An Assessment of the Repeatability, Accuracy, and Time Required to Complete Three Different Egg Counting Techniques Utilized to Determine Pasture Contamination and Anthelmintic Resistance for Gastrointestinal Parasites in Sheep

A Senior Honors Thesis

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Introduction

It is well known that gastrointestinal parasites in sheep cause severe production losses every year. Controlling a worm problem can be extremely difficult for many reasons including the existence of anthelmintic resistance, a periparturient rise in worm egg output, persistence of parasite larvae on pastures, and a lack of knowledge about which stage in the life cycle of the parasite is most vulnerable to attack.[1, 2, 3] Fecal egg counts (FEC) are one of the key sources of information a veterinarian can use to help a farmer prevent a worm problem and to monitor anthelmintic resistance. Although FEC do not accurately estimate the actual worm burden of the sheep, they do reveal the amount of contamination deposited on the pasture, and they allow evaluation of the effectiveness of anthelmintics.[4] If a veterinarian performs a FEC on several fecal specimens from animals on a farm, and finds the counts to be high, she/he can predict that there will be significant pasture contamination during the grazing season. This contamination puts lambs and lambing ewes, whose immune systems are somewhat depressed, at risk for parasitic disease. The veterinarian can suggest treatment and rational prevention strategies based upon the available pasture resources.

Veterinarians often do not utilize fecal egg counting techniques because of cost, equipment needed, time involved, and lack of producer demand. In addition, some techniques, although useful as research tools, are not readily available or used in a private practice setting. In this experiment, three different techniques: the Danish Modified McMaster (DMM)[5], OSU Modified McMaster (OSU MM)[6], and the Advanced Equine Product (AEP)[7] methods were compared. The Danish Modified McMaster technique has the lowest limit of detection, and it is

commonly used in research laboratories. However, it is also more laborious than the other methods, partly because it requires centrifugation, specialized equipment, and a sensitive balance. The OSU MM method is somewhat quicker and easier than the DMM technique, but it too requires a balance. The AEP method does not use a balance or centrifuge, and it is available in a user friendly kit form. The specific objectives of this experiment were to measure the repeatability of each of the three techniques, assess their respective ability to estimate the actual egg count using samples having a known concentration of eggs, and to measure and compare the time needed to complete each technique.

An assessment of the variability found in each technique will be valuable in determining their utility in estimating worm egg output and in potential problems or strengths of each method in measuring anthelmintic resistance in the gastrointestinal worms of sheep. Results of this experimentation can be used to rate the relative strengths and weaknesses of these methods and to assess their potential usefulness in private veterinary practice.

Materials & Methods

Fecal specimens were created to supply two samples of feces with egg counts approximating 50 and 1,000 eggs per gram (epg).

Harvest of Eggs

Fecal samples were collected from animals that were confirmed to be shedding a large number of eggs. Several 45g aliquots of feces were mixed with water to form a slurry and were sequentially poured through a 1.0 mm sieve, a 0.5 mm sieve, and a 0.106 mm sieve to remove excess fecal debris. The remaining liquid was run through a filter with an inner column of 0.025

mm and an outer column of 0.106 mm. This process separated eggs from the fecal material by catching the fecal matter in the 0.025 mm column, while allowing the eggs to be collected from the 0.106 outer column. The washed filtrate was centrifuged for seven minutes at 1500 rpm. The supernatant was siphoned off and the pellet was reconstituted by doubling the volume with 10 % formalin. The number of eggs in the suspension was determined by mixing 0.25 mL of vortexed egg suspension with 3.75 mL of saturated $NaNO_3$ solution, and by then counting the number of eggs in a McMaster slide (3 chambers, 0.3 mL per chamber). In order to estimate the concentration of eggs per mL, the sum of the three chambers was divided by 0.9 mL, and then multiplied by sixteen. This process was repeated until a sufficient number of eggs was harvested.

