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# Serodiagnosis of Visceral Toxocariasis by the Indirect Immunofluorescent Antibody Test: Can it be Revived?

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**Abstract:** Human toxocariasis is one of the most common zoonotic helminth infections in temperate countries worldwide. Serodiagnosis is necessary as direct histological evidence of parasitic infection is rarely forthcoming. The most appropriate currently used serodiagnostic approach is by enzyme-linked immunosorbent assay employing excretory/secretory antigen harvested from *T. canis* larvae (ES-ELISA), followed by a confirmatory analysis by Western blotting. In developing countries, due to the high cost and scarcity of central laboratories with adequate supplies and expertise, few seroepidemiological studies have been carried out to elucidate the prevalence of toxocariasis. Hoping for a more simple, easily applied serological test, the objective was to assess the diagnostic accuracy of an indirect Immuno Fluorescence Antibody Test (IFAT) using *Toxocara canis* Embryonated Eggs (EE), intact Hatched Larva (HL) and Adult Frozen Sections (FSA) as antigens for detection of anti-Toxocara antibodies. Diagnostic sensitivity was assessed using sera from Swiss albino mice post-infection with larvated *Toxocara canis* eggs while diagnostic specificity was assessed using a battery of sera collected from mice experimentally infected with other parasites as well as sera from laboratory bred control mice free from parasitic infections. EE, HL and FSA-IFAT antigens showed sensitivity of 83, 86 and 75%; specificity of 94.1, 97.4 and 88.2% and diagnostic accuracy of 93.4, 96.7 and 86.4%, respectively.

**Key words:** *Toxocara canis*, anti-Toxocara antibodies, IFAT, IFAT, antigens, sensitivity, specificity, diagnostic accuracy

## INTRODUCTION

Human toxocariasis is a common parasitic zoonosis in temperate countries worldwide (Demirci *et al*., 2010; Watthanakulpanich, 2010; Rubinsky-Elefant *et al*., 2010). It is caused primarily by *Toxocara canis* and *Toxocara cati* which are cosmopolitan nematode parasites of canids and felids (Overgaauw, 1997; Despommier, 2003; Smith *et al*., 2009). Transmission is fecal-oral and infections in human hosts can occur upon accidental ingestion of embryonated eggs, e.g., by ingestion of raw vegetables (Overgaauw, 1997; Despommier, 2003). Visceral toxocariasis comprises a heterogeneous group of clinical disorders caused by the migration of infective nematode larvae of Toxocara species through the internal organs of the host (Nash, 2000; Smith *et al*., 2009). Equivocal diagnosis is frequently obtained since it is based primarily upon non-specific criteria. The clinical presentation is not pathognomonic, the eggs are never found in feces and the most suggestive laboratory finding, i.e., eosinophilia is not conclusive. The only confirmatory diagnosis is identification of larvae in biopsies in infected tissue but this is usually impractical (Parsons *et al*., 1986; Schantz, 1989). Serodiagnosis of toxocariasis have become widely accepted as the most appropriate diagnostic approach (Korkmaz, 1998; Nagakura *et al*., 1990; Magnaval *et al*., 2001) and it is usually performed by Enzyme-Linked Immuno Sorbent Assay (ELISA) using Toxocara excretory-secretory antigens released by the tissue-invasive larvae *in vitro* followed by a confirmatory analysis for instance, Western blotting (Smith *et al*., 2009). Despite the fact that such reliable immunodiagnostic methods are currently readily available, few seroepidemiological studies have been carried out to elucidate the prevalence of toxocariasis in developing countries (Smith *et al*., 2009), most probably due to its high cost and scarcity of central laboratories with adequate supplies and expertise.

