DEVELOPMENT OF A RAPID TEST FOR TOXOCARIASIS USING RECOMBINANT *Toxocara cati* TES-120 PROTEIN

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by

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µL	Microliter
µm	Micron
µg	Microgram
ABZ	Albendazole
APS	ammonium persulfate
ARG	Arginine
AUC	area under curve
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	c-type lectin
CT	covert toxocariasis
CV	Cut off value
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
GST	Glutathione S-transferase
His	Histidine
HRP	horseradish peroxidase
i.e.	id est (that is)
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG4	Immunoglobulin G4
IgM	Immunoglobulin M
INFORMM	Institute for Research in Molecular Medicine
IPTG	Isopropanyl-beta-D-thiogalactopyranoside (IPTG) solution
kDa	kilo Dalton

L	Liter
L2	larva stage 2
L3	larva stage 3
LB	Luria-Bertani
Lys	lysine
M	Molar
MALDI TOF/TOF	matrix-assisted laser desorption/ionization time of flight/time of flight
MBZ	mebendazole
mg	milligram
mL	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MUC	Mucin
MWCO	molecular weight cut-off
Ni-NTA	Nickel-Nitrilotriacetic acid
NC	nitrocellulose membrane
NLM	Neural larva migrans
nm	Nanometer
OD	optical density
OI	optical intensity
OLM	ocular larva migrans
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEB-1	Phosphatidylethanolamine (PE)-binding protein-1
pH	“power of hydrogen” (measure of how acidic/basic is the aqueous solution)
pI	isoelectric point
psi	pounds per square inch
RPM	revolutions per minute
ROC	receiver operating characteristic
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Terrific broth
TBS	tris-buffered saline
TBS-T	tris-buffered saline with tween-20

TES	*Toxocara* excretory-secretory
USM	Universiti Sains Malaysia
V	voltage/volts
v/v	volume/volume
VLM	Visceral larva migrans
x g	times gravity of centrifugal force

PEMBANGUNAN UJIAN PANTAS UNTUK TOKSOKARIASIS MENGGUNAKAN PROTEIN REKOMBINAN *TOXOCARA CATI* TES-120

ABSTRAK

Toksokariasis ialah penyakit zoonosis yang terabai dengan kadar penularan di serata dunia dan telah terbukti menjadi sangat lazim dalam kalangan kanak-kanak dari masyarakat sosio-ekonomi yang kurang bernasib baik. Agen penyebab yang telah dikenal pasti ialah *Toxocara canis* dan *Toxocara cati* di mana masing-masing adalah perumah bagi anjing dan kucing. Walaupun diketahui secara umumnya bahawa toksokariasis ke atas manusia disebabkan oleh kedua-dua *T. canis* dan *T. cati*. Kepentingan *T. cati* sebagai agen zoonotik penyakit itu telah dipandang rendah memandangkan *T. cati* TES jarang digunakan untuk serodiagnosis *Toxocara* dan antigen *T. canis* TES umumnya dianggap dapat mengesan jangkitan oleh kedua-dua spesies. Oleh itu, kebarangkalian sesetengah kes jangkitan *T. cati* mungkin tidak dapat dikesan adalah tinggi, dengan ujian menggunakan antigen *T. canis* sahaja. Oleh yang demikian, perkembangan alat diagnostik yang menggunakan antigen rekombinan *T. cati* dan *T. canis* adalah sangat penting untuk membolehkan pengesanan jangkitan yang disebabkan oleh kedua-dua spesies. Terdahulu, protein rekombinan *T. cati* TES-120 telah berjaya dihasilkan dan diklonkan di dalam vektor pGEX-4T-1 yang menunjukkan potensi nilai diagnostik yang memberangsangkan melalui analisis blot Western. Walau bagaimanapun, jumlah penghasilan protein adalah sangat rendah dan tidak sesuai untuk pembangunan ujian pantas. Dalam kajian ini, DNA *T. cati* telah diklon ke dalam vektor pET32 dan dua varian menggunakan tag peptida pendek [lima residu mewakili asid aspartik (D) dan lisin (K)] telah dihasilkan bagi meningkatkan

kadar penghasilan protein rekombinan yang larut. Kesemua tiga varian iaitu TES-120 *cati* / pET32a, TES-120-5D *cati* / pET32a dan TES-120-5K *cati* / pET32a telah diekspreskan dan ditulenkan. Selepas pengesahan identiti rekombinan protein melalui analisis anti-histidine blot Western dan MALDI TOF / TOF, kadar tindak balas imun ketiga-tiga varian terhadap sampel serum telah diuji melalui ujian blot Western dan rTES-120 *cati* telah dikenal pasti sebagai varian yang terbaik. Ujian pantas aliran sisi kemudiannya telah dihasilkan dan diuji ke atas panel serum sampel, dengan konjugasi emas anti-manusia IgG4 sebagai pengesan. Seroreaktiviti diagnostik ujian pantas aliran sisi rekombinan TES-120 *cati* yang baru dibangunkan kemudiannya dibandingkan dengan prototaip ujian pantas aliran sisi yang telah dibangunkan sebelumnya yang terdiri daripada tiga rekombinan *T. canis* antigen iaitu TES-26, TES-30 dan TES-120. Dalam kajian ini, nilai sensitiviti diagnostik rTES-120 *cati*, rTES-26, rTES-30 dan rTES-120 yang diperoleh masing-masing adalah 64.0% (14/22), 82.0% (18/22), 77.0% (17/22) dan 50.0% (11/22). Apabila keputusan individu ujian pantas kesemua empat rekombinan antigen digabungkan, ujian pantas berjaya mengesan 95.0% (21/22) sampel serum positif. Sebagai perbandingan, sensitiviti diagnostik sebanyak 86% (19/22) diperoleh apabila keputusan dipstik *T. cati* dikecualikan. Kesemua dipstik bagi protein rekombinan *T. cati* dan *T. canis* menghasilkan nilai spesifik diagnostik 100% apabila diuji ke atas sampel serum daripada kumpulan individu-individu sihat dan kumpulan jangkitan parasit lain-lain. Kesimpulannya, penglibatan *T. cati* TES-120 dalam ujian pantas aliran sisi untuk toksokariasis meningkatkan nilai diagnostik sensitivitinya dan secara tidak langsung membolehkan pengesanan jangkitan toksokara daripada kedua-dua spesies *T. cati* dan *T. canis*.

