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Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays

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SUMMARY: Composite strains of *Trichostrongylus colubriformis* and *Ostertagia* spp consisting of 0, 1, 10, 25, 50, 75, 90, and 100% of known resistant strains were prepared and tested for benzimidazole resistance using faecal egg count reduction tests, *in vitro* egg hatch assays and tubulin binding assays. All tests detected resistance where the proportion of the resistant strain in the composite was 50% or more, whereas none of the tests unequivocally detected resistance below 25%. Egg count reduction tests were no less sensitive than the *in vitro* tests in detecting low levels of resistance but the egg hatch and tubulin binding assays provided a better quantitative estimate of moderate to high levels of resistance. Faecal egg count reduction therefore, provides a suitable means of detecting resistance in the field but tests, more sensitive to low levels of resistance are required. Results indicate that the use of post-treatment counts alone provides an adequate indication of anthelmintic efficiency.
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Introduction

Efficient use of anthelmintics is an integral part of worm control strategies to prevent production losses from parasitic infections. Consequently, there is a need for sensitive and convenient assays for the detection of resistance to anthelmintics. Four tests are currently available for detecting benzimidazole resistance (Presidente 1985); faecal egg count reduction test; *in vivo* anthelmintic efficiency assay; *in vitro* egg hatch assays (Le Jambre 1976; Coles and Simpkin 1977) and tubulin binding assay (Lacey 1985; Lacey and Snowdon 1988).

The *in vivo* anthelmintic efficiency assay is considered too costly for routine detection of resistance and has been shown to lack sensitivity compared to *in vitro* assays (Johansen and Waller 1989). The faecal egg count reduction test is the usual field procedure for detecting and monitoring resistance. The accepted standard for diagnosis of resistance is a reduction in mean egg count of 90% or less (Presidente 1985). This is somewhat arbitrary and is considerably less than the 99% or more demonstrated in initial reports of anthelmintic efficiency. The percentage reduction is a point estimate and takes no account of the variability in the estimate. Large 95% confi-

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dence intervals for the percentage reduction occur, particularly when resistance is present (for example Anderson *et al* 1988a) thus reducing the value of the point estimate of efficiency.

Egg hatch assays have been widely used in research to measure levels of resistance in nematode strains or crosses of resistant and susceptible worms (for example Le Jambre *et al* 1979a; b; Barton 1983; Martin *et al* 1988a; b). Le Jambre *et al* (1979b) used egg hatch assays to further investigate isolates from properties surveyed by Webb *et al* (1979b) and found resistance on some farms initially classified as susceptible by an egg count reduction test, due possibly to the criteria for resistance applied by Webb *et al* (1979b). In modified form, the same test has been used as a field diagnostic aid, by the choice of a discriminating concentration (Whitlock *et al* 1980; Kemp and Smith 1982).

The tubulin binding assay is a biochemical assay which measures the amount of radioactive benzimidazole that binds to a measured amount of nematode protein. It has been suggested by Donald (1985) that biochemical tests may provide the most specific and sensitive assays for resistance.

The present study was designed to compare the faecal egg count reduction test, egg hatch and tubulin binding assays as means of detecting resistance in composite strains of *Ostertagia* spp and *T. colubriformis* composed of known proportions of resistant and susceptible populations. Comparisons were made between oral and controlled release of albendazole anthelmintic treatment.

Materials and Methods

Various proportions of benzimidazole susceptible (KST81) and resistant (KRT81) *T. colubriformis* were combined and the level of resistance expressed in the progeny of the mixed composite strains was studied in experiment 1. In experiment 2, mixed composite strains of benzimidazole susceptible (KS79) and resistant (KR79) *Ostertagia* spp were studied. The isolation and history of these strains has been presented previously (Martin *et al* 1982; 1984; 1985; 1988b). Composite strains were prepared by mixing different proportions of larvae from susceptible and resistance strains after estimates of their number were obtained from 10 counts of 0.1 ml aliquots of 1 in 100 dilution on each of 6 one ml sub-samples of the stock larval suspension.

Each of the 8 composite *T. colubriformis* strains, or 6 composite *Ostertagia* spp strains, set out in Table 1, was passaged through worm-free sheep, to obtain eggs for the egg hatch assay, larvae for tubulin binding assay and larvae to infect sheep for the faecal egg count reduction test.

