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Immunodiagnosis of Human Toxocariasis

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1. Introduction

Human toxocariasis is a helminthic zoonosis caused by larval stages of *Toxocara canis* and, less frequently, by *T. cati*, the roundworms of dogs and cats, respectively. Accidentally, humans ingest embryonated eggs containing the infective larva which are released in the upper small intestine and then pass through the intestinal epithelium to reach the blood vessels, where they can migrate to the different visceral organs and tissues of the body (Despommier, 2003).

An interesting phenomenon is that these parasites cannot develop into adult forms in humans and are restricted to larval forms, migrating through the soft tissues for months and even years and causing local or systemic inflammatory reactions in the affected organ. Sometimes the immune system can even kill the parasite, however, the immunity generated with the first infection fails to protect against future reinfections. It has been reported that the larvae can survive for many years and even decades in the human host, causing local tissue necrosis, eosinophilic inflammatory reactions and granuloma formation (Schantz & Glickman, 1978; ; Minvielle et al., 1999; Magnaval et al., 2001; Despommier, 2003).

The spectrum of the clinical manifestations in human toxocariasis varies widely from asymptomatic cases to systemic infections. Clinical manifestations and the course of the disease will be determined by the inoculum, the frequency of reinfection episodes in the patient, the location of migrating larvae in the affected organ, and the host response (Pawlowski, 2001).

However, both the inoculum and the frequency of reinfection episodes cannot be measured directly in humans, but they can be assumed through the frequency of contaminated environments with *Toxocara* spp. eggs or by the proportion of children with habits of geophagy in the area or region studied (Magnaval et al., 2001; Pawlowski, 2001; Smith et al., 2009). Migrating larvae may be identified by means of clinical examination and the use of diagnostic imaging tests in order to looking for granulomas, either in the eye, brain or liver (Despommier, 2003). Even when it cannot be directly observed, the imaging diagnostics proves to be of aid to suspect the causative agent; however, all clinical suspicion should be confirmed by additional laboratorial tests (Chieffi et al., 2009; Rubinsky-Elefant et al., 2010), as discussed below.

2. *Toxocara* and toxocariasis in humans

Toxocariasis is more probable in tropical and subtropical areas and it is considered of higher risk in populations from both periurban and rural areas with poor sanitation, and where the people do not usually deworm their pets (Glickman & Schantz, 1981). However, this concept has been reversed as there are confirmed cases of toxocariasis in people who have never had dogs at home, which has led to an awareness of the environmental contamination with parasitized dog feces, especially in public parks, children playgrounds and streets (Holland et al., 1991; Uga et al., 1996; Habluetzel et al., 2003; Avcioglu & Balkaya, 2011).

Adult stages of *Toxocara canis* and *T. cati* have their habitat in the small intestine of their definitive hosts: the canines and felines. The adult gravid female of the parasites release their eggs in the intestinal lumen, which are then released outside with the feces. *Toxocara* eggs are not immediately infective. The larval development inside the egg varies according to both the humidity and temperature from the environment. With a temperature of 15 - 35°C and a relative humidity of 85%, most of the eggs become infective after 2 to 5 weeks (Glickman et al., 1979; Despommier, 2003). However, with temperatures above 35 °C the egg become inactivated, but if the temperature is below 15 °C, the larval development is slow, but not destroyed (O'Lorcain, 1995). Most of the reviewed literature considers that a *Toxocara* egg become infective when it contain the second larval stage. Nevertheless, some authors have reported that two changes occur inside the egg and, therefore, the infective phase would be the third larval stage (Araujo, 1972; Minvielle et al., 1999).

Dogs and cats become infected with *Toxocara* by either ingesting infective eggs, ingesting tissues from paratenic hosts containing larvae, by transplacental migration of larvae to the developing puppies, by transmamary passage of larvae through the milk in young puppies, or by ingesting immature adults stages present in the vomit or feces of infected puppies (Minvielle et al., 1999; Despommier, 2003).

