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The contribution of simple random sampling to observed variations in faecal egg counts

Paul R. Torgerson¹, Michaela Paul², Fraser I Lewis¹

¹Section of Epidemiology Vetsuisse Faculty, University of Zurich, Winterthurestrasse 270, Zurich
8057, Switzerland

²Institut for Mathematics, Faculty of Mathematics and Natural Sciences, Winterthurstrasse 190, CH-
8057 Zürich

Correspondence: P. R. Torgerson, email paul.torgerson@access.uzh.ch

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Abstract

19 It has been over 100 years since the classical paper published by Gosset in 1907, under the
20 pseudonym “Student”, demonstrated that yeast cells suspended in a fluid and measured by a
21 haemocytometer conformed to a Poisson process. Similarly parasite eggs in a faecal suspension also
22 conform to a Poisson process. Despite this there are common misconceptions how to analyse or
23 interpret observations from the McMaster or similar quantitative parasitic diagnostic techniques,
24 widely used for evaluating parasite eggs in faeces. The McMaster technique can easily be shown
25 from a theoretical perspective to give variable results that inevitably arise from the random
26 distribution of parasite eggs in a well mixed faecal sample. The Poisson processes that lead to this
27 variability are described and illustrative examples of the potentially large confidence intervals that
28 can arise from observed faecal eggs counts that are calculated from the observations on a McMaster
29 slide. Attempts to modify the McMaster technique, or indeed other quantitative techniques, to
30 ensure uniform egg counts are doomed to failure and bely the ignorance of the Poisson processes. A
31 simple method to immediately identify excess variation/poor sampling from replicate counts is
32 provided.

33

34 Keyword: McMaster slide, FLOTAC, variability, Poisson distribution, aggregation.

35

36

37 **1. Introduction**

38 The McMaster technique is a widely used technique for the analysis of faecal eggs counts.
39 Nevertheless, there appears to be widespread misconceptions about the processes that lead to
40 variability in the observed eggs counts using this technique and how this relates to the random
41 count distribution known as the Poisson distribution. The Poisson distribution is a discrete
42 probability distribution that expresses the probability of a given number of events occurring in a
43 fixed interval of time, space or volume if these events occur with a known average rate and
44 independently of the time, space or volume since the last event.

45

46 Processes which generate Poisson distributed observations have been known for some considerable
47 time. Indeed one of the classical works by the statistician W. S. Gosset, publishing under the well
48 known synonym “Student” was to examine the distribution of yeast cells suspended in fluid using a
49 haemocytometer (Student, 1907). The conclusion that Gosset reached was that random distribution
50 of yeast cells in a fluid suspension is a Poisson process.

51

52 With the McMasters technique the sample of faeces is first mixed with an appropriate flotation
53 solution. The suspension may then be partially filtered to remove large debris, centrifuged and re-
54 suspended to aid visualisation and the diluted faeces are then observed on a McMasters slide. The
55 eggs float and can be seen lying below a grid and counted. Following counting of the eggs on the
56 slide, the numbers of eggs per gramme of faeces (epg) can be calculated by multiplying the
57 numbers of eggs observed by an appropriate factor which depends on the dilution factor of the
58 faeces with the flotation solution and the volume of the McMaster chamber. Although this is the
59 basic description of the technique, there are many minor variations undertaken in different
60 laboratories. Some of these variations are described in Pereckiene et al. (2008) and Vadlejch et al.
61 (2011). Counting parasite eggs in a McMaster chamber is analogous to that of counting yeast cells
62 in a haemocytometer as described by Gosset in that eggs in a faecal suspension will be randomly
63 distributed in the same way that yeast cells are randomly distributed in a fluid suspension. Thus the
64 random distribution of eggs in a faecal sample or diluted faecal suspension will conform to a
65 Poisson process, provided that the sample is well mixed.