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The diagnostic accuracy of any serological test to detect and quantify the immune response to parasitic infection varies greatly according to the type of antigen preparation and the serological method. Early fluorescent antibody studies on *Toxocara canis* were made and proved to be a simple sensitive method for diagnosis of human toxocariasis (Khalil *et al*., 1989; Eid *et al*., 1991). However, a standardized indirect Immuno Flourescent Antibody Test ( IFAT) has not yet found its way into routine diagnostic practice. So, the present research was designed to standardize a simple reliable IFAT test that can be applicable for serodiagnosis of human toxocariasis in developing countries and when adequate supplies and expertise to do ES-ELISA and Western blotting are not available.

## MATERIALS AND METHODS

**Source of the parasite:** *Toxocara canis* females were collected from stray dogs, washed in saline and dissected for collection of ova. Embryonation of ova was obtained by incubation at room temperature in 1% formalin solution with continuous aeration for 1 month (Zyngier, 1974).

**Laboratory animals:** Two groups of laboratory-bred Swiss albino mice 6 weeks old males were maintained as follows: Group I consisted of 90 mice infected orally with 800 larvated eggs/mouse (Chieffi *et al*., 1995). Batches of 5 infected mice were sacrificed at weekly intervals starting from the 5th up to the 18th week post-infection, sera were collected and stored at -70°C until used to evaluate the sensitivity of the IFAT-antigens. Group II (control group) consisted of 123 laboratory maintained mice experimentally infected with parasites other than *T. canis* as well as 30 laboratory-bred healthy mice free from parasites. Sera collected from sacrificed animals of this group were used to evaluate the specificity (cross reactivity). All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Saudi Arabia).

**Experimental infection of the control mice with different parasites:** A total of 25 mice were infected with *S. mansoni* (Egyptian strain) by subcutaneous injection of 100 carcariae/mouse as described by Peters and Warren (1969), 30 mice by oral inoculation of 300 viable *T. spiralis* larva/mouse as described by Despommier *et al*. (1977), 22 mice by oral inoculation of 1000 viable *H. nana* eggs/mouse obtained from the stool of heavily infected patients (Rifaat *et al*., 1978), 28 mice by oral inoculation of 150,000 *G. lamblia* cysts/mouse (Vinayak *et al*., 1979) and 18 mice by intraperitoneal injection with 0.2 mL/mouse of brain suspension containing 10 cysts of *T. gondii* avirulent strain (Mahmoud *et al*., 1977). Sacrifice of these mice and collection of sera were done at the time of appearance of circulating antibodies i.e., 8 weeks PI for *S. mansoni* (Hammouda *et al*., 1994) and *T. spiralis* (El-Temsahi *et al*., 1992); 4 weeks PI for *H. nana* (Furukawa *et al*., 1984) and *T. gondii* (Handman and Remington, 1980).

**Preparation of *T. canis* IFAT antigens:** Embryonated Egg (EE) antigen was prepared according to Azab *et al*. (1984). Intact second stage larvae hatched from eggs (HL) were prepared as antigen as described by Rajapakse *et al*. (1992). Frozen Sections of Adults (FSA) were prepared for use as antigen according to Ambroise-Thomas and stored unfixed at -70°C until used. The IFAT was performed on pooled sera according to Viens *et al*. (1975).

**Statistical analysis:** Data were computerized and statistically analyzed using the Chi square test (SPSS, 1999).

## RESULTS AND DISCUSSION

Results are shown in Table 1-4. Although, human toxocariasis ranks among the most common zoonotic infections worldwide it remains relatively unknown to the public and few seroepidemiological studies have been carried out (Rubinsky-Elefant *et al*., 2010). Early fluorescent antibody studies on *Toxocara canis* were made by Bisseru and Woodruff (1968) and Woodruff (1970) who used embryonated eggs and partially digested larvae as antigens, respectively. Krupp (1974) used larval lysates and extracts while Viens *et al*. (1975) used sectioned lavae. De Savigny and Tizard (1977) used the larval excretory-secretory antigens in the soluble antigen fluorescent antibody test. Welch and Dobson (1978) used

