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SLIGHT RESISTANCE TO THE RESIDUAL EFFECT OF CLOSANTEL IN A FIELD STRAIN OF *HAEMONCHUS CONTORTUS* WHICH SHOWED AN INCREASED RESISTANCE AFTER ONE SELECTION IN THE LABORATORY

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ABSTRACT

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On the strength of a complaint of suspected resistance to closantel, a field strain of *Haemonchus contortus* was isolated from sheep on a farm near Pretoria and tested for resistance to the remedy, both without exposure to closantel in the laboratory (designated the "unselected" substrain of the parasite) and after a single selection with closantel (called the "selected" substrain). For comparative purpose a few sheep were treated with rafoxanide to ascertain whether the efficacy of this drug was unchanged.

While the unselected substrain appeared to show only slight increased resistance to the residual effect of closantel drenched at a dosage rate of 5 mg/kg, there was a dramatic increase in resistance after one selection. The residual efficacy (determined by the modified NPM test of Groeneveld & Reinecke, 1969, as outlined by Reinecke, 1973), 13–14 days after treatment with closantel was reduced from the registered claim of the remedy in South Africa of > 80% effective in > 80% of the treated flock to "ineffective", or < 50% effective in < 50% of the treated flock. Furthermore, there also appeared to be a slight increase in the resistance of this substrain to rafoxanide, as its efficacy was reduced from 99–100% to about 92%.

This sharp reduction in efficacy after a single selection with closantel seemed to indicate that the slight increase in resistance of the unselected substrain was probably due not to possible variation in the repeatability of the trail method but to repeated treatment with closantel on the farm.

INTRODUCTION

Closantel is registered in South Africa as an anthelmintic drench for sheep at 2 dosage rates, namely 5 mg/kg* and 10 mg/kg live mass**.

The efficacy claims of closantel registered at 5 mg/kg against *Haemonchus contortus* in sheep are Class "A"-efficacy† against 3rd stage larvae (L3), 4th stage larvae (L4) and adult worms, as well as Class "A" residual efficacy against infestation acquired up to 14 days after treatment. At 10 mg/kg the corresponding residual effect is 49 days. The residual efficacy of closantel at 5 mg/kg was tested by the manufacturers by treating sheep with the remedy and infesting them daily with L3 of *H. contortus* on Days 14 and 15 after treatment. The sheep were killed for worm recovery 30–32 days after infestation.

During the autumn of 1980 *H. contortus* ova were recovered from the faeces of sheep during the period of the expected residual efficacy of the remedy. Unfortunately the farmer submitted only a small faecal sample and, since he simultaneously treated the sheep with an anthelmintic having a wide efficacy, the worm strain could not be isolated before winter had set in.

It was decided to isolate the strain during the summer of 1980/1981 and to test whether the residual efficacy of closantel still coincided with the registered claim.

HISTORY OF THE STRAIN OF HAEMONCHUS CONTORTUS

The strain of *H. contortus* used in these investigations originated from sheep on a farm near Cullinan (25°40′S; 28°31′E), neighbouring on Pretoria, South Africa.

During the preceding 2 years the owner of the farm reportedly dosed all sheep on the farm with closantel at 6 mg/kg on 1979-02-17 and 1979-09-11, and closantel at 12 mg/kg on 1980-01-19, 1980-04-14 and 1980-12-13.

On 1981-04-24 sheep faeces were obtained from the farm for isolating the strain in the laboratory. According to the farmer the sheep from which the faeces originated had not been treated with closantel during the preceding 5 months (see dates above).

* Seponver (Janssen Pharmaceutica)

** Flukiver (Janssen Pharmaceutica)

† More than 80% effective in more than 80% of the treated flock (tested according to the modified NPM method of Groeneveld & Reinecke, 1967 as outlined by Reinecke, 1973)

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MATERIALS AND METHODS

Sheep

The 6 Dorper sheep used in the preliminary investigations (see below) were born and raised worm-free.

Fifty Merino sheep (26 ewes and 24 wethers), not raised worm-free, were available for the NPM trials. The wethers and ewes were of different origins, but immediately before commencement of the trials they were kept for more then 14 months and 16 months respectively under conditions of minimal exposure to worms on concrete-floored pens swept twice per week for the removal of accumulating manure. Subsequently, until conclusion of the trial, the sheep were housed under conditions that precluded unintentional infestation with worms.