Experiment 1

Two hundred and forty Merino weaners were randomly allocated into 8 groups of 30 sheep. The sheep in each group were infected with 10,000 3rd stage *T. colubriformis* from each of the composite strains. Twenty-two to 25 d later, faecal samples were taken for egg counts to randomise the 30 sheep infected with each strain into 3 equivalent sub-groups each of 10 sheep. Two days later, sheep in one of the sub-groups received a single oral dose of 3.75 mg/kg of albendazole on an individual body weight basis. Sheep in a second group received a controlled release capsule designed to deliver 32.5 mg of albendazole/d for 20 d and a third group was left untreated. Faecal samples were collected at the time of treatment and 10 d later, after which all sheep were drenched with a double dose of ivermectin and held for re-use.

Experiment 2

The same protocal was used to infect 6 groups of 30 sheep with 60,000 3rd stage larvae from the composite strains of *Ostertagia* spp (KS79:KR79 ratios of 100:0, 90:10, 75:25, 50:50, 25:75 and 0:100).

Egg hatch assays were performed as previously described (Le Jambre 1976; Martin *et al* 1982). The same generation of the composite strains used for the egg count reduction tests was assayed on 2 occasions.

Tubulin Binding Assay

Tubulin binding assays were done by described methods (Lacey 1985; Lacey and Snowdon 1988) using approximately 50,000 infective larvae which yielded 1μg protein/ 3μl extract. ^{3}H mebendazole (MBZ) was prepared and used according to Lacey and Pritchard (1986). Samples were counted for 2 min using standard scintillation methods. Duplicate assays were performed on samples from the composite strains of *Ostertagia* spp and *T. colubriformis*, as shown in Table 2.

Controlled Release Capsule

The controlled release capsule* is based on technology generated by CSIRO (Laby 1978; 1981) and developed into a commercial form by Captec Pty Ltd. It is administered orally by balling gun, lodges in the reticulo-rumen and will continuously release 32.5 mg of albendazole/d for 100 d. The capsule has been designed for sheep up to 65 kg bodyweight. A dose rate of 0.5 mg/kg/d is effective against benzimidazole susceptible strains of nematodes (Anderson *et al* 1988b). For this study, the effective life of the capsule was reduced to approximately 20 d by removing 80% of the matrix. Because the weight of the sheep averaged 38 kg (range 32 to 44) the actual dose rate varied from 0.7 to 1.0 mg/kg/d.

Statistical Analysis

The arithmetic mean of 2 egg counts, one taken 3 to 5 d before and the other immediately before anthelmintic treatment, was used as the pre-treatment count. The first of these counts was used to block animals for randomisation into groups as described above. Analyses were performed on logarithms of the counts, with one being added to all counts in order to avoid problems with zero counts. The geometric means quoted were obtained from:

$$GM = \exp[\Sigma_1 \log (X_1 + 1)/n] - 1,$$

where there are n counts $X_1, \ldots, X_n$ and logarithms base e were used.

Analyses of variance (AOV) were done separately using (i) difference between the post and pre-treatment log counts for each animal (P&P), and (ii) just for the post-treatment log counts (POST). The main effects were STRAINS (Composite strains of resistant and susceptible larvae) and TREATMENTS (control, albendazole oral treatment and albendazole controlled release capsule). The sums of squares for TREATMENTS on 2 degrees of freedom (df) was partitioned into control versus the average for the groups treated with albendazole and oral versus capsule treatment each on 1 df. The interaction investigated was STRAINS X TREATMENTS which was also partitioned into control versus albendazole treated and oral versus capsule.

The estimates of percentage reduction for the two analyses were:

$$PR(P\&P) = 100(1 - (T_2/T_1) * (C_1/C_2)) \text{ and}$$
$$PR\ (POST) = 100(1 - (T_2/C_2)),$$

where

T_1 = GM treated group pre-treatment (day 0),
T_2 = GM treated group 10 days post-treatment (day 10),
C_1 = GM control group day 0, and
C_2 = GM control group day 10.