Table 1: Sensitivity of different *T. canis* IFAT antigens (EE, HL and FSA) in serodiagnosis of toxocariasis

| | Sera of infected mice (No. 90) | | | |
|---|---|---|---|---|
| Type of antigen | No +ve | No -ve | Higher titer | Sensitivity (%) |
| EE | 83 | 7 | 512 | 92.2 |
| HL | 86 | 4 | 1024 | 95.6 |
| FSA | 75 | 15 | 256 | 83.3 |
| Statistical analysis | | | | p<0.01* |

EE: Embryonated Eggs; HL: Hatched Larvae; FSA: Adult Frozen Sections; *Significant difference

Table 2: Number of false positives (cross reactions) among sera of control mice as detected by EE, HL and FSA-IFAT antigens

| | | No. of false positives (%) at 1/16 | | | No. of false positives (%) at 1/24 | | |
|---|---|---|---|---|---|---|---|
| Diagnostic state | No. | EE | HL | FSA | EE | HL | FSA |
| Schistosomiasis | 25 | 4 (16.0) | 2 (8.0) | 5 (20.0) | 3 (12.0) | 1 (4.0) | 4 (16.0) |
| Trichinosis | 30 | 3 (10.0) | 2 (6.7) | 3 (10.0) | 2 (6.7) | 1 (3.3) | 2 (6.7) |
| Hymenolepiasis | 22 | 4 (18.2) | 2 (9.1) | 5 (22.7) | 3 (13.6) | 1 (4.5) | 4 (18.2) |
| Giardiasis | 28 | 2 (7.1) | 2 (7.1) | 3 (10.7) | 1 (3.6) | 1 (3.6) | 2 (7.1) |
| Toxoplasmosis | 18 | 1 (5.6) | 1 (5.6) | 4 (22.2) | 0 (0.0) | 0 (0.0) | 3 (16.7) |
| Total parasitic | 123 | 14 (11.4) | 9 (7.3) | 20 (16.3) | 9 (7.3) | 4 (3.3) | 15 (12.2) |
| Healthy controls | 30 | 2 (6.7) | 1 (3.3) | 5 (16.7) | 0 (0.0) | 0 (0.0) | 3 (10.0) |
| Total no. examined | 153 | 16 (10.5) | 10 (6.5) | 25 (16.3) | 9 (5.9) | 4 (2.6) | 18 (11.8) |

EE: Embryonated Eggs; HL: Hatched Larvae; FSA: Adult Frozen Sections

Table 3: Specificity of *T. canis* IFAT antigens (EE, HL and FSA) using sera of healthy mice and mice infected with different parasites

| | Specificities of IFAT antigens (IFAT titer) (%) | | | | | |
|---|---|---|---|---|---|---|
| | Parasitic infection (123) | | Healthy controls (30) | | Total cases (153) | |
| IFAT antigen | 1/16 | 1/64 | 1/16 | 1/64 | 1/16 | 1/64 |
| EE | 88.6 | 92.7 | 93.3 | 100 | 89.5 | 94.1 |
| HL | 92.7 | 96.7 | 96.7 | 100 | 93.5 | 97.4 |
| FSA | 83.7 | 87.8 | 83.7 | 90 | 83.7 | 88.2 |
| Statistical analysis | $p>0.05^{\#}$ | $p<0.05^{*}$ | $p<0.05^{*}$ | $p<0.05^{*}$ | $p<0.05^{*}$ | $p<0.01^{*}$ |

EE: Embryonated Eggs; HL: Hatched Larvae; FSA: Adult Frozen Sections; *Significant; #Insignificant

Table 4: Diagnostic accuracy of *T. canis* IFAT antigens (EE, HL and FSA) in serodiagnosis of toxocariasis

| IFAT antigens | Sensitivity (%) | Specificity (%) | Diagnostic accuracy (%) |
|---|---|---|---|
| EE | 92.2 | 94.1 | 93.4 |
| HL | 95.6 | 97.4 | 96.7 |
| FSA | 83.3 | 88.2 | 86.4 |
| Statistical analysis | $p<0.01^{*}$ | $p<0.01^{*}$ | $p<0.05^{*}$ |