Sixteen days before commencement of the NPM trials, the 50 sheep were each drenched with 1,5 g of morantel* (constituting a dosage rate of 24–45 mg/mg) to ensure that the sheep were not infested with worms. Examination of faeces by a concentration technique (see below) at the time of treatment failed to reveal any worm eggs, and therefore it is unlikely that these sheep were harbouring worms at the time of treatment.

The parasite

The strain of *H. contortus* was isolated from 2 sheep (Sheep 1 and 2) which had been infested with L3 cultured from sheep faeces obtained from the farm on 1981-04-24.

This was designated the "unselected" substrain of the parasite in contradistinction to the "selected" substrain, which was isolated from the faeces of Sheep 1 after treatment with closantel.

Infestation of the sheep

All the sheep were infested by being dosed *per os* with L3 concentrated on filter paper placed in gelatin capsules (Reinecke, 1973).

Worm recovery and identification

At necropsy the abomasa were collected and mucosal digests were done as described by Reinecke (1973).

In the preliminary trials total worm counts were done with the aid of a steroscopic microscope.

* Banminth (Pfizer)

In subsequent trials the worms were concentrated by the agar recovery method (Van Wyk, Gerber & Groeneveld, 1980) and, while total counts were done of the worms that migrated out of the agar as well as of those in the decanted specimens (Van Wyk *et al.*, 1980), only a 1 × 1/10 aliquot of the ingesta containing the worms that failed to migrate from the agar was examined from each sheep. In some cases, where a large number of adult *H. contortus* occurred in one agar slab, the wire-mesh of the agar frame was exposed by the whip-like movements of clumps of worms, and some worms became entangled in the mesh. Before the slabs were broken up for the purpose of counting the worms that failed to migrate from the agar, the worms that were entangled in the mesh were removed with a pair of thin forceps and included with the worms that did migrate from the agar, since worm counts are more accurate when the worms are not mixed with a large quantity of ingesta.

In each specimen the first 25 worms recovered were identified, but if fewer than 25 worms were recovered, all were identified (Reinecke, 1973).

Faecal egg counts

Unless otherwise stated, faecal egg counts were carried out by the modified McMaster procedure (Reinecke, 1973), sufficient material being examined to detect a minimum of 100 eggs per gram of faeces (epg). Hence, specimens shown in the publication as having zero ova may have contained from 0–100 epg.

An epg count by flotation means total examination of 5 g of faeces by a concentration technique (Whitlock, 1959) so that all the ova present may be counted and the concentration per gram calculated.

I. Preliminary Investigations

Method

On 1981-05-06 Sheep 1 and 2 were each infested with 4 821 freshly cultured larvae of the field strain of *H. contortus*. After faecal egg counts had been done on 3 occasions (Table 1) and sufficient larvae were cultured for use later in the trials, each sheep was dosed on 1981-06-08 with closantel at the rate of 5 mg/kg.

After egg counts on 2 further occasions, the sheep were reinfested with the same strain of parasite, as follows: 1 330 L3 daily per sheep between 1981-06-15 and 1981-06-18, and 3 000 L3 on 1981-06-19 (giving a total of 8 320 L3 per sheep).

Further egg counts were done during July and August of 1981 (Table 1). On 1981-08-21, 4 sheep were infested to act as donors for the larvae required for the 2 subsequent NPM trials: Sheep 3 and 4 were each infested *per os* with 24 000 L3 (designated the "unselected" substrain of *H. contortus*) isolated from Sheep 1 and 2 before these had been treated with closantel; Sheep 5 and 6 each received *per os* 10 280 L3 (called the "selected" substrain of *H. contortus*), isolated from Sheep 1 after the latter had been treated with closantel.

Results

The experimental design and faecal egg counts (epg) of Sheep 1 and 2, infested originally with larvae isolated from the faeces of sheep on the farm, are summarized in Table 1.

