In vitro production of *Toxocara canis* excretory-secretory (TES) antigen

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Abstract *Toxocara canis* is a widespread gastrointestinal nematode parasite of dogs and cause *Toxocara* larva migrans, an important zoonotic disease in humans on ingestion of infective eggs. Toxocarosis is one of the few human parasitic diseases whose serodiagnosis uses a standardized antigen, *T. canis* excretory secretory antigen (TES). The present study describes collection of *T. canis* adult worm, collection and embryonation of *T. canis* eggs, hatching and separation of *T. canis* larvae, in vitro maintenance of *T. canis* second stage larvae for production of TES, concentration of culture fluid TES and yield of TES in correlation with various methods cited in literature.

Keywords *Toxocara canis* · In vitro cultivation · Excretory-secretory antigen

Introduction

Toxocara larva migrans (TLM) or Human Toxocarosis is a helminthic zoonosis due to the infection of humans by ascarid larvae belonging to the genus *Toxocara*, either *Toxocara canis* (Beaver et al. 1952) or *Toxocara cati*

(Nagakura et al. 1990), the adult stages of which are found in the canid and felid intestines respectively. It poses a serious human health problem both in temperate and tropical climates. *Toxocara canis* is widely distributed in most regions of the world where dogs and feral animals like wolves and foxes are infected and is regarded as the main cause of TLM in human beings. Infection results from ingestion of embryonated eggs in soil (Glickman and Schantz 1981) or uncooked vegetables (Vasquez Tsuji et al. 1997) or on dog's hair coat (Aydenizoz-Ozkayhan et al. 2008; Roddie et al. 2008) and ingestion of infective larvae through raw or undercooked meat, giblets or offal (Nagakura et al. 1989; Sturchler et al. 1990; Taira et al. 2004). Seroprevalence surveys indicate that 3–7 % of adults and 15–23 % of children typically have detectable antibodies to *Toxocara* (Rubinsky-Elefant et al. 2008) but higher values have been recorded from the tropics (Mizgajski 2001 and Colli et al. 2010). TLM results in a wide variety of syndromes in humans which includes visceral larva migrans (VLM) (Beaver et al. 1952), ocular larva migrans (OLM), covert toxocariasis (Taylor et al. 1988), common toxocariasis (Magnaval et al. 1994) and cerebral toxocariasis (Magnaval et al. 1997), although most infections are probably subclinical. A definitive diagnosis of toxocarosis is often a significant challenge for the clinician, as clinical pictures of this condition is quite non specific. As clinical manifestations of TLM are often nonspecific, vague and difficult to interpret, serological methods have always been considered for diagnosis. Toxocarosis is one of the few human parasitic diseases whose serodiagnosis uses a standardized antigen, *Toxocara canis* excretory secretory antigen (TES). The present study describes an improved method for production of *T. canis* excretory secretory antigens for serodiagnosis of TLM.

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Materials and methods

Adult *T. canis* worms were collected from non descript pups maintained at Blue Cross Society of India, Velachery. The pups were dewormed with Piperazine hydrate liquid (Virbac, India) at the dose rate of 100 mg/kg. The adult worms expelled on the next day were collected in normal saline and brought to the laboratory of Department of Veterinary Parasitology. The individual female worms were kept in a petridish containing normal saline and dissected using a scalpel at the anterior one-third of the uterus under a stereozoom microscope. The eggs were released from the uterus by applying gentle pressure with forceps. Later the eggs were transferred to 4 % formalin in normal saline and incubated at a temperature of 26 °C for 35 days to induce embryonation (Alcantara-Neves et al. 2008). The embryonated egg suspension was washed and treated with 4 % sodium hypochlorite solution for 2 h to induce decortication. The decorticated eggs were transferred to RPMI-1640 medium containing gentamicin and amphotericin B, mixed gently for about 5 min and kept at 37 °C overnight in an incubator to induce hatching. Next day morning the hatched larvae were centrifuged and the precipitate was placed on 3 ml of Histopaque solution (Sigma Aldrich, USA), density 1.077 g/ml and osmolality 290 m Osm, and then centrifuged at 1,600 rpm for 30 min. Viable larvae were concentrated at the bottom of the tubes (Ponce-Macotella et al. 2011). Viable larvae separated using Histopaque were transferred to RPMI-1640 medium in the 6 well culture plates at a concentration of 10^3 larvae/ml and kept at 37 °C in CO₂ incubator with 5 % CO₂ (Bowman et al. 1987). The culture medium was replaced at five days interval after assessing the viability of larvae. Collected medium was concentrated using PEG 20,000. Protein content was estimated by acid method using BCA kit (Genei, Bangalore) at the absorbance of 562 nm and stored at −20 °C until use.