Fecal specimens from sheep which were not shedding parasite eggs were obtained and their negative status was verified by multiple routine flotations.[8] In order to blind the investigator to the exact concentration of eggs in the two specimens, a third party added sufficient eggs from the suspension containing 10 % formalin to the feces to create a 300 gram specimen containing 1070 epg, and a separate 300 gram specimen which contained 52 epg. Twenty replicates of the high egg count (1070 epg) specimen and twenty replicates of the low egg count (52 epg) specimen were performed using each of the three techniques.

Danish Modified McMaster technique(DMM)[9]

Four grams of feces were weighed out and mixed with 56 mL of tap water. The mixture was stirred and allowed to absorb water for 30 minutes until the fecal material was uniformly suspended. The suspension was stirred again and poured through 2 layers of gauze. Ten mL of the strained suspension was poured into a glass centrifuge tube and centrifuged for seven minutes

at 1500 rpm, the supernatant was aspirated, and enough saturated NaCl/glucose solution was added to bring the volume to 4.0 mL. The suspension was votexed, a four-chambered McMaster slide was loaded , and the eggs were counted under the grid in each chamber (0.5 mL per chamber). The fecal egg count (FEC) was recorded as the sum of the eggs counted in the four chambers multiplied by 3.

OSU Modified McMaster technique(OSU MM)[10]

Four grams of feces were mixed with 26 mL of saturated $NaNO_3$ solution in a small disposable plastic cup, and stirred gently until uniformly suspended. A three-chambered modified McMaster slide was loaded and the eggs under the grid were counted in each chamber (0.3 mL per chamber). The FEC was recorded as the sum of the eggs counted in the three chambers multiplied by 8.33.

Advanced Equine Product Kit technique(AEP)[11]

Saturated $NaNO_3$ solution was added to "Line A" in the calibrated vial supplied with the kit (26 mL), and feces were added until the fluid line was even with "Fill line B". The suspension was stirred until uniformly suspended, a two-chambered slide, supplied with the kit, was loaded with the suspension and eggs were counted under the grid in each chamber (0.15 mL per chamber). The FEC was recorded as the sum of eggs counted in the two chambers multiplied by 25.

The AEP method assumes that fecal material has a specific density such that the amount needed to raise the fluid line from "Line A" to "Fill Line B" weighs 4 grams. To assess potential for variation in this method, 10 random aliquots of fecal specimens from different sheep, which

were placed in cylinders in an amount sufficient to raise the fluid from "Line A" to "Fill Line B", were weighed. The mean and standard deviation were calculated.

Only eggs from the *Trichostrongylus* family were counted. These included, *Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus colubriformis, Nematodirus spathinger,* and *Oesophagostomum columbianum.*

Results

The mean and standard deviation for the high egg count specimen (1070 epg) was 693 ± 42 epg for the DMM technique, 905 ± 108 epg for the OSU MM technique, and 849 ± 211 epg for the AEP technique. The mean and standard deviation for the low egg count specimen (52 epg) was 33 ± 1.0 epg for the DMM technique, 28 ± 15 epg for the OSU MM technique, and 34 ± 28 epg for the AEP technique (see table 1).

The 95% confidence interval around the mean of the high egg count specimen was (674.2, 710.9) for the DMM technique, (857.9, 952.2) for the OSU MM technique, and (756.2, 941.4) for the AEP technique. The 95% confidence interval around the mean of the low egg count samples was (28.7, 36.8) for the DMM technique, (21.7, 34.6) for the OSU MM technique, and (21.3, 46.2) for the AEP technique (see table 1).

The mean and standard deviation for the time required to complete the three techniques for the high egg count specimen (1070 epg) was 12.9 min. ± 1.0 min. for the DMM technique, 17.9 min. ± 1.6 min. for the OSU MM technique, and 8.3 min. ± 0.8 min. for the AEP technique. The mean and standard deviation for the time required to complete the three techniques for the low egg count specimen (52 epg) was 10.6 min. ± 0.9 min. for the DMM technique, 11.4 min. ±

1.6 min. for the OSU MM technique, and 6.5 min. ± 1.2 min. for the AEP technique (see table 2). These times reflect preparation time and egg counting time for the OSU MM technique and the AEP technique only. For the DMM, an extra 41.2 min. must to be added to the mean because the mean and standard deviation given are for counting time only.

