the three different methods performed with low faecal egg counts, four dilutions of each original sample were made for each repetition by mixing the *F. hepatica* positive samples with faeces from non-infected sheep using defined ratios.

*2.2. Mixing procedure*

For the examination of the undiluted *F. hepatica*-positive faeces, 10 g of faeces (standard sedimentation and modified FLUKEFINDER®) or 2 g of faeces (standard FLUKEFINDER®) were weighed into a 250 ml beaker using a digital scale (accuracy: 0.1 g) and a wooden spatula.

In order to compare the EPG in samples containing a lower number of eggs than the original samples using each of the three methods, the *F. hepatica*-positive faeces were mixed and homogenised with *F. hepatica*-negative faeces in defined ratios. For this purpose, a defined amount of *F. hepatica*-positive faeces was individually weighed into a 250 ml-beaker and mixed with a defined amount of *F. hepatica*-negative faeces to obtain proportions of faeces with eggs of 80%, 50%, 20% and 10% (Table 1). Each sample ratio (100%, 80%, 50%, 20%, 10% of *F. hepatica*-containing faeces) was examined five times (technical replicates) with each of the three methods. Such a mixing series was set up on six different days with sample material differing in starting EPG, leading to six biological replicates for each of the five ratios (n=30 in total for biological and technical replicates).

*2.3. Analyses for sensitivity and specificity estimation*

The required amount of faeces for each method was homogenised and weighed as described above to obtain 20 technical replicates of *F. hepatica*-positive samples (mean EPG obtained by five FLUKEFINDER® analyses of 14.6 EPG) and 20 technical replicates of *F. hepatica*-negative samples for each method under investigation. Individual replicates were labelled in a blinded manner by a third party before coproscopic analyses. Samples were only investigated under a microscope until the first *F. hepatica* egg was found and then scored as positive. If no egg was found, the complete sediment was thoroughly inspected.

*2.4. Coproscopic methods*

*2.4.1. Standard sedimentation*

After weighing the defined amounts of faeces (in total: 10 g) into a labelled 250 ml beaker, the faecal material was thoroughly suspended in approximately 50 ml cold tap water using a wooden spatula. The suspension was transferred into a second 250 ml beaker and subsequently sieved through a tea strainer (mesh size: >300 µm) back into the first 250 ml beaker until the beaker was completely filled. The inner surface of the transfer-beaker was thoroughly rinsed to avoid losing eggs. After 30 minutes, the tea strainer was removed from the beaker and the supernatant was decanted. One drop of detergent was added to the sediment and the beaker was replenished with tap water until the 250 ml mark was reached. After a three-minute sedimentation period, the supernatant was decanted again and the beaker was refilled with tap water. This procedure was repeated twice (in total: 3 × 3 minutes of sedimentation).

The sediment usually consisted of two fractions: the lower part containing the faecal components with the highest specific weight (including *F. hepatica* eggs) and a lighter layer on top mostly containing coarse plant components. Since no further sieving followed the decantation, each decantation had to be conducted extensively to remove coarse components of the sediment at this point. Only the bottom part of the sediment remained in the beaker during the decantation process and the upper layer of the sediment was poured off, so that fewer plant components might obscure the microscopical view. Subsequently, the sediment was transferred into a petri dish (6 cm diameter) marked with coloured lines on the bottom. Following the coloured lines, the complete petri dish was microscopically (25x magnification) examined in a meandering pattern and all eggs were counted using a manual counter. The petri dish was reused for multiple samples but thoroughly rinsed with a strong water jet between every sample to avoid cross-contamination.

*2.4.2. Standard FLUKEFINDER® method*

The commercial FLUKEFINDER® device consists of two sections forming a column. Each section contains a sieve: The upper sieve has a larger mesh size, so that *F. hepatica*-eggs can pass through this sieve. The second sieve has a finer mesh size, which holds the eggs back. Howell (2011) indicated the mesh sizes with approximately 125 µm and 30 µm. However, the exact mesh sizes are proprietary and not declared by the manufacturer. The two units of the column fit together with the larger meshed sized sieve on top and the part with the finer meshed sieve on the bottom.