DEVELOPMENT OF A RAPID TEST FOR TOXOCARIASIS USING RECOMBINANT *TOXOCARA CATI* TES-120 PROTEIN

ABSTRACT

Toxocariasis is a neglected zoonotic disease with worldwide distribution and has been shown to be especially prevalent among children from socio-economically disadvantaged populations. The causative agents are *Toxocara canis* and *Toxocara cati* in which the definitive hosts are dogs and cats, respectively. Although it is acknowledged that human toxocariasis is caused by both *T. canis* and *T. cati*, however, the importance of *T. cati* as a zoonotic agent of the disease has been under-recognized since *T. cati* TES is rarely used for *Toxocara* serodiagnosis and *T. canis* TES antigens are generally thought to be able to detect infections by both species. Thus, it is highly likely that some cases of *T. cati* infection may be missed by tests using only *T. canis* antigen. Therefore, it is an urgent need for the development of diagnostic tools that use both *T. cati* and *T. canis* recombinant antigens to enable detection of infections caused by both species. Previously, a *T. cati* recombinant protein TES-120 was successfully produced and cloned into a pGEX-4T-1 expression vector which showed good diagnostic potential in western blot. However, the yield was low and unsuitable for rapid test development. In this study, the *T. cati* DNA insert was cloned into a pET32 expression vector and two short peptides tagged variants [five residues each of aspartic acid (D) and lysine(K)] were designed to increase the chances of obtaining a good yield of the soluble recombinant protein. All three variants namely TES-120 c*ati*/pET32a, TES-120 5D *cati*/pET32a, and TES-120 5K *cati*/pET32a were expressed and purified. After the protein identities were confirmed by anti-histidine western blot

and MALDI TOF/TOF, the immunoreactivities of all three variants against serum samples were tested by western blot and the best variant was found to be TES-120 *cati*/pET32a. Lateral flow dipstick rapid test was then developed and tested against a panel of serum samples, with anti-human IgG4 gold conjugate as the detector. Diagnostic seroreactivity of the newly developed recombinant TES-120 *cati* lateral flow dipstick was then compared to previously developed prototype rapid test which comprised three dipsticks separately dotted with *T. canis* recombinant antigens i.e. TES-26, TES-30, and TES-120. In this study, the diagnostic sensitivity of rTES-120 *cati*, rTES-26, rTES-30 and rTES-120 was found to be 64.0% (14/22), 82.0% (18/22), 77.0% (17/22) and 50.0% (11/22), respectively. When the results of all four dipsticks were combined, the rapid test successfully detected 95.0% (21/22) of the positive serum samples. In comparison, a sensitivity of 86% (19/22) was obtained when results of the *T. cati* dipstick was excluded. All four recombinant dipsticks produced 100% specificity when tested against healthy and other helminthic infection serum samples. Thus, as a conclusion, the inclusion of *T. cati* TES-120 in the lateral flow rapid test for toxocariasis increased its diagnostic sensitivity and enabling the detection of *Toxocara* infection from both species *T. cati* and *T. canis*.

CHAPTER 1

INTRODUCTION

1.1 Human toxocariasis: An overview

Over the years, toxocariasis has acquired increasing worldwide attention and was classified among the five most neglected parasitic infections by the Centers of Disease Control and Prevention (CDC) (Rubinsky-Elefant *et al*., 2010; Macpherson, 2013; Holland, 2017). Toxocariasis is a global neglected zoonosis with worldwide distribution in which the main causal agents are the roundworms in dogs (*Toxocara canis*) and cats (*Toxocara cati*). The disease is usually prevalent worldwide among children in areas with poor sanitary conditions and socio-economically disadvantaged (Macpherson, 2013). In Malaysia, the seroprevalence rate reported was 4.8% to 33.1%. (Hakim *et al*., 1993; Romano *et al*., 2010; Lim *et al*., 2015).

Humans are infected through ingestion of embryonated eggs from contaminated environment and ingestion of encapsulated larvae from raw or undercooked tissues of paratenic hosts. The ingested infected larvae are unable to complete their life cycle in humans, therefore they exist as larvae which migrate throughout the human body, penetrating organs and damaging eyes, pulmonary and neurological tissues (Despommier, 2003; Zahabiun *et al*., 2015). Human toxocariasis manifests itself in two main concerning forms i.e. visceral larva migrans (VLM) and ocular larva migrans (OLM). These manifestations vary from non-specific symptoms to serious health consequences, regarding the migration of larvae in the organ involved, the rate of infection re-occurrence and the immunity responses (Pawlowski, 2001).

A definitive diagnosis of human toxocariasis is rather challenging. It is based on clinical and epidemiological data which is often accompanied by serological tests. Traditional parasitological diagnostic techniques can have significant limitations regarding the specific identification of larvae. At present, serological tests such as enzyme-linked immunosorbent assay (ELISA) and Western blot are the main tools for serodiagnosis of the disease. Additionally, rapid diagnostic tests, particularly lateral flow dipstick test has shown to be one of the most promising approaches for diagnosing this disease. Regarding toxocariasis, no rapid diagnostic test has been commercialized to date. Although understanding the biology and epidemiology of human toxocariasis have been known and can be caused by *Toxocara cati*. However, due to the rare involvement of it in *Toxocara* serodiagnosis, its medical importance is under-recognized. Although *T. canis* and *T. cati* species share many common antigenic fractions, they are not the same (Fillaux & Magnaval, 2013). Thus, some cases of *T. cati* infection may be missed by tests using only *T. canis* antigen. These factors suggest the need for continuous attention to better define the challenge of human toxocariasis, which demands a more effective diagnosis.

1.2 The background of *Toxocara*-excretory-secretory antigens (TES)

Earlier serology efforts in toxocariasis serodiagnosis were based on the utilization of homologs and heterologous antigens derived from extracts of adult worms and eggs of *T. canis*, respectively (Smith & Noordin, 2006) which were highly cross-reactive with *Ascaris* and other parasitic infections (Girdwood *et al,*. 1978; Smith *et al*., 1982; Maizels *et al*., 1987). A study by Savigny (1975) created a major breakthrough for diagnostic detection of human toxocariasis where the introduction of *in vitro* culture technique for *T. canis* second-stage larvae was reported. The technique of maintaining larval culture was later further improved from 18 months and up to three months to

only three weeks with more than 70% of viability (Thomas *et al*., 2016). Protein content in crude of TES antigens was then characterized and used as a diagnostic antigen and utilized in ELISA and Western blot assays. The characterization of TES antigens has been reported in the literature which reported molecular mass variation from 30 kDa to 384 kDa (Badley *et al*., 1987; el-Massry, 1999; Hassanain & Mahmoud, 2008; Sudhakar *et al*., 2014). TES antigens are primarily characterized as mucins, lectins, and other TES products (Holland and Smith, 2006).

