Experimental Parasitology 124 (2010) 247–251

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

# *Teladorsagia circumcincta*: Survival of adults *in vitro* is enhanced by the presence of a mammalian cell line

A. Luque [a], L.R. Walker [a], J.C. Pedley [b], K.C. Pedley [b], K. Hillrichs [a], H.V. Simpson [a], D.C. Simcock [b,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11222, Palmerston North 5301, New Zealand
[b] Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand

ARTICLE INFO

*Article history:*
Received 30 March 2009
Received in revised form 21 September 2009
Accepted 15 October 2009
Available online 21 October 2009

*Keywords:*
*Teladorsagia circumcincta*
Nematodes
*In vitro* culture
Cell tissue culture
*In vitro* egg laying

ABSTRACT

Adult *Teladorsagia circumcincta* survival and motility *in vitro* was examined in a range of different cell culture media, supplements and gas mixes. Under optimum conditions, worms survived for 14 days, exhibiting high motility for 9 days and egg production for 72 h. Optimum conditions involved co-culture of worms with a HeLa cell line in a supplemented cell medium (CEM) and an atmosphere containing 10% $CO_2$, 5% $O_2$ 85% $N_2$, 65% humidity at 37 °C. The incubation medium consisted of Minimum Essential Medium with 10% fetal calf serum, 1% non-essential amino acids, 1% glutamax and 1% penicillin–neomycin–streptomycin cocktail mix. Compared with optimum conditions, incubation in CEM alone, cell conditioned CEM, RPMI alone, Medium 199 alone, reduced $CO_2$ or $O_2$, or when cells were replaced with *Escherichia coli*, both survival and motility were reduced. Optimum conditions for adult *T. circumcincta* maintenance for culture, anthelmintic testing or generation of excretory/secretory products are described.



## 1. Introduction

The adult stage of *Teladorsagia circumcincta* infection in sheep causes profound changes in abomasal function, including elevated abomasal pH, serum pepsinogen and serum gastrin, the loss of parietal cells and generalised hyperplasia (Lawton et al., 1996; Scott et al., 2000). Similar results are observed with other abomasal parasites such as *Ostertagia ostertagi* (Fox et al., 1993) and *Haemonchus contortus* (Simpson et al., 1997). Further, the reduction of food intake by the host appears to coincide with the emergence of adult worms during infection with *T. circumcincta* (Simcock et al., 1999; Fox et al., 2006) and *O. ostertagi* (Fox et al., 2002). While the adult stage is of critical importance, for the parasite in terms of reproduction and for the host in terms of pathology, relatively little is known about the requirements of adult worms for survival and egg production. Further, the study of the adult stage is hindered by their short survival times *in vitro*.

Current studies examining adult stages of nematode parasites utilise short term cultures of adult worms which have been collected from a host *in vivo*. Typically, these cultures utilise mammalian cell culture media containing a balanced salt solution such as Minimum Essential Medium (MEM), Hank's Balanced Salt Solution (HBSS) or Royal Park Memorial Institute medium 1640 (RPMI) with various buffers, antibiotics, fungicides and fetal or newborn bovine serum. Using such media, *T. circumcincta* can be maintained *in vitro* for up to 72 h (Lawton et al., 2002; Przemeck et al., 2005; Craig et al., 2006), though survival time, assessed by monitoring motility, is short *in vitro* compared with *in vivo*. Similar short term cultures have been reported for adult *Ancylostoma caninum* (Zhan et al., 2003), *Ascaris suum* (Islam et al., 2004), *Heligomosmoides bakeri* (Bourgeois et al., 2007), *Heligomosmoides polygyrus* (Stepek et al., 2005), *Nippostrongylus brasiliensis* (Ishiwata and Watanabe, 2007), *O. ostertagi* (Geldhof et al., 2000), *Trichostrongylus colubriformis* (Hounzangbe-Adote et al., 2007) and *H. contortus* (O'Grady and Kotze, 2004; Huber et al., 2005; Przemeck et al., 2005). Although *Strongyloides venezuelensis* can be maintained for up to 9 days *in vitro* (Baek et al., 1998), in the other papers listed, survival times were not observed for longer than four days and did include co-culture with a mammalian cell line. Co-culture of worms with a cell line has been attempted with *Trichinella spiralis*, with results showing that the presence of the cell line can support the entire intestinal phase of the life cycle for this parasite (Gagliardo et al., 2002).