The protocol used here followed the directions given by the manufacturer with slight modifications. The defined amount of faecal material (in total: 2 g) was weighed into a labelled 250 ml beaker (instead of using the 100 ml beaker provided) and suspended in 30 ml cold tap water. The suspension was poured into the top section of the FLUKEFINDER® column. After the suspension had completely passed the upper sieve, the upper section of the FLUKEFINDER® apparatus was half refilled with cold tap water. After repeating this step three times, the two sections of the column were separated. The debris on the upper sieve were removed and the debris on the lower sieve were backwashed with a strong water jet into a labelled 250 ml beaker. After a sedimentation period of three minutes, the supernatant was decanted, and the fine sediment was transferred into a 50 ml-centrifugation tube with conical bottom (instead of using the provided 15 ml tube of equal height). The inner surface of the beaker was thoroughly rinsed using a wash bottle to wash out remaining *F. hepatica*-eggs into the centrifugation tube. The centrifugation tube was filled with cold tap water to the 50 ml mark (water level: approximately 10 cm). After a three-minute sedimentation period, the supernatant was carefully poured off and the centrifugation tube was refilled again up to the 50 ml-mark, and the sediment was well resuspended in 50 ml tap water. After another three-minutes sedimentation period, the supernatant was decanted, and the sediment was transferred into a petri dish. Differing from the instruction manual of the FLUKEFINDER®, a petri dish with a diameter of 6 cm and coloured lines on the bottom was used instead of the small petri dish supplied. This petri dish was also used for the two other methods. The microscopical egg counting was performed using a 25-fold magnification.

The FLUKEFINDER® column and the petri dish were thoroughly rinsed with a strong water jet between every sample to avoid cross-contamination.

*2.4.3. Modified FLUKEFINDER® method*

The first steps were identical to the protocol for the standard sedimentation (see 2.3.1) using 10 g of faeces. Altogether, two sedimentations were performed. Due to the fact that further sieving followed the decanting and the sediment did not have to be fine enough for microscopical evaluation at this point, each decantation was performed very carefully and the decantation was immediately stopped when the thin apex of the top sediment fraction reached the edge of the beaker, meaning that the lighter superficial layer of the sediment was retained. The sediment was then poured into the top section of the FLUKEFINDER® and the process as described under 2.3.2 was conducted.

*2.5. Statistical analyses*

The statistical analyses were performed using GraphPad Prism 5.03 (Graph -
Pad Software, San Diego, CA, USA) and Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). First, the raw egg counts were entered into an Excel spreadsheet and the means and the standard deviations for all technical replicates for each method and each biological replicate were calculated. Linear regressions and Pearson correlations were calculated in GraphPad. In order to determine, if a slope was significantly different from 1, a curve based on the sedimentation data was added on the x and y axis, which results in a perfect line with a slope of 1. Then, slopes of data for FLUKEFINDER® and modified FLUKEFINDER® were compared with this line. A Friedman test followed by the Dunn’s multiple comparison post hoc test were performed to evaluate whether the paired EPG values obtained with the three methods were significantly different from each other.

To determine if the mixing of eggs from *F. hepatica*-positive faeces with non-infected animals resulted in deviations from linearity at low egg counts, data were normalised to the mean EPG in the undiluted sample for a given method. Pearson’s linear regression analyses were performed based on these normalised EPG values and the slopes of the regression lines and Y intercepts for each coproscopic method were compared with the tests implemented in GraphPad. Wald–Wolfowitz runs tests were performed to detect potential deviations from linearity.

The coefficient of variation (CV) was determined for each of the three methods to identify the relative dispersion around the mean EPG value in each level of dilution with *F. hepatica-*negative faeces. Correlation between the normalised mean EPG and the CV values for the different methods were calculated as Spearman’s ρ. Paired CV values for each sample obtained with the different coproscopic methods were pairwise subtracted (sedimentation - FLUKEFINDER, sedimentation - modified FLUKEFINDER®, FLUKEFINDER® - modified FLUKEFINDER®). Then GraphPad was used to conduct a Wilcoxon signed rank test to determine if the median value was significantly different from zero.

The specificity and sensitivity as well as the positive and negative predictive values with their 95 % confidence intervals (95% CIs) were calculated using the 20 true positive and 20 true negative replicates for each method using the BDtest function from the R package bdpv 1.3 and analyses were conducted in R version 4.1.1**.**

**3. Results**

*3.1. Comparison of raw egg counts and EPG values*

The raw egg counts and the calculated EPGs were compared for the three different methods (Fig. 2 and Supplementary Tables S1 and S2). Mean raw egg counts and EPGs were calculated from the five technical replicates for each mixing replicate (n=30) and plotted in Fig. 2. Using the data obtained from the well established sedimentation method as reference, scatter plots for raw egg counts (Fig. 2A) as well as calculated EPG values (Fig. 2B) were plotted. Linear regressions and Pearson correlations were calculated for all combinations of methods (Table 2). All Pearson r coefficients were > 0.978. For raw egg counts, the slope for FLUKEFINDER® was almost twice as high as for modified FLUKEFINDER®. Both slopes were significantly higher than 1 (p < 0.0001) and significantly different from each other (p<0.0001). These data indicate that raw egg counts for the modified FLUKEFINDER® and the FLUKEFINDER® methods were approximately 6- and 14-fold higher than for the sedimentation method. For EPG values, slopes for FLUKEFINDER® and modified FLUKEFINDER® were 2.7 and 6.1, respectively, corresponding to 2.7 and 6.1-fold higher EPG values obtained for these methods compared to sedimentation. Slopes were again significantly different between all methods and also significantly different from 1 (p < 0.0001).

