Using Simulation Techniques To Investigate Methods To Determine Resistance Of Helminths To Anthelmintic Treatment

P.M. Pepper, **A.J. Swain and M. Lyndal-Murphy**

Agency for Food and Fibre Sciences, Department of Primary Industries, Locked Mail Bag No 4,
Moorooka Qld 4105.

Abstract: The widespread and increasing resistance of internal parasites to anthelmintic control is a serious problem for the Australian sheep and wool industry. As part of control programmes, laboratories use the faecal egg count reduction test (FECRT) to determine resistance to anthelmintics. It is important to have confidence in the measure of resistance, not only for the producer planning a drenching programme but also for companies investigating the efficacy of their products. The determination of resistance and corresponding confidence limits as given in anthelmintic efficacy guidelines of the Standing Committee on Agriculture (SCA) is based on a number of assumptions. This study evaluated the appropriateness of these assumptions for typical data and compared the effectiveness of the standard FECRT procedure with the effectiveness of alternative procedures. Several sets of historical experimental data from sheep and goats were analysed to determine that a negative binomial distribution was a more appropriate distribution to describe pre-treatment helminth egg counts in faeces than a normal distribution. Simulated egg counts for control animals were generated stochastically from negative binomial distributions and those for treated animals from negative binomial and binomial distributions. Three methods for determining resistance when percent reduction is based on arithmetic means were applied. The first was that advocated in the SCA guidelines, the second similar to the first but basing the variance estimates on negative binomial distributions, and the third using Wadley's method with the distribution of the response variate assumed negative binomial and a logit link transformation. These were also compared with a fourth method recommended by the International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) programme, in which percent reduction is based on the geometric means. A wide selection of parameters was investigated and for each set 1000 simulations run. Percent reduction and confidence limits were then calculated for the methods, together with the number of times in each set of 1000 simulations the theoretical percent reduction fell within the estimated confidence limits and the number of times resistance would have been said to occur. These simulations provide the basis for setting conditions under which the methods could be recommended. The authors show that given the distribution of helminth egg counts found in Queensland flocks, the method based on arithmetic not geometric means should be used and suggest that resistance be redefined as occurring when the upper level of percent reduction is less than 95%. At least ten animals per group are required in most circumstances, though even 20 may be insufficient where effectiveness of the product is close to the cut off point for defining resistance.

Keywords: *Resistance; anthelmintic control; simulation*

1. INTRODUCTION

The cost of internal parasites to the Australian sheep and wool industry is conservatively estimated at $220 million per year (Anon. 2000). The widespread and increasing resistance of gastrointestinal helminths to anthelmintic control is threatening the viability of the industry. As part of worm control programmes, laboratories use the standard operating procedure, the faecal egg count reduction test (FECRT), to determine resistance to anthelmintics (SCA Technical Report No 28, 1989 and Lyndal-Murphy, 1993).

For the FECRT, the definition of resistance is based on arithmetic means, with the estimate of percent reduction, R, and corresponding approximate 95% confidence limits (CLs) calculated as follows:

$$R=100(1- m_t/m_c)$$

$$CLs = 100(1-(m_t/m_c)\exp(\pm t\sqrt{V})) \quad (1)$$

where $V = [s^2_t/(n_t m_t^2) + s^2_c/(n_c m_c^2)]$, n_t and n_c, m_t and m_c and s^2_t and s^2_c, are the numbers of animals, arithmetic means and variance estimates of the drenched (treated with anthelmintic) and control groups respectively, and t is Student's t for (n_t+n_c-2) degrees of freedom and two-tailed probability 0.05. This utilises the result that the variance of a function, f(x), approximately equals $f'(\mu)$.variance(x), where $f'(\mu)$ is the derivative of f(x) evaluated at μ. Use of the t distribution assumes that the observations are normally distributed.

When the percent reduction is less than 95% and the lower 95% confidence level is less than 90%, resistance is said to occur. Ten to 20 animals are stratified on weight and/or allocated at random to groups when this procedure is applied in practice.

