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In Vitro Culture of *Strongyloides ratti* Larvae

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Introduction

The infective third-stage larvae (L_3) of *Strongyloides ratti* when infected subcutaneously, migrate to the cranial cavity from subcutis of the host and travel down the oesophagus via frontal sinus before entering the intestine(Tada *et al.*, 1979). During this migration and before the first parasitic moult, there are considerable changes in morphology of the parasitic L_3. These include increase in body length, length of the oesophagus, shape of head-end and genital primordium (Mimori *et al.*, 1982). In the present study attempts were made to know: (1) the gas phase requirement and its effect on the survival period *in vitro* of free-living L_3; gas phase requirement and/or effects of secretions of the digestive tract on the survival and development of different parasitic stage of *S. ratti*, (2) to evolve a simple continuous culture technique of the parasitic phase of this nematode, and (3) to explore the possibility of collecting a moulting fluid from a suitable defined medium.

Materials and Methods

1. Host animal, infection and recovery of parasite

The strain of *S. ratti* has been maintained in our laboratory for 6 years by successive passage in Wistar rats. The third-stage larvae (L_3) were harvested from filter paper cultures after 4-day incubation at 27°C (Tada *et al.*, 1979). The recovery of L_3 from the cranial cavity, frontal sinus and intestine was made at appropriate period after a subcutaneous inoculation of 3,000 L_3 into the axilla of rats (Mimori *et al.*, 1982). The larvae were collected in petri-dishes containing Hanks' solution at 37°C. The parasitic L_3 from the intestine and L_4 were sorted out under the stereo-microscope.

2. Axenic culture and preparation of basic media

The general methodology of roller system of axenic culture as applied in cestode, *Hymenolepis microstoma*, by Chowdhury and DeRycke (1979) was adopted here. The Eagle's Medium (BEM), Minimum Essential (MEM), Medium 199, NCTC 135, L 15 (Leibovitz) Medium, RPMI 1634, RPMI 1640 and Hanks' salts were from GIBCO. Except NCTC 135 and RPMI 1640, all media were obtained in powder form. The MEM×2 (Tables 1 and 2) means the medium contained the double concentration (2.058 g/100 ml) of MEM powder. The $NaHCO_3$ (0.085 g/100 ml) and glucose (0.300 g/100 ml) were added in all media. The Hanks' solution contained 0.991 g of Hanks' salts/dl, in addition to $NaHCO_3$ and glucose. All media or constituents of media were either autoclaved or sterilized by filtration. All media contained 300 units of penicillin G, 100 μg kanamycin (Takeda Pharmaceuticals, Japan) and 1.2 μg of fungizone (Squibb)/ml.

3. Preparation of constituents of complex medium and special ingredients

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Serum : Horse serum was obtained in inactive form from The Institute of Microbiology, Osaka University and the rat serum was prepared in the laboratory from male SPF adult Wistar rats. The sera were stored at −20°C and, inactivated at 56°C for 30 min before use.

Vitamin B_6 : 2% vitamin B_6 (Nakarai Chemicals, Japan) in Hanks' solution was sterilised by filtration and stored at −20°C.

Yeast extract : 4% yeast (DIFCO) in Hanks' was sterilized by autoclaving and kept at 4°C.

Liver extract : This was prepared by following the basic technique as described by Sinha and Hopkins (1967). Rat liver available from male SPF rats, or horse liver obtained from slaughter house were cut into small pieces and stored at −20°C. Each time, 50 g of liver was homogenized, with ice cooling, in 200 ml (initially 50 ml) of chilled distilled water. This was centrifuged at 4,000 rpm for 2–4 min. The pH of the total homogenate was adjusted to 4.0 with 1 N HCl and then squeezed through fine cloth to remove coarse particles. The filtrate was centrifuged at 10,000 rpm 1 hr at 4°C. The supernatant thus obtained was sterilized by filtration or directly stored at −20°C in small vials and used within two months.