In addition, the Friedman test followed by Dunn’s multiple comparison post-hoc test detected highly significant differences between all three methods for the raw egg counts as well as the calculated EPG values (*p*<0.0001). While raw egg counts were highest for the modified FLUKEFINDER® followed by FLUKEFINDER® and standard sedimentation, calculated EPG values were highest for FLUKEFINDER® followed by modified FLUKEFINDER® and sedimentation.

*3.2. Dependency of egg enrichment efficacy on egg concentration*

For each dilution series, EPG data were normalised to the mean EPG of the technical replicates of the undiluted sample for the same method. These normalised EPG values were plotted against the fraction of *F. hepatica*-positive faeces in the samples (Fig. 3). Pearson regression analyses were performed for each coproscopic method and revealed a significant linear effect of dilution on the EPG (p<0.0001). Regression lines for all three methods were almost identical although normalised EPGs were slightly higher for FLUKEFINDER® for low fractions of *F. hepatica*-positive faeces in the samples. However, the differences between the three slopes were not significant (*p*=0.3054) and the y intercepts of the regression lines (*p* =0.2351) were not significantly different. The Wald–Wolfowitz runs test did not detect any significant deviations from linearity (*p*=0.5). Therefore, egg recovery of all three methods did not vary depending on the EPG.

3.4. Comparison of reproducibility between methods

Analyses of variances regarding the egg recovery rate between the biological replicates were performed by calculating the CV values from the technical replicates. The CV values were plotted against the mean EPG values followed by calculation of Spearman correlations (Fig. 4A). As expected, Spearman ρ coefficients revealed a significant negative correlation between the mean EPG and the CV in all three methods with ρ=-0.5609 for the standard sedimentation (p=0.001), ρ=-0.5519 for the FLUKEFINDER® method (p=0.002) and ρ= 0.6854 for the modified FLUKEFINDER® method (p<0.0001).

A direct comparison of CVs between methods was used to compare precision of methods and is provided in Fig. 4B. The difference of CVs for each biological replicate was calculated and plotted for the pairwise comparison of the three methods. Using a Wilcoxon rank-sum test it was shown that the median difference was significantly higher than zero for the comparison of the sedimentation with either FLUKEFINDER® or modified FLUKEFINDER® methods. In contrast, the median difference in CV values was not significantly different from zero for the comparison of the two FLUKEFINDER® based methods. This shows that the technical variation is significantly higher when sedimentation is used compared to any of the FLUKEFINDER® methods.

*3.5. Comparison of sensitivity and specificity at low egg concentrations*

*3.5.1. Initial estimation of sensitivities from highly diluted positive samples*

To compare the sensitivity of the three methods in samples with low egg concentration, the FLUKEFINDER® method was taken as a reference and all data from biological replicates with a FLUKEFINDER® mean EPG < 10 in the respective dilution levels (Supplementary Table S2) were pooled. In total, seven biological replicates showed a mean EPG < 10 EPG in the FLUKEFINDER® method, so that 35 biological replicates per method were qualitatively assessed whether the result was positive (≥ 1 EPG) or negative (0 EPG). All technical replicates with a low egg concentration examined using the FLUKEFINDER® and the modified FLUKEFINDER® methods showed a positive result (each 105/105 = 100%). In contrast, for the standard sedimentation two technical replicates showed a negative result and the other 103 replicates were positive (103/105 = 98.1%).

*3.5.2. Systematic evaluation of sensitivity and specificity with defined positive and negative sample*

For each method, twenty true positive samples (mean EPG of 14.6 as determined by five FLUKEFINDER® analyses) and twenty true negative samples were analysed. These data were used to calculate sensitivity, specificity, predictive positive and predictive negative values with 95% CIs. Results are summarised in Table 3 and Supplementary Table S3.

