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STUDIES ON SCHISTOSOMIASIS. 7. A COMPARISON OF VARIOUS METHODS FOR THE INFESTATION OF SHEEP WITH *SCHISTOSOMA MATTHEEI*

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ABSTRACT

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The percutaneous (leg and thorax and abdomen) and subcutaneous routes of infestation with *Schistosoma mattheei* were compared in 29 sheep. Larger percentages of cercariae developed after percutaneous than subcutaneous infestation and the difference was highly significant ($P<0,001$). Furthermore, if the leg was used for percutaneous infestation worm development was significantly higher ($P<0,02$) when the skin was washed thoroughly in water before exposing it to cercariae, than when it was left unwashed. Washing was apparently not necessary if the thorax and abdomen served as the route of infestation.

INTRODUCTION

It is essential to aim at uniform worm burdens in experimental animals in order to interpret the results without using large numbers of animals. Sheep, monkeys and the larger domestic animals are, however, difficult to restrain for the long periods of time required for infestation with schistosomes. This has been one of the reasons for the development of numerous methods and routes of infestation. Fairley & Jasudasan (1929) used a pipette to drip cercarial suspension onto the buccal mucous membrane of goats whereas Fairley & Jasudasan (1929) and Van Wyk, Bartsch, Van Rensburg, Heitmann & Goosen (1974) infested goats and sheep intraruminally by means of a stomach tube. Goats were infested by Fairley & Mackie (1929) by pouring a cercarial suspension into the hollow of the groin. Price (1953) used a board to restrain agouti so that their backs were in contact with cercarial suspension and Heitmann (1969) slowly poured the suspension onto the backs of sheep. McCully & Kruger (1969) and Heitmann (1969) confined the sheep's leg in a measuring cylinder to which the cercarial suspension was added. The ear pinna was used by Malek (1969) for the infestation of a calf, sheep and goats. Malek (1969) and McKenzie (1970) anaesthetized their sheep and the latter author infested them via the axilla, whereas Malek (1969), Erickson, Sadun Lucia, Von Lichtenberg & Cheever (1971) and Foster & Broomfield (1971) poured cercariae into a heavy glass or metal ring placed on the abdominal or dorsal skin of monkeys and larger animals. Cattle were infested by immersion of either the leg in the cercarial suspension contained in a stout polythene bag tied tightly above the knee (Massoud, 1973b), or the tail in a narrow polythene cylinder (Van Wyk & Groeneveld, 1973).

Methods of infestation are increasing in number and consequently the results are becoming more difficult to compare, since no controlled comparisons have been recorded. In this paper 3 routes and 2 methods of infestation with *Schistosoma mattheei* are compared in sheep.

MATERIALS AND METHODS

Parasite

The strain of *S. mattheei* used, its origin and maintenance in the laboratory have been described previously (Van Wyk, 1973).

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Sheep

Twenty-nine Merino wethers which originated from a schistosome-free area, were used. Before infestation they were ranked by live mass and randomized by tables of random numbers into 5 groups. A week before commencement of the trial they were dewormed with thiabendazole* at a dose of 220 mg/kg and were subsequently confined to worm-free stables.

Infestation

Cercariae were collected from a minimum of 36 snails. The methods of sampling of cercariae, preparation of doses for individual sheep, estimation of these doses and determination of their percentage error have been described by Van Wyk & Groeneveld (1973). In no case did the percentage error (95% confidence limit) of the estimated dose exceed 8,7%.

Successive cercarial doses were allocated at random to various groups. None of the cercariae were more than $5\frac{1}{6}$ hours old at the end of the exposure period.

Three different routes and 2 methods of infestation were compared. Infestation time was 30 min for Groups A–D and 15 min for Group E. After infestation the cercariae remaining in the suspension were counted under a stereoscopic microscope. These counts were regarded as the total number of cercariae which had failed to penetrate the sheep (Van Wyk, Van Rensburg & Heitmann, 1975).

Percutaneous infestation via the leg

The sheep in Groups A and B were infested percutaneously by submerging the shorn left fore limb in a plastic measuring cylinder† containing the cercarial suspension. In Group A animals the leg was thoroughly washed with a scrubbing brush and water without soap or detergents immediately before infestation, while in Group B this was omitted. During infestation each sheep was restrained by 2 persons.

Percutaneous infestation via the thorax and abdomen

The sheep in Groups C and D were infested with the aid of plastic halters into which the cercarial suspension was poured. The halters were designed to expose a large tract of skin of the thorax and abdomen to infestation. The area to be exposed was washed before infestation in Group C, but not in Group D. These sheep were also restrained manually.