Pepsin-HCl and trypsin-bile salts solutions: 1 g of pepsin powder (Nakarai Chemicals, Japan ; 1 : 10,000), dissolved in 100 ml of Hanks' solution containing antibiotics, was adjusted to pH 1.6 with 1 N HCl, sterilized by filtration and stored at −20°C. The solution was thawed, gassed with 5% CO_2 gas phase for 1 min and kept at 37°C in the roller drum 1 hr before use.

0.5 g of trypsin and 0.3 g of sodium taurocholate (Wako Chemicals, Japan) were dissolved in 100 ml Hanks' solution and pH adjusted to 7.2 with 0.2 N NaOH, sterilized by filtration and stored at −20°C.

Duodenal fluid : The duodenum obtained from worm-free 7 male adult rats were slit open in petri-dishes surrounded by crushed ice, and each was flushed with 10 ml of 0.015 M PBS (pH 7.2) containing antibiotics (as in the medium) and rinsed in the same solution for 5–10 min. The pooled washings were centrifuged at 4,000 rpm for 30 min at 0°C. The supernatant was sterilized by filtration and stored at −20°C.

4. Preparation of larval inoculum

Third-stage larvae obtained from faecal culture (L_{3f}), cranial cavity (L_{3c}), frontal sinus (L_{3nf}) and duodenum (L_{3i}) or fourth-stage larvae (L_4) recovered from the intestine were washed at least 4–5 times in Hanks' solution before transfer to the roller tube containing pre-incubated medium or constituents of medium. Similary, these were washed in pretreatment solutions when the larvae had undergone pre-treatments (Table 1). Normally, the larval inoculum consisted of 400–500 L_3/5 ml of medium but occasionally for the sake of convenience, the inoculum contained 50–100 L_3/2 ml of medium or constituent of medium (Table 2).

5. Preparation and handling of cultures

The aseptic media or constituents of media and pretreatment solutions were dispensed in 2–5 ml aliquots to roller tubes and, gassed for 30 sec with 5% CO_2 in N_2 (CO_2), 5% CO_2+15% O_2 in N_2 (CO_2+O_2) or 100% O_2 (O_2) (Tables 1 and 2). In some test experiments, 100% CO_2 as gas phase (results not referred to in any table) was also used while few experiments were conducted at atmospheric O_2 (Table 1).

The culture tubes were closed immediately after the gas phase and preincubated in the roller drum (1.5 rev/min) at 37°C for 1–2 hrs. After introduction of the Hanks'-washed larval inoculum, the media were re-gassed for another 30 sec before finally transferred to the roller drum. For prolong culturing, the medium was changed on alternate days except for L_4 where this was done every 24 hrs. Normally, the pH of the complex media ranged between 6.9 and 7.2 while in some cases (Table 1), the pH varied from 3 to 7.4. All experiments were repeated thrice.

6. Evaluation of culture

Growth, morphological changes or survival period of the larvae in media were frequently assessed by observing the stages under an inverted microscope or stereo-microscope.

Table 1 Effect of gas phase, pre-treatment and duodenal fluid on the survival and development *in vitro*, respectively of L_{3f}, L_{3c} and L_{3nf} of *Strongyloides ratti*

L_{3f} in cell-free media		L_{3c}		L_{3nf} in cell-free media (M) plus duodenal fluid (DF) under CO_2	
Medium (gas phase)	Survival period (hrs)	Pre-treatment	Survival period in cell-free media under CO_2+O_2 (hrs)	M : DF	Survival period (hrs)
MEM (air)	48	0.01% Na-Hypochlorite*			
		22°C	1	0 : 100	48†
MEM (CO_2)	24	37°C	10 min		
MEM (O_2)	24	Pepsin-HCl (as such)		MEM×2	
BME (O_2)	24	5 min-1 hr	24	30 : 70	96†
BME (CO_2)	8	M-82 : Pepsin-HCl		50 : 50	120†
Hanks' solution		5 : 1	24	80 : 20	120†
(O_2)	192	10 : 1	24	90 : 10	192†
Hanks' solution		25 : 1	24	95 : 5	168
(CO_2+O_2)	120	50 : 1	24	100 : 0	168
		Pepsin-HCl : Trypsin-Glycocholate (5 : 10 min) and then,		M-199/NCTC 135	
		M-82C	48	95 : 5	168
		M-102C	96	100 : 0	168
		M-112C	96	RPMI 1634	
		M-122C	96	100 : 0	264