TABLE 1 Preliminary trial: experimental design

Date	Procedure	Faecal egg count*	
		Sheep 1 (5373/3)	Sheep 2 (5561/3)
1981-05-06	Infestation with 4 820 L3**	0	0
1981-06-02	—	3 900	1 000
1981-06-03	—	3 000	2 000
1981-06-04	—	5 100	4 300
1981-06-08	Drench with closantel, 5 mg/kg	n.d.‡	n.d.
1981-06-09	—	10 000	8 000
1981-06-10	—	0	0
1981-06-15	Reinfestation with 1 330 L3/day**	n.d.	n.d.
1981-06-16			
1981-06-17			
1981-06-18			
1981-06-19	Reinfestation with 3 000 L3**	n.d.	n.d.
1981-07-03	—	0	0
1981-07-09	—	1 000	100
1981-07-17	—	300	0
1981-07-31	—	0	0
1981-08-03	—	600	0
1981-08-13	—	1 400	100

* Eggs per g of faeces (epg)
** Third stage *H. contortus* larvae
‡ n.d. = not done

The maximum epg before treatment with 5 mg/kg closantel *per os* was 5 100 for Sheep 1 and 4 300 for Sheep 2. On the day after treatment the respective epg counts were 10 000 and 8 000, and on the 2nd day after treatment the counts of both sheep were zero.

After reinfestation, the maximum counts were 1 400 and 100 for Sheep 1 and 2 respectively.

Comment

In South Africa closantel drenched at a dosage of 5 mg/kg live mass has been registered with an efficacy classification of Class "A", that is, >80% effective in >80% of the treated flock, using the NPM method of Groeneveld & Reinecke, 1969, as modified by Clark (Reinecke, 1973), against larval and adult *H. contortus* of sheep and goats, as well as a similar classification for residual efficacy against L3 infesting the sheep up to 14 days after treatment.

While it appeared from the faecal egg counts that treatment was effective when administered 33 days after initial infestation, it appears that at least in 1 of the 2 sheep the remedy did not appear to give the expected residual efficacy, when the sheep were reinfested 7–11 days after treatment, that is, during the period when closantel should have controlled the infestation. It must be borne in mind, however, that since the sheep were not killed for worm recovery after the initial treatment and there were no untreated controls, these results could therefore give only a rough estimate of the susceptibility of the worms.

II. NPM Trials

From the preliminary investigations it appeared that closantel at 5 mg/kg live mass was less effective against this strain of *H. contortus* than the registered claim of >80% effective in >80% of the treated flock for 14 days after treatment.

Consequently, 2 controlled NPM trials were conducted to test the residual efficacy 14 days after treatment, one trial being intended to test the residual efficacy against the unselected parasite and the other against the same parasite after one selection with closantel.

Method

Two controlled trials were conducted simultaneously with closantel drench, using the NPM method of analysis of Groeneveld & Reinecke, 1969, as modified by Clark (Reinecke, 1973), and the unselected substrain being used in one trial and the selected substrain (see Preliminary Investigations) in the other.

The experimental design is summerized in Table 2.

TABLE 2 Experimental design (NPM trials)

Day	Treatment
− 16	Drenched each sheep with 1,5 g morantel
0	Drenched 13 wethers and 12 ewes *per os* with closantel* at a dosage rate of 5 mg/kg each, leaving 11 wethers and 9 ewes as untreated controls and another 5 ewes which were treated with rafoxanide after infestation—see Day +41 below.
+ 13	Dosed 1 700 L3 of the "unselected" substrain daily to each of 24 wethers and 5 ewes and 1 560 L3 of the "selected" substrain to 21 ewes. Total L3—3 400 of the "unselected" and 3 120 of the "selected" substrain
+ 14	
+ 40	Collected faeces for epg count
+ 41	Drenched 5 ewes** *per os* with rafoxanide (7,5 mg/kg live mass)
+ 45	Killed the 21 ewes (infested with the "selected" substrain) for worm recovery
+ 46	Killed the 24 wethers and 5 ewes (infested with the "unselected" substrain) for worm recovery

* Flukiver (Janssen) Lot No. 028A01, Expiry date: February, 1984
** These ewes were infested with L3 on Day +13 and Day +14, but were not drenched with closantel on Day 0

Included in the trial with the unselected substrain were 5 sheep infested with the same parasite but treated with rafoxanide (as shown in Table 2) instead of closantel, to assess the susceptibility of the unselected substrain to rafoxanide, which is chemically related to closantel. The origin of these 5 ewes was the same as that of the sheep used in the trial with the selected substrain.

