

**PRODUCTION AND CHARACTERIZATION OF  
RECOMBINANT MONOCLONAL ANTIBODIES  
AGAINST *TOXOCARA* ANTIGENS**

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**UNIVERSITI SAINS MALAYSIA**

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by

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## LIST OF SYMBOLS

%	percent
°C	Celsius
μ	mu
μL	microlitre
cm	centimeter
g	gram
kg	kilogram
L	litre
M	Molar
mg	milligram
mL	millilitre
mm	millimeter
mM	millimolar
ng	nanogram
pg	picogram
x g	relative centrifugal force
α	alpha
γ	gamma
δ	delta
ε	epsilon
kappa	kappa
lambda	lambda
μm	micrometre
μM	micromolar

## LIST OF ABBREVIATIONS

Ab	antibody
ABTS	2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid
ABZ	albendazole
APS	Ammonium persulfate
BSA	Bovine serum albumin
C	constant
CD4	cluster of differentiation 4
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CDR	complementarity-determining region
cfu	colony forming units
CI	confidence interval
CL-1	cathepsin L-1
CM5	carboxymethylated dextran 5
CT	covert toxocariasis
CV	column volume
DC	detergent compatible
DEC	diethylcarbamazine
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E. coli	Escherichia coli
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	and others
EtBr	Ethidium bromide
F(ab') <sub>2</sub>	minibody
Fab	fragment antigen-binding
Fc	fragment crystalline
Ff	filamentous bacteriophages
FR	framework region

Fv	fragment variable
HC	heavy chain
His	histidine
HRP	Horseradish peroxidase
IFN- $\gamma$	interferon gamma
IgG	Immunoglobulin G
IL	interleukin
IMGT/V- QUEST	V-Query and Standardization
IPTG	Isopropanyl-beta-D-thiogalactopyranoside
$k_a$	association rate constant
$k_d$	dissociation rate constant
$K_D$	equilibrium dissociation constant
kDa	kilodalton
L3	third larval stage
LC	light chain
mAb	monoclonal antibody
MBZ	mebendazole
MPPP	Penang City Council
mRNA	Messenger RNA
MUC	mucin
MWCO	molecular weight cut-off
NaCl	Sodium chloride
NC	nitrocellulose
Ni-NTA	nickel nitrilotriacetic acid
NT	neurotoxocariasis
OD	optical density
OLM	ocular larva migrans
pAb	polyclonal antibody
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
pH	potential of Hydrogen
psi	Pound per square inch

RC	reducing agent
rmAb	recombinant monoclonal antibody
rpm	revolutions per minute
scFv	single-chain variable fragment
scFv-Fc	single-chain variable fragment-fragment, crystallisable
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
spp.	species
SPR	surface plasmon resonance
Strep	streptactin
TAE	Tris acetate EDTA
TB	Terrific Broth
TBA-1	<i>Toxocara canis</i> polyprotein allergen
TBS	Tris-buffered saline
TBZ	thiabendazole
Tc-CTL	<i>Toxocara</i> C-type lectin
Tc-PEB	<i>Toxocara</i> phosphatidylethanolamine (PE)-binding protein
TEA	Triethylamine
TES	<i>Toxocara</i> excretory-secretory
TGF- $\beta$	transforming growth factor beta
Th1	T helper cell 1
Th2	T helper cell 2
TNF- $\alpha$	tumour necrosis factor alpha
Treg	T regulatory
USA	United States of America
USM	Universiti Sains Malaysia
UV	ultraviolet
V	variable
VBASE2	V gene database
V <sub>H</sub>	single-domain antibody fragment of human
VH	Heavy chain variable region
V <sub>HH</sub>	Single-domain variable located on a heavy chain
VL	Light chain variable region
VLM	visceral larva migrans
V <sub>NAR</sub>	Single-domain Variable New Antigen Receptors

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- Appendix A1 Recipes for preparing 10% SDS-PAGE gel
- Appendix A2 Vector map of original and modified version of pET-51b(+)
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**PENGHASILAN DAN PENCIRIAN REKOMBINAN ANTIBODI  
MONOKLON TERHADAP ANTIGEN *TOXOCARA***

**ABSTRAK**

Toksokariasis ialah penyakit parasit zoonotik terabai yang disebabkan oleh cacing gelang parasit usus anjing (*Toxocara canis*) dan kucing (*Toxocara cati*). Toksokariasis manusia mempunyai taburan global dan memberi kesan kepada golongan yang rendah sosioekonominya. Pakar perubatan menghadapi masalah untuk mendiagnosis penyakit toksokariasis manusia kerana tanda-tanda dan simtomnya tidak spesifik dan mungkin serupa dengan jangkitan helmin yang lain. Kebanyakan kaedah serodiagnostik untuk pengesanan penyakit toksokariasis bergantung pada ujian berasaskan antibodi IgG yang menggunakan antigen natif *T. canis*, namun kelemahannya ialah kekurangan spesifisiti diagnostik kerana terdapat kereaktifan silang dengan antibodi terhadap helmin lain. Di samping itu, IgG adalah antibodi yang bertahan lama, oleh itu ujian IgG dapat mengesan jangkitan lampau (telah sembuh). Justeru, terdapat keperluan untuk menambahbaik ujian serodiagnosis bagi penyakit toksokariasis; iaitu dengan membangunkan ujian pengesanan antigen. Untuk membangunkan ujian sedemikian, rekombinan antibodi monoklon (rmAbs) terhadap antigen perkumuhan-rembesan (TES) *Toxocara* boleh digunakan sebagai reagen penangkapan. Dalam kajian ini, dua protein rekombinan iaitu *T. canis* (rTES-26) dan *T. cati* (rTES-120 cati), telah dieskpres dan ditulin. Isolasi mAbs terhadap protein rekombinan tersebut dilakukan melalui kaedah “biopanning” menggunakan perpustakaan imun paparan helmin faj yang dihasilkan sebelum ini. Lima mAb terhadap antigen rTES-26 berjaya diasingkan. Walau bagaimanapun, mAbs terhadap antigen cati rTES-120 menunjukkan urutan scFv yang tidak lengkap; oleh itu, analisis



