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Research article

# Evaluation of new *Toxocara canis* chimeric antigens as an alternative to conventional TES-Ag for anti-*Toxocara* antibodies detection

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ABSTRACT

Human toxocariasis is one of the neglected helminthiases and it is caused by the zoonotic roundworm species *Toxocara canis* and *Toxocara cati*. Diagnosis of human toxocariasis is based on the combination of clinical, parasitological, and epidemiological criteria, as well as serology tests that detect anti-*Toxocara* antibodies. Notwithstanding, due to the absence of pathognomonic symptoms and signs of the disease, serology is the key evidence to support a conclusive diagnosis. TES-ELISA is the most widely used serological test for diagnosis. However, cross-reaction of TES antigens with antibodies produced to other helminth antigens is a major drawback for its application in countries with high parasitic prevalence. *T. canis* recombinant antigens have been described as an alternative to native TES for diagnosis. Nevertheless, the selection of antigenic proteins is a complex process that requires validation. In this paper, we developed an eGFP carrier-based system to express and purify blocks of recombinant polypeptides of *T. canis* antigenic proteins. Intense cross-reaction polypeptides were detected by Immunoblot and avoided to finally produce a chimeric prototype protein. Additionally, a control chimeric protein that harbors the complete tested proteins was produced. Purified chimeric antigens were tested in ELISA and Immunoblot assays with 310 sera samples of negative and positive control individuals. Our results showed that chimeric rCHITC0 and rCHITC1 antigens (with sensitivities of 62% 58%, 38% and 16% in IB-rCHITC0, ELISA-rCHITC0, ELISA-rCHITC1 and IB-rCHITC1 respectively for OLMS) can perform better in terms of specificity (being 91%, 89%, 87% and 76% for ELISA-rCHITC1, IB-rCHITC1, ELISA-rCHITC0 and IB-rCHITC0 respectively for OLMS) than *T. canis* TES-ELISA (with 61% specificity), giving a higher signal with serum samples of infected individuals as well the possibility to discriminate false positive cases with other parasitic infections. Our data suggest that *T. canis* chimeric proteins, represent candidate antigens for phase II studies.

## 1. Introduction

Toxocariasis is one of the most common neglected helminthiasis reported worldwide [1, 2, 3, 4]. Human disease is caused by infection with L3 infective larval stage of zoonotic roundworm species *Toxocara canis* and *Toxocara cati* [4, 5, 6]. *T. canis* is reported as the most prevalent species affecting human tissues and is the most reported species found in a variety of studied paratenic hosts. Furthermore, in most of the studied environments, *T. canis* ova were reported more frequently than *T. cati,* demonstrating his higher zoonotic potential and explaining the major rate of infection in humans [7, 8].

Human infection with *T. canis* L3 larvae can be unnoticed (most common) or can cause illness with mild or severe consequences to health. Diverse signs and symptoms have been associated with infection, however, human disease status depends on affected tissue, parasite inoculum, and host immune response intensity [2, 9, 10, 11, 12]. In this sense, the main and well-characterized forms of the disease have been described: **i)** Ocular larva migrans syndrome (**OLMS**), **ii)** visceral larva migrans syndrome (**VLMS**), **iii)** covert or common toxocariasis (**CT**), and neurotoxocariasis (**NT**) [2, 9, 12, 13, 14, 15, 16]. **OLMS** is frequently diagnosed in older children (11–16 years) and is accompanied by some localized and typically unilateral affectations in eyes such as uveitis,

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endophthalmitis, chorioretinitis, strabismus, and in more severe cases retinal injuries causing detachment with subsequent vision loss [10, 17, 18]. **VLMS** is a frequent entity diagnosed in young children with habits of pica. It is characterized by larvae migration through organs such as the liver, lungs, heart, spleen, and central nervous system, followed by systemic alterations such as fever, hypergammaglobulinemia, eosinophilia, respiratory alterations (asthma), hepatomegaly, cutaneous rash, and diverse nervous system alterations [10, 13, 14]. **Covert toxocariasis** has been described as a common and usually misdiagnosed form of the disease that curse with nonspecific symptoms and signs such as abdominal pain, headache, cough, pulmonary alterations, dermatological disorders, and a normal or slight increase in eosinophil count, that usually are insufficient to classify clinically to patients in previously described ocular or visceral categories [4]. Finally, **neurotoxocariasis** is a less frequent and recent described form of the disease, that occurred after L3 larvae migration into the central nervous system (CNS), causing meningitis, encephalitis, cerebral vasculitis, and myelitis, and is usually accompanied by non-specific signs and symptoms [2, 15, 16].

The absence of pathognomonic symptoms and signs in human toxocariasis make more difficult the clinical-based diagnosis. Furthermore, the absence of *Toxocara* intestinal adult stages and L3 larvae restrictive survival into extra-intestinal body tissues, avoid the possibility of routine diagnosis based on stool examination, like in most soil-transmitted helminthiases (STH) [4, 19].

Laboratory definitive diagnosis of human toxocariasis is based on direct methods for visualization of parasite larvae, such as microscopic visualization in biopsy preparations from affected tissues and it is recognized as the gold standard [4, 20, 21]. Larvae can also be visualized in cerebrospinal or ocular fluids, but its identification could be challenging [20]. However, such methods have limited application in the clinical context due to the difficulty for larvae detection associated with their reduced size and uncertain location into the affected tissues. In general, biopsy-based approaches are very invasive and non-recommended procedures for diagnostic purposes [13, 19, 22, 23]. Those characteristics make toxocariasis diagnosis a cumbersome challenge [21, 23].

Such limitations had driven human toxocariasis definitive diagnosis to a combination of clinical, parasitological, and epidemiological criteria. ELISA IgG using native *T. canis* L3 larvae excretion-secretion antigens (TES-ELISA) is reported to date as the most useful clinical laboratory test for immunodiagnostic purposes and it was reported to have a sensitivity of 78% and a specificity of 92%, according to one study developed in the Graduate School of Public Health of the University of Pittsburgh [24]. TES-ELISA is actually the most studied test and still the reference for human toxocariasis diagnosis [21, 23, 25, 26]. Despite its usefulness, many authors have described important variations in sensitivity and specificity ranging between 45.7% and 100% for sensitivity and 36%–97% for specificity [21, 23, 26, 27, 28]. Differences in performance have been mainly associated with TES native antigens macromolecular composition, and type and number of serum samples used for the validation process [23, 25, 27, 29]. Despite the good performance reported for the commercial and in-house developed TES-ELISA versions in some validation studies, its implementation is extensively questioned by the scientific community since frequent cross-reactions are reported for TES antigens, with antibodies produced in response to antigens of other helminths and protozoa highly prevalent in tropical countries [16, 20, 21, 30]. Furthermore, native *T. canis* TES production is a non-practical and laborious technique that usually involves expensive and non-reproducible methodologies that affect TES antigens in terms of quantity and composition [12, 23, 31, 32]. Despite these problems in TES-ELISA performance and reproducibility, it remains actually as the reference immunological test in routine detection of anti-*Toxocara* sp. antibodies in the healthcare system [4, 20].

Investigation in toxocariasis is a global and local priority, especially regarding the development of modern antigens and tests that offer better performance in terms of sensitivity and specificity in developing countries, where parasitic infections result in high burden diseases for the health system. For this reason, diverse studies in *T. canis* molecular biology have been focused on the identification and characterization of antigenic proteins with potential application as recombinant candidates for vaccination and immunodiagnostic studies. In this way, TES-26 (Tc-PEB-1), TES-32 (TES-30 or Tc-CTL-1) [33, 34], TES-120 (Tc-MUC-1), and Myosin heavy chain have been characterized to the molecular level, synthesized as individual recombinant proteins and evaluated in ELISA/immunoblot for anti-*Toxocara* antibodies detection [35, 36, 37, 38, 39, 40, 41, 42, 43]. These recombinant antigens have been described as reliable, specific, and good candidates to replace native TES antigens. However, some authors report the need for more validation studies with such recombinant proteins to confirm their usefulness in the diagnostic field [4, 21, 28, 36]. Our study aimed to detect intense cross-reaction polypeptides regions inside TES-26, TES-32, TES-120, and Myosin heavy chain *T. canis* proteins using a custom fusion system based on the eGFP reporter gene, and then produce a more specific edited chimeric prototype protein. This low cross-reaction chimeric antigen was then tested in ELISA and Immunoblot assays formats using sera from negative and positive controls. As control chimeric antigen, a recombinant protein that carries the full sequence of the same *T. canis* antigens was produced, purified, and tested under the same conditions.

## 2. Material and methods

### 2.1. Human serum samples

A total of 310 serum samples were collected after obtaining informed consent from the Colombian subjects. Serum samples collection, storing, and processing protocols were previously approved by the human research Bioethical committee from the University of Antioquia research headquarter (CBEIH-SIU) as stated in the minute 5-10-320 of 2010.

Serum samples were grouped as following:

**Group 1:** 21 negative control serum samples from apparently healthy individuals who have no precedent of living with domestic canines and double-negative in serial stool sampling tests for microscopic parasite examination and negative reactivity in an in-house IgG TES-ELISA previously developed and reported by Olave and colleagues in a *T. canis* reactivity study [41].

**Group 2:** 10 control serum samples were obtained from patients with clinical suspicion of toxocariasis (**OLMS or VLMS**) based on symptoms and signs. Samples had previously been confirmed as positive for reactivity (titers $\geq$1:32) in a reference IgG TES-ELISA standardized by the Center for Disease Prevention and Control-CDC.

**Group 3:** 14 serum samples were obtained from patients with suspected ocular toxocariasis (**OLMS**) based on clinical symptoms and signs. Samples had previously been negative for reactivity (titers $<$1:32) in a reference IgG TES-ELISA standardized by the Center for Disease Prevention and Control-CDC.

**Group 4:** 47 serum samples were obtained from patients with suspected toxocariasis based on clinical symptoms and signs (45 patients with **OLMS** suspicion and 2 patients with **OLMS/VLMS** suspicion). From this group, 43 samples were confirmed as positive for reactivity using the in-house IgG TES-ELISA [41].

**Group 5:** A convenience panel of 60 cross-reactivity serum samples was obtained from patients with parasite evidence other than *Toxocara* spp. 37 samples were from patients with confirmatory diagnostic of single intestinal parasite infection in serial stool examination, including *Ascaris* spp. (4), *Trichuris* spp. (5), hookworm (3), *Strongyloides* spp. (5), *Hymenolepis* spp. (5), taeniasis caused by *Taenia saginata* (8) or *Taenia solium* (5), and 2 samples were from patients with evidence of mixed intestinal coinfection including one with *Strongyloides* spp./*Trichuris* spp. (1) and one with *Hymenolepis* spp./*Trichuris* spp. (1). Finally, 23 samples were from patients presumed extra-intestinal parasite infections, including five patients with suspected toxoplasmosis based on clinical signs and symptoms, and with positive reactivity ($>$10 UI/ml) in an IgG IFI for anti-*Toxoplasma gondii* antibody detection (5), four samples were from patients diagnosed with echinococcosis (Hydatid cyst) (4), thirteen

samples were from patients with suspected neurocysticercosis which were confirmed based on clinical and epidemiological criteria and with positive reactivity in an immunoblot test (13) and one sample were from a patient with evidence of free-living Amoeba infection detected by *in vitro* culture and microscopy of an ocular sample (1). All these cross-reactivity sera were also tested with the in-house IgG TES-ELISA [41].