The conditions required for the *in vitro* culture of reproductive adult worms from larvae have been reported for some nematode parasites. *Anisakis simplex* can be cultured from L4 to reproductive adult in RPMI supplemented with Fetal Bovine Serum and pepsin when incubated in 5% $CO_2$ mixed with normal air (Iglesias et al., 2001). *O. ostertagi* adults have been cultured from L3s using a non-commercial medium called API-1 in normal air, but only 30% of L3s develop to mature adults and development is slower than

* Corresponding author. Fax: +64 63 50 56 57.
*E-mail address:* D.C.Simcock@massey.ac.nz (D.C. Simcock).

0014-4894/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2009.10.002

*in vivo* (Douvres and Malaktis, 1977). *H. contortus* adults have also been cultured from L3s *in vitro* using API-1 supplemented with Filde's reagent and sheep gastric contents in a 10% $CO_2$, 5% $O_2$ and 85% $N_2$ environment, though most of the larvae incubated arrested at L4 (Stringfellow, 1986). The *in vitro* conditions reported in these experiments would be expected to also support adult worms in short term culture. Despite this, the conditions reported for *in vitro* culture of *O. ostertagi* and *H. contortus* L3 to reproductive adult have not been widely accepted for the maintenance of adult worms *in vitro*, as subsequent studies utilise different media and adult worms are exclusively harvested *in vivo*.

The limited survival time of adult worms cultured *in vitro* suggests culture conditions are not ideal or that essential ingredients are missing. This is partly overcome by harvesting adult worms *in vivo* and culturing them *in vitro* for short periods of time, usually for 24–72 h. These short term cultures are typically used for experiments screening potential anthelmintics or for generating excretory/secretory (ES) products. However, culturing worms under such conditions, particularly for ES product generation, is compromised by the fact the cultured worms are essentially dying in the *in vitro* environment. If it was possible to maintain adults *in vitro* using culture conditions which could maintain them for extended periods, ES products would be less compromised by artefacts from dying worm cells. In addition, the short duration of current *in vitro* cultures means that experiments which require adult worms always require the sacrifice of animals to harvest these worms, which is ethically and economically costly. With longer worm survival times *in vitro*, the use of host animals could be reduced by utilising adult worms from one animal, particularly for screening, for extended periods of time. Extending the period of adult maintenance, including extension of the duration of egg laying by female worms *in vitro,* would also be a significant step in the development of a full *in vitro* culture system for maintaining parasite cultures without passage through a host.

The following study examined the survival, motility and egg laying by adult *T. circumcincta* using different media, gas mixtures, the addition of a cell line, cell conditioned media or various media additives to determine whether these could enhance adult worm survival *in vitro*.

## 2. Methods

### 2.1. Collection of adult worms

Adult worms were recovered using the technique described by Simpson et al., (1999). Briefly, donor sheep were infected with 50,000 L3 T. circumcincta. After 21 days of infection, the sheep were euthanased and their abomasal contents were mixed 2:1 with 3% agar. When the agar had solidified, it was covered with 0.9% saline. Adult worms were collected as soon as they emerged from the agar into the saline. The process from sheep euthanasia to worm collection took no longer than 40 min. Time from opening the abomasum to collection of adult worms was no longer than 30 min.

