In vitro cultivation of *Toxocara cati* adult worms for production of eggs and evaluation of oviposition

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Summary

Toxocariasis is the clinical syndrome caused by infection of zoonotic roundworms of dogs (*Toxocara canis*) or cats (*Toxocara cati*). Current research on the immunology and pathology aspects of toxocariasis requires *Toxocara* second stage larvae and a ready source of excretory-secretory (ES) antigens. We cultured eleven pairs of both sexes of *Toxocara cati* adult worms maintained in RPMI 1640 medium in order to evaluate the amounts and duration of egg laying. At the first day and last day (day 19), the mean egg counts were 9300 and 250 eggs/ml, respectively. These results showed that this culture method is very appropriate for collection of pure oviposited eggs and/or production of adult ES antigens of *Toxocara cati* that could be used for immunological diagnosis of toxocariasis.

Key words: *Toxocara cati*; cultivation; in vitro; Adult worms

Introduction

Toxocariasis is the clinical term applied to infection in the human host with either *Toxocara canis* (*T. canis*) or *Toxocara cati* (*T. cati*) (Despommier, 2003). Infection can occur by ingestion of embryonated eggs from the soil or ingestion of larvae in uncooked meat of infected paratenic hosts such as chicken, cattle or sheep (Azizi *et al.*, 2007). The prevalence of seropositivity varies from 2.6 to 86 % in different parts of the world (Sadjjadi *et al.*, 2000). Reports on *Toxocara* eggs contamination of soil samples from public areas in various parts of the world showed that the contamination rates ranged from 6 to 92 % (Zibaei & Uga, 2008).

Reports suggest that the spectrum of toxocaral disease goes

beyond the limits of Visceral Larva Migrants (VLM) and Ocular Larva Migrants (OLM) syndrome characterized by chronic weakness, abdominal pain various sign of allergy, mild hypereosinophilia (Magnaev *et al.*, 1991). In the absence of parasitological evidence of infection, diagnosis of larval toxocariasis has relied mainly on immunological methods. Highly sensitive and specific serodiagnostic assays have been developed using *Toxocara* excretory-secretory (ES) antigens (Fife, 1971; de Savigny, 1975; Glickman *et al.*, 1985). The large-scale product of this antigen is recovery of *Toxocara* spp. eggs which unembryonated passed in the feces of dogs and cats and/or in the uteri of female worms. Under optimal temperatures and humidity, these eggs develop into embryonated eggs, that is the principle of *Toxocara* ES antigens (Overgaauw, 1997). None of the reports from trails of in vitro cultivation of *Toxocara cati* adult worms were concerned with egg laying and collection of ES protein; although other helminths have been cultured successfully (Hoti *et al.*, 1994; Chaitong *et al.*, 2001; Walter, 1998; Alarcon de Noya *et al.*, 1992; Hemphill *et al.*, 2002). However, these data are important to evaluate the usefulness of an in vitro model for assemblage of ES antigens of adult worms for comparison with second stage larvae and also eggs laid.

The purposes of this study were to evaluate the duration of in vitro cultivation of *T. cati* adult worms and further to clarify the oviposition in these worms.

Materials and Methods

Recovery of adult worms

Toxocara cati adult worms were obtained by necropsy from stray cats in different parts of Shiraz, Iran. Female worms were isolated and rinsed first in distilled water and then phosphate buffered saline (pH 7.2) to remove adherent faecal material.

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Fig. 1: *Toxocara cati* adult worms were cultured in RPMI 1640 medium

In vitro culture

After three to four washes with Ringer. 1/4 Ringer's solution was prepared by dissolving 1 tablet including Ammonium chloride 0.4; sodium hydrogencarbonate 0.005; calcium chloride-2-hydrate 0.040; potassium chloride 0.0525; sodium chloride 1.125, (Merck, Germany) in 500 ml neutral deionized water and sterilized in the autoclave (15 min at 121°C), pH: 6.9 ± 0.1 at 25 °C. A total of 11 male/female pairs were separated and transferred to 20 ml of sterile RPMI 1640 (Gibco BRL, Netherlands) containing Penicillin/Streptomycin (at 100 U/100 µg/ml) (Sigma - Aldrich Chemie GmbH, Germany), sodium bicarbonate, HEPES buffer (Merck, Germany), Amphotericin B (50 µg/ml) (Bristol-Myers Squibb, Netherlands) and 1 % glucose. The medium was maintained at 37 °C and 5 % carbon dioxide and 95 % nitrogen in a CO₂ incubator (Zibaei *et al.*, 2007).

Egg recovery

The medium was changed twice a day and then daily by tilting the culture flask to allow the adult worms and eggs to sediment and then aspirating of media. The flasks were then replaced with fresh medium. The sediment (eggs were released from females) was centrifuged at 1500 g for 3 minutes for recovery of eggs. The eggs were examined under an inverted microscope and/or stereoscope and counted every 24 hours for 19 days.

Results

Toxocara cati adult worms were cultured in RPMI 1640 for 19 days (Fig. 1). Most of the eggs were recovered in 24 hours with 9300 eggs/ml and after 48 hours, the numbers of eggs were 9000-eggs/ml. Thus after 19 days

the numbers of eggs were decreased dramatically and only a few eggs were recovered (Fig. 2). Changes in the developmental stage of *T.cati* eggs recovered from RPMI 1640 medium are shown in Table 1.

Fig. 2: Daily recovery of *Toxocara cati* eggs from 11 pair adult worms/20ml RPMI 1640 medium within 19 days

Discussion

There have been numerous reports on the development of an in vitro culture technique for helminths to obtain ES products from parasites (Healer *et al.*, 1991), to screen the effects of anthelmintics (Rew *et al.*, 1986), to evaluate nutritional equipments of larvae and adult worm (Urban *et al.*, 1984), to determine biochemical changes associated with parasite development (Gamble & Mansfield, 1996) and for immunodiagnosis and also to evaluate infectivity of egg (Baek *et al.*, 1998).

An in vitro maintenance of adult stage parasite in some of helminthes for experimental investigation requires a serial passage in susceptible laboratory animal, which are not only an expensive work but also a time consuming one.

In some of parasitic diseases including toxocarasis, current diagnosis is serodiagnostic testes that measured total immunoglobulin against ES proteins recovered from in vitro cultured infective larvae (de Savigny, 1975). The production of this antigen need eggs that after full embryonation hatch in vitro according to the methods have reported (Villanueva & Kozek, 1992).

Up until now, have been different reports on the development of in vitro culture techniques for second stage larvae *T. cati* to obtain ES products, but no reports from in vitro cultivation of *T. cati* adult worms. Rodriguez-Caballero *et al.* (2007) have compared the population size of fertilized eggs from *Toxocara canis* female worms kept in culture and those obtained using a uterus excision method. In vitro culture may be useful in order to

Table 1. Situational changes of *Toxocara cati* eggs in medium

Days	Total No. of eggs	No. of eggs		Embryonation (%)
		unembryonated	embryonated	
0 – 5	41292	41172	120	0.3
6 – 10	32700	32338	362	1.1
11 – 15	17580	17135	445	2.5
16 – 19	1973	1792	181	9.2
Total	93545	92437	1108	13.1

harvesting *T. cati* adult worm ES antigen for comparison to L₂ stage and also eggs for future study. However, we did not determine the survival period of adult worms in this medium.

The present study was designed to use a simple *in vitro* culture system for maintenance of *T. cati* adult worms as well as the technique for extending the collection of eggs and ES antigens. The method described in this study is simple to perform and will be available commercially. We feel it may serve as a base for future *Toxocara cati* research projects.

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