**4. Discussion**

The standard sedimentation is a simple and inexpensive method to detect *F. hepatica* eggs in faecal samples. However, the method is generally considered to have limitations regarding examination of faecal samples with low egg counts (Conceição et al. 2002; Becker et al. 2016; Ploeger et al. 2017; Alstedt et al. 2022). Egg shedding by *F. hepatica* as well as by other liver flukes is known to be intermittent and thus, EPGs might show considerable variation over time (Düwel and Reisenleiter,1990; Sargison and Scott, 2011). Hence, sensitive methods to detect infected animals and precise methods to evaluate the efficacy of flukicidal drugs are needed. In human and veterinary medicine, several other methods have been used to detect *F. hepatica* eggs such as Kato-Katz, Mini-FLOTAC and more recently the FLUKEFINDER®. Comparison of FLUKEFINDER® with Kato-Katz and Mini-FLOTAC for human samples spiked with *F. hepatica* eggs revealed that FLUKEFINDER® outcompeted the other two methods in terms of low variation between technical replicates as determined by CV and sensitivity, particularly at low EPGs (Zárate-Rendón et al. 2019). Surprisingly, however, EPGs obtained with FLUKEFINDER® were much lower than expected and also lower than those obtained with the other two methods, resulting in poor accuracy (Zárate-Rendón et al. 2019). In our preliminary investigations, the Mini-FLOTAC method using zinc sulfate (specific gravity: 1.3) as a flotation medium for the detection of *F. hepatica* eggs in faecal samples performed worse than the standard sedimentation (unpublished data), so we did not pursue this method.

Among the methods compared in the present study, FLUKEFINDER® data showed the highest EPG counts. However, since the true EPG was unknown for the samples used here, it was not possible to estimate and compare accuracy between the methods.

Using the initial data set without negative samples, a slightly lower sensitivity of standard sedimentation compared to the other two methods was observed. However, due to the absence of negative samples, this data set was not suitable to calculate sensitivity and specificity values. Therefore, another sample set, which was blinded for the examiner by a third party, was analysed to compare the sensitivity and specificity of all three methods. Using these truly positive and truly negative samples, no differences were detected regarding the performance of the three methods in terms of false positive and false negative outcomes. All truly positive samples showed a positive result for all methods and all truly negative samples showed a negative result for all methods. Since all test results were in complete agreement with the true status of the sample, sensitivity and specificity were 100% for the standard sedimentation, FLUKEFINDER® and modified FLUKEFINDER® in this second data set resulting from the systematic evaluation of sensitivity and specificity. The PPV and NPV were 0.94 for all methods.

Noteworthy, a thorough cleaning process of every repeatedly used equipment after each sample is an absolute prerequisite to preclude cross-contamination when examining many samples in a row. Our experiments showed that a meticulous rinsing of the equipment using a strong jet of tap water is sufficient to clean the FLUKEFINDER® column, beakers and the petri dish between the samples in order to avoid an adulteration of the examination results.

Generally, the EPG values detected with the standard sedimentation turned out to be significantly lower than the EPG values detected with the other two methods in the same biological replicate. This may lead to an underestimation of the severity of infection or even false negative results as seen here for two technical replicates at the highest dilution with faeces from non-infected animals, so that a flukicidal treatment may potentially not appear necessary to the farmer or the veterinarian. Moreover, for evaluating the flukicidal effect of an anthelmintic through a FECRT, coproscopic methods that result in high numbers in raw egg count data have a strong advantage since this decreases the size of the 95% confidence interval of the faecal egg count reduction estimate (Torgerson et al. 2005; Levecke et al. 2015).

Finally, the size of the CV was significantly higher in the standard sedimentation compared to the other two methods, resulting in a higher relative variability of the EPG outcome.

The lower egg counts detected with the standard sedimentation could possibly be caused by eggs remaining in the faecal debris in the tea strainer or by loss of eggs in the decantation process, needed to remove the coarse components of the sediment.

The FLUKEFINDER® method showed the highest EPG values independently of the extent by which the positive samples were diluted with negative faeces, most likely because the mesh sizes of the two sieves are optimally adapted to the size of *F. hepatica* eggs, so that only components with an approximate size above that of *F. hepatica* eggs are filtered out and then *F. hepatica* eggs retained in the two sieving steps, respectively. In contrast to the standard sedimentation, the faecal debris on the sieve, in which the eggs may get caught, is only rinsed three times, with no decanting, so the chance of losing eggs in the sieving process is kept very low. Since the backwashed sediment from the second sieve is very clean once the coarse components have been removed on the first sieve of the FLUKEFINDER®, the subsequent decanting steps in the centrifugation tube have a lower risk of losing eggs when pouring off the supernatant. The sensitivity of this method was 100% in our experiments, even at low egg concentrations. To implement the FLUKEFINDER® method, only the one-time purchase of the FLUKEFINDER® device and centrifugation tubes are necessary aside from the basic equipment which is also required for the standard sedimentation. As no specialised laboratory equipment is needed, it is a simple and low-cost method applicable for the use in basically equipped laboratories.