Results

The results are summarized in Tables 3, 4 & 5

1. *Unselected substrain* (Table 3)

The faecal egg counts of sheep 7 days before they were killed for worm recovery ranged from 0–5 300 epg (mean: 2 500) for the control sheep and from 0–5 700 (mean: 462) for the group treated with closantel.

The mean adult worm burden of the Group A untreated control sheep was 1 041,1 and the burdens of Group B (treated with closantel) and Group C (treated with rafoxanide) were 160,7 and 86,2 respectively. The mean efficacy was therefore 84,6% in the case of closantel and 91,7% for rafoxanide. When analysed by the NPM method of Groeneveld & Reinecke, 1969, as modified by Clark (Reinecke, 1973), a Class "B" residual efficacy rating (>60% effective in >60% of the treated flock), was obtained for closantel drenched at the dosage rate of 5,0 mg/kg live mass.

While in the control sheep the L4 constituted a mean of 1,4% of the total worm burden and the young 5th stage worms a mean of 1,6% of the adult worm burden (Table 5), the respective figures were 7,4% L4 and 0,6% young 5th stage worms in the sheep treated with closantel and 11,0% and 3,5% in the sheep treated with rafoxanide.

The L4 in all 3 groups as well as all the adults in Group C (rafoxanide treated) migrated from the agar gel (Table 6), but in the control and closantel groups 3,9% and 4,3% respectively of the adult worms failed to migrate.

2. *Selected substrain* (Table 4)

The faecal egg counts of the sheep 5 days before being killed for worm recovery ranged from 0–6 800 epg (mean: 2 238) for the untreated controls and from 0–1 300 (means: 255) for the sheep treated with closantel.

The mean adult worm burdens of the control and treated groups were 705,1 and 366,0, respectively, giving a mean efficacy of 48,1%. According to the NPM analysis, the efficacy rating was X, that is "ineffective" or <50% effective in <50% of the treated flock.

The L4 constituted a mean of 2,9% of the total worm burden and the young 5th stage worms a mean of 8,7% of the adult worm burden in the control sheep, and 1,3% and 11,8%, respectively, in the sheep treated with closantel.

As in the trial with the unselected substrain, all the L4 migrated from the agar gel (Table 6) compared with 5,8% and 4,7% of the adult worms of the control and treated groups, respectively, that failed to migrate.

TABLE 3 Unselected substrain of *H. contortus*: numbers of worms recovered

Group	Sheep	Faecal egg count‡ (epg)	Number of *H. contortus*		
			Adults**	L4	Total
A (Untreated controls)	9	0	**0**	0	0
	17	2 000	**31**	12	43
	15	1 600	**404**	17	421
	10	1 200	**775**	8	783
	13	3 500	**868**	12	880
	16	4 100	**887**	11	898
	12	3 600	**1 286**	34	1 320
	7	2 100	**1 586**	0	1 586
	14	5 300	**1 784**	13	1 797
	8	600	**1 795**	51	1 846
	11	3 500	**2 036**	0	2 036
	Mean	2 500	**1 041,1**	14,4	1 055,5
	SD	1 624,2	**714,3**	15,6	718,3
	Median	—	**887**	—	—
	Median× 0,25	—	**222**	—	—
B (Treated: closantel at 5 mg/kg)	25	0	**0**	2	2
	30	0	**0**	4	4
	26	0	**7**	54	61
	23	0	**1**	7	8
	20	0	**20**	17	37
	21	0	**27**	9	36
	22	0	**29**	13	42
	18	0	**37**	32	69
	19	0	**45**	6	51
	29	0	**155**	8	163
	27	200	**170**	5	175
	24	100	**230**	5	235
	28	5 700	**1 368**	4	1 372
	Mean	461,5	**160,7**	12,8	173,5
	SD	1 575,1	**370,5**	14,7	367,3
	NPM efficacy rating	—	**B**	—	—
C (Treated: rafoxanide at 7,5 mg/kg)	33	0††	**0**	12	12
	31	0††	**17**	6	23
	34	500††	**62**	2	64
	32	800††	**172**	25	197
	35	3 600††	**180**	8	188
	Mean	980	**86,2**	10,6	96,8
	SD	1 504	**85,1**	8,8	89,5

‡ Done 7 days before the sheep were killed for worm recovery
** Including young 5th stage worms
†† Epg of faeces taken immediately before treatment with rafoxanide