1.2.1 Mucins

Toxocara mucins are composed of separate segments, a glycosylated and a non-glycosylated domain (Gems *et al*., 1995; Maizels *et al.*, 2000). They typically migrate at molecular mass approximately 120 kDa on SDS-PAGE gel hence named as TES-120 and does not correspond to the actual molecular mass (~ 40 – 45 kDa) due to the heavy glycosylation (Loukas *et al*., 2000). Five distinct mucins have been discovered i.e. MUC-1, MUC-2, MUC-3, MUC-4, and MUC-5. The most dominant mucins are MUC-1, MUC-2 and MUC-3 in which corresponded to the surface coat mucins (Gems & Maizels, 1996; Loukas *et al*., 2000). Meanwhile, MUC-4 secretion was not been reported and showed a discrepancy in the sequence against the surface coat glycoproteins of the family mucins. cDNA MUC-5 has been successfully isolated by using an expression screening method, however, due to its significant difference as compared to other mucins, it is less likely to be identified as *Toxocara* mucin. (Doedens *et al*., 2001).

1.2.2 Lectins

Toxocara lectins are TES glycoprotein also known as C-type lectin (CTL). They have significant roles in recognizing infectious agents (Dodd & Drickamer, 2001) and have a clear function interaction between host and parasite during parasite infestation (Loukas *et al*., 2000). CTL-1 or TES-32 is one of the most prominent larval proteins and its cDNA was first identified and characterized by Loukas *et al*. (1999). There are other identified variants of CTL-1 i.e. CTL-2 and CTL-3 whereby their amino acid is 83-87% identical to CTL-1 (Holland & Smith, 2006). The fourth member of lectins was later identified as CTL-4 which corresponds to 70 kDa protein and differs from CTL-1 or TES-32, and is dominant in mammalian cells (Loukas *et al*., 2000).

1.2.3 Other TES products

Besides mucins and lectins, approximately another 50 distinct molecules were also identified by two-dimensional SDS-PAGE analysis. TES-26 (PEB-1) is known as the most functioning phosphatidylethanolamine-binding protein (Gems *et al*., 1995). Meanwhile, other proteins identified as TES products are TES-400, TES-45, and TES-55 which correspond to their actual molecular mass on SDS-PAGE analysis.

1.3 The organism *Toxocara cati*

1.3.1 Taxonomy

Toxocara cati, a worldwide parasite of the domestic cat was first discovered by Sprent (1956), later named as *Ascaris cati* based on the illustrated figure of a cat worm that had large cervical alae. It belongs to the Animalia Kingdom, a member of the Phylum Nematoda and classified under the Secernentea class. The roundworm is categorized under the Ascaridia order, Toxocaridae family and *Toxocara* genus. Ascaridoid nematodes in the *Toxocara* genus are classified based on their morphological

characteristics and site of infection in host-specific host (Bowman & Atkins, 2009; Borecka *et al*., 2010; Chen *et al*., 2012). Besides *T. cati*, to date, there are 23 other species classified under genus *Toxocara* (Gibbons *et al*., 2001). Among them, the most predominant are i.e. *T. canis*, *T. viturolum*, *T. pteropodis*, *T. lyncus*, which infect dogs, cattle, bats, caracals, respectively. Meanwhile *T. mackerrase*, and *T. apodemi* infect rodents (Skrjabin *et al*., 1991; Borecka *et al*., 2010; Chen *et al*., 2012).

1.3.2 Morphology

T. cati is morphologically closely similar to *T. canis*. *Toxocara* adult worms are normally cream-colored to pinkish and measure up to 10 centimeters in length. Females were reported to be longer than males in the literature whereby they were measured as 6.5 to 10 cm and 4 to 6 cm long, respectively. (Joy *et al*., 2017). The adults have distinct cervical alae positioned at the anterior end of the distinct appearance of an arrow and an esophagus that covers approximately 2% to 6% of the total body length. It stops in a glandular ventriculus that measures about 0.3 to 0.5 mm long (Esch *et al*., 2002). The adult worms are distinguishable from their sexual dimorphism between males and females. The vulva of the females covers about 25% to 40% of the body length and the spicules of the males range from 1.7 mm to 1.9 mm long (Esch *et al*., 2002). The *Toxocara* eggs are brownish, almost spherical and measure approximately 65 µm by 77 µm with smaller pits to compare to the pits observed on *T. canis* eggs (Esch *et al*., 2002; Joy *et al*., 2017). Figure 1.1 shows the light and scanning electron micrographs of eggs and adult *T. cati*.