**Group 6:** 80 serum samples from dog breeders. These samples were not tested for any parasitic infection.

**Group 7:** 78 serum samples from healthy random selected individuals. These samples were not tested for any parasitic infection.

### 2.2. Synthesis and/or cloning of optimized CDS sequences of selected *T. canis* antigens

The complete coding sequences (CDS) for *tes-26*, *tes-32*, *tes-120*, and *myosin heavy chain T. canis* genes, were downloaded from NCBI GenBank (accession numbers: U29761, AB009305, U39815, AJ306290, respectively) and optimized for *Escherichia coli* heterologous expression with the algorithm CODON USAGE ANALYZER [44]. The signal peptide was detected with SIGNALP and eliminated as well [45]. Due to *T. canis* Myosin heavy chain large protein size, we selected two representative fragments, one from the N-terminal region (MyoN) and the second one from the C-terminal region (MyoC). Myosin showed to be the most conserved antigen of the selected ones. For this reason, based on alignment comparative analysis of Myosin heavy chain with other parasitic nematodes available sequences, *Brugia malayi* (XP_001899601.1), *Onchocerca volvulus* (AAA29420.1), *Loa loa* (EJD74963.1), and *Necator americanus* (XP_013290889.1), the highly conserved blocks of the selected fragments were excluded, in order to avoid cross-reactions. Synthetic and optimized nucleotide coding sequences of the *T. canis tes-26*, *tes-120*, *MyoN*, and *MyoC* were ordered to the company BlueHeron (USA), cloned in the pUC19 vector. The *tes-32* synthetic CDS was already produced and cloned into the *pET28a* expression vector in our laboratory as described in a previous work [41].

### 2.3. T. canis polypeptide expression system development

We developed an expression system based on a fusion strategy in the *pET28a* expression vector using enhanced Green Fluorescent Protein (eGFP) as a carrier.

The molecular weight of the target short polypeptides of the selected *T. canis* antigens (TES26, TES32, TES120, MyoN, and MyoC) ranged between 5.9 and 8.2 kDa. The system was designed to express a chimeric protein that harbors the complete eGFP molecule fused with each target short polypeptide at its C-terminal region. The coding sequence of the target short polypeptide should be inserted into a *pET28a-eGFP* expression construct using *Bam*HI/*Hin*dIII restriction sites (Figures 1 and 2).

The eGFP CDS was PCR amplified using oligonucleotides eGFP_*Nde*I_fw and eGFP_*Bam*HI_*Eco*RI_rv (supplementary table s1). Amplification was performed using the following protocol: first, denaturation step at 95 °C for 1 min, followed by 40 cycles of denaturing at 94 °C during 30s, annealing for 30 s at 55 °C and extension for 50 s at 72 °C. Finally, a single final extension step of 15 min at 72 °C was carried out ($MgCl_2$ 1.2mM, dNTPs 0.1mM, *Taq* Buffer 1X, forward oligonucleotide 0.25 μM, reverse oligonucleotide 0.25 μM, *Taq* DNA polymerase at 2.5U/100μL and 0.5μL of template DNA). eGFP product was purified (Qiaquick PCR purification kit, Qiagen), digested with fastDigest *Nde*I/*Eco*RI, and then used for direct cloning into *pET28a* expression vector using Rapid DNA ligation kit (Thermo Scientific). New recombinant expression construct *pET28a-eGFP* was verified using capillary sequencing (Figures 1 and 2).

### 2.4. Cloning of the split versions of the T. canis antigens into the pET28a-eGFP expression construct

*T. canis* selected antigens were "*in silico*" split into short contiguous polypeptides. TES-26, TES-32, MyoN, and MyoC proteins were split into four overlapping polypeptides each one and TES-120 into two non-overlapping polypeptides (protein with repetitive serine/threonine-rich and six-cysteine (SXC) motif) [46, 47]. Short polypeptide length ranged between fifty-seven to seventy-nine amino acids and overlapping regions ranged between ten and twenty-one amino acids (Figure 3, Table 1).

PCR amplification for all *T. canis* polypeptides CDS was carried out (under the same conditions described for eGFP PCR) using primers listed in supplementary table s1 (supplementary figure s1). Synthetic recombinant constructs (*pUC19-tes26*, *pUC19-tes20*, *pUC19-MyoNter*, *pUC19-MyoCter*, and *pET28a-tes32*) were used as template DNA in PCR reactions. Amplified fragments were gel-purified and used for direct cloning into recombinant construct *pET28a-eGFP* by digestion with *Bam*HI/*Hin*dIII restriction enzymes and subsequent ligation using Rapid DNA ligation kit (Thermo Scientific). The eighteen synthetized new recombinant

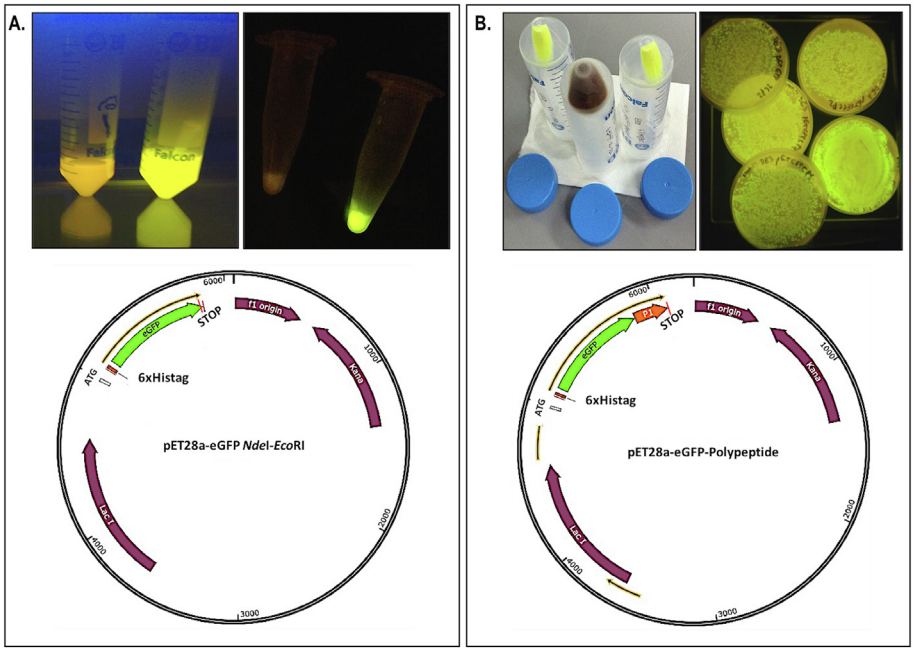


**Figure 1.** eGFP based system for expression and monitoring of *Toxocara canis* TES-26, TES-32, TES-120, MyoN and MyoC derived fusion chimeric recombinant polypeptides. 1A. pET28a-eGFP expression recombinant vector with eGFP CDS cloned *Nde*I-*Eco*RI in pET28a frame. In the top, control *E. coli* BL21(DE3) cells (transformed with pET28a vector) and recombinant *E. coli* BL21(DE3) cells (transformed with pET28a-eGFP vector) expressing N-terminal 6xHis-tagged eGFP. 1B. pET28a-eGFP-polypeptide expression recombinant vector which represent *T. canis* polypeptides fusion at the eGFP C-terminal end (Polypeptides cloned to 3′ end of eGFP CDS using *Bam*HI-*Hin*dIII endonucleases). In the top, control *E. coli* BL21(DE3) cells (transformed with pET28a vector) and recombinant *E. coli* BL21(DE3) cells (transformed with pET28a-eGFP-polypeptide expression vector) expressing *T. canis* polypeptides as chimeric recombinant proteins in fusion to N-terminal 6xHistagged eGFP.

**A. eGFP CDS:**

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCT
ACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACAT
GAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGAC
ACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGC
AGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC
CGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAG
CTGTACAAGTAA

**B. pET28a-eGFP(*Nde*I/*Eco*RI):**

*Nde*I

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCC
CACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACC
ATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCC
TGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT
GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGC
GATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCTAAGAATTCTAAGAATTCGAGCTCCGTCGACAAGCTT

*Bam*HI *Eco*RI *Hind*III

**C. pET28a-eGFP 26P1:**

*Nde*I

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCC
CACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACC
ATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCC
TGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT
GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGC
GATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCATGGACAGTGCGTCAGACTGTGCGGCAAACGCTGGGTCCTGTT
TCACACGACCAGTCAGCCAAGTTCTGCAGAACCGTTGTCAACGTACATGCAATACATGCGACTGCCGGGACGAGGCTAACAATTGTGCAGCTTCAATTAACCTGTGCCAGAACCCCAC
ATTTGAACCTTTAGTTCGTGATCGTTGCCAAAAGACTTGTGGTTTGTGTTAAAAGCTT

*Bam*HI *Hind*III

**D. eGFP:**

MGSSHHHHHHSSGLVPRGSHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERT
IFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKR
DHMVLLEFVTAAGITLGMDELYK*

**E. eGFP-26P1:**

MGSSHHHHHHSSGLVPRGSHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERT
IFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKR
DHMVLLEFVTAAGITLGMDELYKGSMDSASDCAANAGSCFTRPVSQVLQNRCQRTCNTCDCRDEANNCAASINLCQNPTFEPLVRDRCQKTCGLC*

**Figure 2.** Nucleotide and protein sequences of eGFP based system for expression and monitoring *Toxocara canis* fusion chimeric recombinant polypeptides. 2A. Nucleotide coding sequence (CDS) of enhanced green fluorescent protein eGFP (714 bp) including 5′ ATG star codon and 3′ TAA stop codon (Nucleotides in green color). 2B. Nucleotide sequence of recombinant pET28a-eGFP vector including 5′ ATG star codon, 6xHistag epitope coding sequence, eGFP CDS cloned *Nde*I-*Eco*RI in pET28a frame, 3′ TAA stop codon (nucleotides in bold between *Bam*HI-*Eco*RI) and *Bam*HI-*Hind*III endonuclease restriction sequences for *T. canis* polypeptides cloning. 2C. pET28a-eGFP-26P1polypeptide vector with 26P1 polypeptide cloned (*Bam*HI-*Hind*III) in frame with eGFP. 2D. Translated amino acid sequence (261 amino acids) of pET28a-eGFP vector including full length eGFP (amino acids in green) and N-terminal 6xHistag amino acids. 2E. Translated amino acid sequence (331 amino acids) of pET28a-eGFP-26P1polypeptide vector containing *T. canis* 26P1 polypeptide amino acid sequence (amino acids in orange). Asterisk (*) represent stop codon in C-terminal eGFP and C-terminal eGFP-Polypeptide.