* Thibenzole (Merck, Sharp & Dohme)

† Kartell, Milano No. TS 125, inside diameter 9 cm, height 36 cm

Subcutaneous infestation

The sheep in Group E were anaesthetised with ether and the skin incised in the flank. When the haemorrhage had ceased, a subcutaneous pouch of about 0,5 l capacity was formed by careful blunt dissection. The cercarial suspension was then poured into the pouch and left for 15 minutes, after which the remaining fluid was collected. The skin was not sutured and the wounds were not treated until 24 hours after infestation.

Worm recovery

The sheep were killed for worm recovery a mean of 76 days after infestation. The method of perfusion described by McCully & Kruger (1969) for sheep, was modified as follows:

1. No formalin was injected during perfusion to kill the worms.
2. Earle's saline was used for perfusion instead of NaCl only.
3. Pentobarbital sodium* was added to the perfusion fluid to relax the worms (Foster, Cheetham & Mesmer, 1968).
4. After perfusion the entire intestinal tract was scrutinized for worms; those which remained were counted *in situ* and the number expressed as a percentage of the total worm burden of each animal. The worms recovered by perfusion were also counted *in toto*.

The worms recovered were relaxed and killed as described by Van Wyk *et al.* (1974).

Statistical evaluation

The Kruskal-Wallis One-Way Analysis of Variance by Ranks Test (Siegel, 1956) and the Kruskal-Wallis Multiple Comparisons Test (Miller, 1966) were used to determine the significance (95% confidence limit) of differences between groups:

1. Percentage worm development
2. Percentage of cercariae which failed to penetrate
3. Worms remaining *in situ* after perfusion
4. Worm sex ratio.

Results

The results are summarized in Table 1.

Manual restraint of the sheep in Groups A–D during infestation was very difficult and despite careful handling 6 of 24 sheep spilt some cercarial suspension. The maximum number of sheep infested on any day was 9.

Group A (infested percutaneously via the washed leg) had the highest mean infestation rate, namely 63,01% which differed significantly ($P<0,02$) from the 29,44% of Group B (leg, unwashed) and highly significantly ($P<0,001$) from the figure (10,42%) for Group E (subcutaneous infestation). There was, however, no significant difference between the development of worms in Groups A, C and D, all of which had been infested percutaneously.

The feet of the sheep in Group B (leg, unwashed) did not appear excessively dirty, but the cercarial suspension was much dirtier after infestation than that in Group A (leg, washed). The difference was not so marked in Groups C (thorax and abdomen, washed) and D (thorax and abdomen, unwashed).

In 3 of 5 sheep in Group E no cercarial suspension remained in the subcutaneous pouch after 15 minutes of exposure (Table 1). The wounds suppurated probably from infiltration of the hypotonic cercarial suspension which was not aseptic.

The mean percentage of cercariae which failed to penetrate was 0,88% for all 5 groups. More male worms (64,5%) developed than female (35,5%) in all the animals. A mean of 90,68% of the worms was removed by perfusion (9,32% remained *in situ*, Table 1). The differences between the 5 groups were not significant for these values.

The coefficients of variation of the worm development in the 3 groups in which large numbers of worms developed, were very similar (21,9%, 23,2% and 19,1%, in Groups A, C and D respectively). These variations were much smaller than the variations of 36,2% in Group B (leg, unwashed) and 65,3% in Group E (subcutaneous infestation) in both of which few cercariae developed to adult worms.

Discussion

The dose of cercariae used for each sheep was determined by an accurate sampling method (Van Wyk & Groeneveld, 1973). This ensured that errors in estimation of the cercarial doses could have played only a minor role in the variations observed in worm development.

Even though worm development after percutaneous infestation via the thorax and abdomen and the washed leg did not differ significantly, the latter method is easier to apply. It has therefore been adopted as standard and has been retested in numerous groups of Merino and Dorper sheep with consistently reproducible results (Van Wyk, unpublished data, 1974).

Manual restraint of the sheep was unsatisfactory. The number of sheep which could be infested during the relatively short infective life of the cercariae was limited and cercarial spillage during infestation cannot be afforded in experiments of this nature. Because methods of restraint used by previous workers did not appear to offer a satisfactory alternative, an apparatus was subsequently developed for mechanical restraint of sheep during large scale infestation with schistosomes (Van Wyk, 1975.)