lanjut tidak dapat dilakukan. Hanya mAb terhadap antigen rTES-26 dicirikan berdasarkan keluarga gen, panjang jujukan dan taburan asid amino. Dua protein rmAb terpilih (Ab 48 dan Ab 49) telah diekspres dan divalidasi melalui kaedah pemblotan Western. Kemudian, analisis pengikatan rmAbs dengan antigen rTES-26 melalui kaedah pemblotan Western dan ELISA telah disahkan. Dalam kekhususan ELISA, Ab 49 menunjukkan kereaktifan minimum terhadap antigen helmin lain berbanding Ab 48. Selain itu, titrasi ELISA menunjukkan bahawa Ab 49 mengikat dengan kepekatan antigen rTES-26 yang lebih rendah (31.25  $\mu\text{g/mL}$ ) berbanding Ab 48 (62.5  $\mu\text{g/mL}$ ). Kekhususan pengikatan kedua-dua rmAbs dengan antigen natif *T. canis* telah disahkan melalui pemblotan Western dan ELISA. Tambahan pula, analisis resonans plasmon permukaan (SPR) menunjukkan bahawa Ab 48 mempunyai pertalian pengikatan yang lebih kuat dengan antigen rTES-26 berbanding Ab 49. Namun begitu, pertalian pengikatan Ab 49 adalah mencukupi untuk digunakan dalam membangun ujian diagnostik. Kesimpulannya, kajian ini telah berjaya menghasilkan mAbs spesifik terhadap rTES-26 menggunakan perpustakaan imun paparan helmin faj. Dua keluarga gen berbeza bagi mAb khusus *T. canis* telah diasingkan, dan dua protein rmAbs terpilih (Ab 48 dan Ab 49) terhadap antigen rTES-26 telah dihasilkan. Pengikatannya dengan rTES-26 dan antigen natif *T. canis* telah divalidasi, juga kekhususan dan kepekaan pengikatannya. Rekombinan antibodi monoklon, terutamanya Ab 49, berpotensi untuk digunakan bagi pembangunan ujian pengesanan antigen untuk penyakit toksokariasis.

# **PRODUCTION AND CHARACTERIZATION OF RECOMBINANT MONOCLONAL ANTIBODIES AGAINST *TOXOCARA* ANTIGENS**

## **ABSTRACT**

Toxocariasis is a neglected zoonotic parasitic disease caused by intestinal parasitic roundworms of dogs (*Toxocara canis*) and cats (*Toxocara cati*). Human toxocariasis has a global distribution and affects mostly people who are socioeconomically deprived. Clinicians face problems in diagnosing human toxocariasis since the signs and symptoms are non-specific and similar to other helminthic infections. Most serodiagnostic methods for toxocariasis detection rely on IgG antibody-based assays using native antigen of *T. canis*, however the drawback is the lack of high diagnostic specificity due to cross-reactivity with antibodies to other helminths. Additionally, IgG is a long-lasting antibody, thus IgG assays may also detect past (cured) infections. Thus, there is a need to improve the serodiagnosis of toxocariasis and one good way is by developing an antigen detection assay. To develop such an assay, recombinant monoclonal antibodies (rmAbs) to *Toxocara* excretory-secretory (TES) antigens can be used as the capture reagent. In this study, two recombinant proteins namely *T. canis* (rTES-26) and *T. cati* (rTES-120 cati), were expressed and purified. The isolation of mAbs to the recombinant proteins was performed via biopanning using previously produced helminth phage display immune library. Five mAbs against rTES-26 antigen were successfully isolated. However, mAbs against rTES-120 cati antigen showed incomplete scFv sequences; hence, further analysis could not be performed. Henceforth, only the mAbs of the rTES-26 antigen were characterized based on gene family, length of sequence and amino acid distribution. Two selected rmAb proteins (Ab 48 and Ab 49) were expressed and

verified by Western blot. Then, binding analyses of the rmAbs to rTES-26 antigen were verified by Western blot and ELISA. In specificity ELISA, the Ab 49 showed minimal cross-reactivity to other helminth antigens compared to Ab 48. Moreover, titration ELISA showed that the Ab 49 bound to a lower concentration of rTES-26 antigen (31.25  $\mu\text{g/mL}$ ) than the Ab 48 (62.5  $\mu\text{g/mL}$ ). The binding specificity of both rmAbs to the native antigen of *T. canis* was verified by Western blot and ELISA. Additionally, surface plasmon resonance (SPR) analysis showed that Ab 48 had stronger binding affinity to the rTES-26 antigen than the Ab 49. Nevertheless, the binding affinity of Ab 49 was sufficient for use in developing a diagnostic test. In conclusion, this study has successfully enriched specific mAbs against rTES-26 using an in-house helminth phage display immune library. Two distinct gene families of *T. canis*-specific mAbs were isolated, and two selected rmAbs proteins (Ab 48 and Ab 49) against rTES-26 antigen were produced. Their binding against rTES-26 and native antigens of *T. canis* were validated along with their binding specificity and sensitivity. The rmAbs, especially Ab 49, are potentially useful for developing an antigen detection test for toxocariasis.

# CHAPTER 1

## INTRODUCTION

### 1.1 Human toxocariasis: An overview

Toxocariasis is a worldwide zoonotic parasitic disease caused by intestinal parasitic roundworms, mainly *Toxocara canis*, found in dogs and, to a lesser extent, *Toxocara cati* in cats (Glickman & Schantz, 1981). According to the Centers for Disease Control and Prevention (CDC), USA, toxocariasis is among the important neglected parasitic diseases, reflecting its potentially serious impact on public health globally (Moreira et al., 2014). Humans get infected by accidental ingestion of viable embryonated *Toxocara* ova containing the third-stage larvae from contaminated soil, food, water, unwashed vegetables, and undercooked meats from paratenic hosts such as chicken, cattle, and sheep (Chen et al., 2018).

There is a high occurrence of toxocariasis among children due to their closer contact with pets, exposure to several kinds of animals in didactical farms, and accidentally ingesting contaminated soil (Pieroni et al., 2021). Human toxocariasis has a global seroprevalence of 19% (95% confidence interval (CI) 16.6–21.4%), indicating that around 1.4 billion people worldwide are infected with or exposed to *Toxocara* species, particularly in subtropical and tropical regions (Ma et al., 2020). The prevalence varies among different regions of the world, depending on whether living in a rural area; close contact with dogs, cats or soil; consumption of raw meat; drinking of untreated water; geographical locations; and climatic parameters (Rostami et al., 2019a). The overall seroprevalence of human toxocariasis in Southeast Asia countries varies from 3.9% to 84.6%, and in Malaysia, it was reported to be ~20% (Chou & Fan, 2020; Hakim et al., 1993).

Human as paratenic hosts gets infected via accidental ingestion of eggs containing infective third-stage larvae (L3). After ingestion the eggs hatched in the small intestine and the larvae do not undergo maturity but instead carried by the circulation through the somatic tissues and organs of the body causing clinical toxocariasis (Fan et al., 2015). They enter the blood vessels, lymphatic system and migrate into various internal organs such as the liver and lungs, causing visceral larva migrans (VLM). Larval invasion of the eyes causes ocular larva migrans (OLM), and invasion of the central nervous system leads to neurotoxocariasis (NT). The infection also leads to other non-specific symptoms such as fever, anorexia, abdominal pain, vomiting, and behavioural disorders resulting in a syndrome known as covert toxocariasis (CT) (Ma et al., 2018).