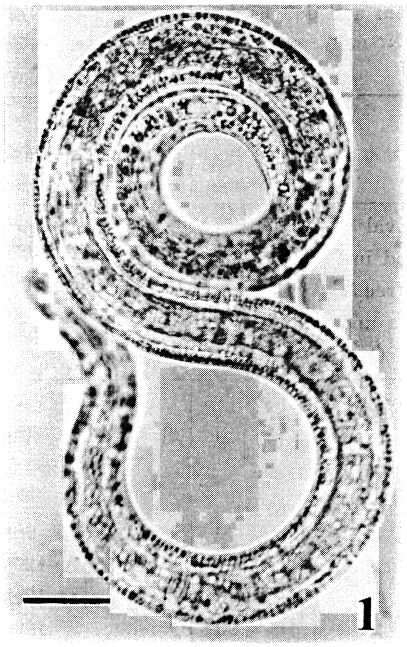
M-82 : NCTC 135 (50 ml)+Horse serum (30 ml)+Vitamin B_6 2% (10 ml)+Yeast extract 4% (6 ml)+Rat liver extract (4 ml).
M-82C : NCTC 135 and horse serum as above+Vitamin B_6 2% (20 ml).
M-102C : Medium 199 (50 ml)+Horse serum and Vitamin B_6 as above.
M-112C : As 82C but L-15 (Leibovitz) medium replaces NCTC 135.
M-122C : As 102C but RPMI 1640 replaces medium 199.
* without a gas phase † treated for 1 hr with Pepsin-HCl

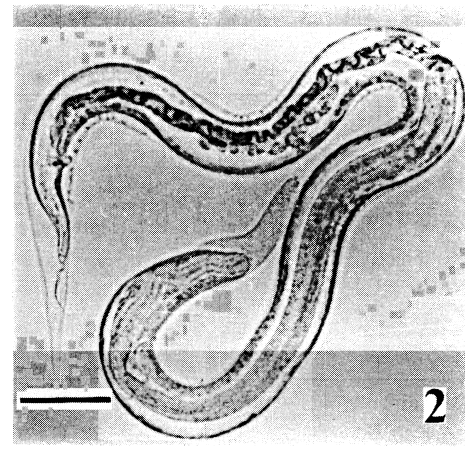
The nature of moulting (beginning, partial or complete), motility, death or degenerative changes of the cuticle of the $L_{3f\text{-}1}$ or L_4 were observed in coverslip preparations.

Results

The various experiments of L_{3f} on the development have shown (Table 1) that the infective larvae survive better in MEM than in BME in a gas phase of O_2 rather than of CO_2. Addition of horse serum to any of the media has no effect on the survival period. The larvae, however, survived best of all in Hanks' solution alone with a gas phase of O_2 or CO_2+O_2.

The parasitic L_{3c} survived better in a gas phase of CO_2+O_2 than that of CO_2. The L_{3c} have poor survival and do not moult by the treatment with pepsin-HCl, bile salts or Na-hypochlorite. Contrary to this, CO_2 gas phase appears to enhance survival of the parasitic L_{3nf} in media providing amino acids,