Although subcutaneous infestation yielded very poor results, worm development was higher than the 1% development reported by McCully & Kruger (1969). The subcutaneous pouch was used as an alternative to subcutaneous injection to exclude the possibility that the cercariae are damaged when injected. Despite this precaution, subcutaneous infestation was a failure in the sheep. In hamsters, on the other hand, one of us (Van Rensburg, unpublished data, 1974) obtained a mean worm development (*S. mattheei*) of 37,2% with this route in 6 hamsters compared to 20,3% in 6 infested percutaneously. It is not clear why such varying results were obtained after subcutaneous infestation with the same strain of *S. mattheei*.

The only feasible explanation for the higher percentage of male than female worms which developed in this trial, is that the original cercarial pool contained more male than female cercariae. Indeed, in one animal (Sheep 1 in Group A—Table 1) 52% of the cercariae to which the sheep was exposed, developed to male worms. This discrepancy is not due to greater viability of males because we (Van Rensburg & Van Wyk, unpublished data, 1971) recovered more female *S. mattheei* than males from this strain in

*Sagatal, May-Baker

TABLE 1 *S. mattheei*. Details of infestation and worm recovery

Group + Method of Infestation	No. of cercariae	% cercariae failed to penetrate	No. of worms recovered	% development	% worms not removed by perfusion	Sex ratio		% cercariae developing to	
						Males (%)	Females (%)	Males (%)	Females (%)
A. LEG WASHED:									
Sheep 1	3 178	0,25	2 418	76,08	5,54	68,5	31,5	52,14	23,95
2	3 642	0,19	2 349	64,49	15,75	68,7	31,3	44,32	20,18
3	3 642	4,84	1 919	52,69	10,84	60,2	39,8	31,74	20,95
4	2 718	0,99	1 107	40,73	10,48	67,3	32,7	36,48	13,32
5	2 916	0,24	2 168	74,35	4,89	57,6	42,4	42,83	31,52
6	3 144	0,1	2 192	69,72	7,76	57,8	42,2	40,30	29,42
Mean±S.D.*	—††	**1,10±1,86**	—	**63,01±13,8**	**9,21±4,0**	**63,3±5,4**	**36,7±5,4**	**41,30±7,0**	**23,22±6,6**
C.V.(**%)	—	—	—	**21,9**	—	**8,5**	**14,7**	—	—
B. LEG UNWASHED:									
Sheep 7	3 178	0,44	1 382	43,49	3,33	68,2	31,8	29,64	13,85
8	3 178	1,18	992	31,21	3,63	65,6	34,4	20,48	10,73
9	3 642	0,58	837	22,98	8,6	65,0	35,0	14,94	8,05
10	2 718	1,42	947	34,84	13,31	66,6	33,4	23,22	11,63
11	2 916	0,31	361	12,38	18,28	54,6	45,4	6,76	5,52
12	3 935	0,74	1 249	31,74	5,28	61,6	38,4	19,54	12,20
Mean±S.D.	—	**0,78±0,44**	—	**29,44±10,7**	**8,73±6,0**	**63,6±4,9**	**36,4±4,9**	**19,10±7,7**	**10,33±3,0**
C.V. (%)	—	—	—	**36,2**	—	**7,7**	**13,5**	—	—
C. THOR-ABD† WASHED:									
Sheep 13	3 178	0,63	1 049	33,01	9,34	65,8	34,2	21,71	11,30
14	3 642	0,22	2 052	56,34	11,6	64,1	35,9	36,13	20,21
15	3 642	0,41	1 543	42,37	14,91	67,3	32,7	28,50	13,87
16	2 718	0,26	1 312	48,27	10,67	65,6	34,4	31,68	16,59
17	2 718	0,44	1 767	65,01	9,28	73,8	26,2	47,98	17,03
18	2 916	0,14	1 300	44,58	15,69	55,8	44,2	24,86	19,72
Mean±S.D.	—	**0,35±0,18**	—	**48,3±11,2**	**11,92±2,8**	**65,4±5,8**	**34,6±5,8**	**31,81±9,4**	**16,45±3,4**
C.V. (%)	—	—	—	**23,2**	—	**8,9**	**16,8**	—	—
D. THOR-ABD UNWASHED:									
Sheep 19	3 178	0,35	1 439	45,28	3,89	67,1	32,9	30,40	14,88
20	3 178	0,73	1 627	51,20	4,06	67,9	32,1	34,77	16,43
21	3 642	0,22	1 491	40,94	12,88	63,2	36,8	25,86	15,07
22	2 718	0,18	1 433	52,72	10,89	67,0	33,0	35,32	17,40
23	2 916	0,38	1 956	67,08	4,91	56,9	43,1	38,17	28,91
24	3 144	0,7	1 343	42,72	8,94	56,1	43,9	23,95	18,77
Mean±S.D.	—	**0,43±0,2**	—	**50,0+9,6**	**7,60±3,9**	**63,0±5,3**	**37,0±5,3**	**31,41±5,7**	**18,58±5,3**
C.V.(%)	—	—	—	**19,1**	—	**8,4**	**14,4**	—	—
E. SUBCUTANEOUS:									
Sheep 25	3 642	0,0	787	21,61	5,84	61,8	38,2	13,34	8,26
26	3 642	0,0	366	10,05	3,28	76,8	23,2	7,72	2,33
27	3 642	0,0	304	8,35	9,21	66,1	33,9	5,52	2,83
28	2 916	6,66	262	8,98	22,9	61,8	38,2	5,56	3,43
29	2 916	2,93	91	3,12	4,4	71,4	28,6	2,23	0,89
Mean±S.D.	—	**1,92±2,9**	—	**10,42±6,8**	**9,13±8,0**	**67,6±6,5**	**32,4±6,5**	**6,87±4,1**	**3,55±2,8**
C.V. (%)	—	—	—	**65,3**	—	**9,6**	**20,04**	—	—
ALL GROUPS:									
Mean±S.D.	—	**0,88±1,5**	—	—	**9,32±5,0**	**64,49±5,4**	**35,5±5,4**	—	—
C.V. (%)	—	—	—	—	—	**8,4**	**15,3**	—	—

