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In vitro culture of tetrathyridia of Mesocestoides corti using a gel based diphasic medium

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Abstract

Tetrathyridia of Mesocestoides corti were cultured in vitro in a diphasic medium consisting of a liquid medium (CMRL Sigma) and a thixotropic nutrient gel (Oxoid). Tests demonstrated that a 50% medium/gel mixture produced optimum conditions for the survival and development of tetrathyridia. Established anthelminthic drugs were inoculated into the gel which demonstrated that this system can be used for preliminary anthelminthic drug screening. The development and survival of the tetrathyridia were influenced by the addition of pepsin, trypsin and liver peptone to the culture media. The development and maturation of proglottids were observed in addition to asexual reproduction by the process of budding. Tetrathyridia maintained in vitro and reinfected into both mouse and rat hosts retained their viability.

Introduction

The second metacestode (larval) stage of the cyclophyllidean cestode Mesocestoides corti is referred to as a tetrathyridium. Tetrathyridia are parasites of various vertebrate hosts including small rodents and lizards (the intermediate hosts) such as the fence lizard (Scalporus occidentalis biseriatus) (Voge, 1967). Adult worms are found in various carnivores. Specht & Voge (1965) established that tetrathyridia isolated from the lizard could be cultured in mice by intra-peritoneal injection. The in vivo culture can also be maintained by per os inoculation (Tallin, 1994). The most important feature of tetrathyridia of M. corti is that they are able to reproduce asexually within the intermediate host.

In vitro culturing of tetrathyridia is normally done in liquid media. There have been attempts to utilize solid agar media as well as diphasic media. Voge & Seigel (1968) used solid agar and found that under culture conditions the tetrathyridia were unable to penetrate the agar.

Ong & Smyth (1985) and Smyth (1987) both indicated that a diphasic medium greatly facilitated the in vitro growth of M. corti but for tetrathyridia to burrow into a coagulated serum base, prepared holes needed to be initially made in the solid agar. Barrett et al. (1982) and Thompson et al. (1982) reported successful development in vitro of tetrathyridia to sexual differentiation. In vivo the tetrathyridia are able to penetrate some tissues, mainly the liver. The understanding of this host–parasite system would be enhanced if penetration into a solid or a combined solid culture media could be achieved. In this paper we describe the use of a new type of growth medium based on a thixotropic nutrient gel.

Materials and methods

Preparation of culture media

A 3.1% thixotropic basal nutrient gel was prepared in the following manner: 31.5 g of powder was added to 1 l of distilled water, mixed and allowed to stand for 10 min, and autoclaved at 121°C for 15 min. Once the gel had cooled to approximately 60°C it was dispensed into sterile 12- and 24-well culture plates. The gel was allowed to solidify and was then stored under sterile conditions until

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required. Gels prepared in this manner constituted the basal gel and were designated '100% gel'.

A liquid culture medium CMRL was prepared as described by Smyth (1979, 1985). The liquid culture medium was used to dilute the basal gel to create 60%, 50% and 40% diphasic gel / medium. To dilute the gel, the CMRL liquid medium was first filter sterilized, followed by the addition of the appropriate volume of medium to the basal gel cooled at 50°C.

Additives such as liver peptone, sodium taurocholate and anthelminthic drugs, when required, were added before dispensing. Heat stable chemicals were added immediately after dilution, thermolabile compounds were added as 'concentrates' to the gel and left overnight to diffuse evenly through the gel. Pepsin, trypsin, peptone (Sigma), liver extract (Oxoid), and sodium taurocholate (Sigma) were used as additives at concentrations of 0.5 mg ml^{-1} and 0.1 mg ml^{-1}.

In vitro culture preparation

Tetrathyridia of *M. corti* were removed with a Pasteur pipette under sterile conditions from the peritoneal cavity of an infected mouse (killed by anaesthesia followed by cervical dislocation). Tetrathyridia were first washed in sterile 0.9% physiological saline to remove any host material, such as mucopolysaccharides found mainly in infected CFLP mice, and then placed in CMRL liquid media in sterile specimen tubes. The procedure was carried out in a class II culture cabinet (Flow).

