Application of a Poisson distribution quality control measure to the analysis of two human hookworm drug treatment studies in Ghana

Andrew C. Kotze a,⇑, Robert J. Dobson b, Debbie Humphries c, Michael Wilson d, Michael Cappello c,e

aCSIRO Animal, Food and Health Sciences, Brisbane, Queensland, Australia
bDivision of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, Australia
cYale School of Public Health, New Haven, CT, USA
dDepartment of Parasitology, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana
ePartnerships for Global Health, Department of Pediatrics, Yale School of Medicine, New Haven, CT, USA

ABSTRACT

We examined faecal egg count reduction tests (FECRTs) conducted with hookworm-infected humans in Ghana in 2007 (study 1) and 2010 (study 2) in order to explore aspects of the test analysis. Some subjects showed increased FEC following drug treatment. This occurred mostly in <150 epg pre-treatment FEC subjects. We sought a means to remove ‘erroneous’ negative drug efficacy cases from the FECRT analysis. Pre- and post-treatment FECs from negative drug efficacy cases were examined to determine whether they represented replicates from a single randomly distributed sample, that is, if they were consistent with a Poisson distribution. Cases where the post-treatment FEC was greater than that expected if it and the pre-treatment sample had been taken from a single random distribution of eggs were excluded from the FECRT. We suggest that these cases most likely represent non-random distribution of eggs in stools, day-to-day variations in egg excretion, or worm patency onset after drug treatment, and hence are not accurate measurements of drug efficacy. This led to exclusion of the most extreme negative drug efficacy cases, with significant increases in overall drug efficacy for study 1 (81.6% vs 89.2%) and study 2 (86.7% vs 89.4%). Excluding FEC <150 individuals from the analysis also increased the study 1 efficacy (81.6% vs 88.9%), however, this resulted in the exclusion of 45% of the study subjects, compared to the exclusion of just 5% using the Poisson distribution method. While low FEC subjects are excluded from livestock FECRTs, the significant prevalence of such subjects in human FECRTs suggests that their exclusion may not be practical. Hence, we suggest that the influence of low FECs can be minimised by excluding ‘erroneous’ negative efficacy cases using a simple Poisson distribution analysis.

© 2014 The Authors. Published by Elsevier Ltd. Open access under CC BY license.

1. Introduction

With the implementation of mass drug administration programs for the control of human soil transmitted helminths (STHs) there is increasing interest in ensuring that drug resistance can be detected should it emerge. While some in vitro bioassay and molecular-based methods for testing drug sensitivity have been examined with reference to STHs and benzimidazole drugs (for example, Albonico et al., 2004, 2005; Diawara et al., 2009; Kotze et al., 2009; Humphries et al., 2013) they are yet to be validated as useful tools for resistance detection in field settings. Hence, the only current means to monitor drug sensitivity in the field is the faecal egg count reduction test (FECRT). This test involves a comparison of faecal egg counts (FECs) in human subjects pre- and post-drug treatment. While such tests have been used for many years in the livestock health sphere to examine changes in drug sensitivity in gastrointestinal parasites, they have only more recently been applied to human STHs. Consequently, a number of recent reports have examined aspects of their design, and made recommendations as to how the tests should be used for human STHs (Levecke et al., 2011a,b; Vercruysse et al., 2011a,b), and the WHO has recently issued revised guidelines for assessing drug efficacy against schistosomiasis and STHs (WHO, 2013).