The 95% confidence intervals for the percentage reduction were obtained by calculating 95% limits for the differences between the means on the log scale using the residual variance from the analyses of variance mentioned above, from the formula:

$$95\%\ CI = 100[1 - P * \exp\{\pm 2 * s * \sqrt{(1/n_c + 1/n_t)}\}],$$

where n_c and n_t are the sample sizes in the control and treated groups respectively, P is either $(T_2/T_1)*(C_1/C_2)$ for P&P, or

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TABLE 1

Faecal egg count reductions calculated on pre- and post-anthelmintic treatment egg counts (P&P) or just post-treatment counts (POST)

Proportion		Geo mean	Albendazole (oral) 3.75 mg/kg P&P		Albendazole (oral) 3.75 mg/kg POST		Albendazole capsule 32.5 mg/day P&P		Albendazole capsule 32.5 mg/day POST	
Susc	Res	EPG	% Red	(95% C.I.)	% Red	(95% C.I.)	% Red	(95% C.I.)	% Red	(95% C.I.)
T. colubriformis										
1.00	0[a]	501	100	(99, 100)	100	(99, 100)	100	(99, 100)	100	(100, 100)
.99	.01[a]	631	100	(100, 100)	100	(100, 100)	100	(100, 100)	100	(100, 100)
.90	.10[a]	645	100	(99, 100)	100	(99, 100)	99	(100, 100)	100	(100, 100)
.75	.25[b]	133	95	(83, 98)	96	(84, 99)	86	(53, 98)	85	(47, 96)
.50	.50[b]	830	89	(66, 96)	91	(72, 97)	84	(50, 95)	86	(56, 96)
.25	.75[c]	94	38	(0, 82)	50	(0, 87)	92	(72, 98)	96	(85, 99)
.10	.90[c]	773	80	(38, 93)	81	(38, 94)	78	(33, 93)	76	(21, 92)
0	1.00[c]	573	74	(19, 87)	71	(8, 91)	65	(8, 83)	56	(0, 86)
Ostertagia										
1.00	0[a]	49	98	(92, 99)	99	(94, 100)	100	(100, 100)	100	(100, 100)
.90	.10[ab]	82	91	(67, 98)	90	(62, 98)	90	(63, 97)	87	(52, 96)
.75	.25[a]	110	99	(98, 100)	99	(97, 100)	98	(92, 99)	98	(91, 99)
.50	.50[c]	80	71	(32, 96)	83	(28, 96)	78	(51, 97)	88	(52, 97)
.25	.75[cb]	286	85	(45, 96)	80	(26, 95)	81	(31, 95)	69	(0, 92)
0	1.00[c]	294	42	(0, 84)	12	(0, 77)	10	(0, 75)	0	(0, 69)

Geometric mean eggs per gram (EPG) was taken from control animals before treatment. In all tests, treated animals are compared to untreated controls. Superscripts indicate statistically similar composite strains within each species.

(T_2/C_2) for POST, and s is the residual standard deviation from the analysis of variance of either the differences in the log counts (P&P) or just the post-treatment log counts (POST).

Probit analysis of the egg hatch data and the calculation of resistance ratios (RR) have been described previously (Martin *et al* 1982; 1988a). 95% confidence intervals for the RR were calculated from the formula:

$$\exp\{\log(RR) \pm 2 * \sqrt{(S_1^2 + S_2^2)}\}$$

where $S_1^2 + S_2^2$ are the variances from the probit analyses.

Tubulin binding ratios (BR) were calculated as the reciprocal of the ratios of the counts/min (cpm) of the ^3H MBZ bound to susceptible and resistant composite strains. Confidence intervals were calculated as the mean cpm $\pm 2 * \sqrt{s^2}$; where s^2 is the pooled variance of the susceptible composite strain and the particular resistant composite strain.

Results

Table 1 shows the geometric mean pre-treatment egg counts, the percentage reductions in *T. colubriformis* and *Ostertagia* spp faecal egg counts and their 95% CIs, for each composite strain treated with either an oral dose of albendazole or an albendazole capsule. The faecal egg count reductions between treated and control animals were estimated using post-treatment counts only and the same data adjusted for pre-treatment counts. Reductions of greater than 84% occurred in *T. colubriformis* composite strains with 50% or less of the resistance genome. Similarly, reductions of 87% or more occurred in *Ostertagia* spp composite strains with 25% or less of the resistance genome. Percentage reductions and their 95% confidence intervals were similar whether or not the post-treatment count was adjusted for pre-treatment count. Similar anthelmintic efficiencies were observed between groups treated with a conventional oral dose of albendazole or given an intraruminal controlled release capsule releasing albendazole at a rate of 32.5 mg/d.