Once the culture plates had been set up they were kept in a incubator at 37°C with an airflow containing 5% CO_2. In experiment 5, however, normal atmospheric conditions were maintained without the addition of 5% CO_2.

Experiment 1. Examination of the diffusion properties of thixotropic gel

Albendazole, praziquantel (both dissolved in dimethyl sulphoxide (DMSO)), sodium taurocholate, pepsin, trypsin and liver peptones were added to the gel. To test the diffusion properties of the gel 1 ml of 0.1% nigrosin was added to the gel and allowed to stand overnight.

Experiment 2. Survival rates of tetrathyridia in thixotropic gel and diphasic media

Tetrathyridia of *M. corti* were placed in wells containing 100% gel, 60% and 40% gel / CMRL mixtures. The original number of tetrathyridia added to each well was recorded. Half the number of living tetrathyridia from three wells with 40% gel / media were transferred after 7 days into wells containing fresh 40% gel mixture. This procedure was repeated every 7 days. Wells were observed for any changes in the morphology and numbers of tetrathyridia.

Experiment 3. The addition of praziquantel and albendazole to the nutrient gel

The anthelminthics were prepared in DMSO (Sigma). Twenty-four-well culture plates comprising six columns of four wells were used. In plate 1 the following amounts of praziquantel were added: column 1, 0.05 ml equivalent to an intra-peritoneal (i.p.) dose of 0.031 mg ml^{-1}; column 2, 0.10 ml equivalent to an i.p. dose of 0.063 mg ml^{-1}; column 3, 0.15 ml equivalent to an i.p. dose of 0.094 mg ml^{-1}; column 4, 0.20 ml equivalent to an i.p. dose of 0.125 mg ml^{-1}. In plate 2 praziquantel was replaced with albendazole, and plate 3 contained only DMSO. Plate 4 contained control diphasic media with no additives.

Experiment 4. Testing the viability of tetrathyridia after in vitro culture transfer from in vitro to in vivo culture

Tetrathyridia of *M. corti* from mice were cultured in *in vitro* in 50% gel / liquid media. After 25 days, 25 individual tetrathyridia were recovered and injected i.p. into a mouse. Tetrathyridia removed from the peritoneal cavity of a mouse were placed in a 50% gel / media culture for 25 days. Two mice were each infected, intraperitoneally (i.p.) with 25 tetrathyridia for 58 days and a total of 575 tetrathyridia were recovered from the two mice. These were maintained *in vitro* for 44 days and then each of four mice was infected with 40 tetrathyridia. After 9 days, 28 peritoneal tetrathyridia were recovered from two of the mice, which were then maintained *in vitro* for 17 days and 11 survived. From the remaining two mice 78 liver and 108 peritoneal tetrathyridia were obtained after 47 days.

Experiment 5. The development and growth of tetrathyridia in vitro

Three 24-well plates (three groups) were prepared with each well containing 1 ml of 100% basal gel and 1 ml of liquid medium containing 2 mg ml^{-1} of liver peptone, 2 mg ml^{-1} trypsin and 2 mg ml^{-1} of pepsin.

Group 1

Tetrathyridia removed from the peritoneal cavity of a mouse were stored in basic CMRL medium at 4°C for 29 days then ±12 tetrathyridia were distributed evenly into the 24 wells. The culture plates were incubated at 37°C and 0.2 ml of medium plus additives were added daily to each well.

Group 2

Tetrathyridia were removed directly from the peritoneal cavity of a mouse and washed three times in CMRL medium to remove any host material such as peritoneal mucus. After washing ±12 metacestodes were added to each well of a 24-well plate containing CMRL with 2 mg ml^{-1} liver peptone, trypsin and pepsin.

Group 3

Tetrathyridia ±12 per well were taken directly from a rat and then treated as in group 2.

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Table 1. The effect of different gel / liquid media ratios on the behaviour of Mesocestoides corti tetrathyridia on (a) day 2 and (b) day 10.