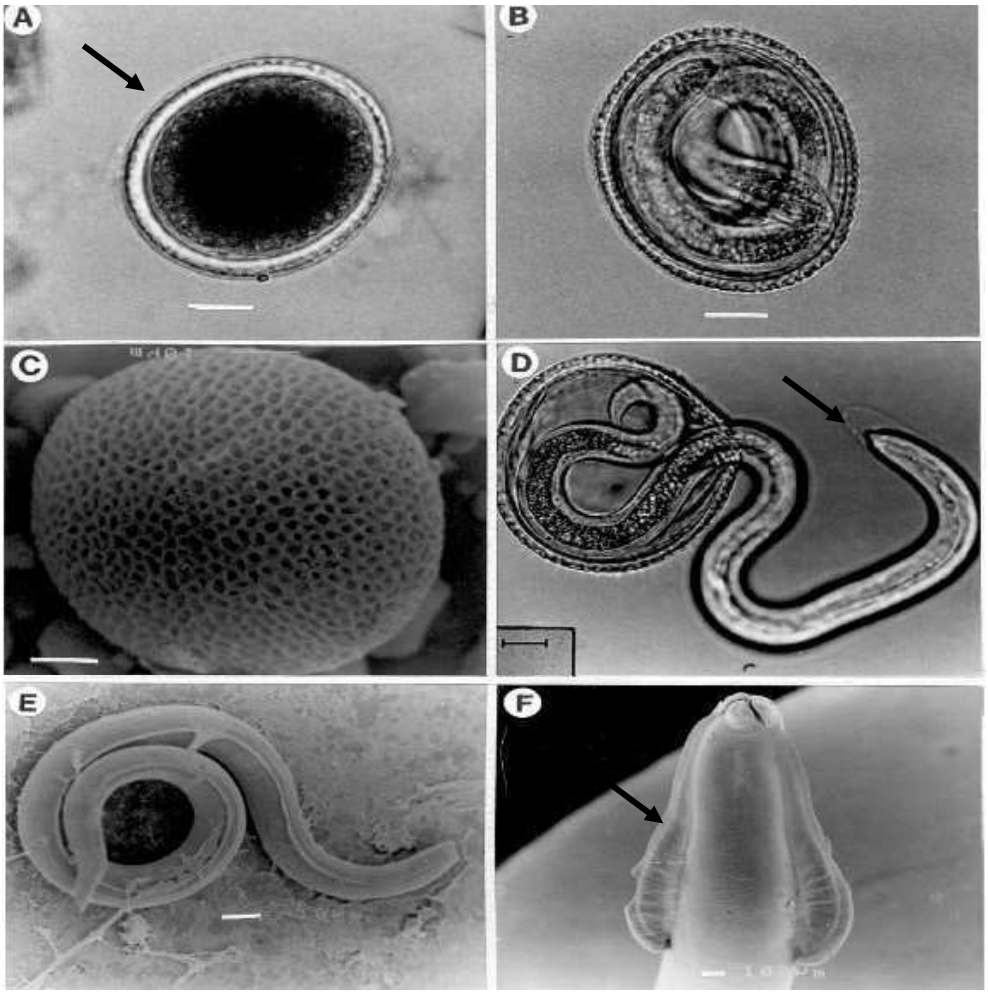

Figure 1.1 Morphological characteristics of *T. cati* under light and scanning electron micrographs (Tekele, 2003).

A: Non-embryonated egg of *T. cati* showing the ovum and corrugated eggshell

B: Embryonated egg of *T. cati* with the appearance of second stage larva within the egg

C: Appearance of pits on the egg of *T. cati*

D: Appearance of a second stage larva hatching from an egg

E: Second stage larva obtained from infected tissue

F: Second stage larva with the appearance of arrow-shaped cervical alae

1.3.3 Life cycle

Toxocara cati has a direct life cycle with cats and other felids as definitive host but rather particularly complex as illustrated in Figure 1.2. Cats are the definitive host in which they acquire the infection by ingestion of infective eggs and tissues from paratenic hosts e.g. rats, mice, chicken, earthworms, and cockroaches. Cats are also infected through transmammary transmission which is one of the major modes of transmission in cats. Unlike dogs, no transplacental transmission was reported (Dubinský *et al*., 1995; Blagburn & Kassai, 2001). In animal host, after the larvae hatch they invade the intestinal lining and start migrating through heart-lung-tracheal route to finally settle in the intestine as adults (Tekele, 2003). *T. cati* eggs are not immediately infectious once shed into the environment since they remain unembryonated until up 2 to 4 weeks for the larval development within the eggs to occur depending on the weather conditions of the environment (Woodhall & Fiore, 2014). Survival of these eggs containing infective larval stage 2 (L2) in the soils can be up to a few months with suitable conditions.

Sprent (1956) described in kittens, ingested eggs shed the L2 larvae in the intestine and migrate via portal veins which later develop into L3 larvae in lungs. The L3 larvae then migrate to the trachea and mouth that often cause coughing or sneezing in kittens. Female adults can acquire the infection when licking their kittens. Meanwhile, in adult cats, the infective L2 larvae migrate to other organs i.e. liver, lungs, brain, muscles and stomach wall where they remain dormant that can last for years and some dormant L2 larvae return to intestines to complete larval development and for offspring production. Transmammary infection occurs by migration of the larvae to mammary glands and lactating females transmit the infection to suckling kitten through milk.

In humans, the hatched larvae are unable to mature and complete the larval development, therefore they penetrate the intestinal lumen and migrate into various organs and tissues e.g. liver, lungs, retina, neurological tissues, and muscles. *Toxocara* larvae in the tissue can cause immediate hypersensitivity reactions resulting in a broad spectrum of clinical manifestations.

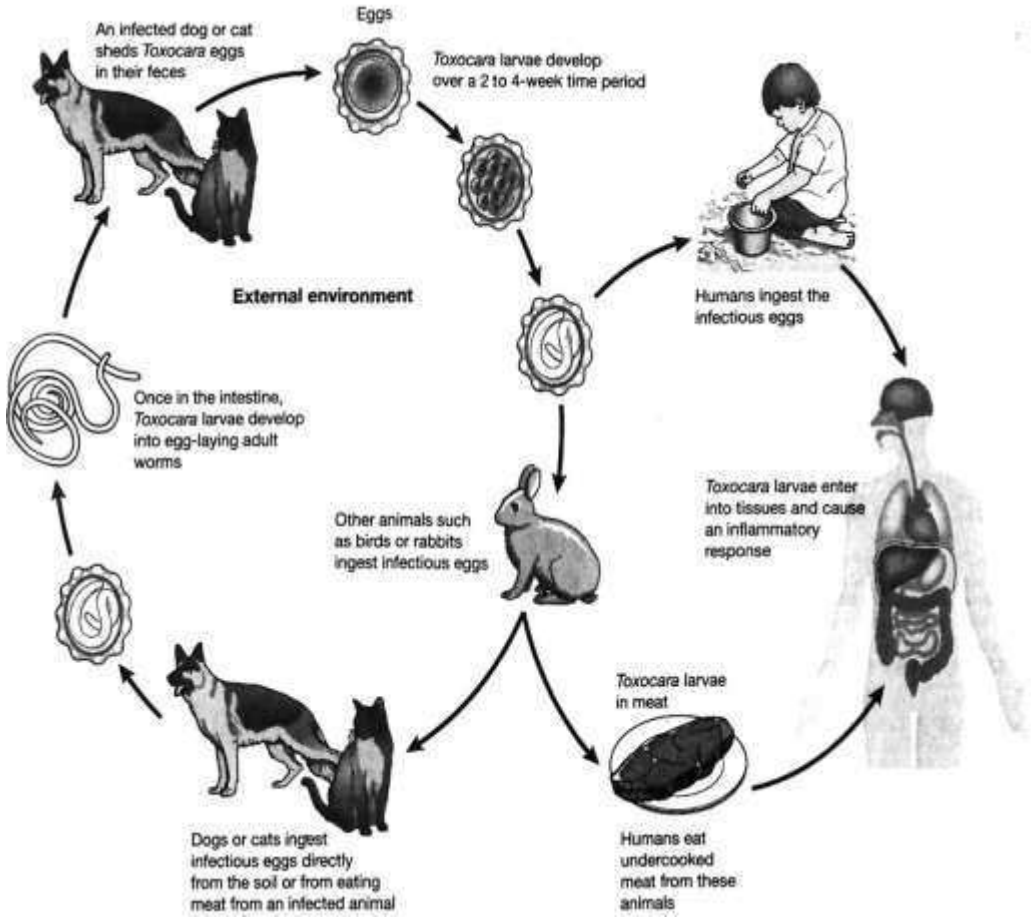

Figure 1.2 The illustrated diagram of *T. canis* and *T. cati* life cycle, the major aetiologic agents of toxocariasis (Woodhall & Fiore, 2014)