The AOVs on *T. colubriformis* and *Ostertagia* spp egg counts indicated no significant difference in the anthelmintic efficiencies estimated from post-treatment egg counts or those estimated from the difference between post and pre-treatment counts. The respective variances for POST and P&P were 1.71 and 1.57 for *T. colubriformis* and 2.22 and 1.95 for *Ostertagia* spp. There were highly significant differences ($P < 0.001$) between TREATMENTS, between STRAINS and the TREATMENTS X STRAINS interaction. Further tests indicated a significant difference ($P < 0.001$) between control and albendazole treatment, but no difference between albendazole oral and albendazole capsule. Similarly, the variance for the interaction between control and albendazole treatment was significant ($P < 0.001$) and that between oral dose and capsule was not significant. Also shown in Table 1 are the statistically homogenous groups. The data for *T. colubriformis* fell into 3 statistically similar groups, those with 10% or less resistance, those with 25 to 50% and those with 75% or more resistance. Although some overlap in the data for *Ostertagia* spp is evident in Table 1, two statistically similar groups were apparent, those with up to 25% or less of the resistant strain and those with 50% or more.

Table 2 shows the estimates of the degree of benzimidazole resistance from egg hatch assays and tubulin binding assays. A significant increase in resistance ratio at the LC_{50} was detected in both *T. colubriformis* and *Ostertagia* spp composite strains with 50% resistance. Tubulin binding ratios for *T. colubriformis* showed significant departure once the resistance

TABLE 2

Resistance ratios at LC_{50} (RR) from egg hatch assays and binding ratio (BR) from tubulin binding assays

Proportion		RR	(95% CI)	BR	(95% CI)
Susc	Res				
T. colubriformis					
1.00	0	1.00	(0.6, 1.6)	1.00	(0.9, 1.1)
.99	.01	.90	(0.5, 1.4)	0.87	(0.7, 1.1)
.90	.10	1.30	(0.9, 2.1)	1.08	(1.0, 1.2)
.75	.25	1.10	(0.7, 1.6)	1.39	(1.2, 1.6)
.50	.50	2.50	(1.8, 3.7)	1.55	(1.4, 1.7)
.25	.75	6.20	(4.2, 9.1)	1.92	(1.6, 2.3)
.10	.90	11.00	(7.8, 15.5)	3.41	(3.0, 3.8)
0	1.00	14.00	(10.0, 19.7)	7.97	(7.3, 8.7)
Ostertagia					
1.00	0	1.00	(0.8, 1.3)	1.00	(0.9, 1.1)
.99	.01	.60	(0.4, 0.8)	—	
.90	.10	.80	(0.6, 1.0)	.95	(0.9, 1.0)
.75	.25	1.30	(1.0, 1.7)	1.02	(0.9, 1.1)
.50	.50	1.80	(1.5, 2.3)	1.16	(1.1, 1.2)
.25	.75	4.50	(3.6, 5.7)	1.94	(1.8, 1.2)
.10	.90	8.20	(6.9, 9.7)	—	
0	1.00	9.50	(8.0, 11.3)	4.81	(4.5, 5.2)