Wood et al, 1995 recommended that claims for anthelmintic efficacy of a product based on geometric means should be expressed against each genus as: highly effective (over 98%), effective (90-98%), moderately effective (80-89%) or insufficiently active (less than 80%). VICH guidelines (Vercruysse et al, 2001) recommended that for sheep and goats the number of adequately infected animals in dose determination and dose confirmation trials of anthelmintic efficacy be a minimum of six animals in each experimental group and that two confirmation studies be conducted. For a claim to be granted, these guidelines advocated the effectiveness, calculated using transformed data (geometric means), should be 90% or higher and the difference in parasite counts between treated and control animals should be statistically significant ($P<0.05$).

The merit of the methods and the effects of their various assumptions were investigated by simulation. Helminth egg counts in faeces for drenched and undrenched groups were generated for distributions with different parameters. Percent reduction and confidence limits were calculated for the methods, together with the number of times in 1000 simulations the theoretical percent reduction fell within the estimated confidence limits and the number of times resistance would be said to occur. Performance of the methods was compared and alternative rules for assessing resistance investigated.

2. METHOD

2.1. Distribution of helminth egg counts

The numbers of helminth eggs were counted in the faeces of 5 flocks of sheep and 2 mobs of goats which had not received anthelmintic treatment in the previous 12 weeks. The distributions of the counts were investigated using Genstat (2000) and negative binomial distributions found to fit the data (Table 1). This distribution has the form

$$\Pr(X=r)=\binom{r+k-1}{k-1}\left(\frac{\mu}{\mu+k}\right)^{r}\cdot\left(1+\frac{\mu}{k}\right)^{-k}$$

with mean μ and variance $\mu+\mu^2/k$. $r=0,1\ldots$

Figures 1 and 2 illustrate typical goodness of fit to the data and the effect of the parameter, k.

2.2. Simulation of helminth egg counts

Helminth egg counts were simulated for n animals per group by sampling from a negative binomial distribution with mean μ_c and parameter k. The negative binomial distributions were generated from gamma and Poisson distributions (Bratley et al, 1983) using Genstat (2002). Egg counts after drenching (ie for each of the treated groups) were generated from binomial distributions with parameters N_i and p, where N_i was the number of eggs generated from the negative binomial distribution for sheep *i* in the drench group and p the proportion of egg counts expected to be present after drenching. One thousand simulations were undertaken for each of the following combinations of parameters:

Number of animals per group, n = 6, 10, 15, 20

Average number of egg counts for animals not drenched, $\mu_c = 400, 1000$

Proportion of egg counts after drenching, p = 0.01, 0.025, 0.05, 0.1, 0.5 (ie R = 99, 97.5, 95, 90 and 50%).

Table 1 Description of negative binomial distributions

Species	No of animals	Deviance	d.f.	Mean μ	k
Sheep	101	15.32	12	3354 ± 242	1.92 ± 0.252
	100	4.49	7	516 ± 41.0	1.60 ± 0.209
	420	25.11	16	722 ± 35.6	0.98 ± 0.063
	105	16.41	16	594 ± 64.7	0.81 ± 0.105
	891	24.36	16	2047 ± 123	0.31 ± 0.013
Goats	194	23.99	17	282 ± 35.9	0.32 ± 0.031
	123	16.70	10	366 ± 35.6	0.86 ± 0.106

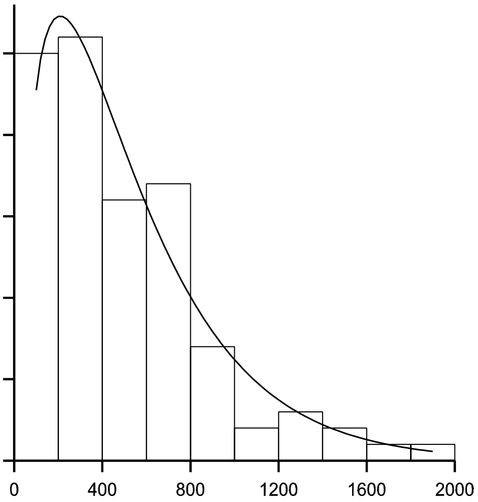

Figure 1. Histogram and fitted-distribution for 100 sheep (estimated k=1.60)