TABLE 4 Selected substrain of *H. contortus:* numbers of worms recovered

Group	Sheep	Faecal egg count* (epg)	Number of *H. contortus*		
			Adults**	L4	Total
D (Untreated controls)	36	0	**30**	3	33
	38	6 800	**78**	63	141
	39	100	**117**	4	121
	37	0	**132**	11	143
	42	3 800	**513**	58	571
	41	900	**851**	15	866
	43	2 700	**1 651**	5	1 656
	40	3 600	**2 269**	8	2 277
	Mean	2 237,5	**705,1**	20,9	726
	SD	2 440,7	**838,5**	24,8	831,7
	Median	—	**323**	—	—
	Median ×0,5	—	**162**	—	—
E (Treated: closantel at 5 mg/kg)	54	900	**3**	0	3
	46	0	**6**	0	6
	50	0	**9**	0	9
	52	0	**14**	10	24
	45	200	**40**	10	50
	48	100	**148**	24	172
	49	0	**194**	0	194
	53	300	**218**	3	221
	51	0	**542**	0	542
	47	0	**759**	5	764
	44	1 300	**2 093**	3	2 096
	Mean	254,5	**366,0**	5	371
	SD	493	**623,4**	7,4	622,6
	NPM efficacy rating	—	×	—	—

* Done 5 days before the sheep were killed for worm recovery
** Mature adults + young 5th stage worms

TABLE 5 Ratios of developmental stages recovered in the NPM trials

NPM trial	Group	Developmental stages (%)		
		L4	5th*	Adults**
Unselected	A (controls)	1,4	1,6	97,0
	B (closantel)	7,4	0,6	92,0
	C (rafoxanide)	11,0	3,5	85,5
Selected	D (controls)	2,9	8,7	88,4
	E (closantel)	1,3	11,8	86,9

* Young 5th stage worms
** Mature adult worms with fully developed sexual organs and/or ova in the uteri

TABLE 6 Migration of the worms from agar gel

NPM trial	Group	Developmental stages			
		L4		5th & adults	
		Number	%* migration	Number	%* migration
Unselected	A (controls)	157	100	10 657	96,1
	B (closantel)	166	100	1 877	95,7
	C (rafoxanide)	42	100	319	100,0
Selected	D (controls)	166	100	4 676	94,2
	E (closantel)	65	100	3 591	95,3
Total	Controls	323	100	15 333	95,5
	Treated	273	100	5 787	95,7

* Only 1/10 of the residual ingesta of each sheep was examined for worms that failed to migrate from the agar gel

DISCUSSION

The L4 present in these sheep at slaughter were probably retarded in development, as they should have developed to the adult stage at the time of slaughter, that is, 31–33 days after infestation (Veglia, 1915).

Closantel has apparently not been tested against inhibited L4, but as the chemically-related remedy, rafoxanide, is not effective against these larval stages (Snijders, Horak & Louw, 1973; Le Jambre & Barger, 1979), closantel was given the benefit of the doubt in this investigation and only adult worm burdens (including the few 5th stage worms) were compared. A similar approach was followed by Hall, McDonell & Graham (1980), who mentioned that they compared adult worm populations in trials involving closantel, but, unfortunately, failed to mention whether or how many immature worms were recovered in their experiments. In the present trials too few L4 were recovered for meaningful comparisons (Tables 3, 4 & 5), but from the results of the NPM trial with the unselected substrain it seems unlikely that closantel is very active against retarded larvae.

The NPM efficacy rating in the case of the unselected strain was Class "B" instead of the Class "A" obtained in the trials conducted by the manufacturers for the purpose of registration in South Africa as a stock remedy (under Act 36/1947). However, the following must be considered: for a Class "A" claim by the NPM method of analysis of Groeneveld & Reinecke 1969, as modified by Clark (Reinecke, 1973), one animal only may have a worm burden exceeding the median count × 0,25 (i.e. 222 worms). Therefore, it will be seen that the critical count, the 2nd highest count in the treated group (Sheep 24 with 230 mature adult *H. contortus*), exceeds this reduced median count (222 worms) by only 8 worms. In addition, the arithmetical mean efficacy was 84,6%. In the original trial conducted for registration, none of the worm burdens of the treated animals exceeded the reduced median burden of the controls and the arithmetical mean efficacy (residual effect 14 days after drenching at a dosage of 5 mg/kg) was 97,0%. While Hall, Kelly, Whitlock & Ritchie (1981) reported a variability of results when the residual effect of closantel was tested 60 days after a dosage rate of 10 mg/kg live mass, the sharp increase in the resistance of this strain of *H. contortus* to closantel after only one selection seems to indicate that there was already a slight decrease in the susceptibility of this unselected substrain of the parasite. Unfortunately, despite the fact that the animals in this trial were infested on the same day (using the same methods) as the animals in the NPM trial with the unselected substrain, worm development was very unsatisfactory and erratic, but it is improbable that these factors affected the efficacy rating.