1.4 Epidemiology of toxocariasis

Toxocariasis has become an emerging zoonosis disease and has been underestimated with worldwide distribution (Robertson *et al.*, 2000). *Toxocara* infections acquired from dogs and cats can cause severe disease in humans (Wilcocks & Manson-Bahr, 1978). Preventive measurement for the disease and treatment are limited due to non-uniformity of the infection with non-specific symptoms that normally do not affect the individual's routine, therefore resulting in the delay of detection.

Thus far, there is no accurate epidemiological data on toxocariasis prevalence and it is primarily dependent on serological data acquired from research conducted worldwide. Besides that, epidemiological studies are also conducted on animals. For cats alone, *T. cati* is one of the most abundant parasites. Prevalences of *T. cati* infection has been well documented worldwide, mainly in the USA, Europe, Japan, Asia, Australia and South Africa where the prevalence rate ranges from 11% to 89% (Baker *et al*., 1989; Oikawa *et al*., 1991; Beelitz *et al*., 1992; Petithory *et al*., 1996; Fei *et al*., 1997; Milstein & Goldsmid, 1997; Tekele, 2003).

The variations of prevalence are attributed by a broad spectrum of factors including geographical, cultural, socioeconomic, immunity and co-infection. Due to the close interaction and exposure to definitive hosts, the behavior of hosts is also a contributing factor (Macpherson, 2013; Viney & Graham, 2013). *Toxocara* infection is more prevalent in tropical settings and socio-economically disadvantaged countries where the treatment and control population of dogs and cats are limited (Smith *et al*., 2009; Macpherson, 2013). The seroprevalence studies conducted in developed countries such as USA, Australia, Denmark, and New Zealand showed that human toxocariasis varies between 2% to 37% (Poulsen *et al*., 2015). Meanwhile, in tropical countries, the seroprevalence rate is generally higher and has been reported to be as

high as 93% (Magnaval *et al.*, 1994). In Malaysia alone, the seroprevalence of toxocariasis was reported to be in the range of 4.8% to 33.1% (Hakim *et al*., 1993; Romano *et al*., 2010; Lim *et al*., 2015). Prevalence studies have also been performed on assessment of egg contamination soil i.e sandpits, backyards, playgrounds, public parks where it is a major source of infection in humans, especially children (Mizgajska, 2001; Lloyd & Morgan, 2011; Manini *et al*., 2012). The rate of soil contaminated with *Toxocara* eggs ranged between 6.6% to 87.1% worldwide (Duwel, 1984; Emehelu and Fakae, 1986; Gunaseelan *et* al., 1992; Oteifa & Moustafa, 1997; Guimarães *et al*., 2005; Rokicki *et al*., 2007; Tiyo *et al*., 2008).

Age is a major risk factor for human toxocariasis where many studies suggested it is more prevalent among children compared to adults (Radman *et al*., 2000; Jones *et al*., 2008; Chieffi *et al*., 2009). This may due to their behaviors which favor the transmission of the infection, the habit of geophagia and exposure to domesticated dogs and cats. On the contrary, a few prevalence studies have claimed that the seroprevalence increased with age where the practice of consuming raw food may have contributed to the increased seroprevalence in adults (Genchi *et al*., 1990; Lee *et al*., 2015).

1.5 Mode of transmissions of human toxocariasis

Transmission of toxocariasis varies worldwide which may be higher in socio-economically disadvantaged populations. Humans are exposed to toxocariasis by unintentional ingestion of soil/dirt defiled with embryonated *Toxocara* eggs of that shed into nature (Fillaux & Magnaval, 2013; Zahabiun *et al*., 2015), or rarely they can acquire through transplacental transmission from infected mother when she ingests infective eggs (Despommier, 2003). Several studies also reported that humans acquire the infection from the habit of consuming undercooked meat (Yoshikawa *et al*., 2008; Strube *et al*., 2013). Lee *et al*. (2015) described an increased incidence of toxocariasis in Korea as a result of Korean cultural practice where individuals like to consume raw food from paratenic hosts.

A new proposed route of transmission involved contacts with infective *Toxocara* eggs on the host's hair coat (Wolfe & Wright, 2003; El-Tras *et al*., 2011) Tavassoli *et al*. (2012) described contact between human and dogs could be a direct transmission of the infective eggs, in contrast, Nagy *et al*. (2011) suggested the newly proposed mode of transmission is a rare case as embryonation progress of *Toxocara* eggs is slower on the hair coat of the host which was supported by a recent study by Keegan and Holland (2013). The *Toxocara* larvae are unable to mature in humans, unlike in their definitive hosts. Therefore they remain as larvae penetrating tissues and migrate throughout the body and eventually will die in situ resulting in immediate hypersensitivity responses within their hosts (Macpherson, 2013).

1.6 Clinical manifestation of human toxocariasis

Human toxocariasis manifests itself in four forms of clinical features i.e. visceral larva migrans (VLM), ocular larva migrans (OLM), covert toxocariasis (CT) and on rare occasions, cerebral toxocariasis which also known as neural larva migrans (NLM) (Taylor *et al*., 1987; Nathwani *et al*., 1992; Smith *et al*., 2009; Fan *et al*., 2015). The signs and symptoms of these clinical features of human toxocariasis are often very non-specific, rather asymptomatic in most cases which causes it to be under-recognized and underdiagnosed unless the abnormality is obvious (Macpherson, 2013).

1.6.1 Visceral larva migrans (VLM)

Visceral larva migrans (VLM) is caused by the migration of parasite through the internal organs or tissues which can result in severe damage to the liver, lungs and other organs. It was first reported by Beaver *et al*. (1952) where *Toxocara* larvae were found in children with high eosinophilia, hepatomegaly, pulmonary infiltration, cough, fever, and hyperglobulinemia. VLM is normally prevalent among children two to seven years old (Magnaval *et al*., 2001; Tekele, 2003) with a history of eating dirt and exposure to domesticated pets at home especially dogs. The association between geophagy and VLM has been reported in several studies where pica is detected in children with VLM (Snyder, 1961; Baldisserotto *et al*., 1999) and it is reported slightly common males rather than females (Glickman & Schantz, 1981; Carvalho & Rocha, 2011). The acute signs of VLM are associated with liver and lungs larval infection and normally include non-specific symptoms such as fever, asthmatic, hypereosinophilia and enlargement of the liver.

