Onderstepoort Journal of Veterinary Research
Volume 29, *Number* 2, *December,* 1962
The Government Printer, Pretoria

STUDIES ON PARAMPHISTOMIASIS. III: A METHOD OF TESTING THE VIABILITY OF PARAMPHISTOME METACERCARIAE

I. G. HORAK, Veterinary Research Institute, Onderstepoort

INTRODUCTION

Laboratory experiments at Onderstepoort, in which sheep were infested with large numbers of metacercariae, proved disappointing. The number of paramphistomes recovered from these sheep varied between 0·8 and 58·3 per cent of the total number of metacercariae dosed (Swart, personal communication, 1961). It consequently became essential to devise a test for determining the viability of metacercariae.

Urquart (1954) used small laboratory animals to determine the viability of metacercariae of *Fasciola hepatica*, Linnaeus 1758, and Lengy (1960) employed this procedure with *Paramphistomum microbothrium*, Fischoeder 1901. This method proved unsatisfactory when tried out at Onderstepoort.

Wikerhauser (1960) described an *in vitro* technique for determining the viability of *F. hepatica* metacercariae. The present paper describes modifications of this *in vitro* technique which proved reliable when applied to *P. microbothrium*.

MATERIALS AND METHODS

Apparatus

(1) Glass petri-dishes, 30 ml capacity.

(2) Incubator adjusted to 38 °C.

(3) Glass pipettes.

(4) Standard dissection microscope.

(5) Two hand tally counters.

Metacercariae

The production, collection and counting of metacercariae have been described by Swart & Reinecke (1962, a, b). In each test approximately 100 metacercariae adherent to small strips of cellulose were used.

Reagents

(1) One hundred ml of fresh ruminal fluid.

(2) One hundred ml of a solution of 1 gm pepsin (B.P.C.) and 0·8 gm NaCl in 100 ml of N/20 HCl (pH adjusted between 1·6 and 1·8 at 25 °C).

Received for publication on 9 May, 1962.—Editor

(3) One hundred ml of a solution of 0·4 gm trypsin*, 1·6 gm bile salts†, 1·0 gm $NaHCO_3$ and 0·8 gm NaCl in 100 ml of distilled water (pH adjusted between 7·9 and 8·1 at 25 °C).

Method

Wikerhauser (1960) incubated metacercariae of *F. hepatica* at 38 °C in a solution of 0·5 gm pepsin (1: 3,000 U.S.P. activity) and 0·8 gm NaCl in 100 ml of N/20 HCl for two to three hours. Thereafter the metacercariae were incubated for two to three hours in a solution of 0·4 gm trypsin (1: 250 U.S.P. activity), 1·0 gm $NaHCO_3$ and 0·8 gm NaCl in 100 ml of distilled water supplemented with 20 per cent ox bile. Viability was determined by microscopic observation of the number of young fluke which had excysted.

This procedure was tested with metacercariae of *P. microbothrium*; but their viability fell below that of *F. hepatica* as described by Wikerhauser (1960).

Modifications

(1) Approximately 100 metacercariae attached to a cellulose strip are incubated at 38 °C for 24 hours in 20 ml of fresh ruminal fluid in a petri-dish. The ruminal fluid is then carefully pipetted off so as not to disturb the metacercariae on the strip.

(2) Twenty ml of the pepsin solution are pipetted into the petri-dish and the whole incubated for a further three to six hours. The pepsin solution is then carefully pipetted off.

(3) Twenty ml of the trypsin solution are then added to the metacercariae with a further incubation period of 16 hours.

(4) Without pipetting off the trypsin solution, the metacercariae on the cellulose strip and those which have become detached are examined under the dissection microscope.

Viability, as expressed by excystation, is determined by counting the number of empty metacercarial cysts. Since the young flukes which emerge from these cysts are liable to be digested within the testing period, they cannot be used as evidence of viability.

Experiment 1

To determine the relative importance of the various digestive reagents of the media used.

Method

Three control batches of five-day old metacercariae were subjected to the complete digestion test, while a further five similar batches were subjected to the above media with the omission of a different digestive reagent in each case.

Results

These are given in Table 1.

* E. Merck A. G., Darmstadt
† Difco Laboratories, Detroit, Michigan

TABLE 1.—*The effect of the various digestive reagents on the excystation of five-day old metacercariae*

Method of digestion	Percentage excystation
All digestive reagents present	87·1
Minus ruminal fluid	54·1
Minus pepsin	80·0
Minus HCl	1·3
Minus trypsin	9·7
Minus bile salts	11·0

Discussion

It appears from the viability recorded in this experiment that HCl, trypsin and bile salts are essential ingredients for the excystation of metacercariae; ruminal fluid is of lesser importance; the omission of pepsin hardly affects the viability at all.

Experiment 2

To determine the effect of various methods of storage on metacercarial excystation.

Method

Metacercariae stored under the following conditions were subjected to digestion:—

(*a*) Normal storage, i.e. metacercariae are stored at room temperature (approximately 21 to 28 °C) on rolled up strips of cellulose placed in test tubes containing a little water. The bottom ends of the cellulose strips touch the surface of the water thus ensuring that the metacercariae are covered by a thin film of moisture.

(*b*) Storage on cellulose strips which were kept dry for varying lengths of time.

(*c*) Normal storage but contaminated by fungus.

(*d*) Normal storage but maintained at 4 °C.

Results

The results are given in Table 2.