When infective *Toxocara* eggs are ingested by puppies, the larvae are released in the small intestine and invade the intestinal mucosa, enter the lymphatic or blood vessels and reach the liver within 24 to 48 hours. Through the bloodstream, the larvae reach the heart and lungs, arrive to the alveolar capillaries and then ascend the respiratory tree to reach the pharynx, where they are swallowed. During the migration, the larvae undergo two changes of their body and finally complete the development process to the adult stage in the small intestine. Egg production occurs between 4 and 6 weeks postinfection (Despommier, 2003).

Interestingly, this process do not occurs in dogs older than 6 months because the migrant larvae do not follow the tracheal route and penetrate into the pulmonary veins and are distributed in almost all the body by means of the systemic circulation, especially in the lungs, liver, kidney, skeletal muscles, but also in the brain. This distribution is known as somatic migration where the parasite is restricted to the larval stage and remains dormant for years (Glickman & Schantz, 1983; Overgaauw, 1997).

Toxocara eggs can also be infective for a number of other species different from canines or felines, a phenomenon known as 'paratenesis' (passage of the infective parasite by one or various hosts with no effect on the completion of its life cycle). Paratenic hosts for toxocariasis include earthworms, mice, rats, rabbits, chickens, pigs, pigeons, sheep, and humans. As the larvae remain alive in the paratenic host for years, a predator may become infected when ingest this parasitized tissues. If a predator is a canine or feline, the larvae

complete their development in the alimentary tract, but it depends of the age (Minvielle et al., 1999; Magnaval et al., 2001; Despommier, 2003).

Parasitized puppies are the main reservoir of *Toxocara*. The contamination level produced by a female dog and her parasitized puppies in the immediate area of their habitat is very high. If we consider that adult stages of *Toxocara* have an average life of 4 months and each female worm produce about 200,000 eggs per day, and the intestinal worm charge may range from one to hundreds of them, therefore, infected puppies may contaminate the environment with millions of eggs each day (Minvielle et al., 1999; Despommier, 2003).

Humans become infected by accidental ingestion of embryonated *Toxocara* eggs containing the infective third larval stage. The larvae are released in the proximal small intestine, penetrate the mucosa and, through the blood vessels, they reach the portal vein and then, they may migrates through the systemic circulation. The larvae are distributed throughout the body causing hemorrhages, inflammatory process and granulomas. Many larvae seem to remain 'dormant' for many years and then continue their migration. Eventually some of them may be destroyed by the host immune response, while others seem to be protected by encapsulating them (Magnaval et al., 2001; Despommier, 2003).

A large proportion of *Toxocara* infections are either asymptomatic or have nonspecific symptoms. The most frequently involved organs are liver, lungs, brain, eyes, heart, and skeletal muscles. Clinically, the chronic forms of human toxocariasis may be widespread or localized; being the latter the most common and it can also lead to blindness (Pawlowski, 2001; Despommier, 2003).

The clinical manifestations may be divided into an acute phase (usually uncertain and unspecified) and a chronic phase. The acute phase of infection takes place immediately after the larvae penetrate the intestinal epithelium and reach the blood vessels and through them, migrate to the liver, which is the first organ to be affected. The inflammatory response degree of the live will depend on the number of migrating larvae to be ingested by the host, because a small number of larvae can be achieved unnoticed into the portal vein without producing signs. From there, the larvae can travel to other organs like heart, lungs, and kidney, starting the chronic phase of the infection. This migration may also include immunologically privileged organs such as the eye and brain (Pawlowski, 2001; Smith et al., 2009). Larval migration may generates nonspecific symptoms such as myalgia, fever, malaise, and may cause wheezing and airway hyperresponsiveness, especially in children or predisposed people.

The spectrum of clinical manifestations in human toxocariasis varies widely from asymptomatic cases to systemic forms of the disease; probably due to the size of inoculum and the host response against the migrating larvae (Taylor et al., 1988, Pawlovski, 2001). During the larval migration, the larva releases a high content of metabolic antigens that leads to the activation of the host immune system which generates an immunopathogenic mechanism that causes the clinical manifestations of the disease. The immune response against the parasite is mediated in different proportions of either T-helper 1 (Th1) cells (which leads to a granuloma formation) and T-helper 2 (Th2) cells (an increased production of IgE antibodies and eosinophils) (Sugane & Oshima, 1984; Kayes, 1997; Pawlovski, 2001). In this point, allergic, atopic or eosinophil-related manifestations may occur and depend on the type of response of each *Toxocara* infected host.