From the epg counts listed in Table 4 it seems likely that at least 2 sheep (Sheep 38 in Group D and Sheep 54 in Group E) had appreciably higher worm burdens at the time the egg counts were done than the worms recovered at slaughter. Turton & Clark (1974) showed that sheep may eliminate *H. contortus* rapidly, and perhaps this phenomenon may have been responsible for the low numbers of worms recovered from some of the sheep in the 2nd NPM trial. Nevertheless, the numbers of worms remaining in the treated animals in this trial are very similar to those of the controls in 7 out of 11 treated sheep, and the maximum worm count in the treated animals is very similar to the maximum of the controls.

This reduced efficacy requires confirmation, preferably both after further selections with closantel, and by further isolations of the strain of *H. contortus* from the farm of origin to monitor possible changes after further use of the remedy on the farm. The indications from the present trials, however, are that pronounced resistance may develop quickly on the farm if the remedy is to be used regularly in future for worm control.

It must be borne in mind that only a few sheep were used for testing the efficacy of rafoxanide, but the results obtained probably also indicate an increased resistance of this unselected substrain to this remedy, which is chemically related to closantel. The efficacy was high (91,7% of the worms being removed—Table 3) but, as discussed by Van Wyk & Gerber (1980), the remedy was previously reported to be almost 100% effective against adult *H. contortus*.

It is interesting that only one case of slight resistance of *H. contortus* to rafoxanide seems to have been reported previously (Van Wyk & Gerber, 1980)*. Snijders *et al.* (1973) showed that rafoxanide appears to have a short residual effect against *H. contortus*, except that hypobiotic L4 are apparently not affected by the remedy. The consensus of opinion seems to be that prolonged exposure of helminths to an anthelmintic (as, for instance, in the case of a slow release formulation or, by inference, a remedy with a residual effect) enhances the changes of increased selection for resistance to the remedy (Hall & Kelly, 1979). Rafoxanide has been widely used in South Africa since its introduction to the local market in 1971 and has been used extensively on some farms. This is, however, the only instance of slight resistance that has been encountered. The residual efficacy of the related closantel is much more marked than with rafoxanide, and, especially at a dosage of 10 mg/kg live mass (with a registered claim of Class "A" efficacy for infestation 49 days after administration of the remedy), it seems likely that very prolonged contact can be expected between the worm and the remedy when closantel is used in routine dosing programmes.

Treatment does not appear to have affected the ability of surviving worms to migrate from agar gel. While all of the 596 L4 recovered from all the groups of sheep did migrate from the agar, 4,5% of 15 333 adults in the untreated control groups and 4,3% of 5 787 in the treated groups failed to migrate. These latter percentages are slightly better than the results of Van Wyk *et al.* (1980) in whose trails 7,9% of 134 205 adult *H. contortus* failed to migrate from the agar gel. The difference may be due to the fact that worms that become entangled with the wire mesh of the agar frames in the small areas where the mesh was denuded of agar by the migration of clumps of worms, were removed by hand and included with the worms that did migrate in the present trials.

* Kelly & Hall (1979) refer to a strain of *H. contortus* in "Southern Africa" resistant to rafoxanide: "P. van Wyk (personal communication, 1977) has also isolated a strain of *H. contortus* resistant to rafoxanide (Tables I & II)". In 1977 one of us (J.A.v.W.) wrote a letter to Kelly, mentioning that "slight resistance" to rafoxanide was being investigated in South Africa. No replies were received to letters written to Kelly in 1977 and 1982, but we presume that the publication of Kelly & Hall (1979) refers to J. A. van Wyk, and that the instance they mentioned is the same as that published by Van Wyk & Gerber (1980)

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