TABLE 2.—*The effect of various methods of storage on the excystation of metacercariae two to 31 days old*

Age of metacercariae in days	Method of storage	Percentage excystation
2 29	Normal storage at room temperature	63–92·9
2	24 Hours dry storage	2·4
3	48 Hours dry storage	0·4
4	More than 48 hours dry storage	—
22	Storage under fungal contamination	12·2–17·1
6–31	Normal storage at 4°C	13·3–92·8

Discussion

The viability of metacercariae was severely affected by dry storage and by fungal contamination. There was little difference in viability between storage at room temperature and at 4 °C.

Experiment 3

To determine the effect of age on viability.

Method

Metacercariae stored at room temperature for varying periods after encystation on cellulose strips were subjected to artificial digestion.

Results

These are incorporated in Table 3.

TABLE 3.—*The effect of age on metacercarial excystation*

Age of metacercariae in days	Number of tests performed	Total number of metacercariae	Total number excysted	Percentage excysted
1	4	1,090	329	30·2
2	3	295	216	73·2
3	3	675	447	66·2
4	3	559	352	63·0
5	3	295	257	87·1
6	4	447	413	92·4
7	5	690	541	78·4
9	1	154	143	92·9
12	1	39	32	82·1
13	2	259	220	84·9
14	5	1,256	1,035	82·4
16	3	216	176	81·5
17	2	141	98	69·5
19	2	241	192	79·7
20	4	427	297	69·6
21	3	310	226	72·9
29	1	121	107	88·4

Discussion

There is apparently a period of maturation before optimal excystation takes place. Prior to the fifth day excystation is poor, thereafter a consistently high level of excystation is observed until the sixteenth day when a gradual but erratic fall occurs until the 29th day. Lengy (1960) made similar observations but suggested that the period necessary for maturation was probably 10 to 20 days. Further experiments will have to be conducted to determine the decrease in viability in metacercariae older than 29 days.

Experiment 4

To correlate the *in vitro* viability with *in vivo* viability.

Method

Seven sheep were infested with 62,000 to 197,000 metacercariae from batches of previously tested viability. Twenty-four hours after dosing, faeces from these sheep were collected individually, mixed with water and the faecal pellets were broken down manually. The faecal suspensions were sieved through sieves, 50 mesh to the linear inch, to remove the coarser fibrous particles. The washings were allowed to settle for 15 minutes, decanted and the sediment was again mixed with water and allowed to settle. This procedure was followed three to four times until the supernatant fluid was fairly clear. Approximately one hundred metacercariae present in the sediment were then examined microscopically and the *in vivo* viability obtained by counting the number of empty cysts in this total.

Results

See Table 4 for the results.

TABLE 4.—*The correlation between the* in vitro *and* in vivo *viability of metacercariae*

Sheep No.	Percentage *in vitro* excystation	Percentage *in vivo* excystation
9252	77·6	71·6
9002	71·4	73·1
8524	52·4	77·0
8540	62·8	38·0
8531	65·1	90·0
9648	63·3	77·6
11025	62·4	75·0

Discussion

In most cases a fairly close correlation between *in vitro* viability and *in vivo* viability is observed. The marked variations that did occur in some instances were possibly because the numbers of metacercariae used to determine both *in vitro* and *in vivo* viability were not always necessarily representative samples of the large doses of metacercariae dosed to the respective sheep.

Experiment 5

To check the *in vivo* viability of metacercariae dosed to sheep.

Method

Five sheep were infested with 62,000 to 170,000 metacercariae. Metacercariae were recovered from the faeces of these sheep by the method already described. The unexcysted metacercariae recovered from three sheep were pooled and those from the other two sheep were kept individually. These unexcysted metacercariae were subjected to the viability test with the omission of ruminal fluid, and the viability determined.

Results

The results are given in Table 5.

TABLE 5.—*The* in vitro *viability of unexcysted metacercariae passed by infested sheep*

Metacercariae recovered from sheep No.	Percentage excysted
8524	2·3
8531	
8540	
9406	0
9675	0

Discussion

The *in vitro* viability of unexcysted metacercariae recovered from infested sheep's faeces varied between 0 and 2·3 per cent. This suggested that the *in vivo* viability of metacercariae, as given in Table 4, is a reasonably accurate figure, as very few, if any, further metacercariae excysted upon being subjected to the *in vitro* viability test.

SUMMARY AND CONCLUSIONS

A comparatively rapid and economical method of assessing the viability of *P. microbothrium* metacercariae was developed by modifying the technique described by Wikerhauser (1960).

The effects of various methods of storage and of age on metacercarial viability were determined.

The highest viability was observed for metacercariae five to 16 days of age

A close correlation between *in vitro* viability and actual *in vivo* viability was recorded.

ACKNOWLEDGEMENTS

The Chief, Veterinary Research Institute, is thanked for facilities to carry out these experiments and permission to publish the results.

REFERENCES

LENGY, J. (1960). Study on *Paramphistomum microbothrium* Fischoeder, 1901 a rumen parasite of cattle in Israel. *Bull. Res. Counc. Israel,* Vol. 9B, pp. 71–130.

SWART, P. J. & REINECKE, R. K. (1962a). Studies on Paramphistomiasis. I: The propagation of *Bulinus tropicus* Krauss 1848. *Onderstepoort J. Vet. Res.* This issue.

SWART, P. J. & REINECKE, R. K. (1962b). Studies on Paramphistomiasis. II: The mass production of metacercariae of *Paramphistomum microbothrium* Fischoeder 1901. *Onderstepoort J. Vet. Res.* This issue.

URQUART, G. M. (1954). The rabbit as host in experimental Fascioliasis. *Exp. Parasitol.* (New York), Vol. 1, pp. 38–44.

WIKERHAUSER, T. (1960). A rapid method for determining the viability of *Fasciola hepatica* metacercariae. *Am. J. Vet. Res.*, Vol. 21, pp. 895–897.