3. Clinical manifestations of human toxocariasis

Several clinical forms of human toxocariasis have been described, but until moment, few attempts were made to classify the clinical expressions derived from *Toxocara* infections. A proposed classification made by Pawlovski (2001) correlate the observed clinical status, the involvement of immunopathologic mechanisms, the intensity of the serological response, and the location of *Toxocara* larvae. This new classification divides human toxocariasis into four major forms namely: systemic, compartmentalized, covert and asymptomatic. However, Smith et al. (2009) still consider that human toxocariasis should be classified in three major forms: visceral larva migrans, ocular toxocariasis, and covert toxocariasis.

Visceral larva migrans, described by Beaver et al. (1952), is a severe systemic form of toxocariasis which is characterized by high eosinophilia, hepatosplenomegaly, pulmonary involvement, fever, hypergammaglobulinaemia, and elevated isohemagglutinins. Cases of LMV are uncommon and occur almost exclusively in children (Schantz & Glickman, 1978; Magnaval et al., 2001; Despommier, 2003). However, this syndrome may be restricted to clinically much less severe cases, in which only some signs of the classic visceral larva migrans form may occur, such as hepatomegaly and eosinophilia (Pawlovski, 2001). Many times the chronic eosinophilia is the main reason to suspect of toxocariasis (Magnaval et al., 2001; Pawlovski, 2001; Despommier, 2003). Hepatomegaly, fever and abdominal pain may be found when the compromise is exclusively hepatic (Magnaval et al., 2001). Dry cough, wheezing, bronchospasm, interstitial pneumonitis, and pleural effusion may occur when a lung involvement is present (Roig et al., 1992; Ashwath et al., 2004). Pruritus and eosinophilic urticaria may also present in some patients with dermatological manifestations (Kim et al., 2010). Other manifestations also include arthralgia, vasculitis, pericardial effusion, etc (Pawlovski, 2001; Despommier, 2003).

Ocular toxocariasis is a localized form of toxocariasis and is a result of the ocular invasion by *Toxocara* larvae, causing a series of clinical conditions, including endophthalmitis (Magnaval et al., 2001; Pawlovski, 2001; Despommier, 2003; Smith et al., 2009), which may be confused with a malignant tumor known as retinoblastoma (Minvielle et al., 1999; Magnaval et al., 2001; Despommier, 2003). The parasite is located within the ocular globe and often causes uveitis and retinal granulomas (Dernouchamps et al., 1990), which is confused with other etiologies and which may pass almost unnoticed, since the patient only afflicts progressive decrease in visual acuity (Pawlovski, 2001; Despommier, 2003); some patients present pain or bleeding due to severe intraocular inflammation (Magnaval et al., 2001; Despommier, 2003).

Another localized form of human toxocariasis which has become more important in recent years is the neurotoxocariasis, a clinical entity resulting from the invasion of the brain by *Toxocara* larvae (Finsterer & Auer, 2007). In the brain, *Toxocara* larvae are not encapsulated and the traces of their migration generally include small areas of necrosis and minimal inflammatory infiltration. Therefore, several cases of neurotoxoxariasis are asymptomatic while in others, the symptoms may vary widely. A case-control study on patients with *Toxocara* infection concluded that larval migration in the human brain does not necessarily induce neurological symptoms or signs (Magnaval et al., 1997), but some symptoms such as neurological deficits, focal seizures, generalized behavioral disorders and eosinophilic meningoencephalitis have been reported in different human cases of toxocariasis (Hill et al., 1985; Skerrett & Holland, 1997).