### 2.2. Gas mixes

Worms were incubated under several different gas conditions. These conditions included incubation in normal atmosphere ("room air") or in a $CO_2$ incubator (5% $CO_2$, 95% room air) or in an anaerobic cabinet with one of the following gas mixtures: 5% $CO_2$, 5% $O_2$, 90% $N_2$ or 10% $CO_2$, 1% $O_2$, 89% $N_2$ or 10% $CO_2$, 5% $O_2$, 85% $N_2$. All incubations were conducted at 45–65% humidity at 37 °C.

### 2.3. Media

Four media types were used to culture adult worms: cell essential medium (CEM), phosphate buffered saline (PBS), RPMI-1640 and Medium 199.

CEM consisted of Minimum Essential Medium (MEM) without glutamine, with phenol red, supplemented with 10% (v/v) Fetal Calf Serum (FCS), 1% non-essential amino acids, 1% glutaMAX, and 1% penicillin–streptomycin–neomycin mixture (PSN) (all products were supplied by GIBCO BRL, from Invitrogen, USA). To this mixture, 2.27 g/L $NaHCO_3$ (Sigma Chemical Co, St Louis, USA) was added to make a 27 mM solution.

PBS contained 4 g NaCl, 0.2 g KCl, 0.575 g $Na_2HPO_4$, 0.1 g $KH_2PO_4$ (all sourced from Sigma Chemical Co, St Louis, USA) in 500 ml adjusted to pH7.2.

RPMI-1640 and Medium 199 were supplied in liquid form (Invitrogen, USA) with no supplements.

### 2.4. Incubation conditions

For the testing of different media and gas conditions, between 10–30 adult worms were incubated in 5 ml Petri dishes in the various environments and media for up to 14 days. Medium pH was monitored and the culturing medium was changed after 7 days, if the worms were still motile. The activity of the worms was monitored at 6 times over the 14 day period, on days 1, 3, 5–7, 9, 12 and 14 after the culture was started.

### 2.5. Worm density and male to female ratio

Using CEM and the gas mixture of 10% $CO_2$, 5% $O_2$, 85% $N_2$, worms were incubated in 5 ml Petri dishes in groups of 5, 10, 30, 50, 100 and around 500. For egg laying studies, 10–30 worms were incubated with ratios of male to female worms of (number of experiments in brackets): 1:1 ($n = 15$), 1:2 ($n = 5$), 1:3 ($n = 10$), 1:4 ($n = 6$) and 1:5 ($n = 10$).

### 2.6. Survival and motility

All worms displaying any form of motility were considered alive. Motility was assessed in four categories: +++: motility similar to that observed at collection, ++: highly motile but less active than when first collected, +: motile but sluggish, I: move when stimulated by touching with pipette, −: no response when touched with a pipette. In tables quoting motility, the activity level of the majority of the surviving worms is stated.

### 2.7. Hela cell cultures, conditioned medium and bacteria cultures

A HeLa cell culture line (ATCC, USA) was maintained in aseptic conditions in CEM medium and 5% $CO_2$, 95% room air. Prior to co-culture with worms, cells were transfected onto a 15 mm microscope slide coverslip and grown to between 20% and 30% confluence before being transferred to a 10 ml Petri dish with new CEM for co-culture with adult worms in 10% $CO_2$, 5% $O_2$, 85% $N_2$, 65% humidity at 37 °C. Conditioned medium was generated by incubating fresh CEM with a population of HeLa cells in a T75 flask for three days. The cells were then removed and the conditioned CEM added to Petri dishes with adult worms for culture.

The OP50 strain of *Escherichia coli* was grown to a lawn on LB broth, set in 4% bacto-agar (both from Invitrogen, USA) in a 10 ml Petri dish. When bacteria covered the surface of the agar, adult worms in fresh CEM were placed on top of the *E. coli* colony. In other experiments LB media was inoculated with *E. coli* and when turbid, the solution was added to CEM to make a 10% final concentration and then incubated with adult worms. Both the bac-