The present study also evaluated the combination of both methods (modified FLUKEFINDER®) for the first time and found that it leads to the recording of higher EPG values compared to the EPG values detected with the standard sedimentation. That is presumably because only two decantation steps must be performed after the coarse sieving through the tea-strainer. Moreover, also the upper layer of the sediment containing mostly lighter plant components is retained in the beaker during these two decantation steps. As the sediment is further filtered through the FLUKEFINDER® column, coarse plant components do not have to be removed through decantation at this point and thereby the chance of losing eggs, which might have not completely settled down to the bottom of the beaker, decreases. In contrast to the standard sedimentation in the initial data set, the sensitivity of the combined method was 100%, even in the samples with the lowest egg concentration.

The calculated EPG values were significantly lower than the EPG values detected with the FLUKEFINDER® method. As already stated above, it is likely that eggs adhere to the faecal debris in the tea strainer as the debris is only rinsed once, so that a proportion of the eggs does not get into the sediment. Both FLUKEFINDER® and modified FLUKEFINDER® methods showed a significantly lower variability in recovering eggs from faecal samples compared to the standard sedimentation and thus, have a higher precision.

Looking at the actual raw egg counts without downscaling the results to EPG level, the highest raw egg counts were found using the modified FLUKEFINDER®. This reveals that the use of 10 g of faeces in the modified FLUKEFINDER® method has a positive effect on the results but that losses during the sedimentation processes are still higher than during the FLUKEFINDER® method alone.

A high raw egg count is crucial for the performance of the FECRT to statistically determine the egg count reduction (Torgerson et al. 2005; Levecke et al. 2015). Considering this perspective, the examination of composite samples instead of individual samples is superior as raw eggs counts increase depending on the number of patently infected animals included in the composite sample. For performing FECRT to evaluate the flukicidal activity of triclabendazole against *F. hepatica*, a protocol using 5 g of faeces from 10 individual sheep (50 g in total) has been validated (Daniel et al. 2012). For the evaluation of anthelmintic efficacy on herd level, this approach was shown to narrow the 95% confidence intervals and it is time-saving compared to the examination of individual samples. However, information on variability of egg shedding and drug efficacy is lost when composite samples are used. Use of so large amounts of faeces is not possible with the FLUKEFINDER® approach. However, pooling of data from multiple FLUKEFINFER® investigations might help to overcome this limitation. The modified FLUKEFINDER® method was able to handle larger amounts of faeces than the standard FLUKEFINDER® by adding two sedimentation steps at the beginning. This led to increased raw egg counts but apparently eggs were lost in the sedimentation step and EPG values were significantly lower. Moreover, the amount of 10 g faeces might sometimes be difficult to collect from small ruminants under field conditions.

**5. Conclusion**

Although the sensitivity of the three methods used in the study was comparable, both FLUKEFINDER® and modified FLUKEFINDER® are superior to sedimentation in terms of egg count data. The FLUKEFINDER® method had its strength in resulting in the highest EPG value, since loss of eggs during enrichment was minimised. The modified FLUKEFINDER® method resulted in higher raw egg counts, which is beneficial for the FECRT. FLUKEFINDER® based approaches should replace sedimentation in all studies for which egg counts are relevant.

**Funding**

This work was conducted as collaborative research project of the Freie Universität Berlin and the Federal Office of Consumer Protection and Food Safety and financially supported by the latter under the reference number 2019000389.

**CRediT authorship contribution statement**

Alexandra Kahl: Conceptualisation, Data curation, Methodology, Formal analysis, Investigation, Writing – Original Draft

Jürgen Krücken: Conceptualisation, Funding acquisition, Methodology, Formal analysis, Writing – Original Draft, Validation, Visualisation; Project administration

Georg von Samson-Himmelstjerna: Conceptualisation, Funding acquisition, Methodology, Formal analysis, Writing – Original Draft, Project administration

Christina Helm: Conceptualisation, Investigation, Writing – Original Draft

Stephan Steuber: Conceptualisation, Funding acquisition, Writing - Review & Editing

Wiebke Weiher: Funding acquisition, Writing - Review & Editing

Werner Terhalle: Funding acquisition, Writing - Review & Editing

Jane Hodgkinson: Resources, Writing - Review & Editing

Diana Williams: Resources, Writing - Review & Editing

**Declaration of Competing Interest**

The authors declare that they have no competing financial interests or personal relationships that are related to the work reported in this publication.