Clinicians have difficulty identifying human toxocariasis because of a wide range of non-specific symptoms, which can also be caused by other helminthic diseases, allergies, and even asthma. It is also difficult to determine toxocariasis in individuals who are asymptomatic, which means that many of these individuals are left misdiagnosed and untreated, resulting in an underestimation of the true worldwide prevalence of toxocariasis (Noordin et al., 2020). Toxocariasis diagnosis using histopathological study of tissue sample is invasive and scarcely warranted, insensitive, and time-consuming. Since the parasite does not mature into an adult stage in humans, parasitological analysis of feces cannot assist with laboratory diagnosis (Wilkins, 2014). Thus, serological and immunological techniques are mostly used to diagnose human toxocariasis (Jasim & Hadi, 2021). Currently, enzyme-linked immunosorbent assay (IgG-ELISA) using *Toxocara* excretory-secretory (TES) antigen is the standard method as serological tests for diagnosis of human toxocariasis and positive findings

should ideally be verified by immunoblot (Western blot) to avoid false-positive results and evaluate cross-reactivity with other infective agents (El-Sayed & Ramadan, 2017).

There is a need to improve the serodiagnosis of toxocariasis via antibody and antigen detection assays. The latter can help differentiate between current and past infections. Antibodies specific to *Toxocara* antigens are needed to develop antigen detection assays and may also be used as quality control reagents for commercial kits (Noordin et al., 2020). The capture antibody can be in the form of polyclonal or monoclonal antibodies. However, increased specificity of sandwich ELISAs for toxocariasis utilizing monoclonal antibodies has been reported when used on multi-parasitized serum samples (Ishiyamna et al., 2009; Zibaei et al., 2010).

## **1.2 The *Toxocara* worm**

### **1.2.1 Taxonomy**

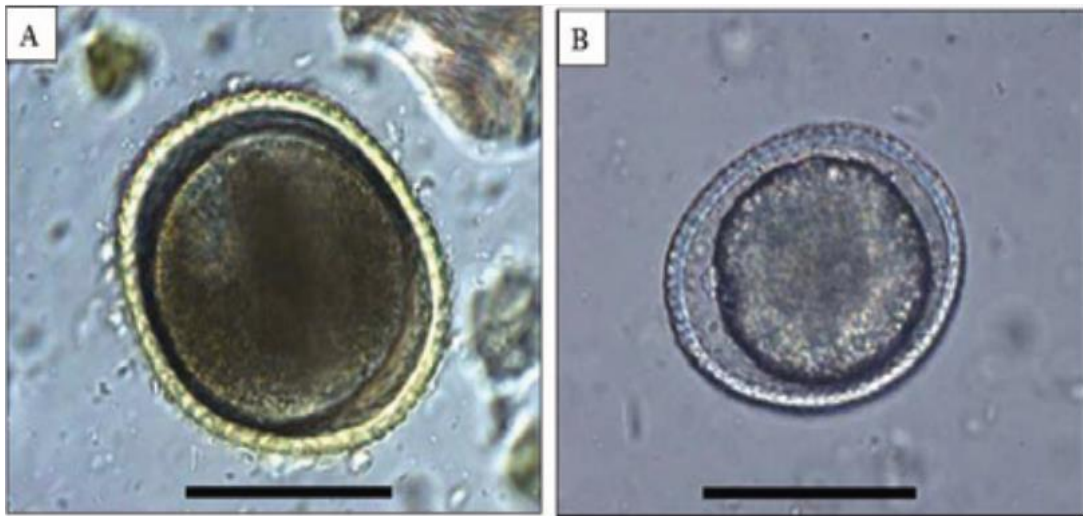
*Toxocara canis* (*T. canis*) and *Toxocara cati* (*T. cati*) are two of the most common intestinal of dogs and cats parasites which have a global distribution (Bowman, 2020). They belong to the Animalia kingdom, member of the Nematoda phylum, classified under the Secernentea class, categorized under the Ascaridia order, part of the Toxocaridae family and grouped under the *Toxocara* genus (Jasim & Hadi, 2021). Identification and categorization of ascaridoid parasites of the *Toxocara* genus are based on their host species and morphological characteristics. To date, there are 23 species classified under *Toxocara* genus (Gibbons et al., 2001). Besides *T. canis* and *T. cati* which infects dogs and cats, several *Toxocara* species infecting other animals have been reported such as *Toxocara vituroolum* (cattle); *Toxocara pteropodis* (bats); *Toxocara lyncus* (caracals); *Toxocara mackerrase* and *Toxocara apodemi* (rodents);

*Toxocara paradoxura* and *Toxocara sprengi* (viverrids); and *T. vajrasthira* (mustelids) (Chen et al., 2012).

### 1.2.2 Morphology

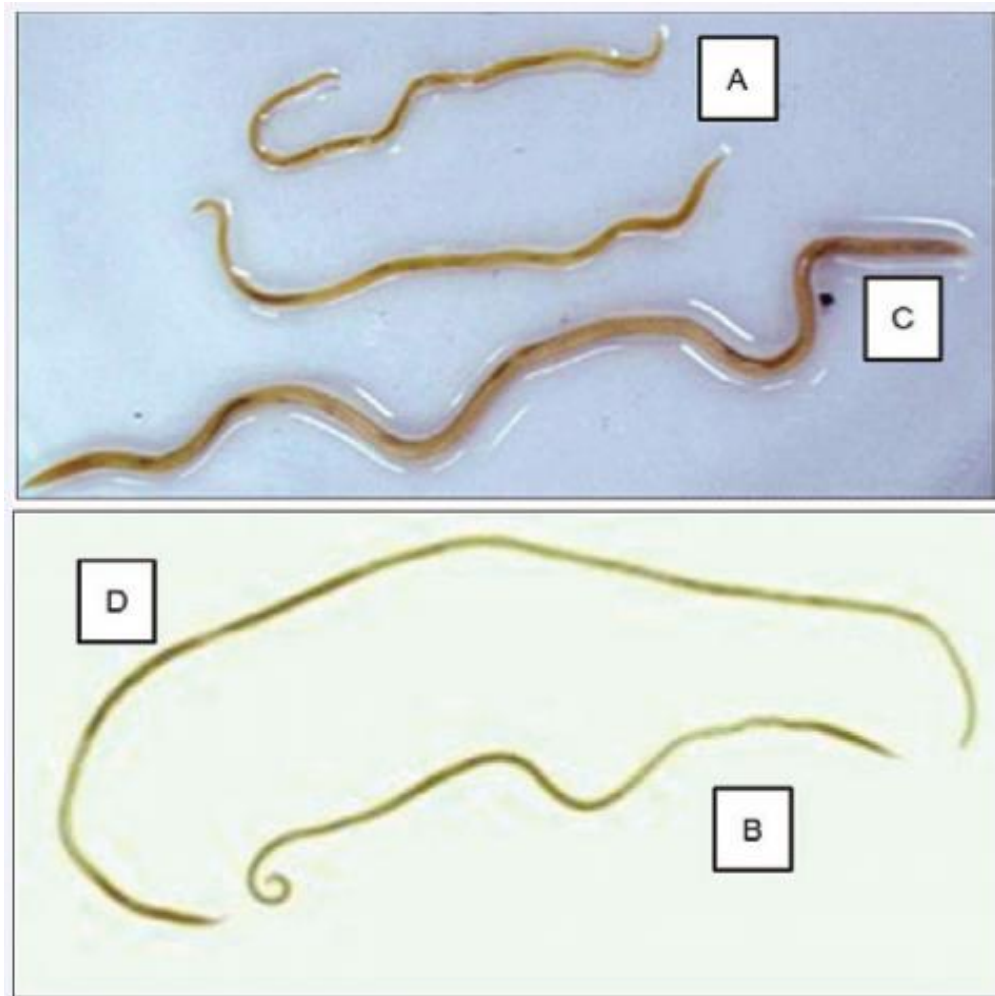
*T. canis* has a morphological resemblance to *T. cati* and both of them have three stages i.e., males and females in their adult stages, eggs and larvae. When the eggs are laid, they are not fully developed. However, when the eggs are eliminated into the environment in dog or cat's faeces, they become embryonated under optimum conditions. They are exceedingly resistant to environmental factors i.e., weather and chemical conditions since the eggs have a protective dense covering which allows them to be infectious for months or years (Joy et al., 2017). The eggs of *T. canis* and *T. cati* are large, brownish, nearly spherical, and the average size is ~ 75 to 90 µm and 65 to 70 µm, respectively (**Figure 1.1**). Females produce around 200,000 unembryonated eggs per day. Under ideal conditions, the eggs grow to infectious stage within timeframe of three to six weeks up to several months and can survive for at least a year (Overgaauw, 1997).