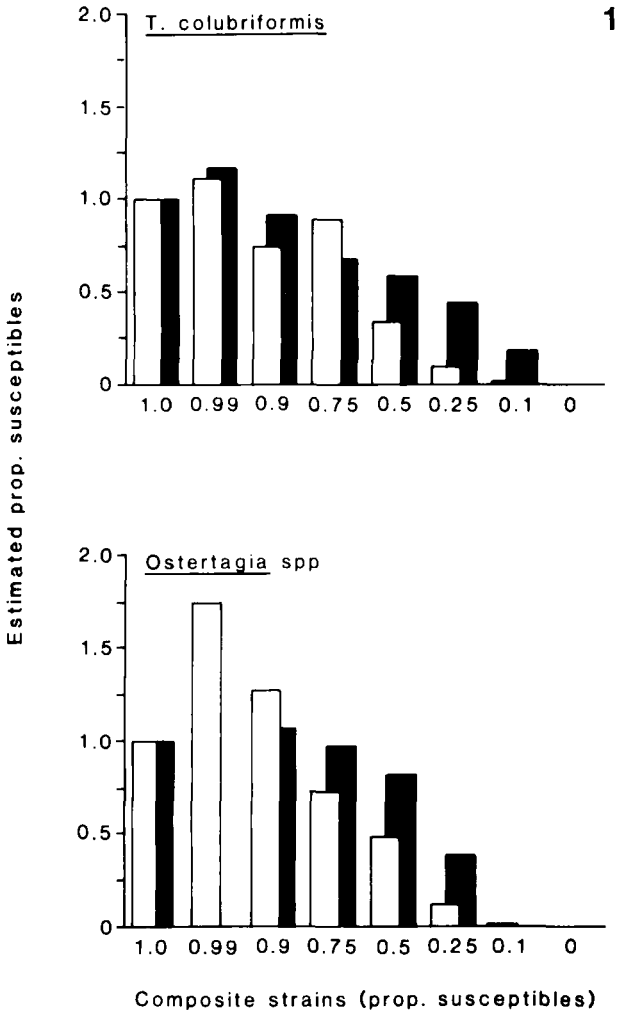

Figure 1. Column graphs of the proportion of susceptibles estimated from egg hatch assays (□) or tubulin binding assays (■) for each of the composite strains of *T. colubriformis* or *Ostertagia*.

reached 25% but 50% resistance was required for *Ostertagia* spp to show a significant increase.

The proportion of susceptibles in the composite strains estimated from the egg hatch and tubulin binding assay results are shown in Figure 1. These data were standardised on a susceptibility scale from 1.0 (100% susceptible) to zero (100% resistant). These assays provided a quantitative estimate, more closely related to the proportion of resistance in the composite strain than could be estimated from the egg count reduction data.

Discussion

The frequency of the benzimidazole resistance genome in the composite strains of *T. colubriformis* and *Ostertagia* spp populations was 25 to 50% before resistance was unequivocally detected by an egg count reduction test, egg hatch or tubulin binding assay. It follows therefore, that under field conditions, detectable levels of resistance would indicate a high frequency of resistance genes. This finding is consistent with resistance being incompletely recessive with respect to fitness in the presence of the anthelmintic (Martin *et al* 1988a). In such a situation, the heterozygotes from the mating of resistant and susceptible parents, are predominantly susceptible.

Furthermore, the rate of increase in resistance genes in a population exposed to anthelmintic, is proportional to the gene frequency, being most rapid in the range from 25 to 75%. This covers the range over which a benzimidazole changes from being an effective, to an ineffective anthelmintic for control purposes.

Egg hatch and tubulin binding assays provided a quantitative estimate of the degree of resistance, whereas the egg count reduction tests provided more qualitative data. Egg count reduction and egg hatch were expected to reflect the phenotypic expression of resistance. Tubulin binding on the other hand, is a measure of the anthelmintic effect at the site of action, and might be expected to provide a more sensitive indication of the presence of resistance in the nematode population (Donald 1985).

One underlying assumption in the interpretation of these data is that the composite strains, when assayed, reflected the same proportion of resistance as the original mixture of resistant and susceptible larvae. Preferential survival or mating of either the susceptible or resistant strain would have altered the actual proportion of resistance. Little is known of preferential mating but the issue of preferential survival is contentious (reviewed by Waller and Prichard 1986). In an effort to minimise these effects, the resistant and susceptible strains used in this study were ecologically similar. Accepting this limitation, it appears that all tests lack the sensitivity to detect levels of resistance below 25%. Where a quantitative estimate of the degree of resistance is required, for example in research studies on changing levels of resistance, egg hatch or tubulin binding assays would be preferable to egg count reduction. Nevertheless, because of the ease of performing a faecal egg count reduction test and its suitability to all drugs, it provides an appropriate test for the detection of resistance. However, a definition of resistance, more stringent than the currently accepted 90% efficiency or less, seems warranted. Based on the percentage efficiencies calculated on geometric mean egg counts presented here, a more realistic definition would be egg count reductions of less than 95% which are associated with a 95% confidence interval extending below the 90% level. At this level of efficiency, a change of anthelmintic group should occur. Continued treatment with the same anthelmintic will result in a rapid increase in resistance and is likely to select fitness modifiers which enhance the expression of resistance and reduce the chance of reversion towards susceptibility (McKenzie *et al* 1982; Martin *et al* 1988b).