One feature of livestock FECRTs is the recommendation to exclude animals with low FEC for statistical reasons (Presidente, 1985; Coles et al., 2006), as low FECs are not accurately measured with existing egg counting techniques which lack sensitivity. The
recommended minimum FECs are 150 for sheep, goats and horses, and 100 for cattle (FECs are usually lower in cattle than in the other species). Satisfying this constraint for inclusion does not generally present a great difficulty in terms of recruitment of suitable animals for livestock studies. However, subject recruitment and compliance issues are more difficult in the human field than for livestock drug efficacy studies. The difficulty in recruiting suitable individuals for human FECRTs is further exacerbated by the high prevalence of subjects with low infection levels at some field sites described in recent reports; for example, over the seven field sites described by Vercruysse et al. (2011a), human subjects with light hookworm infections (that is, epg < 1999, from Montresor et al., 1998) made up 94% of the total number of infections. Further, the proportion of the total study populations at the various sites that had FEC < 150 varied from 6% in Brazil, up to 49% in Cameroon, and 67% in Vietnam. Hence, it may be far more difficult to adhere to a FEC > 150 inclusion limit for human FECRTs compared to the relative ease of following this rule in livestock FECRTs.

Vercruysse et al. (2011a) reported on the influence of inclusion of low pre-treatment subjects on human FECRT data. They found that drug efficacy calculated as the mean of the FECRs in individual study subjects was highly affected by excluding subjects with pre-treatment FECs of <150. However, the inclusion of FEC <150 subjects did not affect the FECRT outcomes at each of their seven field sites when the FECR was calculated on the basis of group means. This indicated that, for their data sets, the responses of the low FEC subjects did not distort the overall study outcomes as long as the analysis was performed at the group level. However, in demonstrating the significant effect of low FEC subjects on FECRT outcomes when calculated using individual FECRs, this study indicated the potential for the influence of low FEC subjects to be significant in terms of the group mean-based FECR if they reached dominant proportions in a study population.

As a part of ongoing efforts to refine human STH FECRTs in order to ensure that they are reproducible, and hence will be able to detect changes in drug sensitivity over time, we aimed to assess the influence of low pre-treatment FECs on the analysis of data from two recently conducted human hookworm FECRTs. We examined data sets collected during trials of albendazole against human hookworms conducted in Ghana in 2007 and 2010 (Humphries et al., 2011, 2013). These studies were of interest as they had been carried out in populations dominated by livestock drug efficacy studies. The difficulty in recruiting suitable animals for livestock studies. However, subject recruitment and compliance issues are more difficult in the human field than for livestock drug efficacy studies. The difficulty in recruiting suitable individuals for human FECRTs is further exacerbated by the high prevalence of subjects with low infection levels at some field sites described in recent reports; for example, over the seven field sites described by Vercruysse et al. (2011a), human subjects with light hookworm infections (that is, epg < 1999, from Montresor et al., 1998) made up 94% of the total number of infections. Further, the proportion of the total study populations at the various sites that had FEC < 150 varied from 6% in Brazil, up to 49% in Cameroon, and 67% in Vietnam. Hence, it may be far more difficult to adhere to a FEC > 150 inclusion limit for human FECRTs compared to the relative ease of following this rule in livestock FECRTs.

Vercruysse et al. (2011a) reported on the influence of inclusion of low pre-treatment subjects on human FECRT data. They found that drug efficacy calculated as the mean of the FECRs in individual study subjects was highly affected by excluding subjects with pre-treatment FECs of <150. However, the inclusion of FEC <150 subjects did not affect the FECRT outcomes at each of their seven field sites when the FECR was calculated on the basis of group means. This indicated that, for their data sets, the responses of the low FEC subjects did not distort the overall study outcomes as long as the analysis was performed at the group level. However, in demonstrating the significant effect of low FEC subjects on FECRT outcomes when calculated using individual FECRs, this study indicated the potential for the influence of low FEC subjects to be significant in terms of the group mean-based FECR if they reached dominant proportions in a study population.