**Acknowledgements**

The authors would like to thank Sabrina Ramünke (Freie Universität Berlin) for assistance in the experimental infection of lambs with *F. hepatica* metacercariae and Paula Ehnert (Freie Universität Berlin) for assistance in the specificity and sensitivity estimation sub-project.

**Appendix**

Table S1. Raw egg counts of all biological and technical replicates for sensitivity estimation

Table S2. Eggs per gram levels of all biological and technical replicates for sensitivity estimation

Table S3. Results of systematic evaluation of sensitivity and specificity with defined positive and negative samples

1. **References**

Alstedt, U., Voigt, K., Jäger, M.C., Knubben-Schweizer, G., Zablotski, Y., Strube, C., Wenzel, C., 2022. Rumen and Liver Fluke Infections in Sheep and Goats in Northern and Southern Germany. Animals (Basel)12.

Alvarez-Sánchez, M.A., Mainar-Jaime, R.C., Pérez-García, J., Rojo-Vázquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec.159, 424-425.

Becker, A.C., Kraemer, A., Epe, C., Strube, C., 2016. Sensitivity and efficiency of selected coproscopical methods-sedimentation, combined zinc sulfate sedimentation-flotation, and McMaster method. Parasitol. Res.115, 2581-2587.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv. Parasitol.7, 95-210.

Charlier, J., Rinaldi, L., Musella, V., Ploeger, H.W., Chartier, C., Vineer, H.R., Hinney, B., von Samson-Himmelstjerna, G., Băcescu, B., Mickiewicz, M., Mateus, T.L., Martinez-Valladares, M., Quealy, S., Azaizeh, H., Sekovska, B., Akkari, H., Petkevicius, S., Hektoen, L., Höglund, J., Morgan, E.R., Bartley, D.J., Claerebout, E., 2020. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. Prev. Vet. Med.182, 105103.

Conceição, M.A., Durão, R.M., Costa, I.H., da Costa, J.M., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Vet. Parasitol.105, 337-343.

Daniel, R., van Dijk, J., Jenkins, T., Akca, A., Mearns, R., Williams, D.J., 2012. Composite faecal egg count reduction test to detect resistance to triclabendazole in Fasciola hepatica. The Veterinary record 171, 153, 151-155.

Düwel, D., Reisenleiter, R., 1990. [*Fasciola hepatica*: coprological diagnosis in comparison to the worm burden in sheep and cattle]. Angew. Parasitol.31, 211-217.

Forbes, A., 2017. Liver fluke infections in cattle and sheep. Livestock22, 250-256.

Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol.187, 436-444.

Hayward, A.D., Skuce, P.J., McNeilly, T.N., 2021. The influence of liver fluke infection on production in sheep and cattle: a meta-analysis. Int. J. Parasitol.51, 913-924.

Howell, A., 2011. Snail-borne diseases in bovids at high and low altitude in Eastern Uganda: Integrated parasitological and malacological mapping. [Dissertation] Liverpool School of Tropical Medicine, University of Liverpool, United Kingdom.

Kahl, A., von Samson-Himmelstjerna, G., Krücken, J., Ganter, M., 2021. Chronic Wasting Due to Liver and Rumen Flukes in Sheep. Animals (Basel)11.

Kamaludeen, J., Graham-Brown, J., Stephens, N., Miller, J., Howell, A., Beesley, N.J., Hodgkinson, J., Learmount, J., Williams, D., 2019. Lack of efficacy of triclabendazole against *Fasciola hepatica* is present on sheep farms in three regions of England, and Wales. Vet. Rec.184, 502.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current Threat of Triclabendazole Resistance in *Fasciola hepatica*. Trends Parasitol32, 458-469.

Levecke, B., Anderson, R.M., Berkvens, D., Charlier, J., Devleesschauwer, B., Speybroeck, N., Vercruysse, J., Van Aelst, S., 2015. Mathematical inference on helminth egg counts in stool and its applications in mass drug administration programmes to control soil-transmitted helminthiasis in public health. Adv. Parasitol.87, 193-247.

Levecke, B., Kaplan, R.M., Thamsborg, S.M., Torgerson, P.R., Vercruysse, J., Dobson, R.J., 2018. How to improve the standardization and the diagnostic performance of the fecal egg count reduction test? Vet. Parasitol.253, 71-78.