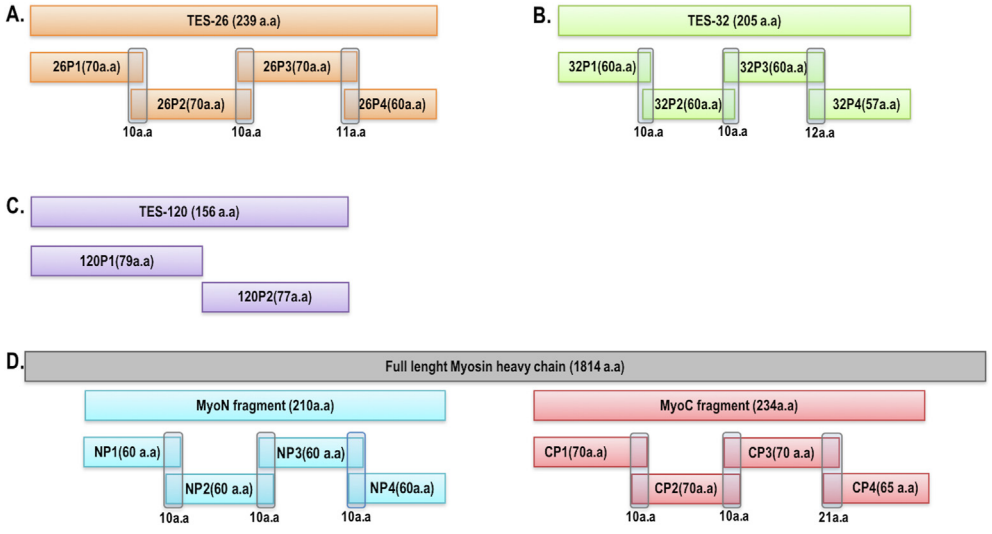


**Figure 3.** Representative strategy used for "*in silico*" split of the *Toxocara canis* rTES-26, rTES-32, rTES-120 proteins into overlapping polypeptides. Horizontal rectangles represent both full length *Toxocara canis* proteins and derived polypeptides. Vertical rectangles in gray represent overlapping amino acids between contiguous polypeptides. 3A. rTES-26 protein with 239 amino acids, was divided into 26P1, 26P2, 26P3 and 26P4 polypeptides. 3B. rTES-32 protein with 205 amino acids, was divided into 32P1, 32P2, 32P3 and 32P4 polypeptides. 3C. rTES-120 protein with 156 amino acids, was divided into 120P1 and 120P2 polypeptides. Due to repetitive domains in 3′ end of 120P1 and 5′ end of 120P2, overlapping amino acids were not included. 3D. MyoN with 210 amino acids and MyoC with 234 amino acids (selected as Myosin heavy chain representative fragments), were divided into NP1, NP2, NP3, NP4 and CP1, CP2, CP3, CP4 polypeptides respectively.

constructs were verified using capillary sequencing and used for subsequent expression experiments.

### *2.5. Recombinant expression for eGFP and T. canis chimeric fusion polypeptides into E. coli*

In order to express the recombinant constructs, *E. coli* BL21 (DE3) cells (Novagen, Germany) were transformed and cultured in LB (Luria-Bertani) liquid medium, supplemented with 50 μg/mL of kanamycin sulfate, and incubated to 37 °C until an optical density (OD) between 0.4 and 0.6 at 600 nm (Mid-log phase). After that, cultures were incubated for an induction period of 5 h at 37 °C, with Isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 0.2 mM (supplementary figures s2–6). After induction, all cultures were harvested by centrifugation at 5000 RCF and bacterial pellets were stored frozen to –20 °C until purification step. Recombinantly expressed eGFP and chimeric

**Table 1.** General description of *Toxocara canis* proteins split into polypeptides for fusion to eGFP.

| | Protein or Polypeptide | Number of overlapping amino acids | eGFP or Polypeptide CDS size | eGFP or Polypeptide CDS molecular mass | Recombinant vector/construct used for expression | eGFP or chimeric polypeptide cloned CDS size | eGFP and chimeric polypeptides calculated molecular mass |
|---|---|---|---|---|---|---|---|
| **0** | **eGFP** | - | 714 bp/238 a.a | 26,8 kDa | pET28a-eGFP | 786 bp/261 a.a | 29 kDa |
| **1** | **TES26-P1** | **TES-26** | 213 bp/70 a.a | 7,6 kDa | pET28a-eGFP-26P1 | 996 bp/331 a.a | 36 kDa |
| **2** | **TES26-P2** | **26P1–26P2:10 a.a** | 213 bp/70 a.a | 7,4 kDa | pET28a-eGFP-26P2 | 996 bp/331 a.a | 36 kDa |
| **3** | **TES26-P3** | **26P2–26P3:10 a.a** | 213 bp/70 a.a | 7,7 kDa | pET28a-eGFP-26P3 | 996 bp/331 a.a | 36 kDa |
| **4** | **TES26-P4** | **26P3–26P4:11 a.a** | 183 bp/60 a.a | 6,6 kDa | pET28a-eGFP-26P4 | 966 bp/321 a.a | 35 kDa |
| **5** | **TES32-P1** | **TES-32** | 183 bp/60 a.a | 5,9 kDa | pET28a-eGFP-30P1 | 966 bp/321 a.a | 35 kDa |
| **6** | **TES32-P2** | **32P1–32P2:10 a.a** | 183 bp/60 a.a | 6,6 kDa | pET28a-eGFP-30P2 | 966 bp/321 a.a | 35 kDa |
| **7** | **TES32-P3** | **32P2–32P3:10 a.a** | 183 bp/60 a.a | 6,9 kDa | pET28a-eGFP-30P3 | 966 bp/321 a.a | 36 kDa |
| **8** | **TES32-P4** | **32P3–32P4:12 a.a** | 174 bp/57 a.a | 6,2 kDa | pET28a-eGFP-30P4 | 957 bp/318 a.a | 35 kDa |
| **9** | **TES120-P1** | **TES-120** | 240 bp/79 a.a | 7,0 kDa | pET28a-eGFP-120P1 | 1023 bp/340 a.a | 36 kDa |
| **10** | **TES120-P2** | | 234 bp/77 a.a | 8,5 kDa | pET28a-eGFP-120P2 | 1017bp/338 a.a | 37 kDa |
| **11** | **MYON-P1** | **MYON** | 183 bp/60 a.a | 7,0 kDa | pET28a-eGFP-NP1 | 966 bp/321 a.a | 36 kDa |
| **12** | **MYON-P2** | **NP1-NP2:10 a.a** | 183 bp/60 a.a | 6,9 kDa | pET28a-eGFP-NP2 | 966 bp/321 a.a | 36 kDa |
| **13** | **MYON-P3** | **NP2–NP3:10 a.a** | 183 bp/60 a.a | 6,8 kDa | pET28a-eGFP-NP3 | 966 bp/321 a.a | 36 kDa |
| **14** | **MYON-P4** | **NP3–NP4:10 a.a** | 183 bp/60 a.a | 6,9 kDa | pET28a-eGFP-NP4 | 966 bp/321 a.a | 36 kDa |
| **15** | **MYOC-P1** | **MYOC** | 213 bp/70 a.a | 8,1 kDa | pET28a-eGFP-CP1 | 996 bp/331 a.a | 37 kDa |
| **16** | **MYOC-P2** | **CP1–CP2:10 a.a** | 213 bp/70 a.a | 8,2 kDa | pET28a-eGFP-CP2 | 996 bp/331 a.a | 37 kDa |
| **17** | **MYOC-P3** | **CP2–CP3:10 a.a** | 213 bp/70 a.a | 8,0 kDa | pET28a-eGFP-CP3 | 996 bp/331 a.a | 37 kDa |
| **18** | **MYOC-P4** | **CP3–CP4:21 a.a** | 198 bp/65 a.a | 7,4 kDa | pET28a-eGFP-CP4 | 981 bp/326 a.a | 36 kDa |

fusion polypeptides were subjected to solubility tests by sonication of bacterial cell pellets treated with native buffer containing $Na_2HPO_4$ 50 mM, NaCl 300 mM, and Imidazole 10 mM (pH 8.0) (supplementary figures s2–6).

### 2.6. Chromatography purification for eGFP and T. canis chimeric fusion polypeptides

Inclusion bodies collected from the bacterial pellets were used for affinity chromatography purification in a Biologic DuoFlow Pathfinder 20 System (BioRad, USA). All purification experiments of the eGFP - *T. canis* fused short polypeptides were carried out under denaturing conditions (supplementary figures s2–6) using urea. After the purification step, inclusion bodies pellets were solubilized by overnight incubation with denaturing buffer (pH 8.0, urea 8 M, $NaH_2PO_4$ 100 mM, Tris 10 mM, Imidazole 10 mM) and then purified by affinity chromatography using IMAC cartridges (BioRad, USA) following manufacturer's instructions (supplementary figures s2–6).

Control eGFP protein (eGFP alone) was purified under native conditions with IMAC columns following the manufacturer's instructions and protocol recommendations.

Chromatography elution fractions containing purified recombinant eGFP and chimeric fusion polypeptides were evaluated by SDS-PAGE and quantified by spectrophotometry at 280 nm using a NanoDrop 2000c spectrophotometer (Thermo scientific) (supplementary figures s2–6). Purity for the IMAC purified recombinant proteins was assessed by capillary electrophoresis using a Bioanalyzer system and High Sensitivity Protein 250 Chips and Reagents (Agilent, USA).

### 2.7. Synthesis and assembly of chitc0 and chitc1 chimeric genes by fusion PCR

The *chitc0* chimeric gene synthesis was carried out through PCR amplification of TES26, TES32, TES120, MyoNter, and MyoCter complete CDS, sequences (excluding signal peptides of TES), followed by assembly in a single chimeric molecule by fusion PCR (Figure 4). In the same way, *chitc1* gene synthesis was carried out, excluding 5 high-cross reaction polypeptides blocks of the protein (previously identified by western blot), and using the 13 remaining short polypeptide coding sequences as blocks for assembly of a single fusion chimeric CDS (Figure 4). A total of 9 PCR reactions using Pwo DNA polymerase (Roche), including 4 fusion PCR reactions, were necessary for the complete assembly of chitc0 chimeric gene. All amplified fragments were purified from agarose gels, using Qiaquick agarose gel extraction kit (Qiagen), and cloned into pJET1.2/blunt cloning vector with ClonJET PCR cloning kit (Thermo Scientific). Analogously, to complete the assembly of *chitc1* chimeric CDS, a total of 13 PCR reactions were carried out, including 5 fusion PCR reactions with *Pfu* DNA polymerase (Thermo Scientific). Amplified fragments were gel-purified and used for direct cloning into pTZ57R vector (InsTAclone PCR Cloning Kit, Thermo Scientific). Recombinant plasmids containing *chitc0* and *chitc1* gene sequences were used for subcloning into *pET28a* expression vector by digestion with *Nde*I/*Hin*dIII restriction enzymes and subsequent ligation using Rapid DNA ligation kit (Thermo Scientific). Final recombinant constructs *pET28a-chitc0* and *pET28a-chitc1* were verified by capillary sequencing and used for transformation and recombinant expression into *E. coli BL21* (DE3) cells (Novagen, Germany) (Figure 4).