Results

About 350 *T. canis* worms including male and female were collected from 30 non descript pups maintained at Blue Cross Society of India, Velachery (Fig. 1). On an average 85,000 eggs were collected from each adult female worm. Twenty plates containing the unembryonated eggs were maintained at a temperature of 26 °C for 35 days to induce embryonation during the study period for larval culture (Fig. 2). Development of eggs was evident from 24 h of incubation. By seventh day 60 % (Average 51,000 eggs/worm) of collected eggs developed to second stage larvae inside (Fig. 3).

Fig. 1 Adult *Toxocara canis* worms

Fig. 2 Unembryonated eggs of *Toxocara canis*

The embryonated eggs with the second stage larvae were washed and decorticated using 4 % sodium hypochlorite solution. It was found that after 2 h of incubation with sodium hypochlorite solution the mamelonated membrane of the eggs got dissolved to a thin membrane around the larvae (Fig. 4). Hatching was induced by incubating the eggs with RPMI-1640 overnight at 37 °C in a CO₂ incubator (Fig. 5). The hatched viable larvae were separated using histopaque solution. 60 % of the viable larvae (Average 30,600 larvae/worm) were recovered by this method.

Hatched larvae were cultured in RPMI-1640 medium containing gentamicin and amphotericin B (Fig. 6) at a concentration of 10^3 larvae/ml with replacement of

Fig. 3 Embryonated eggs containing second stage larvae of *Toxocara canis*

Fig. 5 Hatched out second stage larvae of *Toxocara canis*

Fig. 4 Decorticated eggs of *Toxocara canis* showing larva with thin egg shell layer

medium at 5 days interval and maintained up to three weeks. The plates which were contaminated and those with less than 70 % viable larvae were discarded. About 750 ml of culture fluid containing ES antigen was collected in different batches, concentrated using PEG 20,000 to one tenth of the initial volume and stored at -20°C as crude antigen. Protein content of the crude antigen varied from 2.25 to 3.5 mg/ml by BCA assay.

Discussion

Toxocara canis is a widespread gastrointestinal nematode parasite of dogs and cause TLM, an important zoonotic

Fig. 6 Antigen secreting *Toxocara canis* Larvae in RPMI 1640 medium

disease in humans on ingestion of infective eggs. Since the larvae inhabit internal organs where they are not easily detected, the diagnosis of the infection is mainly serological which detects antibodies against larval excretory/secretory (ES) antigens.

Prior to the use of ES antigens as a diagnostic antigen, most surveys of human infection were based on tests using water soluble, somatic antigens derived from *T. canis* embryonated eggs, larvae or adults. Many of these somatic antigens lacked sensitivity and possessed a high degree of

cross reactivity with other ascarid antigens (Girdwood et al. 1978; Smith et al. 1982).

In the mid 1970s, introduction of in vitro culture technique of *T. canis* second stage larvae for prolonged periods of time and collection of ES antigen (de Savigny 1975) created a breakthrough in diagnosis of TLM, which considerably improved the specificity, sensitivity and reproducibility of various serodiagnostic methods. ES antigens are the first mosaic of antigens to which an antibody response is detectable following infection (Smith et al. 1982) and currently provided us with the most specific target antigen for serodiagnosis in human (de Savigny and Tizzard 1977; de Savigny et al. 1979; Smith et al. 1980; van Knapen et al. 1983; Glickman et al. 1985 and Magnaval et al. 2001).

Toxocara canis eggs were collected by dissection of female worms and incubated in 4 % formal saline at 26 °C for 35 days to induce embryonation. Embryonated eggs were decorticated with 4 % sodium hypochlorite and induced hatching by incubating with RPMI 1640 medium. This procedure was in accordance with that of Alcantara-Neves et al. (2008). Maizels et al. (1984) and Crowcroft and Gillespie (1991) induced embryonation of eggs by incubating the adult worms in formal saline for one month and the latter collected the eggs by homogenisation of the worms instead of dissection of worms. Different concentrations of sulphuric acid for inducing embryonation were also carried out (Oaks and Kayes 1979; Sugane and Oshima 1983; Gupta 1984 and Williamson et al. 1990). Use of formal saline had the advantage of better embryonation and safe usage.