Covert toxocariasis is another form of toxocariasis whose term introduced by Taylor et al. (1987), is less well defined and frequently undiagnosed but it can commonly occur. Covert toxocariasis is characterized by nonspecific signs and symptoms that do not fall into the category of classic visceral larva migrans, ocular toxocariasis or neurotoxocariasis. Covert toxocariasis seems to depend less on a local reaction to the *Toxocara* larvae but is more an organ oriented immunopathological host response to continued stimulation of the host immune system by parasite antigens (Pawlovski, 2001). The clinical expression varies widely and may present as a pulmonary involvement such as asthma, acute bronchitis, pulmonitis with or without a Loeffler syndrome (Feldman & Parker, 1992; Buijs et al., 1997; Inoue et al., 2002), dermatological disorders such as chronic urticaria or eczema (Wolfrom et al., 1995), lymphadenopathy, myositis and a pseudorheumatic syndrome such as arthralgia (le Lauyer et al., 1990; Kraus et al., 1995).

Analysis of the causal relation of *Toxocara* infection with clinically observed symptomatology requires a good clinical knowledge and assessment of laboratory tests including the detection of IgG and IgE specific antibodies, eosinophilia and others (Magnaval et al., 2001; Despommier, 2003). Covert toxocariasis is often confirmed by alleviation or disappearance of specific symptoms and signs after specific anti-helmintic treatment (Magnaval et al., 2001; Pawlovski, 2001; Smith et al., 2009).

Asymptomatic toxocariasis or simply named *Toxocara* infection is often diagnosed by positive serology, occurs mainly in light or old infections and do not require anthelmintic treatment (Huapaya et al., 2009; Smith et al., 2009). Some cases may present mild eosinophilia, but it do not means a hazard for the patient, however, is important to consider this condition, especially in epidemiological studies on this zoonotic disease which are more frequent worldwide (Huapaya et al., 2009).

4. Diagnosis of human toxocariasis

Humans are considered as paratenic hosts within the life cycle of *Toxocara* where the larvae cannot develop into adult worms and, therefore, the parasitological examination of faeces does not contribute to the laboratory diagnosis (Rubinsky-Elefant et al., 2010). The diagnosis of toxocariasis is generally based on clinical signs and symptoms, which are non-specific, epidemiological data (contact with dogs or cats, geophagia, onychophagy, consumption of undercooked or raw meats) and laboratory findings (Chieffi et al., 2009; Despommier, 2003; Magnaval et al., 2001; Watthanakulpanich, 2010).

A definitive diagnosis of human toxocariasis is possible by locating the larvae in infected tissues, using histopathological examination. Tissue biopsy is rarely justified and it is generally insensitive and a time-consuming method (Pawlowski, 2001; Rubinsky-Elefant et al., 2010). Polymerase chain reaction (PCR)-based methods for *Toxocara* identification in clinical and environmental samples have been described (Fogt-Wyrwas et al., 2007; Zhu et al., 2001), but are not widely available. These methods should provide useful tools for the diagnosis and molecular epidemiological investigations of toxocariasis (Li et al. 2007). Parasite antigens can be detected in granulomas by immunohistochemical techniques and are helpful for toxocariasis diagnosis (de Brito et al., 1994; Musso et al., 2007). Medical imaging techniques such as ultrasonography, computed tomography and magnetic resonance imaging have been used to detect granulomatous lesions due to the migration of *Toxocara* larvae in different locations such as liver, nervous system and eye (Degouy et al.,

2001; Magnaval et al., 2001, Watthanakulpanich, 2010). *Toxocara* excreted-secreted products are highly immunogenic and promote a Th2 type immune response, leading to the production of interleukin 4 and 5, and consequently an increase of IgG, IgE antibodies and eosinophilia (Kayes et al., 1980; Del Prete, 1991). In visceral larva migrans, some frequent laboratorial findings are leukocytosis with intense eosinophilia, hypergammaglobulinemia and isohemagglutinin titer elevation (Jacob et al., 1994). However, in ocular toxocariasis, because of larval burden are relatively small, peripheral blood eosinophilia is frequently absent (Glickman & Schantz, 1981).

The diagnosis of ocular toxocariasis is usually based on clinical evaluation with the presence of ocular lesions, such as retinal or peripheral granulomas and endophthalmitis. Anti-*Toxocara* antibody titers are usually higher in visceral larva migrans than in ocular toxocariasis (Elefant et al., 2006). Even low ELISA titres in the serum may be of diagnostic value, but there is no consensus about the cut-off titres for diagnosis (Rubinsky-Elefant et al., 2010).