*S.D. . . Standard deviation
**C.V. = Coefficient of variation
†Thorax and abdomen
††Not calculated

another investigation in which the sex of the cercariae was predetermined by the methods of Cort (1921) and equal numbers of both sexes were used to infest 12 sheep. The abovementioned assumption is also borne out by the results of a subsequent trial in which a different batch of snails was used for cercarial production and more female than male worms developed in 7 sheep (Van Wyk, unpublished data, 1970).

Counting of worms *in situ* is inaccurate unless the blood vessels are opened because the males, being lightly pigmented, are difficult to see through the vessel walls. Hence it is important to remove as many as possible of the worms by perfusion. In this experiment the percentage of worms which were not removed was relatively high. The technique of worm recovery has subsequently been improved by massaging as many worms as possible out of the small blood vessels on the intestine during perfusion (Van Wyk, Van Rensburg & Heitmann, 1975).

The mean percentage development of worms in sheep infested percutaneously via a washed limb (63,01%) compares very favourably with *S. mattheei* recoveries obtained by other workers. McCully & Kruger (1969), working with the same strain, obtained a mean of 28,45% development in sheep they infested percutaneously and killed after a mean period of 71,3 days (calculated from their data in Table 1). To some extent the difference could be due to the fact that they initially counted all the worms *in situ*; moreover, some of their sheep were not infested by leg immersion. However, there must also be other reasons because in only 5/18 sheep involved did the percentage development equal or exceed the lowest worm burden obtained in our experiment. Adaptation of the parasite to sheep as a result of repeated passage, or differences in methods of estimating cercarial doses are also factors which may have played a role. Preston, Nelson & Saeed (1972), who worked with another South African strain and used the leg immersion method of McCully & Kruger (1969), obtained only 16,0% worm development in 4 cross-bred Border-Leicester ewes autopsied 63–70 days after infestation.

Several investigations have been conducted on the development of *S. mattheei* after percutaneous infestation in hosts other than sheep. Van Wyk, Van Rensburg & Heitmann (unpublished data, 1971) obtained 63,08% development in 6 cattle which were very heavily infested and died after 56–59 days. From the data presented in Fig. 1 of the article by Lawrence (1973) it can be calculated that approximately 50% of the cercariae matured to adults in the cattle infested 10–15 weeks previously. Taylor & Andrews (1973) recorded a development of 42% in 10 white mice and 51% in 10 hamsters and Taylor, Nelson, Smith & Andrews (1973) obtained a level of infestation of 41,5% in 2 baboons.

In the case of *Schistosoma bovis*, recoveries of 62,3% in 7 calves, 41,4% in 4 sheep and 67,3% in 2 goats (all infested percutaneously) were obtained by Massoud (1973a). Unfortunately he does not give the individual counts or ranges and hence it is difficult to interpret these important findings to full advantage.

There is clearly a great need for standardisation, not only of the methods and routes of infestation, but also of the techniques for handling of cercariae, worm recovery and counting, etc., to facilitate the comparison of the results of various workers.

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