Decortication of larvated eggs using different concentrations of sodium hypochlorite was tried elsewhere (Oaks and Kayes 1979; Maizels et al. 1984; Bowman et al. 1987; Williamson et al. 1990; Crowcroft and Gillespie 1991; Roldan et al. 2006 and Ponce-Macotela et al. 2011) whereas Iddawela et al. (2007) used saturated calcium hypochlorite. Treatment of eggs with sodium hypochlorite to dissolve chitinous layer tended to mimic physiological conditions (Rajapakse et al. 1992). In the present study embryonated eggs were treated with 4 % sodium hypochlorite for 2 h as it was found to be optimal for subsequent hatching whereas Watthanakulpanich et al. (2008) reported an optimal timing of 3 h. However varied incubation time with different concentrations of sodium hypochlorite has been reported (Gupta 1984; Bowman et al. 1987; Williamson et al. 1990; Shobana 2003; Roldan et al. 2006).

Hatching of the decorticated eggs was induced by incubation with RPMI 1640 medium in the present study whereas Ponce-Macotela et al. (2011) induced hatching of the larvae by treatment with Hank's balanced salt solution with two pH changes. Many authors reported about the hatching of decorticated eggs using mechanical force

(Bowman et al. 1987; Smith 1989; Crowcroft and Gillespie 1991; Roldan et al. 2006; Kim et al. 2008). The procedure adopted in the present study avoided the washing step of the collected larvae with the medium before keeping in cultures and also gave a better recovery of larvae when compared to earlier protocols.

Live larvae for culture were separated from debris, dead larvae and unembryonated eggs using histopaque solution which was a recent method reported by Ponce-Macotela et al. (2011) instead of using a Baermann apparatus (de Savigny 1975; Bowman et al. 1987; Crowcroft and Gillespie 1991; Roldan et al. 2006 and Kim et al. 2008). This recent technique was advantageous since it needed less time of execution, had lesser chance of contamination, reduced handling of samples and there was better recovery of larvae when compared with the traditional Baermann method which required more time (overnight incubation at 37 °C) and with reduced migration of larvae in case of temperature drop.

In the present study on an average 30,600 live larvae/worm were recovered for culture which was higher than 1,400 larvae/worm (Crowcroft and Gillespie 1991) and 6,000 larvae/20 worms (Shobana 2003). Higher recovery of larvae in the present study can be attributed to the use of histopaque method and dissection of individual worms rather than homogenization of entire worms as followed by earlier workers.

RPMI 1640 medium with L glutamine and 25 mM HEPES were utilised for larval culture in the present study (Oaks and Kayes 1979; Bowman et al. 1987; Williamson et al. 1990; Crowcroft and Gillespie 1991; Iddawela et al. 2007; Kim et al. 2008 and Ponce-Macotela et al. 2011). Other media were also found to be good to maintain larval culture such as HMEM (de Savigny 1975), Eagle's minimum essential medium (Gupta 1984), Dulbecco's modified Eagles medium (Boyce et al. 1988 and Morales et al. 2002). However HEPES buffered RPMI 1640 medium containing L glutamine was found to be good for larval culture in terms of better survival of larvae and higher yield of ES products (Badley et al. 1987 and Rajapakse et al. 1992) as observed in the present study.

Larval culture was maintained up to 3 weeks with more than 70 % of larval viability and without any contamination in this study. This was contrary to the report of de Savigny (1975) and (Sugane and Oshima 1983) who maintained the larvae for about 18 months and up to 3 months respectively. This variation may be attributed to the difference in the method adopted like use of histopaque solution or pH change of the medium on incubation.

Concentration of culture fluid containing ES antigen was achieved using PEG 20,000 in this study (Roldan and Espinoza 2009). Many authors reported the usage of ultra centrifugal devices with molecular weight cut off of

10,000 Da for concentration of culture fluid (Badley et al. 1987; Bowman et al. 1987 and Kim et al. 2008).

Protein content of crude ES antigen in the present study was found to be 2.25–3.9 mg/ml. However Bowman et al. (1987) reported a protein concentration of 2–10 mg/ml who maintained 10^7 larvae/ml of RPMI 1640 medium compared to 10^3 larvae/ml of the medium in the present study. de Savigny (1975) reported 0.5–1 mg of lyophilised ES products weekly from a culture of 10^7 larvae maintained in 500 ml of HMEM while Badley et al. (1987) reported a production of 8 ng protein/larva/day. This variation in production of TES antigen is mainly due to different protocols used by various authors.