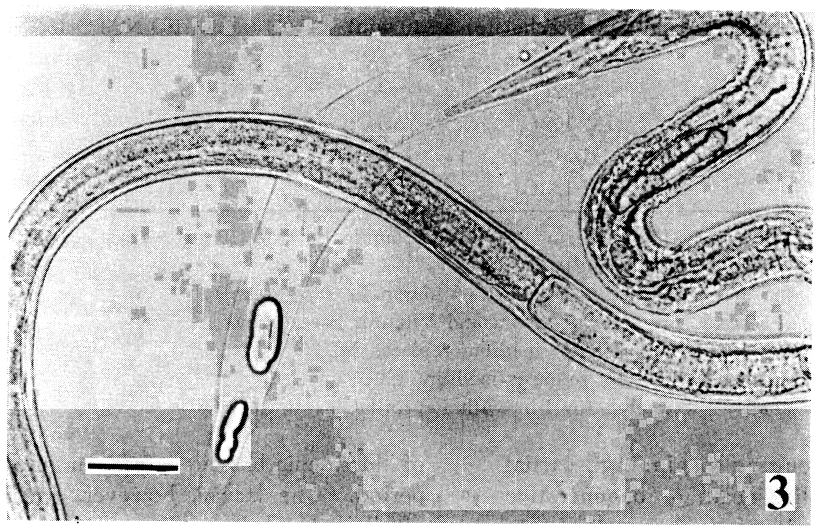


Fig. 1 A parasitic infective larva from naso-frontal portion after 48 hrs of culture in 100% duodenal fluid with a gas phase of CO_2 (Table 1). Note the accumulation of lipid-like granular droplets along the length of the larva close to the basement membrane darkening the worm with age. Bar : 20μm.

Fig. 2 A moulting parasitic third-stage larva (24 hrs after culture) from duodenum grown in Minimum Essential Medium (MEM×2) with a gas phase of CO_2 (Table 2). Note the characteristic shedding of cuticle at either ends. Also observe the digitiform (V-shaped) shed tail-end of the larva and well defiend intestine. Bar : 80μm.

Fig. 3 A moulting fourth-stage larva (24 hrs after culture) from duodenum grown in RPMI 1634 with a gas phase of CO_2+O_2 (Table 2). Note the bluntly-pointed (truncated) tail tip of the young adult and lipid-like globules in the shed cuticle. Bar : 40μm.

Table 2 Pattern of moulting of L_{3i} and L_4 of *Strongyloides ratti in vitro* in different defined media under CO_2+O_2 and CO_2 gas phases 19–24 hrs after inoculum

Media	moulted (%)		dead or degenerated (%)	
	L_{3i}	L_4	L_{3i}	L_4
		CO_2+O_2 gas phase		
MEM×2	60.1	62.1	43.3	27.0
NCTC 135	73.1	64.5	60.9	16.1
Medium 199	65.7	59.1	55.2	6.8
RPMI 1634	61.1	48.0	Nil	20.0
RPMI 1640	70.8	62.7	Nil	19.6
Hanks' solution	56.9*	ND	3.0	ND
Hanks' solution	60.8†	ND	28.0	ND
		CO_2 gas phase		
MEM×2	53.3	20.0	Nil	26.6
NCTC 135	66.6	35.8	Nil	35.8
Medium 199	72.5	54.5	5.8	14.5
RPMI 1634	55.2	38.4	Nil	15.3
RPMI 1640	63.2	48.3	Nil	Nil
Hanks' solution	36.6	40.9	27.1	ND

ND : not done. * within 24 hrs ; † within 48 hrs.

vitamins and salts as buffering agents. The L_{3nf} did not moult or develop further by the treatment with duodenal fluid (Fig. 1), although they survived for 11 days (Table 1).

The results of the moulting pattern of 72-hr-old parasitic L_{3i} and L_4 with CO_2+O_2 and CO_2 gas phases (Figs. 2 and 3) in various defined media indicate that the best results were obtained with NCTC 135, Medium 199 and RPMI 1640 in CO_2+O_2 gas phase so far as the mortality, degenerative changes and percentage of moulting (Table 2) are concerned.