As a part of ongoing efforts to refine human STH FECRTs in order to ensure that they are reproducible, and hence will be able to detect changes in drug sensitivity over time, we aimed to assess the influence of low pre-treatment FECs on the analysis of data from two recently conducted human hookworm FECRTs. We examined data sets collected during trials of albendazole against human hookworms conducted in Ghana in 2007 and 2010 (Humphries et al., 2011, 2013). These studies were of interest as they had been carried out in populations dominated by subjects with low infection intensities (from Montresor et al., 1998). We compared three different analytical approaches for estimating drug efficacy from FECRTs: (a) group FEC averages, (b) group averages excluding subjects with pre-treatment FEC less than 150 epg, and (c) group averages after the use of a Poisson distribution method to identify and exclude subjects with ‘erroneous’ negative drug efficacy values. We also utilized duplicate egg counts from the 2010 field study in order to examine the variability in FEC at low infection intensities.

2. Materials and methods

2.1. FECRT data

We examined data from two recent FECRTs conducted in Kintampo North Municipality, Ghana. The studies were denoted ‘study 1’ (conducted in 2007, Humphries et al., 2011) and study 2 (conducted in 2010, Humphries et al., 2013). Both studies were approved by the Yale Human Investigations Committee and the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) at the University of Ghana. The two studies are summarised below:

(i) Study 1: Egg counts were performed on 258 subjects using the Kato–Katz method (WHO, 1996) on single faecal samples from each subject (egg count sensitivity of 37). Positive counts were recorded for 116 subjects, and 102 of these were treated with albendazole. Post-treatment faecal samples were received from 95 subjects during the period 14–21 days after the drug treatment.

(ii) Study 2: Egg counts were performed on duplicate faecal samples received from 258 subjects using the Kato–Katz method (WHO, 1996) (egg count sensitivity of 24, with two replicate samples per subject, i.e. for the total eggs counted per subject 1 epg = 12 epg). At least one positive count was recorded for 121 subjects, and all of these were treated with albendazole. Duplicate follow up samples were requested from all subjects, and received from 94 subjects during the period 14–21 days after the drug treatment. At least one follow up sample was received from 112 subjects. A total of 84 subjects provided two pre- and two post-treatment samples and were therefore used in the subsequent FECRT analysis.

2.2. Infection intensity

We examined the infection intensities across subjects in Studies 1 and 2 by subdividing the pre-treatment FEC data into infection intensity classes according to the criteria of Montresor et al. (1998) for human hookworms: light, 1–1999 epg, moderate 2000–3999, and high >3999. In addition, we applied a further subdivision of the light infection category into <150 and 150–1999 as (b) group averages excluding subjects with pre-treatment FECs of <150. However, the inclusion of low pre-treatment subjects on human FECRT data. They found that drug efficacy calculated as the mean of the FECRs in individual study subjects was highly affected by excluding subjects with pre-treatment FECs of <150. However, the inclusion of FEC <150 subjects did not affect the FECRT outcomes at each of their seven field sites when the FECR was calculated on the basis of group means. This indicated that, for their data sets, the responses of the low FEC subjects did not distort the overall study outcomes as long as the analysis was performed at the group level. However, in demonstrating the significant effect of low FEC subjects on FECRT outcomes when calculated using individual FECRs, this study indicated the potential for the influence of low FEC subjects to be significant in terms of the group mean-based FECR if they reached dominant proportions in a study population.

As a part of ongoing efforts to refine human STH FECRTs in order to ensure that they are reproducible, and hence will be able to detect changes in drug sensitivity over time, we aimed to assess the influence of low pre-treatment FECs on the analysis of data from two recently conducted human hookworm FECRTs. We examined data sets collected during trials of albendazole against human hookworms conducted in Ghana in 2007 and 2010 (Humphries et al., 2011, 2013). These studies were of interest as they had been carried out in populations dominated by subjects with low infection intensities (from Montresor et al., 1998). We compared three different analytical approaches for estimating drug efficacy from FECRTs: (a) group FEC averages, (b) group averages excluding subjects with pre-treatment FEC less than 150 epg, and (c) group averages after the use of a Poisson distribution method to identify and exclude subjects with ‘erroneous’ negative drug efficacy values. We also utilized duplicate egg counts from the 2010 field study in order to examine the variability in FEC at low infection intensities.