In covert toxocariasis some patients do not present eosinophilia and symptoms are unspecific (Magnaval et al., 2001). Detection of anti-*Toxocara* antibodies in clinically suspected toxocariasis patients in different samples, such as serum, ocular or cerebrospinal fluid is valuable to establish the diagnosis (Magnaval et al., 2001; Vidal et al., 2003; de Visser et al., 2008; Smith et al., 2009;).

5. Immunoserological techniques for diagnosing human toxocariasis

The limitations of the parasitological techniques to diagnosing human toxocariasis have encouraged numerous researchers to develop practical and accurate immunoassays. Several immunodiagnostic tests have been described, such as intradermal reaction, complement fixation, bentonite flocculation, agar-gel diffusion, indirect hemagglutination, immunofluorescence, radioimmunoassay and immunoenzymatic assays (Glickman & Schantz, 1981). The antigens used in these immunoassays included somatic extracts of adult worms, embryonated eggs, intact or sectioned larvae, and metabolic products of larvae collected *in vitro*.

Nowadays, the excretory-secretory antigens of *T. canis* larvae (TES) are widely used in serodiagnostic tests that are used for both the diagnosis and seroepidemiological studies (Roldán et al., 2010). These antigens are obtained from *in vitro* maintenance of infective larvae and are a mixture of highly immunogenic glycoproteins (Maizels et al., 1993). Since the first description of TES antigens production (De Savigny, 1975), few modifications in the method have been made. Recently, modified protocols for TES antigens production has been reported, increasing the parasite yield up to five fold, improving the larval purity and reducing the execution time of the protocol (Alcântara-Neves et al., 2008; Ponce-Macotela et al., 2011).

The use of validated serodiagnostic tests has provided a good understanding on the prevalence of human exposure to *Toxocara*. Toxocariasis is one of the few human parasitic diseases whose serodiagnosis uses a standardized antigen (Smith et al., 2009). Currently, the best serodiagnostic options are using the ELISA-IgG as a screening test and confirm any positive serum with an immunoblot test. In addition, each positive serum may also be confirmed by using an ELISA-IgE (Magnaval et al., 2001; Smith et al., 2009), as discussed below.

5.1 Enzyme-Linked Immunosorbent Assay

The Enzyme-linked immunosorbent assay (ELISA) test using TES antigens is the most common diagnostic method to detect anti-*Toxocara* IgG antibodies (De Savigny, 1979; Magnaval et al., 2001; Elefant et al., 2006; Smith et al., 2009), but it remains problematic in areas where the polyparasitism is endemic and the possibility of cross-reactions is high, reducing its diagnostic value. False positive results may occur in patients with ascariasis, strongyloidosis, trichinellosis, and fasciolosis (Magnaval et al., 2001; Ishida et al., 2003; Chieffi et al., 2009; Roldán et al., 2009; Smith et al., 2009). In order to reduce the cross-reactivity with other parasites, many authors have proposed previous serum absorption with extracts of a variety of nonhomologous parasites, i.e. pre-incubating serum samples with antigenic extracts of adult stages of *Ascaris suum* (Camargo et al., 1992; Nunes et al., 1997; Elefant et al., 2006), while others use a more comprehensive panel of antigen extracts from nematodes, cestodes and protozoa (Lynch et al., 1988).

The human IgG response elicited by *Toxocara* larvae may persist for many years (Cypess et al., 1977; Elefant et al., 2006) and, therefore, a positive result by ELISA-IgG cannot distinguish between past and current infection (Roldán et al., 2009). Moreover, high levels of anti-*Toxocara* antibodies may be found in preschool children, in comparison with older children or adults living in the same community (Rubinsky-Elefant et al., 2008), suggesting that IgG antibody levels tend to decrease when larvae are no longer viable in tissues.

In human toxocariasis, IgM antibodies are also generated and may be detected in both acute and chronic phases, differing from most of unrelated infections, in which they are transient (Smith, 1993).