66

67 **2. Variability in egg counts and random processes**

68 Lack of understanding with regard to the variability of the McMaster technique can be illustrated
69 by two recent papers. In Vidyashanker et al. (2012), unsuccessful attempts were made
70 experimentally to obtain samples with uniform eggs from the same faecal sample by repeating and
71 varying the stirring method of the faecal sample. Such an experiment was an exercise in futility;

72 because of Poisson processes. Levecke et al. (2011) undertook a study comparing variations on the
73 McMaster technique and the FLOTAC (Cringoli et al., 2010) technique to demonstrate that
74 precision increases when analytical sensitivity increases. As we discuss below, the results of Levecke
75 et al. (2011) are entirely predictable, again because of Poisson processes. Both these manuscripts
76 therefore, illustrate misconceptions of the distributions of eggs in a given faecal sample and how
77 this can be described statistically.

78

79 In a well mixed faecal sample, the parasitic elements will have a random distribution in the same
80 way that yeast cells will be randomly distributed in a well mixed fluid suspension. This is a classic
81 Poisson process. However in the McMaster technique the variance of epg estimates between
82 repeated samples of the same faecal sample is inflated due to the multiplication factor when
83 transforming the raw counts to the epg. Additional errors may also arise in the laboratory (such as
84 measurement errors for the weight of faeces or volume of diluting fluid) but these laboratory errors
85 will not be considered further in this manuscript

86

87 Depending on the exact variation of the McMaster technique used, this arithmetical manipulation
88 could be a multiplicative factor of 67, 50, 25, 20, 10 or some other figure. This results in a
89 transformation of the original raw count data and its distribution into something else. As egg
90 counting using a McMaster chamber is a Poisson process, the raw data from repeated samples from
91 the same well mixed faecal sample are unlikely to yield the same result. Rather the results will be
92 variable and will fit a Poisson distribution. Indeed if the raw counts of the parasitic elements from
93 repeat samples of the same faecal sample do not follow a Poisson distribution then that is evidence
94 that the sample was not adequately mixed before processing (Schnyder et al., 2011) rather than
95 some profound biological process. However the calculated epg will not be Poisson distributed
96 because the multiplication factor inflates the variance between samples.

97

98 Simple statistical theory states that if each observation from a sample with mean μ and variance γ is
99 multiplied by a constant n , then the mean of the new sample will be $n\mu$ and the new variance will
100 be $n^2\gamma$. This can be applied to the raw untransformed egg μ_1 counts that are Poisson distributed.
101 These transformed counts are multiplied by the dilution factor n_1 to obtain a sample estimate of epg.
102 Thus:

103

$$\text{epg} = n_1\mu_1$$

104

105 Therefore the variance of the epg, given the variance of the raw count γ_1 ,

106 $= n_1^2 \gamma_1$ (or $n_1^2 \mu_1$ as the mean of a Poisson distribution is equal to its variance)

107

108 Immediately it can be seen that the variance of the epg, $n_1^2 \mu_1$, is not equal to the mean $n_1 \mu_1$ and the
109 transformed data is no longer Poisson distributed. Understanding this will illustrate why the
110 observed eggs in repeated sampling from the SAME faecal sample might appear to be highly
111 variable. As an example, it is possible to examine repeated samples from a single large well mixed
112 faecal sample that has an epg of 200. Using a technique that has a multiplication factor of 50, the
113 expected number of observed eggs on a McMaster slide would be a count of 4. However although
114 this is the expected count an actual count of 4 is observed relatively infrequently. For example if 10
115 independent samples were taken from this faecal sample the actual observed counts could be 2, 0, 6,
116 3, 2, 7, 4, 3, 4, 7. These counts were generated randomly from a Poisson distribution with mean 4.
117 The mean count of this sample is 3.8 with a variance of 5.3. The variance is of a similar magnitude
118 as the mean and hence it can easily be shown that this random sample of 10 values conforms to a
119 Poisson distribution of mean 4. If each is multiplied by the dilution factor of 50, the observed
120 calculated eggs of the 10 samples are: 100, 0, 300, 150, 100, 350, 200, 150, 200, 350. This has a
121 mean epg of 190, but the variance is now 13222. The new variance is related to the variance of the
122 raw counts by a factor of 2500, or the dilution factor squared. Now the variance is much higher than
123 the mean and the calculated eggs are NOT Poisson distributed. This extra Poisson variance is
124 entirely due to the dilution factor. It should also be noted that although the expected eggs is 200, the
125 series of 10 samples varies between 0 and 350 epg. This apparent high variability is entirely due to
126 variability inherent in random processes that has been inflated by the dilution factor.