(ii) Study 2: Egg counts were performed on duplicate faecal samples received from 258 subjects using the Kato–Katz method (WHO, 1996) (egg count sensitivity of 24, with two replicate samples per subject, i.e. for the total eggs counted per subject 1 epg = 12 epg). At least one positive count was recorded for 121 subjects, and all of these were treated with albendazole. Duplicate follow up samples were requested from all subjects, and received from 94 subjects during the period 14–21 days after the drug treatment. At least one follow up sample was received from 112 subjects. A total of 84 subjects provided two pre- and two post-treatment samples and were therefore used in the subsequent FECRT analysis.

2.3. Analysis of drug efficacy

FECRT outcomes for both studies were determined using the R package “eggCounts” (Paul, 2013) via the user friendly web interface at http://www.math.uzh.ch/as/?calc. The approach is based on Bayesian methods, and assumes firstly that the observed egg count is subject to Poisson errors (magnified by the dilution factor), and secondly that the between animal counts are over dispersed (negative binomial or zero inflated). The user enters the pre-treatment and post-treatment FECs, and the dilution factor used to calculate the FECs. Bayesian inference is done via Markov chain Monte Carlo sampling. The output includes the FECR with 95% Confidence Intervals (CIs).

We examined the FECRT data using, (a) the full data sets from studies 1 and 2, as well as, (b) in separate groups showing pre-treatment FECs of < or >150 epg, as we were interested specifically in the effect of excluding FEC <150 epg individuals on the FECRT outcomes. Within study 1 or study 2, drug efficacies derived from these different groups were considered to be significantly different if their 95% CIs did not overlap.

2.4. Poisson distribution quality control test for negative drug efficacy data

We utilised a Poisson distribution-based method (modified from Torgerson et al., 2012) to examine cases in which drug efficacy was negative (FEC increased after drug treatment) as a means of applying a quality control measure to the data set. The FEC results for individual subjects at the pre- and post-treatment time points (n = 2 for study 1, and n = 4 for study 2) were analysed to determine if the group of two or four values for each subject followed a Poisson distribution. The index of dispersion (ID = variance/mean) was calculated for each subject, and then compared...
to the maximum ID values described by Torgerson et al. (2012) for replicate egg counts from a single faecal preparation in which the distribution of eggs followed a Poisson distribution. The subjects whose ID was greater than this maximum value were omitted from the FECRT analysis. The FECRT outcomes for Studies 1 and 2 after application of this method were compared to the drug efficacy outcomes for the full data set, as well as for the < or >150 epg pre-treatment FEC groups.

2.5. Egg count variability

In order to examine the degree of variability among replicate FECs, and particularly the relationship between the degree of variability and the infection intensity, we calculated the coefficient of variation (CV = the standard deviation/mean, expressed as a percentage) for FECs conducted on repeat samples taken from each study 2 subject pre- and post- drug treatment. All subjects who had recorded two FECs, with at least one being greater than zero, were included in this analysis. Some subjects who had submitted two pre-treatment samples, but less than two post-treatment samples (and hence were not included in the FECRT analysis) were included here in terms of their pair of pre-treatment FECs alone. In addition, many subjects who were included in the FECRT analysis showed two zero counts at the post-treatment time point and so were not included in this analysis of egg count variability. Hence, the data set for this analysis did not correspond directly to the FECRT data set. The number of pairs of FECs that were analysed here was 158 (102 pairs of pre-treatment samples, and 56 pairs of post-treatment samples).

3. Results

The distribution of pre-treatment FECs in the subjects from studies 1 and 2 are shown in Fig. 1. Both studies were dominated by subjects in the light infection category (FEC < 1999, from Montresor et al., 1998), with a large number of FECs < 150 in both studies (45% of the total in study 1, Fig. 1A; 39% of the total in study 2, Fig. 1B).