Adult *T. canis* and *T. cati* worms are dioecious, with male and female worms. The tubular testis and spicules of the male worm measure around 1.7 to 1.9 mm long, allowing for direct sperm transmission while the vulva of the female worm covers about one-third the body length and contains very large and extensive ovaries (Bowman et al., 2008). The adult male worms measure nearly 3 to 6 cm length with ventrally arched posterior segment while the female worms reach approximately 10 to 15 cm length with tapered posterior region respectively as shown in (**Figures 1.2 and 1.3**). Moreover, the adult worms have prominent two fin-shaped cervical alae positioned at the anterior end which vary in shape and size and can be used to differentiate between species of the *T. canis* and *T. cati* (**Figure 1.4**).

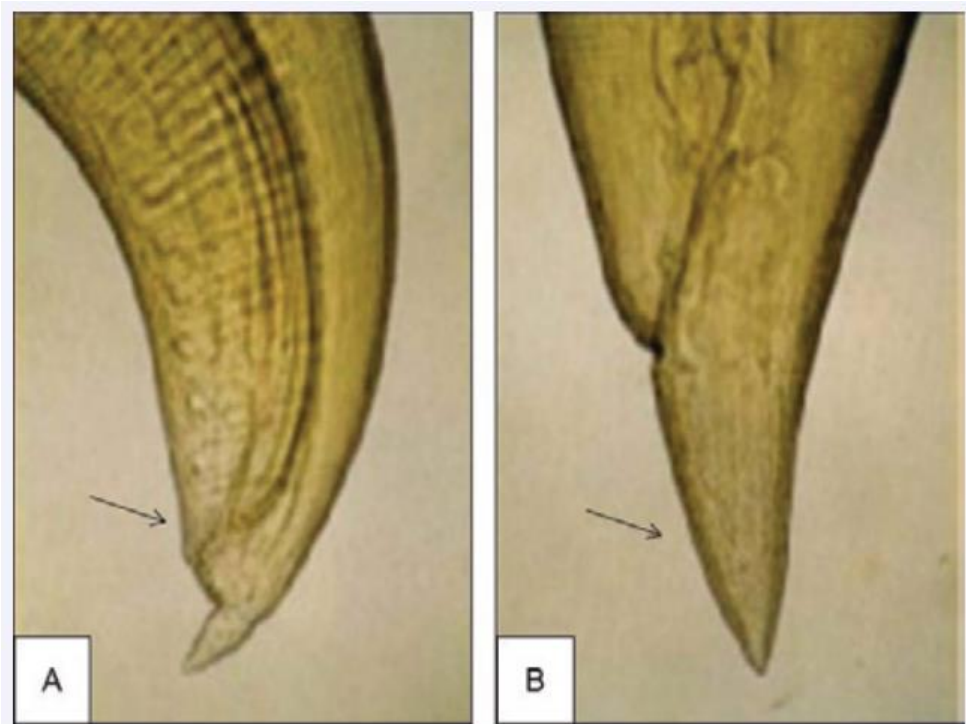


**Figure 1.1** Morphological characteristics of *Toxocara* eggs (A) *T. canis* with bar size (~75 to 90  $\mu\text{m}$ ) and (B) *T. cati* eggs with bar size (~ 65 to 70  $\mu\text{m}$ ) (Machado et al., 2017).

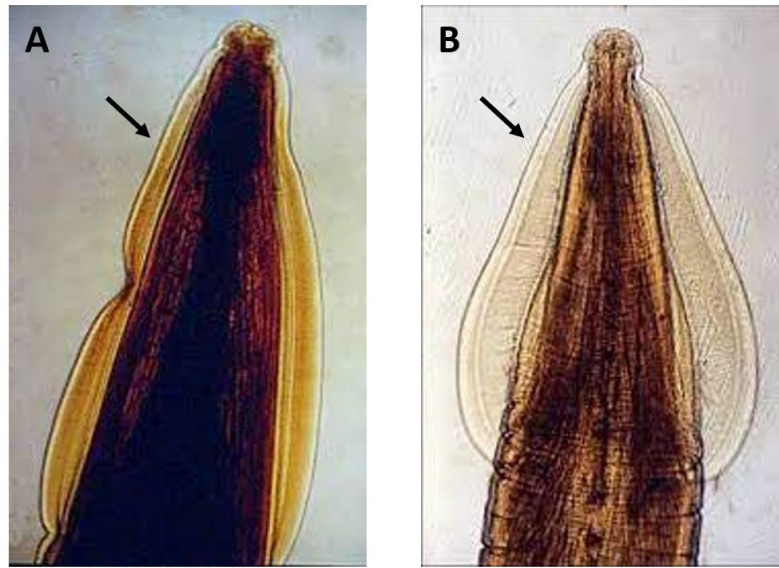




**Figure 1.2** Morphology of adult worms: males (A and B) (3 to 10 cm) and females (C and D) (10 to 15 cm) of *T. canis* and *T. cati*, respectively (Machado et al., 2017).



**Figure 1.3** Morphology of the posterior segments of adult male (A) and female (B) worms of the genus *Toxocara* (Machado et al., 2017).



**Figure 1.4** Morphology of cephalic alae at anterior region of *T. canis* (A) is long and narrow giving the appearance of a spear-shaped alae, while in *T. cati* (B) the cephalic alae is short and wide giving the appearance of an arrow-head alae.