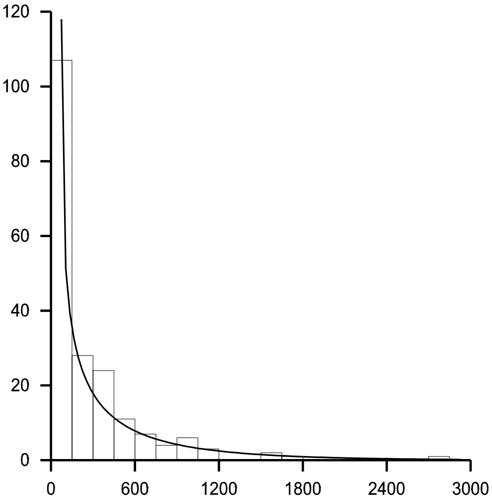

Figure 2. Histogram and fitted-distribution for 194 goats (estimated k=0.32)

2.3. Estimation of percent reduction and confidence limits

Method 1: For each simulation, estimated percent reduction R based on arithmetic means and approximate confidence limits was calculated from (1) with $s^2 = [\sum(x-m)^2]/(n-1)$ (SCA Technical Report No 28, 1989 and Lyndal-Murphy, 1993).

Method 2: Similar to method 1, but assuming the distribution for egg counts follows a negative binomial distribution and using the known values of k, so that $s^2 = m + m^2/k$. This method cannot be used in practice, since k will be unknown, but is included here to indicate the effect of the variance estimation in the other methods.

Method 3: The simulated egg counts were analysed using the Wadley (Smith, 1993) method with the distribution of the response variate as negative binomial, with a logit link transformation and using an estimate of k based on the controls. Confidence limits for percent reduction were calculated from the standard errors given by the procedure.

Method 4: The percent reduction R was based on the geometric means and the confidence limits calculated by analysing the logarithm transformed (log(x+1)) egg counts.

For all methods, the number of times in the 1000 simulations that the true value of the reduction, R, fell within the estimated confidence limits was calculated. If the mean of the drenched counts was greater than the mean of the controls this was recorded and the percent coverage calculated on the remaining simulations.

2.4. Estimation of resistance

For methods one, two and three, the number of occurrences of resistance were calculated, as currently defined by SCA, and also as redefined as occurring when the upper level of percent reduction was less than 95%.

Using the definition of and criteria for resistance based on geometric means as in the VICH guidelines, the number of occurrences of resistance occurring in 500 pairs of simulations were calculated for each set of parameters.

If the mean of the drenched counts was greater than the mean of the controls the percentage was calculated on the remaining simulations.

Table 2. Percentage of times confidence limits cover true R and percentage of times resistance is said to occur when R is calculated from simulated trials with control mean of 400.