Table 1 shows the studies 1 and 2 drug efficacy results for the whole data sets, after division into < or > 150 pre-treatment FEC subjects, and after application of the Poisson distribution exclusion criterion. Drug efficacy values, as measured by FECR, for the group of < 150 FEC subjects from both studies were very low (negative for study 1, and 52.8% for study 2). The study 1 drug efficacy showed a significant increase from 81.6% to 88.9% after exclusion of the 43 subjects (45% of the study subjects) with a pre-treatment FEC of < 150. For study 2, the exclusion of the 33 < 150 epg FEC subjects (39% of the study subjects) did not have a significant effect on the FECR (86.7% vs 88.6%). The application of the Poisson distribution test to study 1 (the identification of the specific subjects excluded for this analysis is described below) resulted in a significant increase in the FECR compared to the whole data set (81.6% vs 89.2%) while only excluding five subjects (5% of the study subjects) from the analysis. Similarly, the Poisson distribution test resulted in a significant increase in drug efficacy for study 2 (86.7% vs 89.4%) with the exclusion of four subjects (5% of the study subjects) from the analysis.

The relationship between drug efficacies and pre-treatment FEC for each individual study subject is illustrated in Fig. 2A. While many subjects showed a drug efficacy of 100% (FEC reduced to zero after drug treatment) (61% for study 1, and 40% for study 2) a large number showed much lower efficacy values. Many subjects showed an increase in FEC after treatment compared to their pre-treatment value (drug efficacy < 0%). These cases were all associated with pre-treatment FECs of < 1000. There was a clear trend to a greater presence of low drug efficacy values as the pre-treatment FEC decreased, with most of these cases occurring for the < 150 FEC subset. The most extreme cases showed efficacies of less than −1000%, representing over 10-fold increases in post-treatment FEC compared to the pre-treatment value.

Index of dispersion (ID) values were calculated using the pre- and post-treatment FECs for each of the study subjects who had shown an increase in FEC after drug treatment (from Fig. 2A). These ID values are shown in Table 2 for the thirteen study 1 subjects, and seven study 2 subjects who had shown a negative drug efficacy. A comparison of ID values with the expected Poisson distribution limits indicated that the variation in FEC values for five study 1 and four study 2 subjects was greater than that expected from a random distribution. The FEC drug efficacy data for these study subjects is illustrated in Fig. 2B. The effect of excluding these subjects on the overall study FECRT outcomes was described above with reference to Table 1.

In order to explore possible explanations for the greater range of FECRT outcomes shown by study subjects with low FEC (from Fig. 2A) we examined the degree of variability in repeat egg counts on separate faecal samples taken from the same study subjects. Study 2 provided an opportunity for this analysis as two faecal samples had been examined for each study subject both pre- and post- drug treatment. Fig. 3 shows that the coefficient of variation (CV) for the pairs of FECs taken throughout the study increased as the mean FEC decreased.

4. Discussion

The present study has analysed data from two recent trials of albendazole efficacy against human hookworms in Ghana in order
highlight the influence of low pre-treatment FECs on human FECRTs. We applied a quality control measure to both data sets in order to identify likely ‘erroneous’ data points, and hence to improve our ability to accurately quantify drug efficacy in these trials. Application of this measure resulted in significantly higher drug efficacy outcomes for both trials, with the removal of only about 5% of the study subjects from the analysis. This method should be a useful quality control measure to apply to all human FECRT data sets in order to ensure greater confidence in FECRT outcomes.

This is particularly important as attention is currently focused on maximising the reproducibility of human FECRTs in order to allow confidence in comparing the results across different geographical areas, or time periods, in order to detect the emergence of drug resistance (Levecke et al., 2011b; Vercruysse et al., 2011a, b).