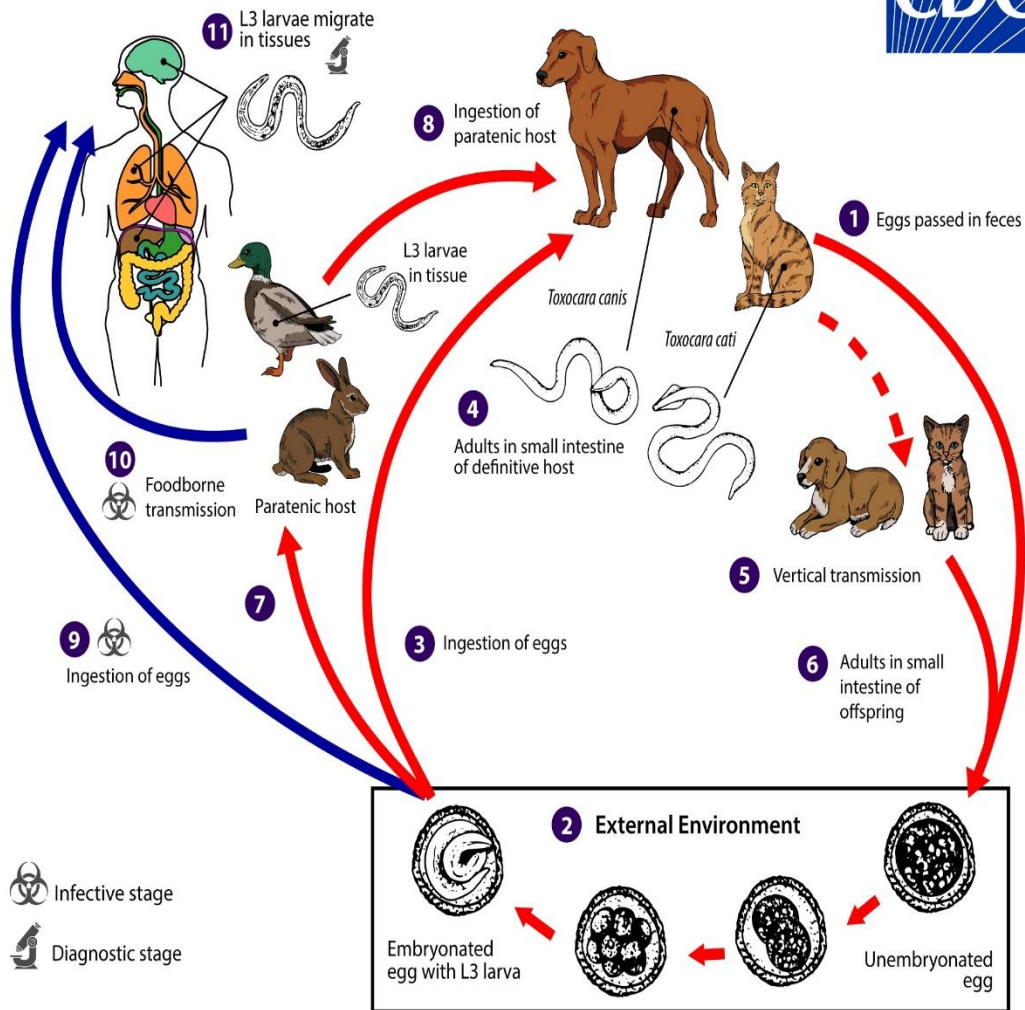
### 1.2.3 Life cycle

*T. canis* and *T. cati* possess two complex life cycles such as direct (one host) and indirect (many hosts) as illustrated in **Figure 1.5**. Infected dogs and cats are the definitive hosts in which they shed unembryonated eggs in their feces. Once eggs released into the environment, depending on the temperature and humidity of the surrounding environment, they undergo embryonation between two to four weeks and become infective containing third-stage (L3) larvae (Woodhall & Fiore, 2014). After being ingested by the definitive host, infective eggs hatch and larvae invade the small intestine, subsequently travel in the bloodstream to the lungs via the liver.

The full cycle usually only happens in puppies (*T. canis*) and in kittens (*T. cati*) where the majority of the larvae continue their migration from the trachea to the oesophagus via the pharynx, reach the stomach and small intestine. The larvae develop into adult worms and oviposit in the small intestine. The adult worms then lay eggs, which are deposited in the feces (Kong & Peng, 2020). However, when older dogs and cats ingest infective eggs, the larvae migrate to various organs in the body and their growth is halted where no further maturation into adult worms occurs. Arrested dormant larvae become revived in female dogs and cats during late gestation and may infect the puppies and kittens by transplacental (pass through the placenta into the foetus) and transmammary (suckling through milk) routes (Ma et al., 2018).

*T. canis* and *T. cati* can also be transmitted indirectly via the accidental ingestion of infective eggs by paratenic hosts for example duck, rats, and rabbits. The eggs hatch and the larvae invade the gut wall, where they become encyst in diverse tissues. When definitive hosts ingest encysted larvae within the paratenic host tissue, the larvae mature into adult worms in the small intestine and the life cycle is complete (Joy et al., 2017).

Humans as paratenic hosts can become infected by ingesting infective larvae from paratenic hosts or infective eggs from contaminated soil, soil, food or water. The eggs hatch after ingestion, and the larvae enter the intestinal wall, where they are transmitted to various tissues such as liver, lungs, neurological tissues, muscles and retina by the blood circulation. The larvae are unable to mature further in these sites, and can induce host inflammatory responses and mechanical damage, resulting in a broad spectrum of toxocariasis clinical manifestations (Ma et al., 2018).



**Figure 1.5** Life cycle of *T. canis* and *T. cati* in definitive and paratenic hosts. Source: <https://www.cdc.gov/dpdx/toxocariasis/index.html> (accessed on 7 August 2022)

### 1.3 Mode of transmission of human toxocariasis

Humans get infected with *Toxocara* spp. by accidental ingestion of embryonated infective eggs of *T. canis* and *T. cati* found in soil contaminated with feces of dogs and cats, respectively (Sazmand et al., 2020). Playgrounds, sandpits, gardens, parks and beaches are places visited by animals, thus likely to be prone to contamination (Beugnet et al., 2018). There is a high occurrence of toxocariasis among children as they are exposed to several kinds of animals in farms and other areas, and accidentally ingesting soil contaminated with infected *Toxocara* eggs due to their play habits and poor hygiene (Pieroni et al., 2021).

Humans can also become infected by ingesting encapsulated third-stage larvae of *Toxocara* spp. in water, raw or undercooked meats of paratenic hosts such as chicken, cattle, and sheep or by eating unwashed contaminated fruits and vegetables (Chen et al., 2018; Zibaei et al., 2017). Another method of transmission is human who are in close contact with dogs or cats as embryonated eggs could attach on the hairs of these definitive hosts (Bakhshani et al., 2019). However, a study showed that relatively low quantity of embryonated eggs were found on the host hair as embryonation progress is slower on the hair coat, particularly among well-cared dogs (Keegan & Holland, 2013).