Discussion

The experiments (Tables 1 and 2) with various gas phases for culturing parasitic L_3 in simple defined and complex media, indicated that L_{3c} behaved in the same way as L_{3f} i.e. when subjected to absolute CO_2 or 5% CO_2 they died instantly or within few hrs (Table 1). Contrary to this, the parasitic L_{3c} with or without pre-treatments survived a considerable period in a gas phase of 100% O_2 or CO_2+O_2 ; those cultured from the frontal sinus appear to withstand CO_2 much better than L_{3c} even though further development or moulting did not occur. The L_{3i} survived well, moulted and underwent further development both in CO_2+O_2 and CO_2 gas phases. Our experiments (unpublished data) by ligations of various sites of digestive tract have shown that the larvae undergo first parasitic moult near the pylorus, evidently there is an influence of CO_2 as well as pepsin-HCl.

In many nematodes, the infective L_3 which enter the host *per os* moult quickly or the percentage of larval moulting is enhanced by CO_2, pH, sodium taurocholate, horse serum, chick embryo extract and sodium hypochlorite (Rogers and Sommerville, 1963, 1968 ; Rose, 1973). From our earlier *in vivo* (Mimori *et al.*, 1982) and *in vitro* studies (unpublished data), we presume that L_3 of *S. ratti*, recovered from the duodenum, physio-anatomically are the same as L_3 which infect the host *per os* and moult on entering the gut. "Physiological metamorphosis" (Rogers and Sommerville, 1963), therefore, is a pre-requisite for *S. ratti* and probably for many other

parasitic nematodes which enter the host via the skin. An example of this is the infective larvae of *Ancylostoma caninum*, which even on entering orally mostly reach lungs (a place of high O_2 tension), follow the tracheal route, before reaching the host's gut (a place of high CO_2 tension) for the final moults. In fact, early parasitic stages of this species appear to be dependent on O_2 and subsequently CO_2+O_2 or CO_2 gas phases.

Weinstein and Jones (1957) cultured parasitic stages of *Nippostrongylus muris* to sexually mature adults in a complex medium but failed to grow those of *S. ratti* in the same medium. In the present study, the percentage of development *in vitro* of the intestinal L_3 to adult or L_4 to adult in complex media is very low, ranging from 6% to 15% (unpublished data). However, we were able to keep 7 and 10-day-old adult female alive in defined media for 48 hrs or more with CO_2+O_2 or CO_2 gas phase and moulting of L_{3i} to L_4 or L_4 to adult were achieved in Hanks' solution alone (Table 2).

Summary

Infective third-stage larvae of *Strongyloides ratti* from faecal culture and parasitic phase of third-stage larvae from the craninal cavity, frontal sinus, duodenum and, fourth-stage larvae from the intestine of rat hosts have been cultured axenically in roller tubes in various defined and complex media with different gas phases, at 37°C. The results have indicated that infective larvae of this species at free-living phase are aerobic. Parasitic stage larvae from the cranial cavity and frontal sinus could be maintained for 11 days in media providing amino acids, vitamins, glucose and salts as buffering agent. Parasitic third and fourth-stage larvae from the intestine moult best in both NCTC 135, Medium 199 and RPMI 1640 in CO_2+O_2 gas phase.

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Strongyloides ratti 幼虫の培養に関する研究

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濾紙培養により得た *S. ratti* 第3期幼虫（L_{3f}），をラットに投与して得た寄生相の第3期幼虫（L_{3c}・L_{3nf}・L_{3i}）及び腸管から得た第4期幼虫（L_4）を37 °C で無菌的に培養し，培地組成，ガス相についてその条件を検討した．その結果，濾紙培養して得た L_{3f} は好気的であることが明らかとなった．更に寄生相の L_{3c}・L_{3nf} をアミノ酸，ビタミン，グルコース，各種塩の存在下で11日間培養し得た．小腸から回収した L_{3i} 及び L_4 は NCTC 135と199培地，及び RPMI 1640に CO_2+O_2 ガス相下で最もよく脱皮が起こった．