k	R%	n	No. $m_c>m_t$	%coverage of true R				% judged resistant						
								$R<95\%$; $R_L<90\%$			$R_U<95\%$			$R<90\%$ $m_t \neq m_c$
				Method				Method			Method			Method
				1	2	3	4	1	2	3	1	2	3	4*
2	99	6	1000	95.3	97.6	95.1	91.4	0	0	0	0	0	0	0
		10	1000	95.0	96.2	94.2	89.0	0	0	0	0	0	0	0
		15	1000	95.2	96.4	94.2	86.2	0	0	0	0	0	0	0
		20	1000	95.1	96.4	95.1	79.3	0	0	0	0	0	0	0
	97.5	6	1000	95.0	96.6	93.0	94.7	6.5	6.9	6.5	0	0	0.1	0.2
		10	1000	94.8	95.6	93.8	93.9	2.4	2.5	1.8	0	0	0	0
		15	1000	94.6	95.9	94.2	93.4	0	0	0	0	0	0	0
		20	1000	95.7	96.5	95.2	93.4	0	0	0	0	0	0	0
	95	6	1000	94.7	96.3	93.1	95.1	54.3	55.8	51.9	3.1	2.0	4.3	14.2
		10	1000	94.3	95.3	93.0	94.8	44.9	51.5	41.5	3.6	3.1	4.5	7.8
		15	1000	94.4	96.0	93.4	94.1	30.6	32.8	29.1	3.2	2.5	3.4	2.8
		20	1000	95.9	96.4	94.4	94.5	20.9	20.3	18.8	2.6	2.3	3.6	1.0
	90	6	1000	93.9	96.6	93.9	95.9	96.5	96.6	96.1	41.0	34.2	41.6	80.4
		10	1000	94.4	95.4	93.3	94.5	98.2	98.7	97.6	56.2	54.4	55.0	74.2
		15	1000	95.3	95.8	93.8	95.0	98.0	98.4	97.1	74.6	75.4	72.4	76.0
		20	1000	95.3	96.7	95.2	95.9	98.1	98.6	98.2	88.0	88.4	86.4	78.6
	50	6	940	97.6	98.8	95.7	98.0	100	100	99.8	99.9	99.9	87.7	100
		10	977	96.7	98.0	96.3	96.5	100	100	100	100	100	96.4	100
		15	995	94.6	96.5	95.9	95.4	100	100	100	100	100	99.3	100
		20	1000	96.1	96.7	97.3	96.4	100	100	100	100	100	100	100
0.5	99	6	1000	92.0	95.1	90.4	87.1	4.7	4.7	4.7	0.2	0	0.3	14.8
		10	1000	91.1	95.2	90.6	74.9	1.9	1.9	1.9	0	0	0	3.4
		15	1000	93.0	95.2	92.2	65.8	0.2	0.2	0.2	0	0	0	1.2
		20	1000	94.6	95.5	93.2	54.0	0	0	0	0	0	0	0.2
	97.5	6	1000	92.1	95.7	89.5	93.2	26.9	26.9	26.9	1.3	0.3	0.9	40.4
		10	1000	91.1	95.0	90.7	90.4	19.1	19.1	19.1	0.6	0.1	0.4	19.8
		15	1000	92.8	95.2	91.6	86.6	10.2	10.2	10.2	0.2	0	0.2	12.8
		20	1000	94.3	95.5	93.1	81.3	8.4	8.4	8.1	0	0	0	7.2
	95	6	998	91.5	95.3	91.4	94.4	55.8	55.8	55.8	5.0	2.9	5.0	67.5
		10	1000	91.6	95.3	91.2	94.5	53.6	53.6	53.5	5.1	3.2	5.6	53.2
		15	1000	92.7	95.0	91.2	91.9	54.0	54.0	54.0	4.3	2.4	4.6	46.8
		20	1000	94.1	95.5	92.8	89.9	54.5	54.7	53.8	3.8	2.9	4.5	45.0
	90	6	992	92.0	95.5	94.7	95.8	84.6	84.6	84.6	21.2	11.8	15.1	89.5
		10	999	91.7	95.4	92.7	95.0	88.0	88.0	88.0	25.7	20.7	22.1	83.6
		15	1000	92.9	95.0	92.5	94.0	92.2	92.2	91.9	33.7	28.6	30.6	87.0
		20	1000	93.8	95.6	93.2	92.8	95.5	95.6	95.1	39.8	37.3	36.6	88.8
	50	6	740	95.5	97.4	92.2	98.1	99.5	99.5	98.9	76.1	69.1	27.6	100
		10	810	95.9	98.0	92.5	98.2	100	100	99.4	94.1	93.7	61.6	100
		15	894	96.5	97.4	93.7	96.4	100	100	99.8	99.1	98.8	78.2	100
		20	921	97.4	98.3	95.1	97.5	100	100	99.4	99.7	99.8	85.5	100

*resistance said to occur in 500 pairs of simulated trials

3. RESULTS AND DISCUSSION

The results for the runs with control mean of 1000 were very similar to those with control mean of 400 so only those for mean 400 are presented (Table 2).

The number of times the mean of the treated group is greater than that of the controls when R is 50% underlines the importance of having an adequate number of animals per group.