Several transmission pathways suggest that there is a high chance of human infection (Ma et al., 2018). Nevertheless, the *Toxocara* larvae are unable to develop in humans, unlike in their definitive hosts, since humans do not provide adequate conditions for the larvae to continue their growth and maturity. Thus, the larvae enter tissues, migrate throughout the human body and remain alive for several years resulting in a wide range of clinical symptoms in the hosts (Woodhall & Fiore, 2014).

## **1.4 Clinical manifestation of human toxocariasis**

*T. canis* and *T. cati* infections are frequently linked with a wide spectrum of clinical manifestations, ranging from asymptomatic to non-specific clinical manifestations, making it challenging to diagnose clinical cases of toxocariasis (Chen et al., 2018). Additionally, the majority of individuals are asymptomatic which may go undetected and untreated. As a result, the true worldwide prevalence of toxocariasis is likely to be underestimated (Noordin et al., 2020). Human toxocariasis has been systematically categorized into four clinical forms based on which organs that are afflicted known as visceral larva migrans (VLM), ocular larva migrans (OLM), neurological toxocariasis (NT), and covert or common toxocariasis (CT) (Jasim & Hadi, 2021). The severity of clinical symptoms vary influenced by the larvae burden in the tissue invaded, the duration of larval migration, and the immune-mediated response as well as the age of the infected individual (Joy et al., 2017).

### **1.4.1 Visceral larva migrans (VLM)**

Visceral larva migrans (VLM) is the result of *Toxocara* larvae entering blood vessels and lymphatic system and subsequently migrating systemically through human visceral tissue which can cause immense damage to the liver, lungs, and other organs. The first VLM was reported in three children with hypereosinophilia, hepatomegaly, pulmonary infiltration, cough, fever, and hyperglobulinemia (Beaver et al., 1952).

The clinical disease generally manifests among children aged two to seven years old caused by recurrent infection with *Toxocara* larvae at a high intensity, like having geophagia as well as having close contact with pet animals (Chen et al., 2018). The disease is characterised by fever, abdominal pain, hepatosplenomegaly, and necrosis, bronchospasm and asthma (Despommier, 2003; Strube et al., 2013). Some infected



individuals may also show leucocytosis, eosinophilia up to 70%, hypergammaglobulinaemia, and organs involvement such as myocarditis, myalgia with eosinophilic polymyositis, arthritis, and nephritis. Dermatological alterations such as rash, pruritus, eczema, panniculitis, urticaria, and vasculitis have also been linked to VLM (Holland & Smith, 2006; Joy et al., 2017).

Recently, a study showed that toxocariasis can affect not just youngsters but also people as old as 80 years and above. The infection does not require direct contact with pet animals and infection and can also be acquired through oral consumption of undercooked meat (e.g. fowl, hares, even snails) containing *Toxocara* larvae. Thus, contamination of the environment with *Toxocara* eggs and their airborne dissemination must be regarded a major cause of infection (Auer & Walochnik, 2020).

#### **1.4.2 Ocular larva migrans (OLM)**

Ocular larva migrans (OLM) occurs when the infective larvae invade the posterior pole or peripheral of the retina, causing the formation of a granuloma that can lead to impaired vision, heterotopia, and macula ablatio (Small et al., 1989). The syndrome is common in older children and adults and manifests as unilateral vision loss which is typically coupled with strabismus (Dinning et al., 1988). Bilateral ocular involvement has been documented, however it is rare (Ahn et al., 2014; Jasim & Hadi, 2021). The first case of OLM was identified in 1956 in a children with presumed retinoblastoma, in which *Toxocara* larvae were found in enucleated eyes with granulomatous lesions (Badri et al., 2021; Taylor, 2001).

A study revealed three possible ocular manifestations: chronic endophthalmitis (generally appears between the ages of 2 and 9 years), posterior pole granuloma (normally occurs between the ages of 6 and 14 years), and peripheral granuloma (usually arises from adolescence to adulthood) (El-Sayed & Ramadan, 2017).

Furthermore, the alterations are associated with uveitis, papillitis with or without granuloma in the eyes, cataract; and delay in diagnosis can lead to blindness (Machado et al., 2017). The severity of visual impairment is dependent on migratory or dead larvae, the extent of eosinophilia as well as the immune response to the worm in the eye (Pivetti-Pezzi, 2009).

### **1.4.3 Neurotoxocariasis (NT)**

Neurotoxocariasis (NT) occurs when *Toxocara* larvae migrate into the brain and spinal cord (Janecek et al., 2017). The invasion of the larvae cause cerebral lesions and neurological impairment, mostly in the cerebral and cerebellar white matter, as well as obstruction of cerebral blood vessels (Chen et al., 2018).

This disease is rare and mostly affects middle-aged persons and more prevalent in adult males; about 76% of cases were men with a mean age of  $42.3 \pm 15.2$  years (Deshayes et al., 2016; Sánchez et al., 2018). The first case of NT was discovered in humans in an autopsy investigation in which *Toxocara* larvae were identified in the left thalamus of a child with poliomyelitis (Beautyman & Woolf, 1951). Although NT is thought to be uncommon and poorly reported, over the last three decades with the developments of toxocariasis diagnostics, an increasing number of clinical NT cases related to larval invasion of central nervous system have been recorded (Fan et al., 2015).

The clinical symptom of NT involves diverse neurological manifestations namely meningitis, encephalitis, myelitis and cerebral vasculitis associated with non-specific symptoms such as headache, fever, weakness and epileptic seizures (Nicoletti, 2020). Moreover, there are peripheral nervous system symptoms of NT that have been recorded, including radiculitis, cranial nerve affection, and musculoskeletal involvement (Sánchez et al., 2018). A rare clinical manifestation was reported in a

healthy five-year-old boy who had NT with distinctive symptoms and multi-site involvement of both the central and peripheral nervous systems. The boy might have consumed contaminated food or experienced geophagia since his neighbourhood had poor living conditions and multiple dogs and cats (Salvador et al., 2010).

#### **1.4.4 Covert and common toxocariasis (CT)**

Covert and common toxocariasis (CT) is more common but less severe and represent a non-specific clinical syndrome as caused by infection with *Toxocara* larvae that could not be classified as VLM, OLM, or NT (Joy et al., 2017; Moreira et al., 2014). There are two types of syndrome namely covert toxocariasis which is most frequently seen in children, and common toxocariasis often found in adults (Ma et al., 2018).

The findings of a case–control research in Ireland on a group of children contributed to the definition of "covert toxocariasis" as a distinct clinical entity among seropositive individuals. The clinical signs for this syndrome are fever, anorexia, headache, behavioural and sleep disturbances, cough, abdominal pain, hepatomegaly, nausea and vomiting, with or without eosinophilia, and moderate titers of *Toxocara* specific antibodies (Taylor et al., 1987).