Other antibody isotypes, such as IgE, may be detected by ELISA and result more specific than IgG; however, they are less sensitive for the diagnosis of human toxocariasis (Magnaval et al., 1992). In a follow-up of 23 children with visceral toxocariasis, Elefant et al., (2006) found that the levels of IgE antibodies were significantly decreased at one year post-treatment with thiabendazole in comparison with IgG levels which declined only four years post-treatment.

The measurement of the specific IgG avidity by ELISA suggests that it can help in distinguishing between acute and chronic infections (Hubner et al., 2001; Dziemian et al., 2008; Fenow et al., 2008). In the follow-up study after chemotherapy, Elefant et al. (2006) found high-avidity IgG antibodies at the time of the diagnosis, without further increase over the following years.

As an alternative to the ELISA, dot-ELISA has been standardized, presenting comparable sensitivity of standard ELISA. The advantages of the test are related to stability, lower cost and shorter execution time (Camargo et al., 1992; Roldán et al., 2006).

Regarding to the IgG subclasses, IgG2 and IgG3 antibodies yield sensitivities of 98% and 78%, respectively (Watthanakulpanich et al., 2008). On the other hand, the detection of IgG4 antibodies contributes to increase the specificity of the immunoassay (Noordin et al., 2005).

In ocular toxocariasis cases, probably due to the low number of infective larvae, serum anti-*Toxocara* antibodies may be present in very low titres or even undetectable (Sharkey & McKay, 1993; Glickman & Schantz, 1981; Magnaval et al., 2002). However, titers of specific antibodies in intraocular fluids, such as vitreous or aqueous humor, are usually higher than

those in serum, suggesting a local antibody production (Biglan et al., 1979; De Visser et al., 2008; Rubinsy-Elefant et al., 2010).

Because of the cross-reactions occurring in populations from tropical areas, some recombinant TES antigens have been expressed and used for the detection of specific IgG (Yamasaki et al., 2000; Wickramasinghe et al., 2008; Mohamad et al., 2009) and IgE antibodies (Norhaida et al., 2008) with promising results. Two of these recombinant antigens named rTES-30 (Yamasaki et al., 2000) and rTES-120 (Fong & Lau., 2004) provide more reliable diagnostic results and may be used for the development of serodiagnostic assays for human toxocariasis.

The detection of circulating *Toxocara* antigens has been reported by a capture enzyme-linked immunoassay using monoclonal antibodies (Robertson et al., 1988; Gillespie et al., 1993). The assay detects a carbohydrate epitope of TES antigens and proved to be useful in confirming acute visceral larva migrans diagnosis. Due to lack of specificity, the test was not recommended to be used alone in diagnosis (Gillespie et al., 1993). A monoclonal antibody to the TES-120 kDa antigen has been described and may be useful for determining both the parasite burden in early infection and the efficacy of chemotherapy (Yokoi et al., 2002).

5.2 Immunoblot assay

The immunoblot or Western blotting assay is a test that combines the high sensitivity of the immunoenzymatic tests with the high resolution of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This method has been successfully adapted for the confirmatory serodiagnosis of various parasitic diseases, including schistosomiasis, hydatidosis, cysticercosis, taeniasis, fasciolosis and strongyloidosis (as described in Roldán & Espinoza, 2009).

Immunoblot assays based on TES antigens have been proposed as confirmatory tests after screening by ELISA tests (Magnaval et al. 2001; Roldán & Espinoza, 2009). Two clusters of bands have been defined: low molecular weight bands (from 24 to 35 kDa) were reported as highly specific, while the high molecular bands (from 132 to 200 kDa) are unspecific (Magnaval et al., 1991). Morever, Nunes et al. (1997) found that a band between 55 to 66 kDa seems to be the responsible for the cross-reactivity between *T. canis* and *A. suum*.

An immunoblot was standardized for monitoring IgG, IgE and IgA antibodies after-chemotherapy in patients with toxocariasis. IgG antibodies to >205 kDa fractions, IgA to 29–38, 48–54, 81–93 kDa and IgE to 95–121 kDa were suggested as candidates for monitoring the treatment. Further identification of antigen epitopes related to these markers will allow the development of sensitive and specific immunoassays for the diagnosis and therapeutic assessment of toxocariasis (Rubinsky-Elefant et al., 2011).