In a case study, Snyder (1961) disclosed these clinical signs in 20 children with VLM aged 16 and 48 months; 55.0% with a history of dirt-eating and fever; 40.0% with pallor, 20.0% with bronchospasm, 85.0% and 45% with liver and spleen enlargement, respectively. Such clinical findings were supported by a case-study report by Baldisserotto *et al*. (1999) where 18 cases of toxocariasis presented with clinical findings as follows: with enlargement of liver (72.7%) and spleen (50%), history of exposure to dogs (38.0%), cervical adenitis (33.0%), lung symptoms (27.7%), fever (22.2%), pallor and geophagy (16.6%), limb pain (11.1%), and skin lesions (5.5%). It was also reported that patient with persistent eosinophilia and unexplained febrile illness should be suspected of having VLM (Gónzalez *et al*., 2000; Despommier, 2003).

1.6.2 Ocular larva migrans (OLM)

Ocular toxocariasis results from migration of the parasite into the posterior of the eye, causing decreased vision, red-eye, retinal damage, and in severe cases, loss of vision (Despommier, 2003). The OLM is relatively rare which often affects children (Ahn *et al*., 2014), rarely in adults (Roth & Gleckman, 1985; Ljungström & van Knapen, 1989; Holland & Smith, 2006). The prevalence of OLM occurrence is yet to be well documented since the reports on this clinical manifestation are limited (Ahn *et al*., 2014). A study by Maetz *et al*. (1987) reported 11 cases of OLM per 1000 patients recorded over a six months period one case per 1000 persons was estimated to have OLM in the population of Alabama. Meanwhile, in Asia, the number of OLM occurs in adults is increasing which may due to their cultural practice of eating raw food (Choi *et al*., 2008, 2012; Ahn *et al*., 2014).

The clinical features of OLM are classified into four groups based on their anatomic localization namely posterior pole granuloma, peripheral granuloma,

nematode endophthalmitis, and atypical presentations (Woodhall *et al*., 2012; Ahn *et al*., 2014). The posterior granuloma is recognized by a high whitish inflammatory mass with or without pigmentation in the posterior eye pole (Ahn *et al*., 2014). In some cases, patients may encounter a decreased sight (Harley *et al*., 2005) and it is the most clinical presentation 44% of cases observed in OLM patients (Wilkinson & Welch, 1971). Woodhall *et al*. (2012) described peripheral elevated white nodule is found in peripheral granuloma that may occur with degrees variation of adjacent membranes and pigmentary alterations. Nematode endophthalmitis presented with inflamed painful red eye causing hypopyon development and in serious cases it may lead to retinal detachment (Woodhall *et al*., 2012). Meanwhile, the atypical presentations described as the optic nerve head inflammation, mobile subretinal larvae and diffuse chorioretinitis (Stewart *et al*., 2005; Smith *et al*., 2009; Rubinsky-Elefant *et al*., 2010). The occurrence of ocular toxocariasis cases are documented mostly as unilateral, and the bilateral incidents of bilateral are relatively uncommon (Castillo *et al*., 1995; Overgaauw and Knapen, 2008; Woodhall *et al*., 2012; Ahn *et al*., 2014). However, a bilateral OLM with distinct clinical presentations in each patient's eye confirmed by ELISA intraocular fluids was reported (Lee *et al*., 2015).

1.6.3 Covert toxocariasis (CT)

Covert toxocariasis is more common as compared to VLM and OLM. It is similar to VLM, but its symptoms are very non-specific and less severe (Moreira *et al*., 2014), therefore, the majority of infected individuals are underdiagnosed. The non-specific symptoms include headache, abdominal pain, cough, insomnia, and behavioral changes. Patients who are positive for anti-*Toxocara* antibodies but are asymptomatic have been successfully identified in cross-sectional community seroepidemiological studies (Taylor *et al*., 1987; Magnaval *et al*., 1994; Pawlowski, 2001). In a study conducted in France, toxocariasis patients with breathing difficulties, skin problem, fatigue, and stomach cramps showed high titers of anti-*Toxocara* antibodies, eosinophilia and elevated levels of total IgE levels (Glickman *et al*., 1987). Meanwhile, Taylor *et al*. (1988) described symptoms such as pyrexia, loss of appetite, headache, nausea, behavior and sleep disturbances, pulmonary problems, skin problems, and hepatomegaly were found in infected children. Covert toxocariasis is often accompanied by the association of other chronic diseases such as diabetes, chronic kidney disease, and cardiovascular disease (Yoshida *et al*., 2016). Meanwhile, Mazur-Melewska *et al*. (2015) described the association of pulmonary involvement including asthma, acute bronchitis, pneumonia, urticaria, enlargement of the lymph nodes, myositis and pseudorheumatoid syndrome in with CT diagnosis. The authors also stated that it is crucial to perform a radiological examination for confirmatory of the presence of *Toxocara* larvae in the affected tissue (Mazur-Melewska *et al*., 2015).

1.6.4 Neural toxocariasis

Neural toxocariasis or neural larva migrans (NLM) is another serious clinical presentation of toxocariasis resulted from migration of *Toxocara* larvae to the brain and central nervous system which regulated by several aspects such as host genetic factors, the number of ingested eggs and previous exposure (Xinou *et al*., 2003; Fan *et al*., 2015). NLM in humans was first reported in an autopsy study where *Toxocara* larva was found in the left thalamus of a child with poliomyelitis (Beautyman & Woolf, 1951). NLM is believed to be rare and not well-documented. However, due to advancements in diagnostic of toxocariasis to date, the increasing number of clinical NLM cases due to the larval invasion of the brain or CNS have been recorded over the last three decades (Fan *et al*., 2015).

NLM symptoms are non-specific, while infected patients may have fever, lethargy, light sensitivity, headache, visual impairment (Finsterer & Auer, 2007) and seizures (Hotez & Wilkins, 2009). NLM is described to be associated with neurological damage and epilepsy, neuropsychologic deficiency, eosinophilic meningoencephalitis, myelitis and cerebral vasculitis (Magnaval *et al*., 2001; Finsterer & Auer, 2007; Holland & Hamilton, 2013; Deshayes *et al*., 2016). The association between NLM with schizophrenia was also reported where the prevalence rate of toxocariasis in schizophrenic patients was recorded to be 23.3% as compared to only 2.2% in control subjects ((El-Sayed & Ismail, 2012). Knowledge and understanding of the pathogenesis of NLM in humans remain non-established. However, seroepidemiological data suggest high levels of exposure to *Toxocara* in general population indicates that the disease has been under-recognized.