Meanwhile, another case–control research among adults in France resulted to the description of "common toxocariasis" which is a condition characterized by respiratory problems, skin rash, pruritus, weakness, abdominal pain, commonly accompanied with eosinophilia, increased levels of IgE, and high titers of *Toxocara* specific antibodies (Glickman et al., 1987). The terms "covert" and "common" toxocariasis most likely refer to the clinical range of mild *Toxocara* infections in children and adults, respectively. Thus, anthelmintic therapy is generally not required

in patients with these mild types of toxocariasis (Nicoletti, 2013; Rubinsky-Elefant et al., 2010).

### **1.5 The *Toxocara* excretory-secretory antigens (TES)**

Earlier attempts in human toxocariasis serodiagnosis relied on somatic antigens obtained from extracts of *T. canis* embryonated eggs, infective larvae or adult worms and showed minimal sensitivity and cross-reactivity with other ascarids and parasitic infections (Girdwood et al., 1978; Smith et al., 1982).

Helminth persistence is mediated by active immune suppression in both definitive and paratenic hosts. The *Toxocara* spp. release soluble antigens known as *Toxocara* excretory-secretory products (TES), which have an effect on host immune cells (Raulf et al., 2021). Hence, TES antigens are utilised in both diagnosis and seroepidemiological studies (Kavitha et al., 2019).

Native TES antigens comprise a combination of highly immunogenic glycoproteins obtained from *in vitro* culture of infective larvae (Magnaval et al., 2001; Maizels, 1984). When used in conjunction with ELISA, the use of TES antigens has significantly improved the sensitivity and specificity of toxocariasis serodiagnosis compared to prior approaches that used somatic antigens derived from embryonated eggs or adult worms (Watthanakulpanich, 2010). The first description of the *in vitro* culture method for producing TES antigens showed that the amount of antigen generated is proportional to the number of larvae (De Savigny, 1975). Hence, improvements to the approach have been reported, including a five-fold increase in parasite production, improved larval purity, and a reduction in execution time (Ponce-Macotella et al., 2011).

TES antigens secreted by *Toxocara* spp contain a huge number of glycosylated molecules and proteins; and primarily characterized as mucins [e.g. TES-120 (MUC-1 to 5)], lectins [e.g. TES-32/30 (Tc-CTL-1), TES-70 (Tc-CTL-4)] and other TES products [e.g. TES-26 (Tc-PEB-1)] (Gems et al., 1995; Gems & Maizels, 1996; Maizels et al., 1984). TES antigens activate and control the host immune system, protect the parasite and allow it to evade the human immunological responses, thus enabling them to persist for several years in the host tissues (Maizels, 2013). The use of native TES antigens in serodiagnosis yields good results because of its high sensitivity. However, it is time-consuming and tedious, and the culture volume restricts output capacity. Furthermore, in tropical regions where infections with different helminths are prevalent, cross-reactions with antibodies to other parasites can occur when using native TES, albeit to a lesser extent than with *Toxocara* somatic antigen. Thus, specificity of serodiagnosis is affected when using native TES (Mohamad et al., 2009).

## **1.6 Pathogenesis of human toxocariasis**

The level of tissue damage in the host along with the generation of signs and symptoms varies depending on larvae that invading different tissues (Jasim & Hadi, 2021). In the intestinal tract, larvae hatched from embryonated eggs or released by the digestion of the tissues of paratenic hosts enter the somatic cycle. When the cycle is complete, the larvae enter development arrest and migrate throughout the human body for a varying duration of time. Once stalled in tissues or organs with an abundance of immunological and/or phagocytic cells, the larvae may become encapsulated inside eosinophilic granulomas, causing it to be destroyed or remain in a viable state for many years (Fillaux & Magnaval, 2013).

The liver, lungs, heart, central nervous system (CNS) and eyes are considered the most susceptible organs in which the hatched larvae can be identified. The migrating larvae are often associated with immunological reactions such as haemorrhage, necrosis and inflammation with eosinophils (Magnaval et al., 2001). Moreover, amount of migrating juveniles and age of the host are crucial aspects in determining the extent of the clinical manifestations (Despommier, 2003).

The viability of the migrating larvae inside the paratenic host is dependent on the soluble TES antigens secreted by the larvae. Hence, the pathology in human happens when immunological responses are targeted against the TES antigens rather than the somatic antigen that are only exposed during larval demise (Fillaux & Magnaval, 2013). The larvae persistently release about 2 ng/larva/day of the TES antigens which are lacking in the somatic antigenic panel of larvae and adults (Fernando et al., 1970). Part of the TES antigen is internally released by the larvae's oesophageal gland and excretory column, while the other part consists of glycoproteins discharged by the larval outer epicuticular layer which is constantly and rapidly regenerated (Page et al., 1992a; Page et al., 1992b). In addition, the TES antigens also contain potent allergenic substance called TBA-1 that causes allergic reaction in individuals with toxocariasis (Yahiro et al., 1998).

The TES antigens secreted by the third-stage infective larvae initiate host immune responses (Del Prete et al., 1991). The involvement of TES antigens in inducing granulomatous inflammation has been described (Fan et al., 2015). *Toxocara* infection triggers delayed-type hypersensitivity that is a polarised T helper cell 2 (Th2) response, characterised by the involvement of a varies of interleukins (IL), including IL-4, IL-5, and IL-13 cytokines, which are implicated in the activation of mast cells, eosinophils, and macrophages, as well as elevated levels of IgE. Normally, activation

of the Th2 response occurs simultaneously with downregulation of the T helper cell 1 (Th1) inflammatory response, as revealed by a decrease in tumour necrosis factor alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ), and interleukin-17 (IL-17) production (Fan, 2020; Maizels, 2013).

TES antigen is also responsible for the parasite evading the host immune system by activating T regulatory (Treg) cells, which trigger the production of downregulating cytokines such as IL-10 and transforming growth factor beta (TGF- $\beta$ ) (Allen & Maizels, 2011). There is a precise balance of pro-, anti-inflammatory, and regulatory immune responses towards *Toxocara* spp. in the immunopathogenesis of toxocariasis. However, the immune system frequently fails in its attempts to eliminate long-term survival larvae in paratenic hosts since TES antigens can orchestrate immune evasion or manipulation (Fan, 2020).

## **1.7 Global seroprevalence of toxocariasis**

Human toxocariasis is one of the most prevalent and significant zoonotic parasitic diseases with worldwide distribution. It is a neglected disease with millions of individuals highly exposed or infected with the parasite. There are information gaps in the epidemiology of human toxocariasis at the global, regional, and national levels (Ma et al., 2018).