The mean and standard deviation of the 10 aliquots of different fecal specimens used to raise "Line A" to "Fill Line B" in the cylinder provided in the AEP kit were 3.66g ± 0.36g.

Discussion

Because of its extremely small variability, the DMM technique is superior in repeatability to the OSU MM and the AEP techniques. The DMM technique has the lowest limit of detection because more fecal matter is examined (2 mL), thereby allowing the use of a lower multiplier. Furthermore, the DMM technique requires weighing the sample to a specific weight every time. This process helps control variability by keeping the amount of fecal material used the same for every fecal egg count. In addition, the DMM technique is easier to read because of reduced fecal debris in the slide. The OSU MM technique has a relatively small multiplier, and intermediate variance, however, it is generally more difficult to read than the DMM technique because of increased fecal debris.

The AEP technique has a wide variability caused, in part, by the smaller amount of suspension examined resulting in a relatively high multiplier (25), and by utilizing fecal volume to bring "Line A" to "Fill Line B" in the kit. Because the samples are not weighed, the amount of fecal material used for the AEP technique can be variable. Unlike the DMM and OSU MM techniques which both use a specific weight of 4 g of feces prior to suspension, the AEP

technique depends upon volume of the feces as measured by fluid displacement. When a balance was used to measure the weight of feces actually placed in the container, it was found that an average of 3.66 g ± 0.36g of feces were added to the AEP technique, 8.4% less than the assumed 4 grams. Fecal dry matter and diet may influence weight and volume. As with the OSU MM technique, the repeatability of the AEP technique may be negatively effected by the difficulty with visualizing worm eggs in the presence of fecal debris.

For the high egg count specimen, there was no real difference between the AEP and OSU MM techniques with respect to their ability to estimate the true mean egg count as shown by their overlapping confidence intervals. The estimated mean of the DMM technique was significantly lower than the other two techniques. For the low egg count specimen there was no real difference in estimated means between the three techniques, however, the confidence interval was narrower for the DMM technique reflecting its superior repeatability.

All methods underestimated the actual count of both the high and low egg count specimens. One possible explanation is that the third party used to blind the experimenter underestimated the number of eggs placed in the negative fecal samples. However, all methods of sample preparation have inherent traps where eggs could be lost because of adherence to glass, plasticware, fecal debris, or gauze. The DMM technique uses gauze to strain out coarse fecal debris from the suspension prior to the centrifugation step. This may explain the somewhat lower counts observed for this technique even though the use of the gauze improves visualization of the eggs in the counting chambers. In addition, the spiked specimen tended to dry in the plastic container during the process of counting the sixty replicates in spite of efforts to prevent

this. It is likely some eggs remained stuck to the side of the container.

The relatively small underestimation of the true number of eggs present in the fecal sample is of little practical importance if one is consistent in the use of a specific method. All three techniques were relatively close to the estimated "true" number in both the high and low count specimens. In practice, one usually establishes a cutoff point or "thumb rule" where sheep are wormed in order to prevent pasture contamination if counts rise above a predetermined level. In this study the three methods are comparable and reasonably repeatable. It is unlikely that the degree of underestimation would cause a mistake to be made if a reasonable number of fecal samples from the group are examined.

When egg count reduction trials are used for detecting anthelmintic resistance, all samples are examined using the same method. Therefore, the small underestimation of egg counts seen in this study should have no effect on results for the percent fecal egg count reduction between the control and treatment groups, regardless of the method used.[12]

As a research tool, the DMM technique appears to be a strong method because it allows the experimenter to store the processed sample up to a week, it has the lowest limit of detection, and it has the lowest variability. In addition, the DMM technique is the easiest of the three techniques to read because it has the least fecal debris in the slide chambers. Nevertheless, the DMM technique does require a balance and a centrifuge, and it takes about 45 minutes longer to set up than the other two methods. The OSU MM technique, which takes much less time to set up and read than the DMM technique, gives a good estimate of the "true" number of eggs present in the feces, and it has relatively low variability. It should be useful as a research tool, as a

relatively fast way to conduct anthelmintic resistance testing, and as a useful tool for the practitioner to estimate mean fecal egg counts of a group of sheep.