1.7 Diagnosis of human toxocariasis

1.7.1 Clinical diagnosis

Toxocariasis diagnosis is often difficult due to its non-specific symptoms and non-uniformity of clinical presentations, and it is primarily based on clinical, serological, epidemiological, and laboratory data including radiological imaging, serology, eosinophilia and IgE level (Zibaei & Sadjjadi, 2017). Clinical findings such as clinical and epidemiological data i.e exposure to hosts, intake of raw meats and geophagy have to be supported by laboratory tests that are often laborious and not suitable for low resource settings areas. Biopsy examination is the definitive test to confirm the presence of *Toxocaral* larvae in the affected tissues, however, this method is very invasive, less sensitive and time-consuming (Pawlowski, 2001; Rubinsky-Elefant *et al*., 2010; Carvalho & Rocha, 2011). The radiological imaging procedure is believed to be an easier and less invasive tool to detect the disease (Ishibashi *et al*., 1992; Gónzalez *et al*., 2000). However, this method is not suitable since it requires a lab setting to be done. Nevertheless, the diagnosis of symptomatic toxocariasis was concluded to be based on five factors by Pawlowski (2001) which were patient's characteristics and history, clinical signs and symptoms, positive serology, eosinophilia counts and increased levels of IgE.

1.7.2 Laboratory diagnosis

Due to limitations and inefficiency of clinical diagnosis, many researchers have proposed that serological tests are the most appropriate tool for diagnostic of human toxocariasis (Bruckner, 1985; Lynch *et al.*,1988; Nunes *et al*., 1999; Dubinský *et al*., 2000; Yamasaki *et al*., 2000; Tekele, 2003). Over the years, numerous serological tests have been developed for detection of anti-*Toxocara* IgG antibody in human. ELISA and Western blot assays are the two most useful and preferred laboratory diagnostic

tests, the former is more commonly used. The use of TES antigens in both assays have long been recognized as a standard diagnostic reagent, and widely utilized for diagnosis and seroepidemiological studies. Molecular approaches such as polymerase chain reaction (PCR) was used to analyze clinical and environmental samples (Fogt-Wyrwas *et al*., 2007). *Toxocara* larvae in liver biopsies were successfully detected by using the method, however it was unable to differentiate the *Toxocara* species (Rai *et al*., 1997; Ishiwata et al., 2004). *T. canis* DNA was detected in cerebrospinal fluid (CSF) of human patient which was previously tested *Toxocaral* positive via IgG-Western blot (Caldera *et al*., 2013). Thus far, this method has not been used to detect larvae from human tissue.

1.7.2 (a) Enzyme-linked immunosorbent assay (ELISA)

ELISA is the most common technique used in routine toxocariasis diagnosis. The first ELISA developed for the diagnosis of toxocariasis was based on soluble embryonic eggs and adult larvae as well as somatic extracts of adult worms (Holland & Smith, 2006). Introduction of a method to preserve *Toxocara* larvae and extraction of TES (Savigny, 1975) has enabled the development of diagnostic tools for toxocariasis (Fillaux & Magnaval, 2013). Since then, more ELISA assays have been developed using native *Toxocara* TES antigens to detect specific anti-*Toxocara* IgG (Rudzińska *et al*., 2017). Indirect ELISA and dot-ELISA (Camargo *et al*., 1992; Magnaval *et al*., 2001; Roldán *et al*., 2006) are two formats of ELISA that have been described in the literature. Dot-ELISA test is believed to be more efficient for epidemiological studies, primarily in developing countries due to it is being more stable and built in the lower setting resource as compared to the standard indirect ELISA (Roldán *et al*., 2006; Bojanich *et al*., 2012).

Several commercial IgG-TES-ELISA kits are available in the market e.g. Novalis *Toxocara* IgG ELISA (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany), *Toxocara* IgG ELISA (Cypress Diagnostics, Belgium) and *Toxocara canis* ELISA kit (Bordier Affinity products, SA, Switzerland) and performance level of these ELISA kits reported to be good with diagnostic sensitivity and specificity (> 90% and > 90%, respectively) (Jacquier *et al*., 1991; Smith & Noordin, 2006; Fillaux & Magnaval, 2013). However IgG-TES-ELISA often encounter cross-reactivity problems and unstable seroreactivity problems especially in the areas where poly parasitic helminth infections are common (Fan *et al*., 2015) indicating that the use of native TES antigens may be less relevant to be used in the regions where other nematode diseases are predominant (Noordin *et al*., 2005). This claim was supported by a few studies where cross-reactivity of native TES-ELISA assays was reported when tested with serum samples from patients with strongyloidiasis, trichinellosis and ascariasis (Lynch *et al*., 1988; Jacquier *et al*., 1991).

Moreover, diagnostic seroreactivity issue of native TES-ELISA emerged when the assay was employed on multi-parasitized, therefore suggesting the need for a more specific diagnostic tool. Most commercial kits also use anti-human IgG as the detector antibody which contributes to the non-uniformity of the detection of the infections since IgG assays do not discriminate past and recent infections. The diagnostic potential of IgG subclasses in serodiagnosis of toxocariasis has been reported in the literature whereby of all immunoglobulin subclasses, the use of IgG2 antibody was reported to have the highest diagnostic sensitivity (Watthanakulpanich *et al*., 2008). Meanwhile, the use of IgG4 antibody was reported to have improved specificity of the native TES- ELISA assays (Noordin *et al*., 2005; Mohamad *et al*., 2009). Thus,

promoting significant reduction of cross-reactivity problem in serodiagnosis of toxocariasis despite it is being less sensitive than the standard IgG TES-ELISA.

The inability of routine ELISA to differentiate current from resolved infections has led to the further development of antigen detection assay in which enables the detection of antigens circulated by the dormant *Toxocara* larvae that remain metabolically active in human body (Savigny, 1975). It is primarily based on the antigen-antibody interaction to a specific antigen in which implied as idealistic method in differential serodiagnosis of active from past infections. Antigen capture ELISA uses stationary phase bounded antibody to specifically bind the antigen and has been proven to be able to identify parasite-secreted substances in roundworm infections (Robertson *et al*., 1988).