We found that drug efficacies were much lower in the groups of subjects showing low pre-treatment FECs (<150 epg). However, the impact of this on the FECRT outcome differed between the two studies: in study 1 omission of <150 FEC subjects had a significant impact on the drug efficacy (81.6% for all data, vs 88.9% for >FEC 150 group), while for study 2 there was no significant difference between the two efficacy values (86.7% for all data vs 88.6% for >FEC 150 group). This comparison of studies 1 and 2 is informative as to the potential impact of low FEC subjects on interpretation of whether a FECRT outcome may be indicative of the presence of drug resistance. In study 1 the interpretation of the study outcome may be quite different depending on whether the low FEC subjects are included: while the value of 88.9% (<150 FEC excluded) is comparable to the hookworm drug resistance threshold of 90% recommended by Vercruysse et al. (2011b) and the WHO (2013), and may therefore not trigger concern as to the presence of resistance, the value of 81.6% (<150 FEC included) is more suggestive of the presence of drug resistance in the population.

This contrast between the two studies in terms of the impact of exclusion of FEC <150 subjects indicates that the impact of low FECs on the overall FECRT outcome in different studies will not be easy to predict. Both studies 1 and 2 had a similar percentage of subjects with FEC <150 (45% for study 1, and 39% for study 2), however, the most extreme cases of FEC increases following drug treatment were seen in study 1 (Fig. 2). Hence, while the distorting effect of the low FEC subjects in study 1 could be detected and highlighted after the FECRT was complete, the effect could not be predicted by simply noting the percentage occurrence of low pre-treatment FECs in the study population. Vercruysse et al. (2011a) found that exclusion of <150 subjects had very little effect on mean group drug efficacy results in Vietnam (remained at 100%) and Cameroon (changed from 93.0% to 93.6%) despite these sites having 67% and 49%, respectively, of the study subjects showing FEC <150. Hence, the distortion of FECRT outcome by low FEC subjects seen in study 1 was not observed at the Vietnam and Cameroon sites despite the low FEC subjects forming a larger percentage component of the study populations at these two sites.

A significant number of study subjects showed increases in FEC following drug treatment (14% in study 1, and 8% in study 2). These cases occurred mostly in the <150 FEC subjects. The spectra of effects of a drug treatment range from complete removal of a worm population from an infected host, to having no effect on the population. Hence, cases where FEC increases following treatment are not a reflection of the drug action, but rather are most likely related to inaccurate egg counting techniques (low sensitivity, variability due to Poisson distribution effects, discussed below), non-random distribution of eggs in stools at low infection levels (discussed below), worm biology (for example, day-to-day variation in egg excretion, the onset of patency, discussed below), or a laboratory error (mislabelling etc.). These cases therefore do not represent the effects of the drug against the worm population, and hence we believe that they should not be included in a FECRT which aims to accurately measure drug efficacy.

We therefore sought a means to examine a FECRT data set with a view to determining if specific instances of increased FEC following drug treatment should be excluded from the study. We suggest that one means to address this issue is to apply a modification of the quality control measure proposed by Torgerson et al. (2012) for replicate egg counts on single faecal preparations. FECs based on repeat samples from thoroughly mixed faecal suspensions are expected to be variable due to the Poisson distribution of eggs in the suspension (Torgerson et al., 2012). These authors describe a means to determine if such replicate measurements from a single faecal preparation follow a Poisson distribution. If the replicate values did not satisfy this criterion, the authors argued that they represented samples from a non-random distribution, and therefore that there had been a problem with sample processing, mixing or counting, and, hence, that data set should be discarded, and the counts repeated. We have applied this test across pre- and post-treatment samples from individuals rather than to replicate egg counts from a single faecal preparation as described by Torgerson et al. (2012). We have considered repeat samples from the same subject at different time points to simulate repeat samples from a single faecal preparation that may be expected if the worm population in the host does not change in response to the drug, that is, if the drug has a zero efficacy. If the worm population in the host is unchanged, and hence their egg output is also unchanged, the concentration and distribution of eggs in the pre-treatment sample should be equivalent to the post-treatment sample, assuming that the distribution in the stool at both time points is random.