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References

- Alcantara-Neves NM, dos Santos AB, Mendoca LR, Figueiredo CA, Pontes de Carvalho L (2008) An improved method to obtain antigen excreting *Toxocara canis* larvae. *Exp Parasitol* 119:349–351
- Aydenizoz-Ozkayhan M, Yagu BB, Erat S (2008) The investigation of *Toxocara canis* eggs in coats of different dog breeds as a potential transmission route in human toxocariasis. *Vet Parasitol* 152:94–100
- Badley JE, Grieve RB, Bowman DD, Glickman LT, Rockey JH (1987) Analysis of *Toxocara canis* excretory secretory antigens: physiochemical characterization and antibody detection. *Parasitol* 73:593–600
- Beaver PC, Snyder CH, Carrera GM (1952) Chronic eosinophilia due to visceral larva migrans. *Pediatrics* 9:7–19
- Bowman DD, Grieve M, Grieve RB (1987) Circulating excretory antigen levels and specific antibody responses in mice infected with *Toxocara canis*. *Am J Trop Med Hyg* 36:75–82
- Boyce WM, Branstetter BA, Kazacos KR (1988) Comparative analysis of larval excretory-secretory antigens of *Bayliascaris procyonis*, *Toxocara canis* and *Ascaris suum* by western blotting and enzyme immunoassay. *Int J Parasitol* 18:109–113
- Colli CM, Rubinsky-Elefant G, Paludo ML, Falavigna DLM, Guilherem EV, Mattia S, Araujo SM, Ferreira EC, Previdelli ITS, Flavigna-Guilherem AL (2010) Serological, clinical and epidemiological evaluation of toxocariasis in urban areas of South Brazil. *Rev Inst Med Trop Sao Paulo* 52:69–74
- Crowcroft NS, Gillespie SH (1991) Hatching of second stage larvae of *Toxocara canis*: a rapid method for processing large numbers of worms. *J Helminthol* 65:311–312
- de Savigny DH (1975) In vitro maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigen for use in serodiagnostic tests for visceral larva migrans. *J Parasitol* 61:781–782
- de Savigny DH, Tizzard IR (1977) *Toxocara* larva migrans: the use of larval secretory antigens in haemagglutination and soluble antigen fluorescent antibody tests. *Trans Roy Soc Trop Med Hyg* 71:501–507
- de Savigny DH, Voller A, Woodruff AW (1979) Toxocariasis: serological diagnosis by enzyme linked immunosorbent assay. *J Clin Pathol* 32:284–288
- Girdwood RWA, Smith HV, Bruce RG, Quinn R (1978) Human *Toxocara* infection in the West of Scotland. *Lancet* 1:1318
- Glickman LT, Schantz PM (1981) Epidemiology and pathogenesis of zoonotic toxocariasis. *Epidemiol Rev* 3:230–250
- Glickman LT, Grieve RB, Lauria S, Jones DL (1985) Serodiagnosis of ocular toxocariasis: a comparison of two antigens. *J Clin Pathol* 38:103–107
- Gupta AK (1984) A simple method of artificial hatching of *Toxocara canis* in vitro and preparation of excretory secretory antigen. *Curr Sci* 53:529–530
- Iddawela RD, Rajapakse RPVI, Perera NAND, Agastuma T (2007) Characterization of *Toxocara canis* species specific excretory secretory antigen and development of a double sandwich ELISA for diagnosis of visceral larva migrans. *Korean J Parasitol* 1:19–26
- Kim Y, Huh S, Chung Y (2008) Seroprevalence of toxocariasis among healthy people with eosinophilia. *Korean J Parasitol* 1:29–32
- Magnaval JF, Glickman LT, Dorchie PH (1994) La toxocarise, une zoonose helminthique majeure. *Rev Med Vet* 145:611–627
- Magnaval JF, Galindo V, Glickman LT, Clanet M (1997) Human *Toxocara* infection of the central nervous system and neurological disorders: a case control study. *Parasitol* 115:537–543
- Magnaval JF, Glickman LT, Dorchie P, Morassin B (2001) Highlights of human toxocariasis. *Korean J Parasitol* 39:1–11
- Maizels RM, de Savigny DH, Ogilvie BM (1984) Characterization of surface and excretory secretory antigens of *Toxocara canis* infective larvae. *Parasite Immunol* 6:23–37
- Mizgajski H (2001) Eggs of *Toxocara* spp in the environment and their public health implication. *J Helminthol* 75:147–151
- Morales OL, Lopez MC, Nicholls RS, Agudelo C (2002) Identification of *Toxocara canis* antigens by western blot in experimentally infected rabbits. *Rev Inst Med Trop Sao Paulo* 44:213–216
- Nagakura K, Tachibana H, Kaneda Y, Kato Y (1989) Toxocariasis possibly caused by ingesting raw chicken. *J Infect Dis* 160:735–736
- Nagakura K, Kanno S, Tachibana H, Kaneda Y, Ohkido M, Kondo K, Inoue H (1990) Serologic differentiation between *Toxocara canis* and *Toxocara cati*. *J Infect Dis* 162:1418–1419
- Oaks JA, Kayes SG (1979) Artificial hatching and culture of *Toxocara canis* second stage larvae. *J Parasitol* 65:969–970
- Ponce-Macotela M, Cabellero AR, Abarca GEP, Gordillo MNM (2011) A simplified method for hatching and isolating *Toxocara canis* larvae to facilitate excretory-secretory antigen collection in vitro. *Vet Parasitol* 175:382–385
- Rajapakse RPVI, Vasanthathilake VWSM, Fernando ST (1992) Collection of eggs and hatching and culturing second stage larvae of *Toxocara vitulorum* in vitro. *J Parasitol* 78:1090–1092
- Roddie G, Stafford P, Holland C, Wolfe A (2008) Contamination of dog hair with eggs of *Toxocara canis*. *Vet Parasitol* 152:85–93
- Roldan WH, Espinoza YA (2009) Evaluation of an enzyme linked immunoelectrotransfer blot test for the confirmatory serodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz* 104:411–418
- Roldan W, Cornejo W, Espinoza Y (2006) Evaluation of the dot enzyme linked immuno sorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz* 101:71–74
- Rubinsky-Elefant G, da Silva-Nunes M, Malafronte RS, Muniz PT, Ferreira MU (2008) Human toxocariasis in rural Brazilian Amazonia: seroprevalence, risk factors and spatial distribution. *Am J Trop Med Hyg* 79:93–98
- Shobana M (2003) Immunodiagnosis of *Toxocara canis* infection. M.V.Sc. thesis submitted to the Tamilnadu Veterinary and Animal Science University, Chennai
- Smith HV (1989) A rapid method for hatching infective eggs of *Toxocara canis*. *Trans Roy Soc Trop Med Hyg* 83:215