### 2.8. Expression and purification of the T. canis rCHITC0 and rCHITC1 chimeric proteins

Synthetized *pET28a-chitc0* and *pET28a-chitc1* recombinant expression constructs were used to transform *E. coli* BL21 (DE3) cells (Novagen, Germany). Recombinant bacteria were cultured in LB (Luria-Bertani) liquid medium containing 50 μg/mL of kanamycin sulfate and incubated to 37 °C until an optical density (OD) between 0.4 and 0.6 at 600 nm (Mid-log phase) was reached. After that, cultures were incubated overnight with 0.5 mM IPTG at 30 °C or 37 °C for rCHITC0 and rCHITC1 chimeric protein expression, respectively (Figure 5). After induction, all cultures were harvested by centrifugation at 5000 RCF and bacterial pellets were stored frozen at −20 °C until purification procedure. Recombinantly expressed rCHITC0 and rCHITC1 were submitted to a solubility test carried out by sonication of the bacterial cell pellets with native buffer containing $Na_2HPO_4$ 50 mM, NaCl 300 mM, and Imidazole 10mM (pH 8.0) (Figure 5). Due to the lack of solubility of the his-tagged chimeric proteins, inclusion bodies were collected from the pellets under denaturing conditions and then used for subsequent chromatography purification.

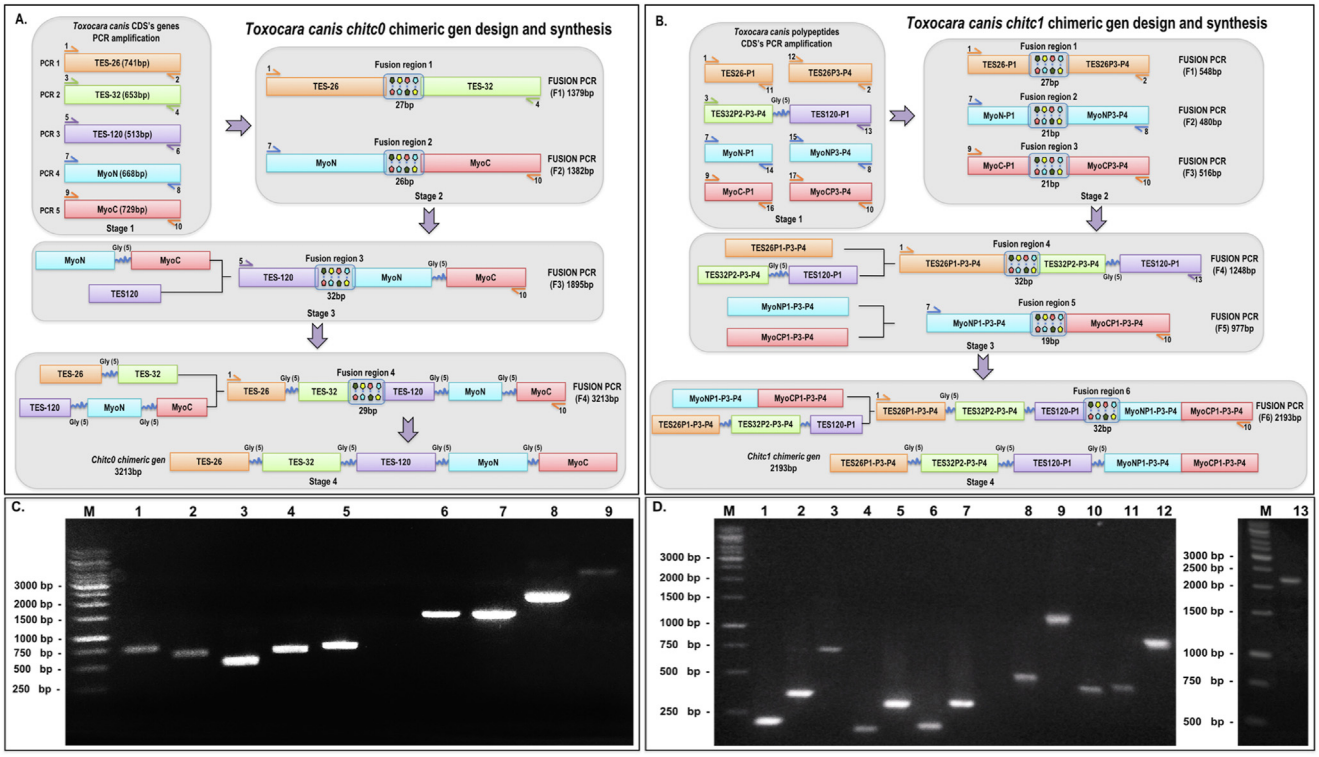


**Figure 4.** *Toxocara canis chitc0* and *chitc1* chimeric gens design and synthesis. Synthesis of *chitc0* and *chitc1* chimeric gens involves PCR for amplification of different *T. canis* individual coding sequences and fusion PCR for assembly of pre-amplified coding sequences into single chimeric DNA molecules using overlapping fusion regions. 4.A, Stage 1. PCR amplification of individual *T. canis* tes-26, tes-32, tes-120, MyoN and MyoC coding sequences using 1 to 10 oligonucleotides; Stage 2. Assembly of pre-amplified tes-26/tes-32 and MyoN/MyoC coding sequences by fusion PCR, using oligonucleotides 1 and 4 (for F1 fusion fragment synthesis), oligonucleotides 7 and 10 (for F2 fusion fragment synthesis) and fusion regions 1/2 (overlapping regions with 27 and 26 complementary bases respectively); Stage 3. fusion PCR for F2 fusion fragment and tes-120 coding sequence Assembly of, using oligonucleotides 5 and 10 (for F3 fusion fragment synthesis) and fusion region 3 (overlapping region with 32 complementary bases); Stage 4. fusion PCR for F1 fusion fragment and F3 fusion fragment assembly, using oligonucleotides 1 and 10 (for F4 fusion fragment (chitic0) synthesis) and fusion region 4 (overlapping region with 29 complementary bases). Full length 3213 bp *chitc0* chimeric gen include *T. canis* tes-26, tes-32, tes-120, MyoN y MyoC coding sequences separated by fifteen base spacers (Gly) conformed by five glycine codons (GGT/GGC); 4.B, Stage 1. PCR amplification of individual *T. canis* tes26-P1, tes26-P3-P4, tes32-P2-P3-P4-tes120-P1, MyoN-P1/MyoN-P3-P4, MyoC-P1/MyoC-P3-P4 polypeptide coding sequences, using oligonucleotides 1 to 3 and 7 to 17; Stage 2. Assembly of pre-amplified tes26-P1/tes26-P3-P4, MyoN-P1/MyoN-P3-P4, MyoC-P1/MyoC-P3-P4 polypeptides coding sequences by fusion PCR, using oligonucleotides 1–2 (for F1 fusion fragment synthesis), 7–8 (for F2 fusion fragment synthesis), 9–10 (for F3 fusion fragment synthesis) and fusion regions 1, 2 and 3 (overlapping region with 27, 21 and 21 complementary bases,respectively); Stage 3 Fusion PCR for F1/tes32-P2-P3-P4-tes120-P1 and F2/F3 fusion fragments Assembly, using oligonucleotides 1 and 13 (for F4 fusion fragment synthesis), oligonucleotides 7 and 10 (for F4 fusion fragment synthesis) and fusion regions 4 and 5 (overlapping regions with 32 and 19 complementary bases, respectively); Stage 4. fusion PCR for F4 fusion fragment and F5 fusion fragment assembly, using oligonucleotides 1 and 10. Full length 2193 bp *chitc1* chimeric gen is a modified prototype of *chitc0* which exclude *T. canis* tes26-P2, tes32-P1, tes120-P2, MyoN-P2 and MyoC-P2 polypeptide coding sequences and preserve three of the base spacers (Gly) conformed by five glycine codons (GGT/GGC). 4.C. 1% Agarose gel electrophoresis showing PCR amplified *T. canis* tes-26, tes-32, tes-120, MyoN and MyoC coding sequences, F1, F2, F3 and F4 fusion fragments. Line M, 1 kb DNA ladder; lines 1, 2, 3, 4 and 5, *Pfu* DNA polymerase amplified tes26 (741 pb), tes32 (653 pb), tes120 (513 pb), MyoN (668 pb), MyoC (729 bp) *T. canis* polypeptides CDS respectively; lines 6, 7, 8 and 9, *Pfu* DNA polymerase amplified F1 (1379 bp), F2 (1382 bp), F3 (1895 bp) and F4 (3213 bp) fusion fragments respectively. 4.D. 1% Agarose gel electrophoresis showing PCR amplified *T. canis* polypeptides fragments coding sequences, F1, F2, F3, F4, F5 and F6 fusion fragments. Line M, 1 kb DNA ladder; lines 1, 2, 3, 4, 5, 6 and 7, *Pfu* DNA polymerase amplified tes26-P1 (210 pb), tes26-P3-P4 (365 bp), tes32-P2-P3-P4/tes120-P1 (732 bp), MyoN-P1 (182 bp), MyoN-P3-P4 (319 bp), MyoC-P1 (204 bp) and MyoC-P3-P4 (333 bp) *T. canis* polypeptides CDS respectively; lines 8, 9, 10 11 and 12, *Pfu* DNA polymerase amplified F1 (548 bp), F2 (480 bp), F3 (516 bp), F4 (1248 bp) and F5 (977 bp) fusion fragments respectively; line 13, *Pfu* DNA polymerase amplified F6 (2193 bp) fusion fragment after purification step.

Previous to chromatography purification, rCHITC0 and rCHITC1 chimeric proteins inclusion bodies were subjected to an additional washing protocol (three washing steps using native buffer supplemented with 1% Triton X-100 and 1 M Urea, pH8.0), then solubilized with urea 8 M buffer. Purification was developed by affinity chromatography under denaturing conditions (as described before in the purification methodology) (Figure 5). Chromatography elution fractions containing purified chimeric proteins were quantified by spectrophotometry at 280 nm using a NanoDrop 2000c spectrophotometer (Thermo scientific) and then purity was tested by capillary electrophoresis was mentioned above (Figure 5). Fractions containing purified chimeric proteins were then used for immunoblotting and ELISA tests development (Figure 5. C, F).