In antigen detection assays, polyclonal (Poly-Ab) and monoclonal antibodies (mAB) have been commonly used over the years. Poly-Ab antigen-antibody interaction occurs on one or more antigen epitopes, meanwhile, while mAb occurs on one specific antigen epitope (Lipman *et al*., 2005). Early efforts to detect circulating TES antigen using Poly-Ab for detection of active infections showed unsatisfactory diagnostic sensitivity (Robertson *et al*., 1988; Gillespie *et al*., 1993) in which attributed to the low larval burdens. Cross-reactivity was also reported whereby 10 out of 40 serum samples from patients with other parasitic infections were positive (Gillespie *et al*., 1993). On the other hand, the application of mAb provides a more promising potential in immunodiagnosis where the assay's diagnostic performance has been reported to have increased. The limit of detection by mAb reported in the literature vary from as low as 440 pg/mL to 78 ng/mL (Robertson *et al*., 1988; Gillespie *et al*., 1993; Ishiyamna *et al*., 2009; Zibaei *et al*., 2010; Rodríguez-Caballero *et al*., 2015). Nanobodies, naturally occurring antibodies which composed of heavy chains, have a

comprehensive antigen-binding range that can be easily manufactured and are therefore used in the development of many types of immunoassays for better diagnostic performance in antigen detection-based assays (Helma *et al*., 2015; Dmitriev *et al*., 2016; Morales-Yanez *et al*., 2019). To date, only one study has reported the use of nanobodies to detect TES antigen (Morales-Yanez *et al*., 2019). The authors revealed the use of three *T. canis*-specific nanobodies that were applied in sandwich ELISA to detect TES antigen in which it showed a limit of detection of 0.650 ng/mL. The application of nanobodies in the serodiagnostic would offer such a major breakthrough for further development of a more promising tool and a better understanding of the biology of *Toxocara* infections.

1.7.2. (b) Western blot

Western blot assays are often used to confirm serological data obtained from ELISA (Magnaval *et al*., 2001; Roldán & Espinoza, 2009). The earlier attempt of IgG-Western blot demonstrated serum from infected rabbits and humans primarily with ocular larva migrans where they were tested using TES-Western blot in which revealed a group of four bands in the lower molecular mass i.e. 24, 28, 30, and 35 kDa, and three protein bands at the higher molecular mass i.e. 132, 147 and 200 kDa (Magnaval *et al*., 1991; Fillaux & Magnaval, 2013). It was also revealed that the group of three bands found in the lower molecular mass were *Toxocara* specific (Magnaval *et al*., 1991; Park *et al*., 2000; Fillaux & Magnaval, 2013), which were later confirmed by subsequent studies which described the same banding patterns (Zarnowska & Jastrzebska, 1994; Morales *et al*., 2002; Roldán & Espinoza, 2009). When compared to commercial TES-ELISA, in-house Western blot was found to have a 55% higher sensitivity (Gueglio *et al*., 1994; Courtade *et al*., 1995). The most common commercial Western blot kit available in the Europe market is *Toxocara* WB-IgG (LDBIO Diagnostics, Lyon,

France) in which diagnostic performance varies in various studies (Macpherson, 2013).

1.7.2. (c) Rapid test

The application of rapid detection kits in the industrial diagnostics rose over the recent years. Rapid lateral immunoassay is believed to be one of the most promising approaches which have shown the most remarkable success in various fields. It presents simpler and more reliable techniques that do not require advanced devices. It also provides visible outcomes in about 15 minutes, making it ideal to be used in low-resource settings (Dubinský *et al*., 2000). The approach has long been adapted to detect many parasitic infections such as filariasis (Rahmah *et al*., 2001), Chagas' disease (Cardinal *et al*., 2006), and leishmaniasis (Schallig *et al*., 2004).

In the literature, thus far, only one report has described a commercial rapid test namely ToxocaraCHEK which is a flow-through-based assay with native TES antigen immobilized on nitrocellulose membrane of the test device (Akao *et al*., 1997). However, the production status of the kit remains unknown. A report published by Lim *et al.* (2015) on the field of evaluation of a rapid test namely iToxocara utilizing recombinant TES-30 antigen revealed a diagnostic sensitivity and specificity of 85.7% and 90.1% respectively. However, the test requires either only serum or plasma as a sample since the binding of antigen to an antibody can easily be interfered by lysed blood cells. A laboratory prototype of IgG4-lateral flow immunoassay utilizing three recombinant *T. canis* antigens i.e. rTES-26, rTES-30 and rTES-120 was developed whereby the test obtained a diagnostic sensitivity of a combination of the results from *T. canis* antigens was 93.1% and 100% specificity (Yunus *et al*., 2018). To date, there is no report of a commercialized dipstick assay utilizing recombinant antigen for the detection of human toxocariasis.

The development of analytical devices that provide reliable and fast diagnostics through digitalization and quantification of biological responses offers the potential for more sophisticated advancement in the industrial diagnostics. One study has described the application of microfluidic immunosensor using laser-induced fluorescence (LIF) incorporated with TES antigen which coupled to silica nanoparticles for detection of IgG anti-*T. canis* antibodies revealed had a limit of detection of 0.12 ng/mL^{-1} (Medawar *et al*., 2017). The latest research, using nanobodies (Nbs) in an electrochemical magneto-sensor showed that the approach had improved the diagnostic sensitivity by 10-fold greater than the sandwich ELISA method (Morales-Yanez *et al*., 2019). The authors also revealed that the analytical sensitivity of the sensor was 15 pg/mL in serum spiked with TES antigen and no cross-reactivity was observed with antigens from other nematode infections. Both reports highlighted highly potential assays for the development of toxocariasis diagnosis.

1.7.3 Recombinant TES antigens in serodiagnosis of human toxocariasis

In recent years, efforts have been focused on producing recombinant TES antigens (rTES) that can improve serodiagnosis of toxocariasis, as a substitution for native TES antigen. The use of recombinant TES antigen has improved diagnostic specificity and reduced cross-reactions with other parasitic diseases (Yamasaki *et al*., 2000; Wickramasinghe *et al*., 2008; Mohamad *et al*., 2009; Yunus *et al*., 2018). The production of recombinant TES antigens is much simpler and represents a more controlled process compared to the laborious native TES antigens. The amount of produced antigen relies on the amount of parasite culture (Yamasaki *et al*., 2000).

Thus far, four types of recombinant TES proteins i.e. rTES-26, rTES-30, rTES-120, and *T. cati* rTES-120 have been reported in the literature to exhibit diagnostic