- Smith HV, Quinn R, Bruce RG, Girdwood RWA (1980) A paper radioimmunosorbent test (PRIST) for the detection of larva specific antibodies to *Toxocara canis* in human sera. *J Immunol Methods* 37:47–55
- Smith HV, Girdwood RWA, Quinn R, Bruce RG (1982) The development of early serological responses in rabbits infected with *Toxocara canis* and *Toxascaris leonina*. *Trans R Soc Trop Med Hyg* 76:89–94
- Sturchler D, Weiss N, Gassner M (1990) Transmission of toxocariasis. *J Infect Dis* 162:571–572
- Sugane K, Oshima T (1983) Purification and characterization of excretory secretory antigen of *Toxocara canis* larvae. *Immunology* 50:113–120
- Taira K, Saeed I, Permin A, Kapel CM (2004) Zoonotic risk of *Toxocara canis* infection through consumption of pig or poultry viscera. *Vet Parasitol* 121:115–124
- Taylor M, Keane C, O' Connor P, Mulvihill E, Holland C (1988) The expanded spectrum of toxocaral disease. *Lancet* 1:692–695
- van Knapen F, van Leusden J, Polderman AM, Franchimont JH (1983) Visceral larva migrans: examinations by means of enzyme linked immunosorbent assay of human sera for antibodies to excretory secretory antigens of the second stage larvae of *Toxocara canis*. *Z Parasitenkd* 69:113–118
- Vasquez Tsuji O, Barbabosa IM, Zavala JT, Hernandez AR, Torres HP (1997) Vegetables for human consumption as probable source of *Toxocara* spp. infection in man. *Bol Chil Parasitol* 52:47–50
- Watthanakulpanich D, Smith HV, Hobbs G, Walle AJ, Billington D (2008) Application of *Toxocara canis* excretory secretory antigen and IgG subclass antibodies (IgG 1–4) in serodiagnostic assays of human toxocariasis. *Acta Trop* 106:90–95
- Williamson HJE, Allardyce RA, Clemell RS, Hidajat RR (1990) Serum and neutrophils alter the rate of excretory secretory antigen release by *Toxocara canis* infective larvae in vitro. *Parasite Immunol* 12:175–187