Gel / Media	Plate	Activity	Dividing	Strobilization	Penetration	Elongation
(a)						
gel only	A	+++	–	+	–	–
83 / 17	B	+++	++	+	–	++
67 / 33	C	+++	–	–	+++	–
75 / 25	E	+++	–	–	+	–
50 / 50	D	+++	–	–	++	–
(b)						
gel only	A	+++	–	+	–	–
83 / 17	B	+++	++	+	–	++
67 / 33	C	+++	–	–	++++	++
75 / 25	E	+++	–	–	+	–
50 / 50	D	+++	–	–	+++++	–

+, Some activity; ++, moderate activity; +++, active; –, no change.

Results

Diffusion properties

All plates that were stained with nigrosin turned blue after 12 h, demonstrating that if the nigrosin could diffuse through gel then additives, such as drugs or enzymes, are also likely to diffuse through the different gel preparations.

Survival rates

Table 1 shows the effect of the various gel to media ratios on the growth and development of tetrathyridia, on days 2 and 10. In the growth column the measurements represent the maximum and minimum size tetrathyridia. Tetrathyridia were observed to be active in both the solid and diluted gels. Asexual reproductive activity was most frequently observed in the least diluted gel. Active penetration of tetrathyridia was observed in equal volumes of the gel and liquid media. Table 2 shows the survival rates and the effect of transferring tetrathyridia to fresh gel / media that were first kept at 4°C for 14 days after removal from the host. These results show that after four transfers tetrathyridia maintain their activity and growth but after the second transfer dividing appears to cease.

Table 2. The effect on the growth and longevity of Mesocestoides corti tetrathyridia by transferring them to fresh media on days 7, 15, 22 and 32.

Day	Transfer	Activity	Dividing	Growth (mm)
0				
5		+++	+++	0.5–1.75
7	1st	+++	+++	
12		+++	+++	
15	2nd	+++	+++	0.1–1.7
19		+++	+	
22	3rd	++	+	
25		+++	+	0.2–2.0
32	4th	+++	–	
34		+++	–	0.2–3.0
79		++	–	0.2–4.5

–, No change; +, some activity; ++, moderate activity; +++, active; ++++, very active; growth = min and max length.

Addition of praziquantel and albendazole to cultures

After 2 h on plate 1 with praziquantel, tetrathyridia were severely contracted with little or no movement whereas on plates 2, 3 and 4 (albendazole, DMSO and control, respectively) tetrathyridia were all active. On day 7 all tetrathyridia in plate 1 (praziquantel) had died. On plate 2 (albendazole) there was some activity with signs of tegumental damage. On plates 3 (DMSO) and 4 (controls) all tetrathyridia were live and active. On day 12 all tetrathyridia on plate 2 (albendazole) had died whereas on plates 3 and 4 (controls and DMSO) the metacestodes were still alive.

Transfer from in vitro to in vivo cultures

Tetrathyridia removed from the peritoneal cavity of a mouse and placed in a 50% gel / media culture for 25 days survived and multiplied in vivo when injected i.p. into mice. Recovery and further passage (see experiment 4

Table 3. The survival and development of tetrathyridia of Mesocestoides corti following maintenance at 4°C (experiment 5, group 1).

Days in culture	LT (%)	SW (%)	SB (%)	SA (%)
0	283 (100)	–	–	–
14	243 (86)	47 (19.4)	27 (11.1)	–
21	188 (77)	43 (22.9)	10 (5)	–
28	163 (58)	47 (47)	11 (11)	–
35	108 (38)	28 (25.9)	9 (9)	–
42	91 (32)	24 (26.9)	10 (10.9)	3 (3.3)
49	38 (13)	13 (4.2)	4 (10.5)	9 (23.7)

LT, living tetrathyridia; SW, with 2+ scoleces; SB, tetrathyridia with buds; SA, without a scolex.

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Table 4. The survival and development of tetrathyridia of Mesocestoides corti removed directly from the mouse (experiment 5, group 2) and cultured in vitro.