### *2.9. Immunoblotting assay using T. canis chimeric recombinant polypeptides and rCHITC0/rCHITC1 chimeric antigens*

Purified eGFP, *T. canis* fusion polypeptides, or chimeric rCHITC0 and rCHITC1 were separated by 10% SDS-PAGE (A total protein concentration of 5 μg/gel was used) and transferred into nitrocellulose membrane using trans blot tank blotting system (BioRad). Protein transference was monitored with Ponceau red staining solution (Sigma) and pre-stained protein molecular weight marker (Thermo Fisher scientific). Membrane blocking was carried out with saline-tris (ST) buffer, containing 5% skim milk during an overnight incubation at 4 °C. Blocked membranes were washed three times, once with ST-tween 0.05% and twice with ST (5 min each one) and divided into strips. The strips were incubated with serum

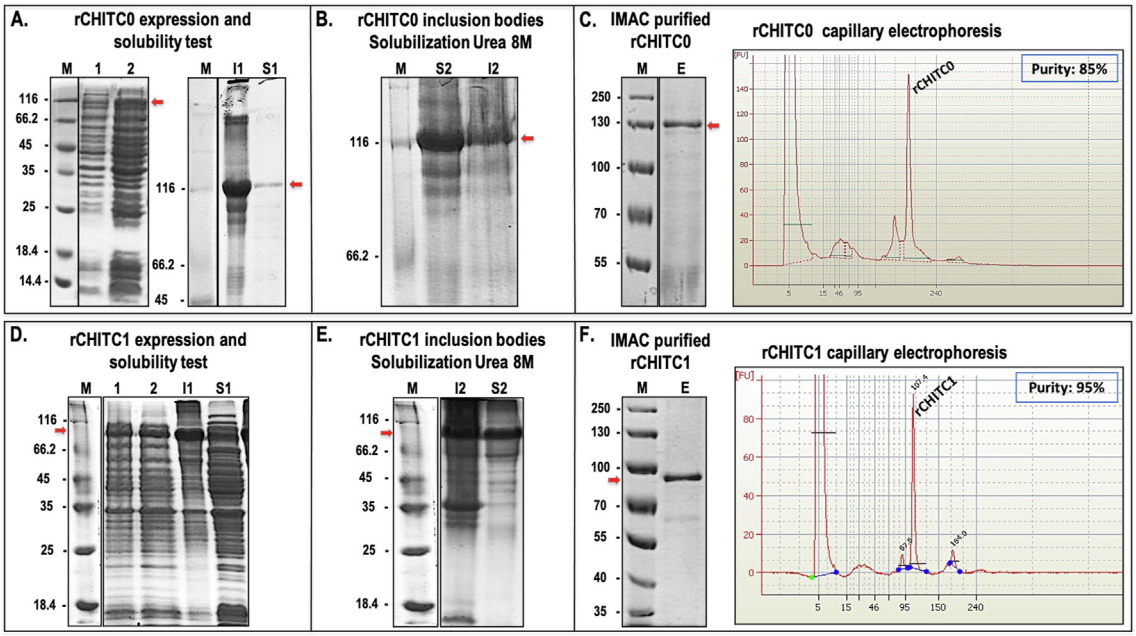


**Figure 5.** SDS-PAGE with *T. canis* rCHITC0 and rCHITC1 chimeric recombinant antigens expression, solubility test and IMAC purification. 5. A, D Lines M, molecular weight marker (kDa); Lines 1, *E. coli* BL21(DE3) whole lysates before expression (0 horas); Line 2, *E. coli* BL21(DE3) lysates after rCHITC0 (5.A) and rCHITC1 (5.D) chimeric antigens expression; Lines I1, Insoluble fractions (pellet with inclusion bodies) of *E. coli* BL21(DE3) expressing rCHITC0 (5.A, I1) and rCHITC1 (5.D) chimeric antigens after lysis by sonication; Lines S1, Soluble fractions (supernatant) of *E. coli* BL21(DE3) expressing rCHITC0 (5.A) and rCHITC1 (5.D) chimeric antigens after lysis by sonication. 5.B, E. Recombinant expressed rCHITC0 and rCHITC1 chimeric antigens Urea solubilization. Line M, molecular weight marker (kDa); Lines I2, insoluble fractions containing solubilized rCHITC0 (5.B) and rCHHITC1 (5.E) chimeric antigens inclusion bodies after treatment with buffer urea 8M pH 8.0, Lines S2, soluble fractions containing solubilized rCHITC0 (5. B, S2) and rCHHITC1 (5. E, S2) chimeric antigens inclusion bodies after treatment with buffer urea 8M pH 8.0. 10.C, F. Recombinant expressed rCHITC0 and rCHITC1 chimeric antigens denaturing IMAC purification and purity test. Line M, molecular weight marker (kDa); Lines E, elution fraction containing purified rCHITC0 (5.C) and rCHITC1 (5.F) chimeric antigens. Electropherograms from capillary electrophoresis shows calculated purity for purified rCHITC0 (85%) and rCHITC1 (95%) chimeric antigens peaks. SDS-PAGE gels summarize similar bands patterns obtained in three independent experiments. Supplementary figure s13 uncropped version (13U) in supplementary material, contain original non-adjusted image of Figure 5 and shows *T. canis* rCHITC0 and rCHITC1 chimeric recombinant antigens expression, solubility tests and IMAC purification.

samples diluted 1:50 in ST containing 1% skim milk and then incubated 1 and a half hours at 37 °C and shaking gently. Then, strips were washed again (as described before) and incubated with goat anti-human IgG peroxidase-conjugated (diluted 1:1000) for 1 h at 37 °C. Finally, 3,3′-diaminobenzidine tetrahydrochloride metal-enhanced (DAB) and stable peroxide substrate buffer were used for antibody detection. As a control for reactivity assessment in the immunoblot assays, we used a monoclonal anti-6xHistag epitope IgG antibody and/or a polyclonal mouse anti-rMyoCter to detect recombinant his-tagged eGFP, *T. canis* polypeptides, and chimeric recombinant antigens rCHITC0/rCHITC1 in the nitrocellulose membranes. A serum sample was considered as positive if reactivity was observed as a band of the expected molecular mass over

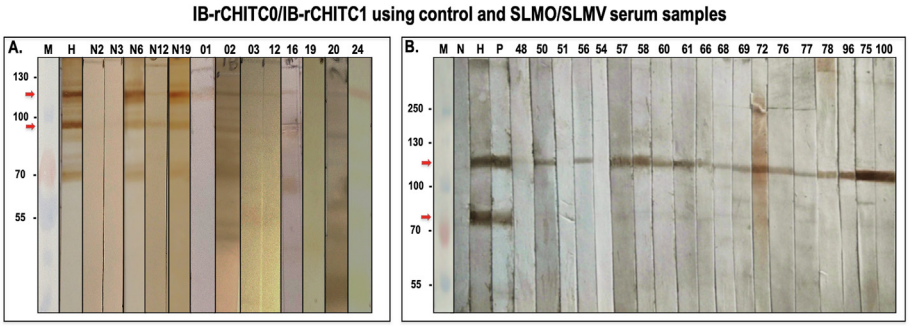


**Figure 6.** Immunoblot analysis showing summarized *T. canis* chimeric rCHITC0 and rCHITC1 reactivity against 152 infected and non-infected human serum samples. Red arrows indicate reference bands used for reactivity analysis in the nitrocellulose membranes. 6.A. Line M, molecular weight marker (kDa); 6. A. Line H, control Anti-6xHistag IgG antibody; Lines N2, N3, N6, N12 and N19, negative control serum samples (group 1); Lines 01–24, clinical suspected *T. canis* serum samples confirmed as reactive in the inhouse TES-ELISA and TES-ELISA CDC reference tests; 6.B. Line M, molecular weight marker (kDa); Line N, negative control Pooled serum samples, 6. A. Line H, control Anti-6xHistag IgG antibody; Line P, control Anti-rMYO-Cter polyclonal serum; Lines 48–100, clinical suspected *T. canis* serum samples confirmed as reactive in the inhouse TES-ELISA (group 4). Supplementary figure s14 uncropped version (A1-A2) and supplementary figure s15 uncropped version (B) in supplementary material, contain original non-adjusted image of Figure 6 and shows the *T. canis* chimeric rCHITC0 and rCHITC1 immunoblot reactivity analysis using infected and non-infected human serum samples.

the nitrocellulose membranes (Table 1, Figures 5 and 6 and supplementary Figures s7–12). Reactivity against bands with other than expected molecular weight mass (*E. coli* co-purified proteins or truncated 6x-his-tagged recombinant proteins) was not considered for the reactivity analysis. All the immunoblot experiments were performed in triplicates and results were summarized in Figure 6 and supplementary figures s7–12.

### 2.10. ELISA using purified rCHITC0 and rCHITC1 chimeric antigens

A total of 307 serum samples were assessed in ELISA format (groups 1–7 serum samples). The optimal concentration of antigen and the combination of serum samples or conjugated polyclonal antibody (goat anti-human IgG peroxidase-conjugated polyclonal antibody) were stablished by checkerboard titration experiments, using chimeric antigens at seven different concentrations (0.125, 0.25, 0.5, 1, 5, 10 and 20 μg/mL), serum samples at three different dilutions (1:10, 1:50 and 1:100) and polyclonal antibody at three different dilutions (1:1000, 1:5000 and 1:8000). Briefly, 96-well flat-bottom microtiter plates (Nunc immune Maxisorp) were coated with 100 μL of either rCHITC0 or rCHITC1 recombinant chimeric antigens at a concentration of 10 μg/mL in 0.05 M bicarbonate buffer, pH 9.6. Then, microtiter plates were incubated in a humid chamber for 1 h at 37 °C followed by 4 °C overnight. Plates were washed using phosphate-buffered saline (PBS), pH 7.2, containing 0.05% (vol/vol) Tween 20 (PBS-T), for unbound antigen removal. After five washing steps (5 min each with PBS Tween 20 (PBS-T)), wells were blocked using PBS buffer containing 5% skim milk for 90 min at 37 °C. Plates were washed again three times (as described before) and then were incubated at 37 °C for 90 min with 100 μL of human serum samples diluted at 1:50 (serum dilution in PBS-T buffer containing 2.5% skim milk). After incubation, serum samples were discarded, and plates were washed three times with PBS-T. Afterward, an incubation step with goat anti-human IgG peroxidase-conjugated polyclonal antibody (pAb) was carried out. 100 μL of antibody was added to each well, at a dilution of 1:8000 (pAb diluted in PBS buffer containing in 2.5% skim-milk) followed by incubation to 37 °C for 60 min. A final wash cycle (three times) followed by the addition of 100 μL o-phenylenediamine dihydrochloride (OPD) substrate and 20 min incubation was carried out at 37 °C. The colorimetric reaction was halted adding 50 μL stop solution (0.5 M HCl and 0.3 M $H_3PO_4$) to each well. Optical densities were measured immediately at 493 nm (reference, 490–493 nm) using a Multiskan FC microplate photometer (Thermo scientific). All the ELISA experiments were performed in triplicates and optical density values for each evaluated sample correspond to the average of triplicates.

### 2.11. In-house TES-ELISA based on T. canis L3 larvae native TES antigens

To evaluate the presence of anti-*Toxocara* sp. IgG antibodies in all evaluated serum samples, we used the in-house standardized TES-ELISA test (in-house TES-ELISA), previously developed and reported by Olave and colleagues in a seroreactivity study [41]. Native TES antigens used for in-house TES-ELISA standardization were collected from *T. canis* L3 larvae following the Savigny method [29, 41, 48]. Performance of the TES-ELISA was calculated using 41 control serum samples (positive and negative) from Colombian individuals with clinical suspicion of ocular toxocariasis (**OLMS**), which were evaluated for toxocariasis by the Center for Disease Prevention and Control-CDC using as a reference a standardized IgG TES-ELISA test. Using these serum samples, the test performance was reported with a sensitivity of 95% (95% CI: 75.13%–99.87%) and specificity of 61% (95% CI: 38.44%–81.89%) for **OLMS** diagnosis.