3.1. Confidence interval coverage of true R

If the assumptions underlying the methods are satisfied one would expect the true percent reduction to fall within the 95% confidence limits 95% of the time. The results for methods 1 and 3 are very similar, though with those for method 3 tending to be slightly below those for method 1. For k=2, when the distributions are tending towards Normal, the coverages are close to the expected 95% but when k=0.5 and the distributions are very skewed, the coverages are generally lower than expected, though still exceeding 90%. The coverages for method 2 for k=0.5 are much closer to 95% suggesting that underestimating variance is occurring for methods 1 and 3.

The coverage for method 4 is poor when the percentage reduction is high and so counts for the treated group are small. This appears worse as the number of animals increases, ie as the width of the confidence intervals decreases, indicating severe bias in the estimation of R by method 4 and the inappropriateness of assuming a lognormal distribution when the percentage reduction is high. The situation is worse when k=0.5 than when k=2.

3.2. Percentage judged resistant

With the parasites considered free of resistance when the true percentage reduction is 95% or more, the methods should ideally determine the percent of cases that are resistant, to be zero when the true percent reduction is 99% and 97.5%. Similarly when the true R is 90% or lower, the methods should judge the parasites to be resistant. The two rules used with methods 1, 2 and 3 for assessing resistance use quite different approaches to controlling the types of judgement errors they allow and this is clearly reflected in the results (Table 2). The first rule (estimated R<95%; R_L<90%) judges a case to be resistant if the researcher cannot be more than 97.5% confident that the true reduction is not less than 90%. This rule therefore favours judging a product as resistant when uncertain. Thus for a product with true R of 95% and only 6 animals in each group the confidence intervals are wide and cases are judged resistant about 55% of the time. With 20 animals in each group this improves to about 20% of the time when k=2 but remains worse than 50% when k=0.5. When k=0.5 this rule even judges products with true R of 97.5% to be resistant a substantial proportion of the time, especially with 6 animals in each group.

The second rule (R_U<95%) judges a case to be resistant if the experimenter is more than 97.5% confident that the true reduction is less than 95%. This rule therefore favours judging a product as not showing resistance when uncertain. Thus for a product with a true R of 95% the percentage judged resistant is close to the expected 2.5%. The effect of increasing the number of animals, so reducing the confidence intervals, is to improve the power of the method to judge as resistant products with true R less than 95%. For a true R of 90% increasing the number of animals in each group from 6 to 20 increases the chance of it being judged as resistant from about 40% to almost 90% when k=2 and from about 20% to 40% when k=0.5.

The rule used with method 4 (basically, estimated R<90% in both of a pair of trials) does not use the confidence intervals to control the frequency of errors made in judging resistance. If method 4 gave unbiased estimates of R, when the true R is 90% the probability of the estimates of R in both trials being < 90% is 0.25 so that 25% of cases should be judged resistant. In fact the bias in the estimates increases this to 80%. This also means that products with higher true R can be judged resistant a substantial proportion of the time (for example 40% of cases when true R is 97.5%, k=0.5 and number of animals in each group is 6).

4. CONCLUSION

Whether or not helminths are judged to be resistant to an anthelmintic product has important implications for both the user and the manufacturer. Neither would wish to go to the expense of changing or developing new products if there is really no resistance to them. These results show that with the current SCA definition of resistance, resistance is likely to be declared more frequently than advisable especially when the distribution is highly skewed, which is not uncommon from the historical data. The authors suggest that resistance be redefined as occurring when the upper level of percent reduction is less than 95%. However, it should be noted that when the true reduction is 90%, resistance would not be declared two thirds of the time unless the number per group is greatly increased. Given the distribution of helminth egg counts existing in Queensland flocks, the method based on geometric means would not be an appropriate method to determine resistance as demonstrated by the results of the percent coverage and the frequency of declaration of resistance, especially from the highly skewed distribution. Comparing the results of the three methods based on arithmetic means confirms that method 1 is adequate. All methods and definitions of resistance illustrate the need to sample adequate numbers of animals. At least ten per group is required in most circumstances but even twenty may be insufficient where effectiveness of the product is close to the cut off point for defining resistance.

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