The AEP technique is the quickest and easiest method to use. However, the method does have significant variability. Anthelmintic resistance testing, using fecal egg count reduction techniques, relies upon estimation of percent reduction from a calculated mean.[3] One criteria for assigning resistant status is a lower limit of less than 90% reduction after calculation of a 95% confidence interval around the estimated percent reduction.[13] Inherent variability and a high multiplication factor in the AEP technique might cause an error if the calculated lower limit is below 90% simply because of the variability of the method. This concern will require further investigation. However, the technique may still be valuable in estimating fecal egg output when making decisions about worming the flock.

References

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Table 1: Mean, standard deviation, and confidence intervals for tests with specimens having a known amount of eggs utilizing the OSU Modified McMaster (OSU MM), Danish Modified McMaster (DMM), and the Advanced Equine Product Kit (AEP) methods.

Specimen	OSU MM	DMM	AEP
High*** Counts	905 ± 108* (857.9, 952.2)**	693 ± 42 (674.2, 710.9)	849 ± 211 (756.2, 941.4)
Low**** Counts	28 ± 15 (21.7, 34.6)	33 ± 9 (28.7, 36.8)	34 ± 28 (21.3, 46.2)

*Mean ± Std. Dev.

** 95% Confidence Interval

***Actual eggs in high count specimens: 1070 epg.

****Actual eggs in low count specimens: 52 epg.

Table 2: Mean and standard deviation of the time required to complete tests with specimens having a known amount of eggs utilizing the OSU Modified McMaster (OSU MM), Danish Modified McMaster (DMM), and the Advanced Equine Product Kit (AEP) methods.

Specimen	OSU MM (min.)	DMM (min.)	AEP (min.)
High Counts**	17.9 ± 1.6*	12.9 ± 1.0	8.3 ± 0.8
Low Counts***	11.4 ± 1.6	10.6 ± 0.9	6.5 ± 1.2

*Mean ± Std. Dev.

**Actual eggs in high count specimens: 1070 epg.

***Actual eggs in low count specimens: 52 epg.

Note: For the DMM, an extra 41.2 minutes need to be added to account for set up time.

APPENDIX

Materials

AEP:
Advanced Equine Product Kit
Tongue depressors
$NaNO_3$

OSU MM:
Plastic cups
Graduated cylinder
$NaNO_3$
Tongue depressors
Balance
McMaster Slide-3 chambers
(0.3mL each)

DMM: Table top centrifuge
Plastic cups
Tongue depressors
4 x 4 Gauze
Balance
Tap water
15 mL Centrifuge tubes
Water suction pump NaCl/glucose solution
Universal slide--4 chambers (0.5 mL each)

Harvesting:
Feces collecting devices
1.0 mm, 0.50 mm, 0.106 mm sieves
Visser filter with inner column 0.025 mm and outer column at 0.110mm
Table top centrifuge
Formalin
McMaster slide--3 chambers (0.3 mL each)
50 mL centrifuge tubes
200 mL beakers
Tap water
Tongue depressors

Methods:

Danish Modified McMaster technique (DMM)[2]

-Weigh out 4 g feces
-Mix with 56 mL of tap water
-Stir and let sit for 30 minutes until fecal material is uniformly suspended
-Stir again and pour through 4 x 4 in. gauze
-Pour 10 mL of strained suspension into centrifuge tube
-Centrifuge for 7 minutes at 1500 rpm on table top centrifuge
-Siphon off supernatant using a water suction pump
-Add enough saturated NaCl/glucose solution to make a volume of 4.0 mL
-Mix well, fill universal slide (4 chambers, 0.5 mL per chamber)

-Count all eggs in the four chambers using a microscope at 100X, and sum together
-Fecal egg count = sum x 3

The NaCl/glucose solution is prepared by dissolving 500 g of glucose in one liter of saturated NaCl solution.