EE: Embryonated Eggs; *Significant difference; HL: Hatched Larvae; AFS: Adult Frozen Sections

pure adult worm antigens purified by affinity chromatography. Khalil *et al.* (1989) used embryonated eggs and frozen sections of adults. In spite of all these methods, a standardized IFAT antigen has not yet found its way into routine and practical diagnosis of visceral toxocariasis. In an attempt to identify an easy applicable IFAT antigen for current use in ordinary laboratories, the present research presents a comparative evaluation of three unsophisticated IFAT antigens, namely EE, HL and FSA. The mouse model was used to assess sensitivities and specificities of such antigens. Out of 90 sera samples collected from experimentally infected mice the HL antigen provided positive results in 86 (95.6%) compared to 83 (92.2%) and 75 (83.3%) by EE and FSA antigens (Table 1), respectively with statistically significant difference ($p<0.01$) (Table 1). The HL antigen provided positive results up to a dilution of 1024 compared to 512 and 256 by EE and FSA antigens, respectively. This indicates that HL antigen was more sensitive in detecting small amounts of serum antibodies that is more sensitive in diagnosis of early infection. This is in close agreement with Viens *et al.* ( 1975) and Glickman *et al.* (1985) who reported that larval antigens were more sensitive than adult worm antigens. Also, Khalil *et al.* (1989) found that embryonated egg antigen was more sensitive than frozen sections from adult worms. The negativity of four samples by using HL antigen in the present research was recorded 1 week post-infection and was most probably due to inapparent antibodies at that early post-infection time. This may be in agreement with Chieffi *et al.* (1995) who reported that the IFAT detected *T. canis* antibodies within 2 weeks after infection. The high sensitivity obtained by EE antigen (92.2%) in the present research indicates its permissibly in serodiagnosis of toxocariasis. Also inspite of the less sensitivity recorded by FSA antigen (83.3%) Eid *et al.* (1991) encouraged the use of adult Toxocara antigen due to presence of shared antigens with the larvae.

In the present research, cross reactions between *T. canis* and other parasites were detected by the three antigens. However, HL antigen was the least to show false positives. Using a serum dilution of 1/64 , only 4 out 123 parasitic cases (3.3%) gave false positive reactions compared to 9 cases ( 7.3%) and 15 cases (12.2%) detected by FSA antigen, respectively. Among healthy controls and at serum dilution of 1/64, no false positives were recorded except 3 out of 30 cases (10%) detected by FSA antigen. In other words and among the total control cases , HL antigen gave a specificity of 97.4% which was significantly ($p>0.01$) higher than that provided by EE (94.1) and FSA (88.2%) antigens (Table 1). These results are in conformity with Bowman *et al.* (1987) who found that immunoflourescence on the cuticle of whole

undamaged larvae was specific. In published literatures, cross reactions between *T. canis* and other parasites were infrequently reported in except with Ascaris (Woodruff, 1970; Stevenson and Jacobs, 1977; Cypess *et al*., 1977; Page *et al*., 1991); *W. bancrofti* (Woodruff, 1970); *T. spiralis* (Cypess *et al*., 1977); other *Toxocara* species namely *T. vitulorum*, *T. leonine* and *T. cati* (Page *et al*., 1991) and *T. tricura* (Hakim *et al*., 1992).

## CONCLUSION

The result of this study show that HL-IFAT proved to be an easy, sensitive, specific test with high diagnostic accuracy and its applicability for diagnosis of visceral toxocariasis has to be revived and standardized at least in developing countries and when adequate supplies and expertise to do ES-ELISA and Western blotting are scarce. The use of intact *Toxocara canis* hatched larvae as an antigen in the IFAT provided a sensitivity of 95.6%, specificity of 97.4% and a diagnostic accuracy of 96.7%. So, it constitutes a reliable method for diagnosis of visceral toxocariasis and its applicability has to be revived and standardized at least in developing countries and when adequate supplies and expertise to do ES-ELISA and Western blotting are scarce.

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