### 2.12. Anti-rMyoCter polyclonal mouse antiserum production

A total of 10 eight-weeks-old male C57BL/6 mice were divided into a non-immunized group (negative control group with 5 mice) and an immunization group (a group with 5 mice). Purified *T. canis* MYOC recombinant protein (32kDa) was used as antigen (final concentration of 15 μg/50 μl) mixed with complete Freund's adjuvant (CFA) in a first subcutaneous injection (day 0). After that, three boosts of antigen mixed with incomplete Freund's adjuvant (IFA) were applied at two weeks intervals. Five days after the last injection, mice were sacrificed, and total blood was collected and used for serum extraction by centrifugation step of 3000 RCF for 15 min, followed by storage at −20 °C. Mice of the negative control group were similarly immunized with PBS with no antigen and used for serum extraction as described before. Finally, mouse polyclonal antiserum was used for antibody titers determination by immunoblot, using *T. canis MyoCter* recombinant antigen. Polyclonal antiserum was reactive until a dilution of 1:10,000 for the immunization group, compared with the negative control group (not reactive), using a goat anti-mouse peroxidase-conjugated secondary antibody for detection.

### 2.13. Statistical analysis

Data were tabulated and analyzed using Microsoft Excel®. In-house TES-ELISA and Immunoblot with chimeric recombinant antigens (IB-rCHITC0 and IB-rCHITC1) performances were calculated using MedCalc V16.8.4 (MedCalc statistical software). For this calculation, we used an overall toxocariasis seroprevalence of 7.3% previously described by Acero et al. (2001), in a seroprevalence study developed in one Colombian school children population with similar epidemiological findings to this from our study [49]. ELISA-rCHITC0 and ELISA-rCHITC1 cutoff values and performances were obtained using R statistical software version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and IB-rCHITC0 test reactivity data for 149 serum samples from groups 1 to 5. Serum samples N6, N12 and N19 from the negative control group were excluded as true negative controls for ROC curve analysis, due to their strong reactivity detected in the IB-rCHITC0, and IB-rCHITC1 assays (Table 2, Figure 6). pROC package was used to calculate the area under the ROC curve (AUC) and 95% confidence intervals (95%CI) following the bootstrap method (1000 replicates). The most probable cutoff point value was detected using the Youden index [50] (Figure 7). Association between *T. canis* IgG seropositivity in ELISA or Immunoblot assays with chimeric antigens and history of contact with dogs or toxocariasis suspicion by clinical and epidemiological findings was established using IBM statistical software version 19 (IBM® SPSS statistics) and Chi-squared test-based statistical associations.

## 3. Results

### 3.1. Design of a custom eGFP-fusion recombinant expression system for T. canis polypeptides and seroreactivity assessment

To map high cross-reactivity fragments inside *T. canis* TES-26, TES-32, TES-120, and Myosin heavy chain (MyoN and MyoC fragments) proteins, we develop a custom system for the expression of *T. canis* segmented proteins as short chimeric recombinant polypeptides fused to carrier eGFP. The design involved the synthesis of a new recombinant expression vector based on the pET28a plasmid with the eGFP-coding sequence (*pET28a-eGFP*) for expression of *T. canis* polypeptides (Figures 1 and 2). Accordingly, the CDSs of the *T. canis* target proteins were divided into a total of 18 partial CDSs that encodes 18 polypeptides that ranged between 5.9 and 8.2 kDa. TES-26, TES-32, MyoN, and MyoC were divided into 4 partial CDSs that encode 4 polypeptides each one, and TES-120 was divided into 2 partial CDSs that encode 2 polypeptides (Figure 3, Table 1).

We successfully amplified 18 fragmented *T. canis* polypeptide coding sequences, using oligonucleotides listed in supplementary table s1 (supplementary figure s1). All amplified CDS fragments were cloned at the 3′ end of eGFP CDS (Figures 1 and 2). All synthesized recombinant vectors were verified by capillary sequencing. Control eGFP and chimeric

**Table 2.** Comparison of sensitivity between In-house TES-ELISA and Immunoblot/ELISA with rCHITC0/rCHITC1 chimeric antigens.

| Number of positive samples/Total number of samples (%) | | | | | |
|---|---|---|---|---|---|
| Assay type (total IgG) and antigen source | In-house TES-ELISA | IB-rCHITC0 | IB-rCHITC1 | ELISA-rCHITC0 | ELISA-rCHITC1 |
| **Sensitivity (%) for OLMS** | **95** | **62** | **16** | **58** | **38** |
| Healthy individuals (group 1) | 0/21 (0) | 3/21 (14) | 3/21 (14) | 0/18 (14) | 0//18 (14) |
| Clinical suspected OLMS patients reactive in TES-ELISA CDC (group 2) | 9/10 (90) | 7/10 (70) | 3/10 (30) | 2/10 (20) | 0/10 (0) |
| Clinical suspected OLMS/VLMS patients (groups 3, 4) | 45/61 (73.7) | 29/61 (47.5) | 2/61 (3.3) | 8/61 (13.1) | 1/61 (1.6) |
| Parasite infected patients (group 5) | 52/60 (86.6) | 38/60 (63.3) | 14/60 (23.3) | 16/60 (26.6) | 17/60 (28.3) |
| **Total serum samples** | **152** | **152** | **152** | **149** | **149** |

eGFP-fused polypeptides constructs were successfully transformed and expressed in *E. coli* BL21 (DE3). The recombinant proteins were purified through 6x-Histag IMAC affinity chromatography in an FPLC instrument and then used for the reactivity immunoblot assays (supplementary figures s2–6).

Recombinant eGFP and chimeric polypeptides were initially detected with anti-6x-Histag IgG antibody to confirm its respective molecular mass and to generate references that allow defining the standard for a positive reaction in the forthcoming immunoblot assays (Table 3, supplementary figures s7–12). Non-specific reactivity against carrier purified recombinant eGFP was discarded because no reactivity was observed in all tested human serum samples (Table 3, supplementary figure s**7**).

The eighteen fusion polypeptides were not recognized by any of the negative control sera (group 1) (Table 3, supplementary figures s8–12, line 16). Whereas most of them (TES26-P2/P3/P4, TES32-P1/P2/P4, TES120-P1/P2, MYONP1/P2, and MYOCP1/P2) were recognized by sera of the individuals with suspected toxocariasis (group 4). The remaining fusion polypeptides (TES26-P1, TES32-P3, MYONP3/P4, and MYOCP3/P4) showed no reactivity with the same sera group (Table 3, supplementary figures s8–12, lines 2–7).

When the fusion polypeptides were tested with cross-reaction sera (group 5), only 5 polypeptides showed strong positive reactions: TES26-P2, TES32-P1, TES120-P2, MYON-P2, and MYOC-P2 (Table 3, supplementary figures s8–12, lines 8–15). From this, TES26-P2, TES120-P2, MYON-P2, and MYOC-P2 polypeptides were reactive when tested against *Ascaris* spp. pooled sera. TES26-P2, TES32-P1, TES120-P2, MYON-P2, and MYOC-P2 polypeptides were reactive when tested against pooled sera of *Trichuris,* hookworm, *Strongyloides* and *Hymenolepis* infected patients. In the case of taeniasis serum samples, TES32-P1, TES120-P2, MYOC-P2, and TES26-P2 polypeptides were detected as reactive. Being TES32-P1 and TES120-P2 associated with reactivity against *T. solium* infected patients, and MYOC-P2 and TES26-P2 with reactivity against both *T. saginata* and *T. solium* infected patients.

Additionally, the fusion polypeptides TES26-P2, TES32-P1, MYON-P2, and MYOC-P2 were reactive when tested against serum samples of individuals with toxoplasmosis (Table 3, supplementary figures s8–12, lines 8–15). Finally, the pooled sera of the individuals infected with *Strongyloides* spp. only recognized the polypeptide MYOC-P4 (Table 3, supplementary figure s12, line 11). Out of all the tested polypeptides, only 7 showed no reactivity with any of the cross-reaction sera (group 5).

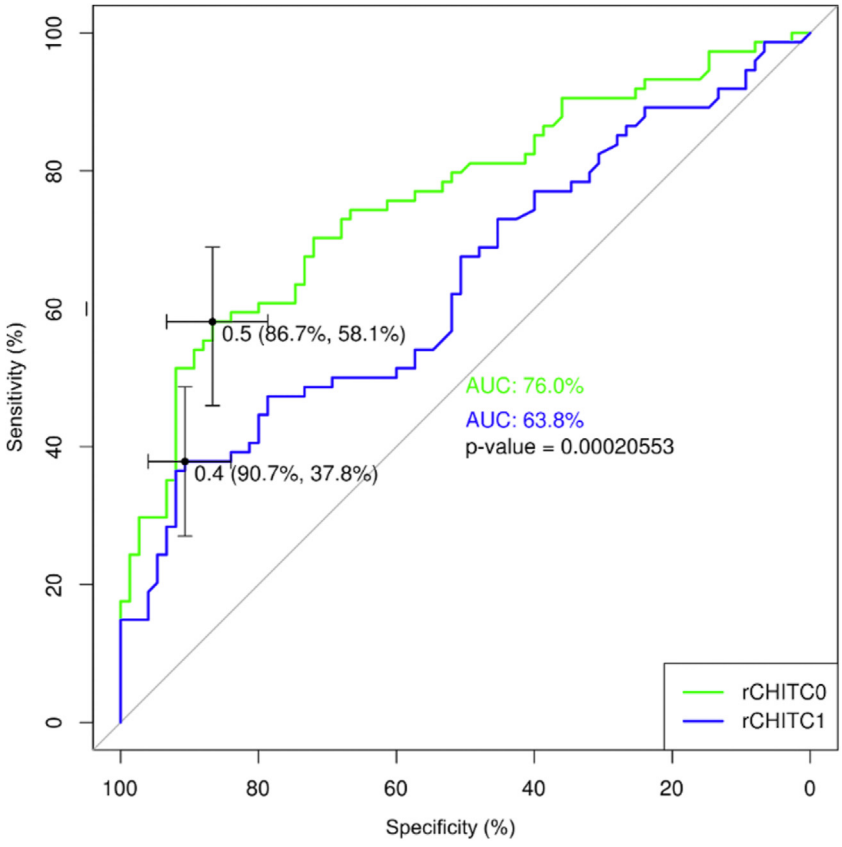


**Figure 7.** ROC curve analysis for rCHITC0 and rCHITC1 chimeric antigens and cutoff for *T. canis* ocular larval migrans syndrome (OLMS). ROC curve analysis was conducted using mean optical density values obtained for 149 serum samples (groups 1–5) in ELISA-rCHITC0 and ELISA-rCHITC1 assay. Reactivity data of immunoblot using rCHTC0 assay (IB-rCHITC0) was used for serum samples classification as positive or negative. AUC of 76% (IC95%: 68,06%–83,57%) and 63,8% (IC95%: 54,62%–72,38%) were calculated for ELISA-rCHITC0 and ELISA-rCHITC1 respectively. Best cutoff points were calculated using bootstrap method (1000 replicates) being >0,491 for ELISA-rCHITC0 with a sensitivity of 58,1 (IC95%: 47,3%–68,92%) and specificity of 86,67 (IC95%: 78,67%–93,33%) and >0,394 for ELISA-rCHITC1 with a sensitivity of 37,84 (IC95%: 27,03%–48,65%) and specificity of 90,67 (IC95%: 84,0%–96,0%).