Nowadays, Immunoblot assay is useful to confirm any positive serum by the ELISA test (where pre-absorption is not carried out) in patients with suspected toxocariasis (Magnaval et al., 1991; Roldán & Espinoza, 2009; Rubinsky-Elefant et al., 2011).

In order to give an idea of how to develop an immunoblot assay using TES antigens to detect specific IgG antibodies, we describe a protocol developed by Roldán & Espinoza (2009), however, we recommend to revise in detail other procedures described by other authors (Magnaval et al., 1991; Nunes et al., 1997; Rubinsky-Elefant et al., 2011).

The TES antigens may be obtained by *in vitro* maintaining larval stages of *T. canis* in RPMI 1640 cell culture medium supplemented with HEPES and glutamine, as described by Bowman et al. (1987). However, it is important to mention that some authors also use the traditional method described by De Savigny (1975), which uses Eagle's minimal essential medium supplemented with HEPES and glutamine (Nunes et al., 1997; Elefant et al., 2006). As a consequence of cultivating the parasite in different kind of culture media, it may generate different antigenic bands at the time of separating the TES antigens by SDS-PAGE.

The TES antigens should be diluted to a final concentration of 200 µg/mL with sample buffer (2.5 mM Tris-HCl, pH 8.0, containing 1% SDS, 50 mM dithiothreitol, 0.4% glycerol and 0.025% bromophenol blue), and then heated at 65°C for 15 min, as recommended by Tsang et al. (1991). In this part, it is important to mention that other authors use Laemmli's sample buffer to dilute the TES antigens.

The TES antigens are separated by SDS-PAGE at a constant voltage of 100V for 15 min (to move the proteins through the stacking gel) and then at 200V (to move the proteins through the resolving gel), until the bromophenol blue reach the end of the gel. Many authors usually have used 10% or 12% resolving polyacrylamide gels (Magnaval et al., 1991; Elefant et al., 2006), but others prefer use gradient resolving gels, such as 5-15% (Nunes et al., 1997; Rubinsky-Elefant et al., 2011) or 4-16% (Roldán & Espinoza, 2009). The relative molecular weight (MW) of the TES antigens may be calculated by using the wide-range molecular weight markers that are commercially available.

The separated TES antigens may be transferred to nitrocellulose sheets (0.2 µm pore size) using an electrotransfer blotting apparatus with a constant current of 2.0 A or a constant voltage of 100V. The time of blotting depends on the model of apparatus and it is very important to follow the instructions described in the manual of each apparatus.

The nitrocellulose sheets containing the TES antigens should be washed for 30 min with 0.01 M phosphate-buffered saline containing 0.3% Tween 20 (PBS-T), and then cut into 3-mm-wide strips and stored at -20°C until use.

These nitrocellulose strips may be incubated with human serum samples diluted at 1:50 in PBS-T containing 5% non-fat milk for 2 hours at room temperature or overnight at 4°C. After this period of incubation, the strips must be washed 3 times for 5 min each with PBS-T and then, the strips must be incubated for 2 hours with an anti-human IgG horseradish peroxidase conjugate diluted 1:1000 in PBS-T. After washing 3 times with PBS-T as above, the strips must be incubated with a freshly prepared substrate solution (15 mg of 3,3' -diaminobenzidine tetrahydrochloride, 30 mL of PBS and 10 µL of 30% hydrogen peroxide). After 5 minutes of incubation, the enzymatic reaction must be stopped by washing the strip with tap water. Positive reactions on the strip are determined by the visualization of defined brown bands judged using the naked eye.

Usually, a total of 9 antigenic bands (24, 28, 30, 35, 56, 67, 117, 136, y 152 kDa) may be detected using this procedure, but the diagnostics bands for the confirmatory serodiagnosis of human toxocariasis are the low molecular weight bands (from 24 to 35 kDa) (figure 1).