Levecke, B., Rinaldi, L., Charlier, J., Maurelli, M.P., Bosco, A., Vercruysse, J., Cringoli, G., 2012. The bias, accuracy and precision of faecal egg count reduction test results in cattle using McMaster, Cornell-Wisconsin and FLOTAC egg counting methods. Vet. Parasitol.188, 194-199.

Levecke, B., Rinaldi, L., Charlier, J., Maurelli, M.P., Morgoglione, M.E., Vercruysse, J., Cringoli, G., 2011. Monitoring drug efficacy against gastrointestinal nematodes when faecal egg counts are low: do the analytic sensitivity and the formula matter? Parasitol. Res.109, 953-957.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The netherlands. Vet. Parasitol.91, 153-158.

Mooney, L., Good, B., Hanrahan, J.P., Mulcahy, G., de Waal, T., 2009. The comparative efficacy of four anthelmintics against a natural acquired *Fasciola hepatica* infection in hill sheep flock in the west of Ireland. Vet. Parasitol.164, 201-205.

Nielsen, M.K., 2021. What makes a good fecal egg count technique? Vet. Parasitol.296, 109509.

Novobilský, A., Amaya Solis, N., Skarin, M., Höglund, J., 2016. Assessment of flukicide efficacy against *Fasciola hepatica* in sheep in Sweden in the absence of a standardised test. Int J Parasitol Drugs Drug Resist6, 141-147.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against Triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an in vivo efficacy test in sheep. Vet. Parasitol.195, 118-121.

Ploeger, H.W., Ankum, L., Moll, L., van Doorn, D.C.K., Mitchell, G., Skuce, P.J., Zadoks, R.N., Holzhauer, M., 2017. Presence and species identity of rumen flukes in cattle and sheep in the Netherlands. Vet. Parasitol.243, 42-46.

Reigate, C., Williams, H.W., Denwood, M.J., Morphew, R.M., Thomas, E.R., Brophy, P.M., 2021. Evaluation of two *Fasciola hepatica* faecal egg counting protocols in sheep and cattle. Vet. Parasitol.294, 109435.

Sargison, N.D., Scott, P.R., 2011. Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. Vet. Rec.168, 159.

Stuen, S., Ersdal, C., 2022. Fasciolosis-An Increasing Challenge in the Sheep Industry. Animals (Basel)12.

Torgerson, P.R., Paul, M., Furrer, R., 2014. Evaluating faecal egg count reduction using a specifically designed package "eggCounts" in R and a user friendly web interface. Int. J. Parasitol.44, 299-303.

Torgerson, P.R., Paul, M., Lewis, F.I., 2012. The contribution of simple random sampling to observed variations in faecal egg counts. Vet. Parasitol.188, 397-401.

Torgerson, P.R., Schnyder, M., Hertzberg, H., 2005. Detection of anthelmintic resistance: a comparison of mathematical techniques. Vet. Parasitol.128, 291-298.

Williams, D., 2020. Update on liver fluke in sheep. In Pract.42, 341-347.

Zárate-Rendón, D.A., Vlaminck, J., Levecke, B., Briones-Montero, A., Geldhof, P., 2019. Comparison of Kato-Katz Thick Smear, Mini-FLOTAC, and Flukefinder for the Detection and Quantification of *Fasciola hepatica* Eggs in Artificially Spiked Human Stool. Am. J. Trop. Med. Hyg.101, 59-61.

**Table 1**

Procedure to generate artificial samples with different levels of low *F. hepatica* faecal egg counts.

|  |  |  |  |
| --- | --- | --- | --- |
| *F. hepatica*-positive fraction | Standard sedimentation (faeces per replicate) | Standard FLUKEFINDER® (faeces per replicate) | Modified FLUKEFINDER® (faeces per replicate) |
|  | *F. hepatica*-positive (g) | *F. hepatica*-negative (g) | *F. hepatica*-positive (g) | *F. hepatica*-negative (g) | *F. hepatica*-positive (g) | *F. hepatica*-negative (g) |
| 100% | 10 | 0 | 2 | 0 | 10 | 0 |
| 80% | 8 | 2 | 1.6 | 0.4 | 8 | 2 |
| 50% | 5 | 5 | 1 | 1 | 5 | 5 |
| 20% | 2 | 8 | 0.4 | 1.6 | 2 | 8 |
| 10% | 1 | 9 | 0.2 | 1.8 | 1 | 9 |

**Table 2**

Correlation and linear regression results between sedimentation, FLUKEFINDER® and modified FLUKEFINDER® for raw egg counts and EPG values.