**Table 3.** eGFP and *T. canis* chimeric recombinant polypeptides reactivity results in the Immunoblot assay.

| Serum samples reactivity | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Anti 6xHistag | *Toxocara spp* | *Ascaris spp* | *Trichuris spp* | Hookworm | *Strongyloides spp* | *T. solium* | *T. saginata* | *Hymenolepis spp* | *Toxoplasma spp* | Negative control | High reactivity polypeptides |
| **Evaluated serum** | mAb | 6 | 1* | 1* | 1* | 1* | 1* | 1* | 1* | 1* | 1* | - |
| **eGFP WT** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES26-P1** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES26-P2** | **R** | **R (2/6)** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **NR** | ✓ |
| **TES26-P3** | R | R (2/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES26-P4** | R | R (2/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES32-P1** | **R** | **R (5/6)** | **NR** | **R** | **R** | **R** | **R** | **NR** | **R** | **R** | **NR** | ✓ |
| **TES32-P2** | R | R (2/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES32-P3** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES32-P4** | R | R (1/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES120-P1** | R | R (2/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **TES120-P2** | **R** | **R (5/6)** | **R** | **R** | **R** | **R** | **R** | **NR** | **R** | **NR** | **NR** | ✓ |
| **MYON-P1** | R | R (1/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **MYON-P2** | **R** | **R (4/6)** | **R** | **R** | **R** | **R** | **NR** | **NR** | **R** | **R** | **NR** | ✓ |
| **MYON-P3** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **MYON-P4** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **MYOC-P1** | R | R (3/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **MYOC-P2** | **R** | **R (5/6)** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **NR** | ✓ |
| **MYOC-P3** | R | NR (0/6) | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| **MYOC-P4** | R | NR (0/6) | NR | NR | NR | R | NR | NR | NR | NR | NR | - |

mAb: Monoclonal Antibody. 1*: Serum samples used as a pool; R: Reactive serum sample; NR: Non-reactive serum sample; ✓: Polypeptides showing high reactivity to serum samples from *Toxocara canis* and other helminths and protozoa parasites (non-specific or cross reactivity polypeptides).

Those were polypeptides TES26-P1, TES26P3/P4, TES32P2/P3/P4, TES120-P1, MYON-P1, MYON-P3/P4, and MYOC-P1/P3 (Table 3, supplementary figures s8–12, lines 8–15). Based on these reactivity results, TES26-P2, TES32-P1, TES120-P2, MYON-P2, and MYOC-P2 were identified as high cross-reaction polypeptide regions, which potentially would be involved with false-positive reactions.

### 3.2. Design and synthesis of T. canis rCHITC0 and rCHITC1 chimeric recombinant antigens

Based on *T. canis* chimeric polypeptides reactivity data, we decided to synthesize a chimeric prototype gene (CDS), termed *chitc1*, that avoided the detected fragments with high cross-reactivity of the analyzed antigens (TES26-P2, TES32-P1, TES120-P2, MYON-P2, and MYOC-P2). As a control, another version of the chimeric gene was generated, *chitc0*, in which the full CDS sequences of the studied antigens were kept as originally cloned. Schematic illustration representing design and synthesis of *T. canis chitc0* and *chitc1* chimeric genes (coding for rCHITC0 and rCHITC1 chimeric antigens) is shown in Figure 4 (figure 4. A–D). The design included 10 and 14 oligonucleotides used in PCR and fusion PCR reactions for chimeric *chitc0* and *chitc1* synthesis, respectively (Figure 4. A-B; supplementary table s2). Agarose gel electrophoresis shows purified F1, F2, F3 fusion fragments and F4 full size assembled *chitc0* chimeric gene (Figure 4. C, lines 6–9). Synthetized chimeric *chitc0* was cloned to obtain recombinant expression vector *pET28a-chitc0.* Similarly, *chitc1* synthesis was carried out in a total of thirteen PCR reactions which were divided into four stages (Figure 4. B). Agarose gel electrophoresis confirmed the purified F1, F2, F3, F4, and F5 fusion fragments and F6 full size assembled *chitc1* chimeric gene (Figure 4. D, lines 8–13). Synthetized chimeric *chitc1* was cloned to obtain recombinant expression vector *pET28a-chitc1*.

### 3.3. T. canis rCHITC0 and rCHITC1 chimeric antigens expression and purification

Synthetized *pET28a-chitc0* and *pET28a-chitc1* recombinant expression vectors were able to successfully express 118kDa rCHITC0 and 81kDa rCHITC1 chimeric recombinant proteins, although they were found insoluble forming inclusion bodies in *E. coli BL21 (DE3)* cells (Figure 5. A, D). Collected inclusion bodies containing rCHITC0 and rCHITC1 were urea solubilized and then used for standard denaturing IMAC purification (Figure 5. B, E). Purified rCHITC0 and rCHITC1 antigens with 85% and 95% purity were used for further evaluation in the immunoblot and ELISA assays (Figure 5. C, F).

### 3.4. T. canis rCHITC0 and rCHITC1 chimeric recombinant antigens reactivity assessment

Chimeric rCHITC0 and rCHITC1 reactivity was tested using immunoblot (IB-rCHITC0/IB-rCHITC1) and ELISA (ELISA-rCHITC0/ELISA-rCHITC1) assays (Figures 6 and 7). Sensitivity, specificity, and cross-reactivity are shown in Tables 2 and 4. Regarding sensitivity, in-house TES-ELISA showed the highest yield (95%) compared to Immunoblot and ELISA with chimeric antigens, which presented minor sensitivities in decreasing order for IB-rCHITC0 (62%), ELISA-rCHITC0 (58%), ELISA-rCHITC1 (38%), and IB-rCHITC1 (16%) assays (Table 2). Sensitivity performance was mainly associated with the reactivity results obtained for 71 serum samples from patients with clinical suspicion of toxocariasis (groups 2, 3 and 4), of which 76% (54/71) were reactive with in-house TES-ELISA, and only 50% (36/71), 7% (5/71), 14% (10/71), and 1.4% (1/71) were reactive with IB-rCHITC0, IB-rCHITC1, ELISA-rCITC0, and ELISA-rCHITC1, respectively (Table 2, Figures 6 and 7). The same results were observed for reactivity by specific groups. In this analysis, 90% (9/10) of the control serum samples from individuals with clinical suspected OLMS that were previously confirmed as reactive using CDC TES-ELISA (group 2 serum samples) and 73.7% (45/61) of the individuals with clinical suspected OLMS/VLMS from groups 3 and 4 showed reactivity in the in-house TES-ELISA, compared with a lower reactivity detected for this same groups of samples in the assays with both chimeric recombinant antigens (Table 2, Figures 6 and 7). In contrast, calculated specificity was superior for both chimeric recombinant antigens with obtained values of 91%, 89%, 87%, and 76% for ELISA-rCHITC1, IB-rCHITC1, ELISA-rCHITC0, and IB-rCHITC0, respectively, compared to the one showed for in-house TES-ELISA (61%) (Table 4). Better specificity

**Table 4.** Comparison of the specificity and cross reactivity between In-house TES-ELISA and Immunoblot/ELISA using rCHITC0/rCHITC1 antigens.

| Assay type (total IgG) and antigen source | Cross-reactivity (False positive results) | | | | |
|---|---|---|---|---|---|
| | Number of positive samples/total number of samples (%) | | | | |
| | Inhouse TES-ELISA | IB-rCHITC0 | ELISA-rCHITC0 | IB-rCHITC1 | ELISA-rCHITC1 |
| **Specificity (%) for OLMS** | **61** | **76** | **87** | **89** | **91** |
| **Clinical suspected OLMS patients (group 3)** | 2/14 | 5/14 | 0/14 | 0/14 | 0/14 |
| ***Ascaris* sp.** | 3/4 | 2/4 | 2/4 | 0/4 | 2/4 |
| **Free living Amoeba** | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| ***Echinococcus* sp.** | 4/4 | 0/4 | 0/4 | 0/4 | 1/4 |
| ***Hookworm*** | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| ***Hymenolepis* sp.** | 4/5 | 4/5 | 4/5 | 1/5 | 3/5 |
| ***Strongyloides* sp.** | 5/5 | 3/5 | 4/5 | 1/5 | 4/5 |
| ***Toxoplasma* sp.** | 2/5 | 4/5 | 1/5 | 2/5 | 3/5 |
| ***Trichuris* sp.** | 5/5 | 4/5 | 5/5 | 2/5 | 4/5 |
| ***Taenia saginata*** | 7/8 | 4/8 | 6/8 | 1/8 | 5/8 |
| ***Taenia solium* (taeniasis)** | 5/5 | 3/5 | 1/5 | 2/5 | 2/5 |
| ***Taenia solium* (cysticercosis)** | 11/13 | 10/13 | 5/13 | 3/13 | 2/13 |
| **Mixed intestinal infection** | 2/2 | 1/2 | 2/2 | 0/2 | 1/2 |
| **Healthy individuals (group 1)** | 0/21 | 3/21 | 1/18 | 3/21 | 1/18 |
| **Total cross reactivity** | 54/95 (**57**) | 46/95 (**48**) | 34/92 (**37**) | 18/95 (**19**) | 31/92 (**34**) |
| **Dog breeders (group 6)** | - | - | 3/80 (3.7) | - | 0/80 (0) |
| **Randomly selected and apparently healthy people (group 7).** | - | - | 4/78 (5.1) | - | 1/78 (1.2) |

showed for both chimeric recombinant antigens was associated to the lower reactivity detected for the parasite-infected patient group (IB-rCHITC0 with 63.3% (38/60), ELISA-rCHITC1 with 28.3% (17/60), ELISA-rCHITC0 with 26.6% (16/60), and IB-rCHITC1 with 23.3% (14/60)), compared with the high reactivity 86% (52/60) detected for this same group of samples in the in-house TES-ELISA (Table 2). This lower number of false-positive reactions detected for most sera of the different groups of parasitic infections, explains the reduction in overall cross-reactivity rate from 57% (for in-house TES-ELISA) to 19% and 48% (for IB-rCHITC1 and IB-rCHITC0, respectively), and 34% and 37% (for ELISA-rCHITC1 and ELISA-rCHITC0 respectively) (Table 4). On the other hand, among the 80 individuals with exposure to canines (group 6), we detected a total reactivity of 3.7% (3/80) using ELISA-rCHITC0 and no reactivity using ELISA-rCHITC1 (Table 4). Additionally, there was no statistically significant association between contact with canines and reactivity detected in assays with chimeric antigens using this group of sera. Finally, from 78 randomly selected and apparently healthy people, we detect a global reactivity of 5.1% (4/78) using ELISA-rCHITC0 and 1.2% (1/78) using ELISA-rCHITC1 (Table 4).