127

128 **3. Estimation of Confidence Intervals of eggs**

129 It is also possible to construct confidence limits of single epg calculations from the McMaster
130 technique utilizing the Poisson distribution. An easy example is when an epg of 100 is calculated
131 from an observed count of 2 eggs on a McMaster slide: using a dilution factor of 50 to transform
132 the raw count to epg (see Table 1). A random sample from the Poisson distribution with a mean of
133 0.242 has a 2.5% probability of returning a value as high or higher than 2. This gives a lower 95%
134 confidence interval of $0.242 * 50$ or 12.1 epg. Likewise a random sample with a Poisson distribution
135 of mean 7.22 has a 2.5% probability of returning a value as low or lower than 2. This gives an upper
136 confidence interval of $7.22 * 50$ or 361.2 eggs. Using this methodology, Table 1 gives some
137 illustrative confidence limits of observed eggs given the dilution factor. This can readily be shown
138 using the Poisson cumulative distribution function in Excel. The cumulative Poisson probability of
139 0.975 or lower confidence interval is shown by entering '=POISSON((count-1), 0.242, TRUE)' into
140 a cell on the spreadsheet. Here the count is 2. The cumulative Poisson probabilities of 0.025 or the

141 upper confidence limit is shown by '=POISSON(count,7.22,TRUE)', again the count is 2.

142

143 Calculators for Poisson confidence intervals are available on the internet; for example GraphPad
144 (www.graphpad.com/quickcalcs/ConfInterval1.cfm). Thus the raw untransformed count can be
145 entered into this calculator which will give the confidence interval for this count. The
146 untransformed count and the confidence intervals can then be multiplied by the dilution factor to
147 give the estimate of the epg and its 95% confidence interval. Thus, whenever epgs are reported for
148 individual animals it is very easy to report the 95% confidence interval of that epg.

149

150 Table 1. Observed untransformed egg counts seen on a McMaster slide and the resultant estimate of
151 the epg with 95% confidence intervals given the appropriate dilution factor.

Untransformed egg count observed on the McMaster slide	Dilution factor	Calculated epg	Lower 95% confidence interval of epg	Upper 95% confidence interval of epg
0	50	0	0	184.5
1	50	50	1.3	278.6
2	50	100	12.1	361.2
5	50	250	81.2	583.4
10	50	500	239.8	919.5
20	50	1000	610.8	1544.4
0	10	0	0	36.9
1	10	10	0.3	55.7
2	10	20	2.4	72.2
5	10	50	16.2	116.7
10	10	100	48.0	183.9
20	10	200	122.2	308.9

152

153

154 Another inevitable conclusion can be drawn by the conformation with a Poisson distribution of raw
155 egg counts on a McMaster slide. The variation of the estimated epg calculated by repeated samples
156 from the same faecal sample decreases, as the numbers of samples taken to estimate that epg are
157 increased. Indeed it can be predicted in a precise mathematical form. If repeated single samples are
158 taken from a faecal sample with mean epg $n_1\mu_1$ then the coefficient of variation (standard
159 deviation/mean) can be shown simply to be $1/\sqrt{\mu_1}$ and is not dependent on the dilution factor.