A systematic review and meta-analysis estimated that toxocariasis has a global seroprevalence of 19% (95% confidence interval [CI], 16.6–21.4%) in human populations. When extrapolating to the global population in 2016, it was predicted that 1.4 billion people are infected with or exposed to *Toxocara* species. As shown in **Figure 1.6** which represent the seroprevalence of human toxocariasis values (%) in the year 2019 reported that the seroprevalence of human toxocariasis vary substantially in

different geographical regions, ranging from 8.2% to 37.7% with 37.7% (95% CI, 25.7–50.6%) in Africa, 34.1% (95% CI, 20.2–49.4%) in South-East Asia, 24.2% (95% CI, 16.0–33.5%) in Western Pacific, 22.8% (95% CI, 19.7–26.0%) in the Americas, and 10.5% (95% CI, 8.5–12.8%) in Europe (Ma et al., 2020; Rostami et al., 2019a). The majority of epidemiology surveys of *Toxocara* infection in Southeast Asia had been conducted in Malaysia; the seroprevalence in Malaysia ranged from 3.9% to 35.5% and varied according to ethnicity and lifestyle (Chou & Fan, 2020). Studies have been demonstrated that Indians had the highest positive rate (35.5%), followed by Malaysian aborigines, Orang Asli (31.9%), Muslim Malays (14.8%), and Chinese (10.9%) (Hakim et al., 1992, 1993). Based on a reported IgG4-ELISA results among indigenous people, male revealed a higher seroprevalence than females, which were 9.5% and 1%, respectively (Romano et al., 2010). Comparable seroprevalence differences between males and females were also reported in another study (Lim et al., 2015). In two studies conducted in Malaysia, the positive rate among children was higher compared to adults (Hakim et al., 1993; Romano et al., 2010). Interestingly, regardless of whether the test employed was the rTES-30-ELISA or a rapid diagnostic test, it was shown that middle-aged Serendah Orang Asli Village individuals have the highest infection rate of *Toxocara*. followed by adolescents, and then children (Lim et al., 2015).

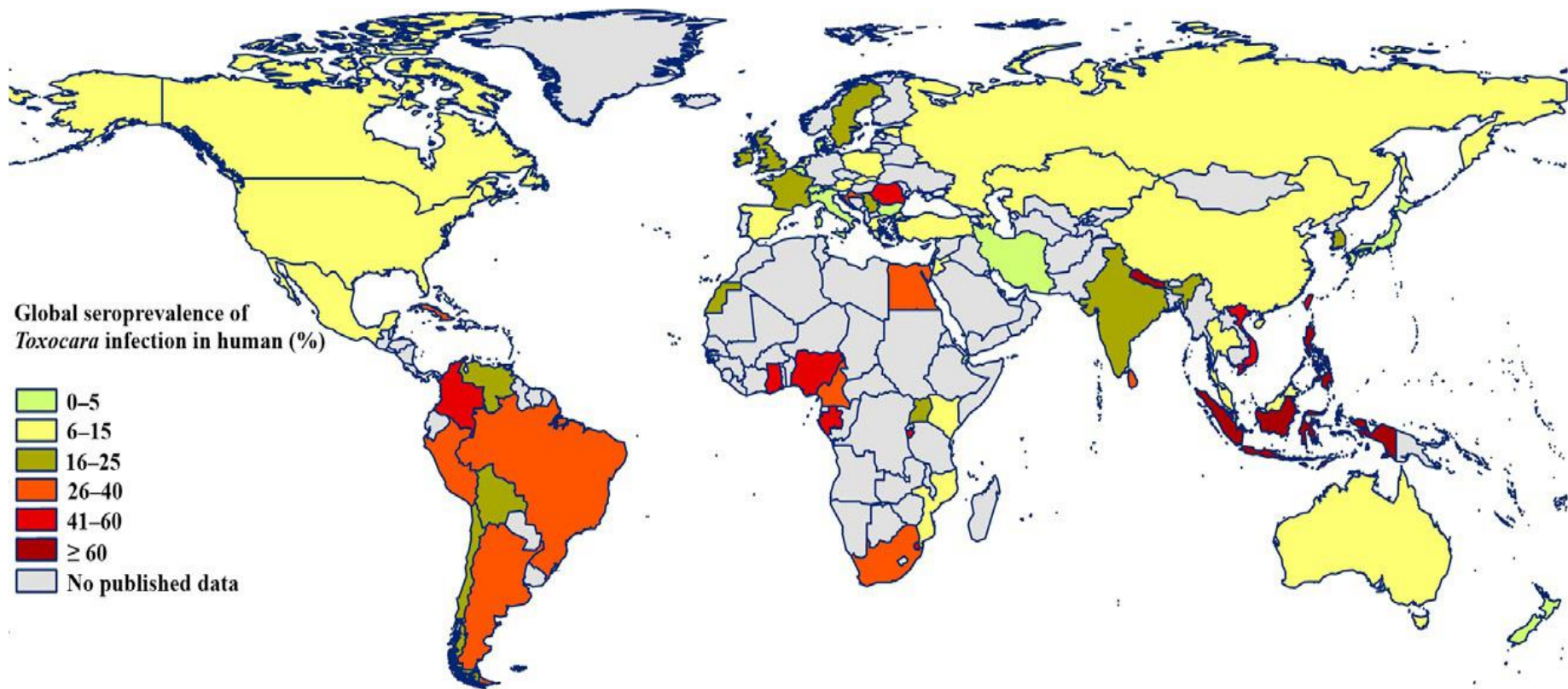
Recently, a study reported that the global prevalence of *Toxocara* infection in children was 30% (95% CI, 22–37%) and the estimated prevalence in Asia was the highest at 35% (95% CI, 3–67%) due to many different socioeconomic levels, and many populations with poor hygiene practices (Abedi et al., 2021) Since many countries have significant rates of infection or exposure to *Toxocara* species, there is a need for better awareness of human toxocariasis and enhanced strategies to minimise the harmful health effects of this disease (Rostami et al., 2019a). The county or region with high



seroprevalence of *Toxocara* in human population is associated with lower-income level, lower human development index, lower latitude, higher humidity, higher temperature and higher precipitation. Meanwhile, there are number of possible risk factors influencing *Toxocara* seropositivity such as male gender, living in rural areas, young age, close contact with dogs, cats or soil; consumption of raw meat, and consumption of untreated drinking water (Rostami et al., 2019a).

Environmental conditions greatly affect the embryonation of *Toxocara* eggs, which may influence infection transmission (Gamboa, 2005). Globally, it is predicted that  $\geq 100$  million dogs are infected with *T. canis* with a prevalence of 11.1% (95% CI, 10.6–11.7%) (Rostami et al., 2020a). Meanwhile, ~118 to 150 million cats worldwide are predicted to be definitive hosts of *T. cati* with a prevalence of 17.0% (95% CI, 16.1–17.8%), thus serving as sources of human infection (Rostami et al., 2020b). A study revealed that the global prevalence of *Toxocara* species eggs found in public places such as beaches, parks and playgrounds was 21% (95% CI, 16–27%) with highest prevalence of 35% (95% CI, 15–58%) in the Western Pacific, and the lowest (13% [95% CI, 8–23%]) in the North and Central Americas 13% (95% CI, 8–23%). Since public places are commonly contaminated with *Toxocara* eggs, it signifies a serious threat to human health (Fakhri et al., 2018).





**Figure 1.6** Global seroprevalence estimation of human toxocariasis. Prevalence values are based on published study (Rostami et al., 2019a).