OSU Modified McMaster technique (OSU MM)[7]

-Weigh out 4 g of feces
-Mix with 26 mL saturated $NaNO_3$ and stir gently until uniformly suspended
-Fill McMaster slide (3 chambers, 0.3 mL per chamber)
-Fecal egg count = sum x 8.33

Advanced Equine Product Kit technique (AEP)[1]
-Add 26 mL of saturated $NaNO_3$ solution to calibrated vial - "Line A"
-Add feces until the solution is even with "Fill line B"
-Stir for 20-30 seconds, or until uniformly suspended
-Fill both chambers with sample (2 chambers, 0.15 mL per chamber)
-Fecal egg count = sum x 25

Harvesting Eggs
-Collect fecal material from animals shedding eggs
-Mix 45 g of fecal material with tap water and mix into a slurry
-Pour that mixture separately through a 1.0 mm sieve using fingers to crush any intact pellets, and water to wash eggs through
-Pour that solution through a 0.5 mm sieve, then through a 0.106 mm sieve
-Pour remaining liquid through a visser filter with inner column at 0.025 mm and outer column at 0.110 mm
-Open visser filter and collect the remaining liquid in a 50 mL centrifuge tube
-Centrifuge for 7 minutes at 1500 rpm with a table top centrifuge
-Siphon off supernatant using a water suction pump
-Reconstitute the pellet by doubling the volume with 10% formalin
-Repeat as many times as need to get desired amount of eggs
-Determine number of eggs per mL by mixing 0.25 mL of vortexed egg solution with 3.75 mL $NaNO_3$.
-Mix well and fill a McMaster slide (3 chambers, 0.3 mL per chamber)
-Sum/(0.9 mL) x 16 to estimate eggs per mL

Determining negative feces:
-Mix a fecal sample until eggs within are uniformly suspended
-Mix 2g of fecal sample with some $NaNO_3$
-Pour mixed sample through gauze into a pill bottle

-Add enough $NaNO_3$ to get a positive meniscus
-Place cover slip over the meniscus
-Wait 3 minutes and place cover slip on a slide
-Check sample for eggs under the microscope
-Repeat process
-If zero eggs found, sample is considered negative

Spiking feces:
-Take known negative fecal sample and add the appropriate amount of egg suspension to get desired epg, taking into account, every mL of egg solution equals 1 g.

RAW DATA

Replicate	Time (min.)	Grid 1	Grid 2	Grid 3	Total	EPG
1	20.4	42	42	32	116	963
2	23.1	34	30	39	103	855
3	20.2	28	25	35	88	730
4	19.1	44	40	30	114	946
5	17.4	33	31	33	97	805
6	18.6	31	49	29	109	905
7	17.3	34	29	42	105	872
8	17.3	27	28	29	84	696
9	16.6	45	37	40	122	1013
10	16.9	33	37	45	115	955
11	16.0	44	42	43	129	1071
12	17.7	36	40	36	112	930
13	16.4	29	50	32	111	921
14	17.3	36	36	53	125	1037
15	18.7	21	42	33	96	797
16	15.7	23	34	32	89	739
17	17.3	51	34	28	113	938
18	16.7	45	43	23	111	921
19	19.0	50	43	35	128	1062
20	16.7	42	38	34	114	946

Raw data: OSU Modified McMaster--High egg count (1070 epg) specimen

Replicate	Time (min.)	Grid 1	Grid 2	Grid 3	Grid 4	Total	EPG
1	15.0	56	51	54	57	218	654
2	12.1	72	50	59	50	231	693
3	15.1	52	49	61	81	243	729
4	13.0	62	55	57	65	239	717
5	11.8	41	50	66	60	217	651
6	14.0	56	68	52	56	232	696
7	13.0	59	58	71	64	252	756
8	11.3	51	64	61	52	228	684
9	14.2	57	54	55	77	243	729
10	12.6	61	56	68	53	238	714
11	13.1	55	55	57	56	223	669
12	12.9	61	57	56	54	228	684
13	12.6	57	56	55	57	225	675
14	12.5	51	55	56	48	210	630
15	12.9	55	47	51	60	213	639
16	12.7	59	52	58	57	226	678
17	12.5	59	54	53	69	235	705
18	12.1	60	64	50	52	225	678
19	12.2	66	66	67	70	269	807
20	12.4	50	54	51	66	221	663