160 However if repeated multiple samples are made (eg 10 samples or 10 McMaster slides), you are
161 effectively observing raw count data which has a Poisson distribution with a mean of the expected
162 sum of the 10 observations (as the sum of independent Poisson variables is also Poisson).
163 Consequently the coefficient of variation will vary with $1/\sqrt{10 * \mu_1}$. Thus the coefficient of
164 variation will be proportional to $1/\sqrt{\alpha}$ where α is the number of repeat samplings on the same
165 faecal sample. This again can be illustrated with the 10 samples simulated earlier. This has a total
166 count of 38 and pooling these count would give a confidence interval of 27 to 52 counts. The epg
167 can now be calculated as 38×5 (as we have 10 repeat samples from the same faecal sample) or 190
168 as expected. The confidence limits of the of the epg can be shown to be 135 to 260 epg. If the
169 simulation was repeated with just 5 random samples from a Poisson distribution of mean 4, this
170 might generate the values 4, 1, 5, 7, 3. This gives a total count of 20 with an estimated epg of $20 \times$
171 10 or 200 eggs. The confidence limit (and hence coefficient of variation) is much wider at 122 to
172 309 eggs.

173

174 **4. Results that inevitably arise due to Poisson processes.**

175 With a knowledge of the Poisson process it is possible to replicate the data of some experiments
176 studies quite easily with mathematics in a highly predictable manner. For example, Levecke et al.,
177 (2011) undertook a study to investigate if analytical sensitivity was important when monitoring
178 drug efficacy against gastrointestinal nematodes when faecal egg counts are low. The study
179 involved 30 calves, which had eggs of less than 200. From each calf 4 samples were examined by
180 McMaster with an analytic sensitivity (or dilution factors) of 10,15, 33.3 and 50. A fifth sample was
181 examined by the FLOTAC technique which has an analytic sensitivity of 1 (i.e. the dilution factor is
182 1). The mean epg of the 30 samples was then calculated and the number of zero counts recorded.
183 The mean eggs were in the range of 60.5 to 75.6, with no zero counts being recorded for the
184 FLOTAC and up to 11 zero counts for McMaster with an analytic sensitivity of 50. The eggs of
185 individual calves were not recorded but a range given for each test. The FLOTAC technique had a
186 minimum of 9 epg and a maximum of 160 epg and mean epg of 61.1. The following methodology
187 can be used to demonstrate that the expected results for the McMaster examinations of Levecke et
188 al. (2011) are entirely predictable with just the knowledge of the FLOTAC results. First it is
189 possible to simulate the results of the 28 eggs for the calves not reported with individual FLOTAC
190 results. This can be done using computer generated random numbers. In the example we report here
191 we used a uniform random number generator which gives 28 samples. The mean of these 28
192 samples plus the additional samples of 9 and 160 reported (i.e. 30 samples in total) is constrained to
193 a mean of 61.1. Alternatively 28 negative binomial distributed samples with mean 61.1 and a
194 modest k of approximately 2.5 will suffice as Levecke et al. (2011) restricted their experiment to

195 non-zero counts below 200. This can then model variability between the calves. Each simulated epg
 196 is divided by the dilution factor to give a simulated raw count observed on the McMaster. For each
 197 simulated raw count, the expected proportion of zeros, using the Poisson distribution can be
 198 calculated and these summed over the 30 animals to obtain an expected number total number of
 199 zeros. The results are illustrated in table 2 (on this occasion for the generated data of uniformly
 200 distributed eggs) and compared to the actual results obtained by the various McMaster analytical
 201 sensitivities. Between animal negative binomial eggs give similar results (data not shown). What is
 202 clear is the predicted number of zeros conforms almost exactly to those observed in the study. Thus
 203 the answer to the question Levecke et al. (2011) posed “do the analytic sensitivity and formula
 204 matter?” is most certainly “yes”! However, you do not need to undertake a McMaster analysis of
 205 120 samples to demonstrate this. But rather it can be easily shown by the application of the
 206 mathematics of Poisson processes.