Delaying the evolution of resistance can be achieved by highly efficient anthelmintics (Donald 1983; Martin *et al* 1988a). Strategic worm control programs such as "Wormkill", "Drenchplan" and "Wormplan" are best implemented after egg count reduction tests on individual farms have determined which anthelmintic groups are effective. Subsequent tests can be done to monitor their ongoing effectiveness. In these situations, there is a need for the detection of low levels of resistance. The difficulty shown here in detecting low levels of resistance, indicates there is still a need for more sensitive tests for field use.

Statistical analyses indicated no significant difference in egg counts, from groups infected with each composite strains, taken 10 d after treatment with albendazole either orally, at 3.75 mg/kg, or from a controlled release capsule delivering 32.5 mg/day.

The estimates of anthelmintic efficiency from egg count reduction tests were not greatly affected by adjustment for pre-treatment counts. Similar variances from the AOVs were observed using either method, which indicates little difference in the precision of these estimates. These data support the argument developed by Vizard and Wallace (1987), from which they concluded that the extra labour required for pre-treatment counts was not worthwile in view of the small loss of precision from using only post-treatment counts. The pre-treatment counts were used as a blocking factor for randomising animals into groups to ensure that control and treated groups had animals representing the range of counts.

The strains used in this study were selected for resistance with thiabendazole. Based on the communications of Webb *et al* (1979a) and Berger (1980) which reported an increased efficiency of the longer acting benzimidazole oxfendazole against thiabenzadole resistant strains, it could be argued that albendazole may have a high efficiency against the thiabenzadole selected composite strains. However, the KR79 strain

of *Ostertagia* spp has been previously studied and found to be highly resistant to a range of previously unencountered long acting benzimidazoles (Martin *et al* 1985). In addition, other reports have demonstrated side resistance within the group of benzimidazole anthelmintics (Hotson *et al* 1970; Colglazier *et al* 1975; Hogarth-Scott *et al* 1976; Le Jambre *et al* 1981). It is expected therefore, that the findings reflect a general phenomenon and highlight the difficulty in detecting low levels of benzimidazole resistance and the need for more sensitive tests for the detection of resistance in the field.

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Inhibited development of trichostrongylid worms in grazing cattle

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SUMMARY: Inhibition of development of gastro-intestinal trichostrongylid worms was studied using successive groups of tracer calves and groups of continuously grazed calves over one year in the Tully area of North Queensland lowland wet tropics. The results, assessed by means of worms from these calves recovered at necropsy 3 weeks after their removal from pastures, showed inhibition in the development of *Haemonchus placei* and *Cooperia punctata* at the early fourth stage at the approach to and during the relatively dry period in the area. Inhibition was however minor and inhibited larvae formed but only a small percentage of worm burdens in both categories of calves, indicating that they were not in any way of major epidemiological importance. It was suggested that the minor nature of inhibition was due to the mild climatic conditions which could not produce appropriate conditioning treatment, or caused only mild selection pressure for inhibition in the area.
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Introduction

Increased recognition of the importance of inhibited larvae as a precursor to clinical disease (Anderson *et al* 1965) or massive pasture contamination (Hart 1964) for cattle in addition to its apparent primary role of carrying over populations through unfavourable conditions in the external environment has emphasised the desirability of knowledge of the status of inhibited development in any particular area for effective planning of worm control. The importance of such knowledge is accentuated by the fact that, in contrast to normally developing worms, inhibited larvae of a considerable number of cattle trichostrongylids are not readily removed by most of currently available anthlemintics (Gibson 1980) or are removed only at increased dosage (Fabiyi *et al* 1979b).

It was therefore decided to ascertain the status and seasonal patterns of inhibited development of trichostrongylid worms in cattle in the wet tropical Tully area of North Queensland. Such studies have not hitherto been chronicled in the region, and were carried out as part of a basis for formulation of effective control of parasitic gastro-enteritis in this area.

The Tully area, where the study was conducted, has a humid tropical climate with an annual rainfall of approximately 2500

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