Raw date: Danish Modified McMaster--High egg count (1070 epg) specimen

Replicate	Time (min.)	Grid 1	Grid 2	Total	EPG
1	8.8	12	13	25	625
2	8.3	10	13	23	575
3	7.9	18	26	44	1100
4	9.7	19	14	33	825
5	8.1	15	18	33	825
6	9.8	10	20	30	750
7	8.1	10	16	26	650
8	7.3	26	23	49	1225
9	7.6	21	22	43	1070
10	7.8	9	6	15	375
11	7.4	18	15	33	825
12	8.2	17	16	33	825
13	7.8	23	22	45	1125
14	9.4	18	18	36	900
15	7.9	15	15	30	750
16	9.1	16	27	43	1075
17	8.6	12	17	29	725
18	7.5	24	12	36	900
19	7.9	10	22	32	800
20	7.9	24	16	41	1025

Raw date: Advanced Equine Product--High egg count (1070 epg) specimen

Replcate	Time (min.)	Grid 1	Grid 2	Grid 3	Total	EPG
1	13.1	4	1	0	5	42
2	16.8	1	3	0	4	33
3	12.6	0	1	0	1	8
4	10.3	1	0	3	4	33
5	11.0	1	2	2	5	42
6	12.1	1	1	2	4	33
7	11.0	5	1	1	7	58
8	11.2	1	0	1	2	17
9	11.7	1	1	1	3	25
10	12.6	2	2	1	5	42
11	10.0	1	0	1	2	17
12	11.0	2	0	0	2	17
13	11.1	1	1	1	3	25
14	11.6	2	4	1	7	58
15	10.6	3	1	0	4	33
16	10.7	2	1	0	3	25
17	11.6	0	1	1	2	17
18	10.2	0	0	1	1	8
19	10.0	2	0	0	2	17
20	9.5	0	1	1	2	17

Raw data: OSU Modified McMaster--Low egg count (52 epg) specimen

Replicate	Time (min.)	Grid 1	Grid 2	Grid 3	Grid 4	Total	EPG
1	10.8	4	2	3	1	10	30
2	11.7	4	3	2	1	10	30
3	10.8	4	2	1	3	10	30
4	10.9	2	2	1	4	9	27
5	11.5	2	3	3	5	13	39
6	10.7	0	3	0	3	6	18
7	10.8	4	2	2	5	13	39
8	9.7	1	5	0	1	7	21
9	11.8	6	3	3	2	14	42
10	10.1	1	3	2	2	8	24
11	9.2	2	3	2	4	11	33
12	9.9	0	3	3	3	9	27
13	10.5	2	1	2	2	7	21
14	9.9	4	1	3	3	11	33
15	11.3	7	3	2	2	14	42
16	11.5	6	3	2	3	14	42
17	11.7	6	3	4	2	15	45
18	11.3	4	1	0	3	8	24
19	9.2	4	7	2	4	17	52
20	9.5	4	4	3	1	12	36

Raw date: Danish Modified McMaster--Low egg count (52 epg) specimen

Replicate	Time (min.)	Grid 1	Grid 2	Total	EPG
1	9.8	0	1	1	25
2	7.2	0	1	1	25
3	7.6	0	1	1	25
4	9.4	1	0	1	25
5	6.5	1	0	1	25
6	6.9	0	0	0	0
7	6.2	1	0	1	25
8	6.2	2	0	2	50
9	5.7	1	1	2	50
10	5.8	1	3	4	100
11	6.8	0	1	1	25
12	6.1	2	1	3	75
13	5.9	0	0	0	0
14	5.8	0	3	3	75
15	6.6	2	1	3	75
16	5.6	0	1	1	25
17	5.8	0	1	1	25
18	5.1	0	1	1	25
19	5.9	0	0	0	0
20	5.4	0	0	0	0

Raw data: Advanced Equine Product--Low egg count (52 epg) specimen