207

208 Table 2. Observed data from Levecke et al. (2011) from a study of 30 calves with low faecal egg
 209 counts, together with the expected number of samples with zero counts calculated from the Poisson
 210 distribution, with between animal variation as described in the text. The expected mean of observed
 211 epg was calculated with FLOTAC with the various dilution factors (analytic sensitivity).

Analytic sensitivity (epg)	Mean observed (epg)	Min/ max epg	Number of samples with zero counts	Expected number of samples with zero counts calculated using the Poisson distribution.
1	61.1	9 to 160	0 out of 30	0 out of 30
10	64.7	0 to 230	3 out of 30	2 out of 30
15	60.5	0 to 150	4 out of 30	4 out of 30
33.3	75.7	0 to 399.6	8 out of 30	7 out of 30
50	71.5	0 to 350	11 out of 30	10 out of 30

212

213 Importantly and perhaps widely misunderstood is an observed zero count could still have come
 214 from a faecal sample that has a positive epg. This is due simply to the random sampling of the zero
 215 term in an appropriate Poisson distribution that has an mean >0 . This is analogous to calculating
 216 exact binomial confidence intervals for disease prevalence in a sample where there are no diseased
 217 animals. The very real possibility, of an observed zero count actually coming from an animal with a
 218 considerable level of parasitism (Table 1), illustrates why zero counts cannot be ignored in any
 219 circumstance. Nevertheless Vidyashanker et al. (2012) (in the group discussion appendix) suggested
 220 ignoring zero counts when working with groups of horses numbering 50 or more. Furthermore,
 221 even with a high count of 1000 epg, using a dilution factor of 50 there is a 5% probability of over,
 222 or underestimating, the eggs by as much as 39.1% or 54.4%, respectively. In the same group

223 discussion appendix there was the suggestion that increasing the actual number of eggs being
224 counted would reduce the variability in the counting process. This is true and consistent with
225 variability introduced by Poisson processes. Nevertheless it was subsequently argued for a less
226 stringent FECRT protocol because macrocytic lactone efficacies are high and further illustrates the
227 lack of understanding of random variability inherent in FECs.

228

229 This also illustrates that the FLOTAC technique (Cringoli et al., 2010), where the dilution factor can
230 be as low as 1 can reduce substantially the errors that inevitably occur when evaluating eggs.
231 However, even with an analytical sensitivity of 1, with a low egg counts of say 1 or 2 eggs, the
232 diagnostic sensitivity will still only be 63% and 86% respectively because of Poisson processes.

233

234 **5. Quality control**

235 It is possible to introduce a simple quality control method when undertaking egg counts that can
236 immediately identify excess variation or poor sampling from replicate counts. A fundamental
237 property of the Poisson distribution is that the mean is equal to its variance. When taking small
238 numbers of random samples from a Poisson distribution there can be quite large variations in the
239 variance to mean ratio or Index of Dispersion (ID). How far the ID can depart from 1, whilst still
240 being consistent with a Poisson distribution, depends on both the mean and the number of
241 replicates. A goodness of fit test is available in many statistical software packages and this could be
242 used to test if replicate samples could have arisen from the same Poisson distribution. An alternative
243 and relatively straightforward method is by examining the size of the ID. The ID is distributed as a
244 χ^2 distribution with $n-1$ (where n is the number of replicates) degrees of freedom providing the
245 mean or number of replicates is sufficiently high (Selby, 1965). For low numbers of replicates
246 and/or low expected mean there are departures from the χ^2 distribution. Therefore, by using a
247 Poisson random number generator, we have estimated the upper limits of ID using a simulation
248 study by undertaking 10,000 simulations of series of replicates. From these simulated replicates we
249 have calculated the upper 97.5 percentile of the distribution of the IDs. These are reported in Table
250 3 and can be used as a quick check that the variance to mean ratio of replicate samples is not too
251 large. For example, if just two replicates were taken and these were 1 and 9, it gives a mean count
252 of 5 and a variance of 32. The ID is $32/5 = 6.4$. The maximum ID consistent with a Poisson
253 distribution of mean 5 of two samples is 4.6. Indeed no two random samples from any Poisson
254 distribution should have an ID greater than 5. Hence there is a problem with processing, mixing or
255 counting of the sample and the replicates should be repeated. Similarly replicate counts of 1, 12 (ID
256 =9.3); 2, 20 (ID=14); 68, 100 (ID=6.1) are unlikely to be Poisson distributed. However, replicate
257 counts of 0, 2 (ID = 2); 2, 7 (ID=2.8) and 57, 74 (ID = 2.2) have sufficiently low ID to be consistent