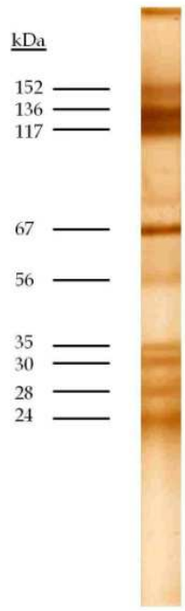

Fig. 1. An immunoblot strip showing the 9 antigenic bands used for the confirmatory serodiagnosis of human toxocariasis.

6. Final considerations

Traditional parasitological diagnostic approaches in toxocariasis, regarding the identification of the larvae, have innumerous limitations. Therefore, laboratory tests are very important to help the clinicians may give a reliable diagnosis. Human toxocariasis has been widely investigated, but many questions about to the diagnosis, effectiveness of chemotherapy, and prognosis remains to be elucidated.

Undoubtedly, the use of the TES antigens in ELISA tests has contributed to improve the immunodiagnosis and to carry out several epidemiological surveys. However, the levels of serum IgG antibodies may remain present for a long time, even in the absence of the disease. When using native unfractionated TES, the probability of cross-reactions may occur in regions where polyparasitism is endemic. In some ocular toxocariasis cases, the serum sample may be repeatable negative without excluding the infection (Smith et al, 2009).

There are some factors that can influence the diagnostic efficiency, such as variation in methods among laboratories, lack of universal units for expressing titers, disagreement in the definition of cut-off lines, differences among surveyed populations and interference of unrelated diseases, such as asthma, in the reactivity of anti-*Toxocara* antibodies (Smith et al. 2009).

Until moment, the ELISA assay is the most widely accepted serodiagnostic test for the detection of anti-*Toxocara* IgG antibodies. Although the ELISA may detect infections by both *T. canis* and *T. cati* (Hotez & Wilkins, 2009), the relative contribution of *T. cati* as etiologic agent of toxocariasis cases has been underestimated (Fisher, 2003). A development of a *T. cati* recombinant antigen would be useful for a better understanding on *T. cati* infection (Smith et al., 2009).

There are many points to be improved in the immunodiagnosis of human toxocariasis, such as the establishment of a standard cut-off value, the definition of true toxocariasis cases and true negative samples.

To establish the final diagnosis of visceral or ocular toxocariasis, clinical signs, symptoms, laboratory data and eosinophil count should be considered (Roldán et al., 2010; Rubinsky-Elefant et al., 2010). On the other hand, the definition of the true negative cases is also complicated and it should be taken in account at the moment of calculating the cut-off values. A positive result in a serological test does not necessarily indicate a causative relationship between *Toxocara* infection and current disease (Pawlowski, 2001). Moreover, in developing countries with a high prevalence of soil-helminth zoonoses is difficult to find true negative serum samples to be used as controls in immunoassays for toxocariasis. Further studies are crucial to improve the sensitivity and specificity of the immunoassays for toxocariasis in order to determine the course of the disease. Tools for distinguish between current or past infection, to improve the diagnosis of ocular toxocariasis, and to define the effectiveness of the therapeutics are needed. Probably, a mixture of recombinant antigens will improve the efficacy of the immunoassays (Mohamad et al., 2009).

7. References

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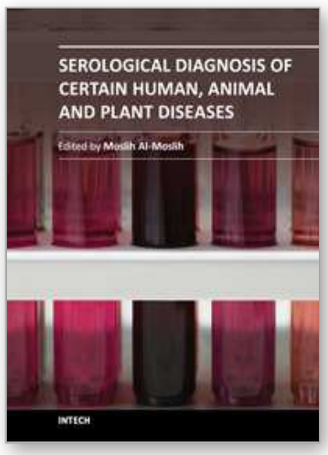

Serological Diagnosis of Certain Human, Animal and Plant Diseases

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This book explains the concept of serological methods used in laboratory diagnoses of certain bacteria, mycoplasmas, viruses in humans, animals and plants, certain parasitic agents as well as autoimmune disease. The authors present up-to-date information concerning the serological methods in laboratory diagnosis of such infectious diseases. Section one deals with the serological methods for bacteria. Section 2 deals with serological methods in human, animal and plant viruses. Section 3 is concerned with the serological laboratory diagnosis of echinococcus and human toxocariasis agents. The last section deals with serological laboratory methods in the diagnosis of coeliac disease.

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