We sought to be able to distinguish between those subjects whose FEC increased by a magnitude within expectations of a Poisson distribution (hence the two time points represented replicates from a single randomly distributed sample) as distinct from those

<table>
<thead>
<tr>
<th>Study</th>
<th>Data set</th>
<th>Number of subjects</th>
<th>Mean FEC (epg)</th>
<th>Drug efficacy, 95% CIs (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-treatment</td>
<td>Post-treatment</td>
</tr>
<tr>
<td>1</td>
<td>All data</td>
<td>95</td>
<td>735</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Pre FEC &lt; 150</td>
<td>43</td>
<td>81</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Pre FEC &gt; 150</td>
<td>52</td>
<td>1275</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>After negative efficacy exclusionsb</td>
<td>90</td>
<td>770</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>All data</td>
<td>84</td>
<td>531</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>pre FEC &lt; 150</td>
<td>33</td>
<td>72</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>pre FEC &gt; 150</td>
<td>51</td>
<td>828</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>After negative efficacy exclusionsb</td>
<td>80</td>
<td>550</td>
<td>58</td>
</tr>
</tbody>
</table>

a Within a study, drug efficacies followed by the same letter are not significantly different, based on overlap of 95% CIs.
b Denotes adjustment of data by exclusion of subjects showing negative drug efficacies in which pre- and post-treatment FECs did not lie within expected Poisson sampling variation limits (see text for details).
individuals whose FECs increased by a greater magnitude than expected if the repeat samples followed such a distribution. We applied this test to all subjects who had shown an increase in FEC following the drug treatment. Subjects whose repeat samples did not follow the expected distribution were excluded from the FECRT analysis as we suggest that the observed increase in FEC represents the result of an influence other than drug action (e.g., non-random distribution of eggs in the stool, labelling or mixing error, day-today egg excretion variability, onset of patency). This resulted in the most extreme cases of negative drug efficacy being removed from the overall FECRT data set (Fig. 2B), with a significant impact on the FECRT outcome for both studies 1 and 2 (Table 1). Notably, the subject showing the greatest increase in FEC after drug treatment in study 2 (Fig. 2B, subject 59032 from Table 2) was not removed from the analysis using this method. However, their inclusion would be expected to have little consequence on the FECRT outcome as the FEC for this subject was so low as to have little or no impact on the overall FECRT result on a group basis.

We suggest that this test is an appropriate quality control measure to apply to subjects showing increased post-treatment FEC in human FECRTs. It is very simple to apply as it is done after the collection of all field data. ID values are calculated using the mean and variance of the pre- and post-treatment FECs for the individual subjects who show increased FEC following the drug treatment, and then these values are simply compared to ID values shown in a single reference Table in order to assess whether the repeat (pre- and post-treatment) FECs follow a Poisson distribution. The test cannot be applied to subjects whose FEC decreases following drug treatment, no matter how small the difference, as such an effect could be due to drug action, and hence the pre- and post-treatment samples would not be expected to represent replicates from a random distribution.

Interestingly, the impact of applying this quality control method was similar to the outcome of the analysis in which <150 FEC subjects were omitted (88.9% vs 89.2% for study 1, and 88.6% vs 89.4% for study 2). However, the Poisson analysis resulted in the exclusion of only five subjects from the study 1 data set, and four from the study 2 data set, compared to exclusions of 43 and 33 subjects from studies 1 and 2 if the <150 epg pre-treatment FEC exclusion criterion was applied.