Days in culture	LT (%)	SW (%)	SB (%)	SA (%)	PR (%)
0	273 (100)	7 (2.6)	39 (14.3)	0	–
4	332 (118)	8 (2.4)	39 (11.7)	70 (21.1)	–
6	343 (126)	12 (3.5)	55 (16)	89 (25.9)	–
13	254 (93)	6 (2.4)	46 (18.1)	55 (21.7)	–
20	192 (70)	12 (6.3)	36 (18.8)	27 (14.1)	18 (9)
27	135 (50)	13 (9.6)	16 (11.9)	14 (10.4)	34 (25)
34	79 (29)	4 (5)	9 (11.3)	7 (8.9)	31 (39)

LT, living tetrathyridia; SW, with 2+ scoloces; SB, tetrathyridia with buds; SA, without a scolex; PR, proglottid development.

protocol) indicate quite definitely that tetrathyridia when kept in vitro in the gel/media retain their viability.

Development and growth in vitro

In the plates from groups 1, 2 and 3, 13%, 29% and 18% of the tetrathyridia survived for 49, 34 and 18 days respectively (tables 3, 4 and 5).

In all three groups (tables 2, 4 and 5) metacestodes had developed two scoleces and buds. Acephalic forms were also observed. The largest number (49.6%) of double scolex or 'two-headed' forms were seen on day 0 in group 3 (table 5). However, 34.2% of metacestodes in group 1 (table 3) had developed a second scolex by day 49, whereas in group 2 (table 4) the metacestodes contained the least number of double scolex forms.

Acephalic forms were not observed in groups 2 and 3 (tables 4 and 5) at day 0 and in group 1 not until day 42 but by day 49 the highest proportion (23.7%) of acephalic forms was observed.

The maturation of metacestodes as indicated by the onset of proglottid development occurred in groups 2 and 3 (tables 4 and 5) after 20 days in culture.

Discussion

The results demonstrate that a nutrient thixotropic gel is a medium in which tetrathyridia of M. corti can survive especially in varying strengths of gel from 100% gel; 60% gel/medium and 40% gel/medium (table 1). However 60% and 40% diphasic media facilitate the most penetration.

All the chemical additives as well as praziquantel and albendazole were able to diffuse through the gels hence allowing the metacestodes to make contact with the additive following a process of diffusion rather than being immersed in a solution containing a soluble form of the additive. The metacestodes can also be transferred from one culture to another (table 2). Transfer to a fresh culture medium avoids the build up of metabolic waste products of M. corti, which otherwise might become toxic and thus prolongs parasite survival in vitro.

An important aspect of an in vitro culture apart from demonstrating parasite survival under such conditions, is to provide a way of investigating a number of biological characteristics of the parasite. In addition, this system is useful as a preliminary drug screen assay. Established anthelminthics such as praziquantel and albendazole, 'injected' into the gel killed the tetrathyridia.

If tetrathyridia of M. corti can survive and undergo some development, how viable are they? The present results demonstrate that tetrathyridia cultured in vitro for at least 25 days are viable and a sequence of in vitro and in vivo cultures does not affect parasite viability. This then may challenge the fact that many helminth parasites establish themselves in the host by acquiring host proteins onto their outer tegument.

Tetrathyridia can readily survive for at least 14 days and when liver peptone, trypsin and pepsin are used as additives to culture media, the survival rate is prolonged for up to 48 days (tables 3, 4 and 5). In group 2, 14.1% of surviving tetrathyridia showed signs of proglottid development by day 20. This relatively low number of maturing tetrathyridia in culture suggests that once a predator eats an intermediate host only a small number of

Table 5. The survival and development of the tetrathyridia of Mesocestoides corti taken from the peritoneal cavity of a rat (experiment 5, group 3).

Days in culture	LT (%)	SW (%)	SB (%)	SA (%)	PR (%)
0	131 (100)	2 (49.6)	20 (15.3)	46 (35.1)	–
7	100 (76)	7 (7)	46 (46)	55 (55)	–
14	54 (41)	3 (5.6)	15 (5.6)	15 (27.8)	–
21	39 (30)	3 (7.7)	5 (12.9)	5 (12.9)	2 (4)
27	24 (18)	3 (12.5)	3 (12.5)	3 (12.50)	2 (8)

LT, living tetrathyridia; SW, with 2+ scoloces; SB, tetrathyridia with buds; SA, without a scolex; PR, proglottid development.

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larval stages of M. corti develop into adult worms. The high egg potential of each worm is likely to overcome the possibility that only a few worms reach maturity and maintain the life cycle and hence survival of M. corti is ensured.

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