|  |  |  |
| --- | --- | --- |
|  | FLUKEFINDER® | Modified FLUKEFINDER® |
| Raw egg counts |
|  | Slope (95% CIa) | Intercept (95% CIa) | r | P valueb | Slope (95% CI) | Intercept (95% CI) | r | P valueb |
| Sedimentation | 13.58 (12.45 – 14.70) | 0.54 (-7.33 – 8.41) | 0.978 | <0.0001 | 6.06 (5.65 – 6.46) | 2.13 (-0.71 – 4.97) | 0.985 | <0.0001 |
| FLUKEFINDER® |  |  |  |  | 0.44 (0.42 – 0.46) | 2.28 (0.06 – 4.51) | 0.991 | <0.0001 |
| EPG values |
| Sedimentation | 2.72 (2.49 – 2.94) | 1.08 (14.66 – 16.81) | 0.978 | <0.0001 | 6.06 (5.65 – 6.46) | 2.13 (-0.71 – 4.97) | 0.985 | <0.0001 |
| FLUKEFINDER® |  |  |  |  | 2.19 (2.08 – 2.31) | 22.83 (0.55 – 45.11) | 0.991 | <0.0001 |

aCI, confidence interval.

bSignificance of correlation (slope ≠ 0)

**Table 3**

Sensitivity, specificity, predictive positive (PPV) and predictive negative values (NPV) for standard sedimentation, FLUKEFINDER® and modified FLUKEFINDER® methods on samples with low EPG

|  |  |  |  |
| --- | --- | --- | --- |
|  | Standard sedimentation | FLUKEFINDER® | Modified FLUKEFINDER® |
|  | True pos. | True neg. | True pos. | True neg. | True pos. | True neg. |
| n/N | 20/20 | 0/20 | 20/20 | 0/20 | 20/20 | 0/20 |
|  | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Sensitivity | 1 | 0.83-1 | 1 | 0.83-1 | 1 | 0.83-1 |
| Specificity | 1 | 0.83-1 | 1 | 0.83-1 | 1 | 0.83-1 |
| PPV | 0.94 | 0.75-0.97 | 0.94 | 0.75-0.97 | 0.94 | 0.75-0.97 |
| NPV | 0.94 | 0.75-0.97 | 0.94 | 0.75-0.97 | 0.94 | 0.75-0.97 |

EPG, eggs per gram faeces; pos., positive; neg., negative; n, number of positive samples; N, number of investigated samples; 95% CI, 95% confidence interval.

For positive samples: Mean EPG: 14.6; median EPG: 14.0; range: 12-18.5

**Figure legends:**

**Fig. 1.** Cotton bag placed underneath a sheep’s tail for collecting faecal samples. Strips below (1) and above (2) the hind legs from both sides were brought together at the hind back and knotted together (3).

**Fig. 2.** Pearson correlation and linear regression of raw egg counts (A) and calculated EPG values (B) for each biological replicate from six mixing series with five mixing steps (n=30) using standard sedimentation (10 g faeces), FLUKEFINDER® (2 g faeces) and modified FLUKEFINDER® (10 g faeces). Lines and dashed lines indicate regression lines and 95% confidence bands, respectively. The dotted black lines indicate a slope of 1.

**Fig. 3.** Comparison of linearity of normalised EPG data between methods. EPG values for each replicate were normalised to the mean EPG of the same dilution series obtained with the same method. Pearson regression analyses revealed a linear relationship between the fraction of *F. hepatica*-positive faeces in the sample and the normalised EPG. Slope and y intercepts were not significantly different between data sets. The calculated linear equations (and correlation coefficients) were y=-0.054+1.011x (0.839), y=-0.015 + 0.968x (0.895) and y=-0.071+1.031x (0.930) for sedimentation, FLUKEFINDER® and modified FLUKEFINDER®, respectively.

**Fig. 4.** Dependency of the coefficient of variation (CV) on the mean egg concentration (A) and the coproscopic method used (B). (A), The CV was calculated from the five technical replicates analysed for each biological replicate and plotted against the mean EPG of the biological replicate. Spearman correlation analyses revealed that variation decreased with increasing egg counts. (B), The paired CV values were compared for each biological replicate using the different methods as pairs. For this purpose, pairwise comparisons were made by subtracting from the CV for sedimentation the CVs for FLUKEFINDER® or the CV for modified FLUKEFINDER® for the same biological replicate. For the third comparison, the CV for modified FLUKEFINDER® was subtracted from the CV for FLUKEFINDER®. The values for differences were plotted (B) and a Wilcoxon signed rank test was conducted to determine if the median of the differences was significantly different from 0. \*\*\*, p < 0.001.