258 with a Poisson distribution. With 2 replicates no samples with a mean greater than 10 should have
 259 an ID of greater than 5. Similarly with 5 replicates 2,5,4,7, 8 (mean =5.2, variance =5.7, ID
 260 =5.7/5.1=1.1) and 5 replicates of 20, 17, 14, 24, 30 (ID = 1.9) are consistent with a Poisson
 261 distribution whilst 0, 4, 6, 9, 11 (ID = 3.1) or 10,18,12, 28, 30 (ID=4.2) show statistically
 262 significant deviations from a Poisson distribution. For samples with greater number of replicates or
 263 higher sample means of the replicates, the maximum ID can be estimated from the appropriate χ^2
 264 distribution. If 20 replicates the ID could be no more than 1.73. This figure is derived from the the
 265 upper 2.5 percentile of the χ^2 distribution with n-1 (20-1) or 19 degrees of freedom – 32.9. This is
 266 then divided by n-1, or 19 to arrive at 1.73. For 20 replicates, 1.73 is the limit regardless of the
 267 sample mean. Finally, if all the replicate counts are 0, there is no need to check the goodness of fit.
 268 Rather the upper CI of the epg can be estimated directly from the methods described above in
 269 section 3.

270

271 Table 3. Maximum index of dispersion (variance/mean) of the raw count data between replicates
 272 from the same faecal sample if the replicates are Poisson distributed.

Expected (mean) count of replicates	Number of replicates					
	2	3	4	5	7	10
1	3	3	2.9	2.4	2.3	2.1
2	4	3.5	2.9	2.7	2.3	2.1
3	4.5	3.5	3	2.7	2.4	2.1
5	4.6	3.5	3	2.7	2.4	2.1
7	4.8	3.6	3.1	2.7	2.4	2.1
≥10	5	3.7	3.1	2.8	2.4	2.1

274

275 6. Conclusions

276 The distribution of egg counts and parasites between different animals within a group is well known
 277 to be over-dispersed. For examples see Shaw and Dobson, (1995), Grenfell et al. (1995), Wilson et
 278 al. (1996) and Shaw et al. (1998). Likewise statistical methods that encompass this over-dispersion
 279 have been recommended to analyse anthelmintic efficacy (Torgerson et al., 2005). With the
 280 exceptions of Morgan et al. (2005), Dobson et al. (2009), El-Abdellati et al. (2010) and Schnyder
 281 et al. (2011) there appears to be little appreciation of the sampling errors that will inevitably arise
 282 from the use of faecal egg count techniques such as the McMaster, despite this being an extremely
 283 widely used diagnostic procedure. As illustrated in Table 1, the errors can be surprisingly large and
 284 cannot be overcome by further mixing the faecal suspension. Undertaking repeat counts on the
 285 same sample would help to ameliorate this problem. This latter option is effectively examining a
 286 larger mass of faeces and repeat counts should be Poisson distributed. Alternatively use of a

287 technique such as the FLOTAC which does not have a high dilution factor would reduce (but not
288 eliminate) between count variability. Nevertheless, regardless of the quantitative technique used, the
289 challenge remains to fully incorporate the diagnostic errors which inevitably arise from the Poisson
290 process that occur in quantifying egg counts in faeces with the over-dispersed distributions that
291 occur between separate animals and separate faecal samples.

292

293

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297

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