Two early human hookworm studies specifically examined the distribution of eggs in human stools by comparing the variability in egg counts from replicate samples from single stools (eggs counted using the smear method) to the variability in replicate samples from a single thoroughly mixed faecal suspension from each stool (the dilution method from Stoll, 1923) (Beaver, 1949; Melvin et al., 1956). Beaver (1949) found that the variability was similar for the two types of measurements, suggesting a random distribution of eggs in the stool. Melvin et al. (1956) found that both the replicate smear method counts from single stools, and the replicate dilution method counts from single faecal slurries, followed a Poisson distribution (based on index of dispersion values), indicating that the eggs were also distributed in a random manner in the faeces of their study subjects. However, importantly these studies utilised human subjects with FEC >1000 epg in the case of Melvin et al. (1956) and at least 1000 eggs per ml in the study of Beaver (1949), that is, significantly higher than many of the pre-treatment FECs recorded from the Ghanaian subjects in studies 1 and 2. Hence, while the early studies indicate that the potential source of variation in FEC data due to non-random mixing of human hookworm eggs in stools may not be significant when the FECs are above 1000 epg or 1000 eggs per ml, the potential remains for them to be more significant for low FEC subjects that dominated the studies 1 and 2 populations. It may be expected that there is an increased likelihood of a non-random distribution of eggs in the stool as the egg count decreases to very low levels. Egg production may become limited to more discrete intervals of time and location within the intestine when only a low number of adult females are present. Hence, two sources of variability associated with low egg counts are expected to be present, with one certainly expected to become more important as the FEC decreases (variation due to Poisson sampling errors, assuming a random distribution of eggs in the stool), and the other also potentially becoming important as egg counts decrease (due to possible non-random mixing of eggs in the stool).

Another possible explanation for the low drug efficacy values observed in some individuals, and negative efficacies in others, is that some hookworms which survived the drug treatment only reached patency after the pre-treatment sampling time. In this way they would not have contributed to the eggs present in the pre-treatment sample, but may have contributed significantly to the post-treatment sample. This is an unavoidable issue with human FECRTs in environments where there is significant transmission occurring at all times in the study population. The impact of...
this is likely to be greater in subjects with low pre-treatment FECs than those with high FECs as the onset of patency in a given number of worms in these two types of subjects will be more significant in terms of its magnitude relative to the pre-treatment egg excretion rate in the subjects which showed only a low egg output before the drug treatment. The FECRT can only measure efficacy against egg laying worms at the time of treatment and provides no information on efficacy against developing stages. While exclusion of animals with FEC <150 is recommended for livestock FECRTs, such a criterion has not been applied strictly to human studies, largely because of the difficulties associated with recruiting enough study subjects with sufficiently high FECs for such studies. The present study has illustrated the impact that inclusion of low FEC subjects may have on a FECRT outcome, particularly with study 1. This issue has the potential to become even more important as ongoing mass drug administration programmes, alongside health education and improved sanitary conditions, reduce infection levels in areas where the possible emergence of resistance will need to be monitored (Humphries et al., 2012). That is, the testing for resistance will become more problematic as drug treatments continue over time. As suggested by several authors (Knopp et al., 2011; Levecke et al., 2011b,c; Torgerson et al., 2012) one means to overcome this is to increase the sensitivity of the counting technique (for example, counting more McMaster slide chambers, or using FLOTAC) such that a count of 150 represents the observation of many more eggs in the counting procedure. In this way, the application of a >150 FEC cut-off for inclusion in a study becomes less relevant as the sensitivity of the egg counting increases. The greater impact of low FEC subjects observed in study 1, with an egg detection sensitivity of 37, compared to study 2, with a sensitivity of 12, highlights this point. In addition, we suggest that the Poisson distribution quality control measure described here is applied across pre- and post-treatment samples for subjects showing negative drug efficacies in order to remove ‘erroneous’ data points from human FECRTs. The study subjects showing negative drug efficacies in studies 1 and 2 were mostly present in the <150 FEC group, and hence, the adjustment proposed here may be one means to at least partly counter the potentially distorting influence of inclusion of low FEC study subjects in human FECRTs.

Acknowledgments

This work was supported by USA National Institutes of Health Grant AI099623 and the Ghana–Yale Partnership for Global Health. The authors would like to thank the field research team at the Noguchi Memorial Institute for Medical Research for technical support, and the people of Kintampo North Municipality for their participation. The authors have no conflicts of interest concerning the work reported in this paper.

References