## 4. Discussion

The actual burden due to toxocariasis is largely underestimated in many countries around the world [1, 2, 13, 51]. Diagnostic limitations make most human toxocariasis studies based just on seroepidemiological surveys. TES-ELISA is actually the reference immunodiagnostic test for human toxocariasis diagnosis [21, 23, 25, 26]. However, cross-reactivity using TES-ELISA has been extensively reported in several studies developed around the world, being much higher in tropical countries with a high prevalence of parasitosis [27, 31, 32, 52, 53, 54, 55]. Lynch reported cross-reactions for TES antigens using serum samples from individuals infected with *Ascaris* spp, *Strongyloides* spp, hookworms, *Trichuris* spp, *Enterobius* spp, *Taenia* spp, and *Schistosoma* spp [52]. In 1991, Magnaval also reported cross-reactions against Ascaris spp, *Anisakis* spp, and *Enterobius* spp in 33% (39/118) of the studied samples using TES-ELISA [56]. Similar results were published by Roldan and coworkers in 2009. Using conventional TES-ELISA, authors reported cross-reactivity for 48% of the patients infected with *Ascaris* spp., *Ancylostoma* spp., *Trichuris* spp., *Enterobius* spp., *Strongyloides* spp., *Hymenolepis* spp., *Dipylidium* spp, *Taenia* spp. (taeniasis and cysticercosis), *Fasciola* spp, and *Echinococcus* spp [55]. More recently, another study developed by Olave and colleagues, reported similar results using in-house TES-ELISA and parasitized serum samples. In this study, cross reactivity was reported for 90% (19/21) of the evaluated samples from patients with *Ascaris* spp., *Trichuris* spp., *Strongyloides* and *Toxoplasma* spp. [41]. Our study using the same standardized in-house TES-ELISA for OLMS diagnostic, also reveals cross-reactivity rates similar to those previously reported by other authors [23, 25, 27, 29, 52, 53]. Our experiments with 60 positive control cases from parasitic confirmed or serological suspicion serum samples, allowed us to detect a high reactivity rate (86%) and cross-reactions with *Ascaris* spp., free-living amoeba, *Echinococcus* spp., *Hymenolepis* spp., *Strongyloides* spp., *Toxoplasma* spp., *Trichuris* spp., *Taenia saginata* (taeniasis), *Taenia solium* (taeniasis/cysticercosis), and hookworm infected patients (Tables 2 and 4). It is globally reported that TES-ELISA does not perform as well as initially thought, and our finds support such observations. This situation leads to the actual decline in the confidence in TES-ELISA in countries with a high prevalence of parasitic infections due to the striving interpretation and implementation of its results as a confirmatory diagnostic test [4, 23].

The use of immunodiagnostic tests based on recombinant antigens has been defined by researchers and the world scientific community as the most promising alternative for the specific diagnosis of human toxocariasis [4, 10, 20, 57]. *T. canis* recombinant proteins represent an important antigen source and have been described as the best alternative to native TES, due to the advantages in their controlled production and the increased sensitivity and specificity reported when they were tested in ELISA and immunoblot formats [4, 20, 21]. *T. canis* TES-26, TES-32, TES-120, and Myosin heavy chain have been described as the antigens with greater potential for specific human toxocariasis diagnostic [21, 36, 37, 40, 41]. Despite these advantages, some studies have reported that cross-reactions for these recombinant antigens are still and important issue. In 2009, Mohamad described moderated cross-reactivity for TES-26, TES-32 and TES-120, evaluated in ELISA format with serum samples from patients with confirmed parasitic infections 37. Another study developed by Anderson in 2015, reported cross-reactions for TES-26 (amebiasis, E. *nana*, ascariasis, baylisascariasis, cysticercosis, echinococcosis, hookworm, malaria, paragonimiasis, schistosomiasis, trichinellosis, and trichuriasis) and Tc-CTL-1 (Amebiasis and *E. nana*), using 120 serum samples from the U.S. parasitized patients [36]. Similarly, Obwaller and colleagues reported cross-reactions for some Myosin heavy chain fragments in their immunoblot experiments using serum samples from patients with other helminthiasis [35]. Experiments performed by Yamasaki in 1988/2000 and more recently by Olave in 2016, also reported cross-reactions using recombinant TES-32 [33,41,42].

Based on the cross-reactivity reported for these recombinant antigens and the lack of information about amino acidic sequences involved in such as nonspecific reactions, further studies are needed to validate the level of cross-reactivity of these candidate recombinant antigens, using patient samples from tropical regions, where helminth infections are endemic. That's why, we report here an innovative methodology to map and identify polypeptidic regions potentially involved in residual cross-reactions inside *T. canis* TES-26, TES-32, TES-120, and Myosin heavy chain candidate antigens. Using a custom-designed pET28a-eGFP cloning system, we successfully expressed and purified fragmented *T. canis* TES-26, TES-32, TES-120, MyoN, and MyoC as 18 short recombinant polypeptides individually fused to a carrier eGFP (Table 1, Figures 1, 2, and 3 and supplementary figures s1–6). Immunoblot experiments using serum samples from positive control cases (group 5) and purified *T. canis* polypeptides as antigens, allowed us to identify for the first time TES26-P2, TES32-P1, TES120P2, MYON-P2, and MYOC-P2 as high cross-reactivity polypeptidic regions (against *Ascaris* spp., *Hymenolepis* spp., *Strongyloides* spp., *Trichuris* spp., *Taenia saginata/solium*, *Toxoplasma* spp., and hookworm) inside the evaluated recombinant *T. canis* antigens (Table 3, supplementary figures s7–12).

In previous studies, different authors have evaluated *T. canis* recombinant proteins as individual antigens. Notwithstanding the reduction in sensitivity when recombinant antigens were used, authors reported superior performance in terms of specificity compared to native TES [33, 40, 41, 46, 58]. Some studies have proposed and developed assays using multiple recombinant antigens in a single assay as a measure to improve sensitivity and specificity of *T. canis* immunodiagnostic tests [36, 37]. Despite the advantages of this strategy and the better performance of this type of assays, authors have reported the need for more validation studies using this approach [36]. In this study, we proposed a methodology based on chimeric recombinant proteins for custom design of highly specific antigens. Using information about cross-reactivity inside previously studied *T. canis* antigens, we designed a novel methodology to produce two chimeric genes coding for *T. canis* chimeric proteins rCHITC0 and rCHITC1 (Figure 4). Chimeric rCHITC0 used as a control antigen was designed with the complete coding sequences of TES-26, TES-32, TES-120, rMYON, and rMYOC fused in tandem as a single chimeric *chitc0* gene (Figure 4. A, C). Chimeric rCHITC1 used as a high specificity prototype antigen was designed by eliminating the coding regions of the polypeptides that previously showed strong cross-reactivity (TES-26P2, TES32-P1, TES-120P2, MYON, and MYOC).

We conducted a phase I study using chimeric recombinant antigens, seropositive individuals and seronegative individuals, with the aim to assess the capacity of the test to differentiate between them [59, 60, 61]. Our obtained results are comparable to those reported by other authors. Immunoblot and ELISA using rCHITC0 and rCHITC1 chimeric recombinant antigens performs with a lower overall sensitivity (16%–62%) compared to in-house TES-ELISA (95%) using native antigens (Table 2).

This behavior has been previously described in other *T. canis* antibody measurements studies that used single and combined recombinant antigens and could be explained due to their less antigenic complexity compared to TES antigens. The presence of multiple antigenic proteins (including some highly glycosylated) can lead to a greater reactivity with *T. canis* suspected patients sera [36, 37]. This same reduction in sensitivity was reported by Anderson and colleagues using rTES-32 and rTES-26 antigens and a panel of 50 OLMS serum samples in Luminex-based assays. With their experiments, Anderson reported a very similar performance for sensitivity (64% sensitivity using both antigens combined in a single assay) as we reported here for IB-rCHITC0 (58%) and ELISA-rCHITC0 (62%) assays [36]. It must be pointed out that the reduction in the recombinant antigens' reactivity is not bad *per se*, since as we have observed in our TES-ELISA analysis, the false-positive reactions are common.

Another possible cause for the lower sensitivity calculated in our study using chimeric antigens could be associated with the use of OLMS positive control serum samples for performance evaluation. It could be explained because *T. canis* ocular infections have been usually associated with mild infections with *T. canis* L3 larvae, which usually induce low levels of circulating *T. canis* specific antibodies [62, 63, 64, 65]. It is possible that using positive control sera from SLMV patients (usually with greater *T. canis* specific circulating antibodies) [26], chimeric antigens sensitivity could be significantly improved as Anderson described in their studies using SLMV positive control serum samples and rTES-26/rTES-32 antigens [36].

The low reactivity of OLMS sera cited before was observed when clinically suspected ocular toxocariasis samples (groups 2 and 3) were tested with chimeric antigens (Table 2). Our results show lower reactivity for this samples in IB-rCHITC0 (50,7%, 36/71), ELISA-rCHITC0 (14%, 10/71), IB-rCHITC1 (7%, 5/71) and ELISA-rCHITC1 (1,4%, 1/71), in comparison with that detected using in-house TES-ELISA (76%, 54/71) (Table 2, Figure 6). In OLMS-suspected patients, a negative serology using chimeric antigens is not enough evidence to categorically discard ocular toxocariasis. In such cases, and taking into account a possible lower immune response against *T. canis* antigens, authors suggest the measurement of specific intraocular antibodies to confirm or rule out a clinically-suspected OLMS case [19, 62, 63, 64, 66].

In terms of specificity, most of the chimeric recombinant assays displayed a superior performance compared to the in-house TES-ELISA, which showed a 61% specificity (Table 4). rCHITC1 antigen showed the best specificities being 91% in ELISA and 89% in immunoblot, being followed by rCHITC0 with 87% and 76% in ELISA and immunoblot, respectively (Table 4). This better specificity can be explained by the use of recombinant chimeric antigens with stringently selected polypeptidic regions (instead of TES), that were expressed in the *E. coli* heterologous systems that doesn't perform the eukaryote protein glycosylation process [67]. In the case of the rCHITC1 antigen, the highest specificity was expected due to the elimination of strong cross-reactive polypeptidic regions from the control rCHITC0 chimeric antigen (Tables 3 and 4, Figure 4). Although the specificity values obtained for rCHITC0 and rCHITC1 antigens are not optimal, as reported in other *T. canis* recombinant proteins based studies [36, 37, 38, 40, 41, 42, 43], we achieved an important reduction in overall cross-reactivity, even though a panel of sera from parasite positive control cases living in a tropical region (group 5), with high exposure to parasitic infections, was used (Table 4).

One of the limitations of this study lies in the fact that we cannot exclude the possibility that individuals analyzed in this work were previously exposed to helminths or that they have active parasite co-infections not detected. Additionally, the screening of the serum samples used, was carried out using a low-specificity (61%) in-hose TES-ELISA test [41]. That's why the calculated specificities for our chimeric antigens must be carefully analyzed and assumed as relative values until a more robust phase II study could be developed. These issues represent a typical limitation in developing a serological test for tropical parasitosis.

Taking into account that our study was based on anti-*Toxocara* total IgG detection, it is possible that using specific $IgG_4$ subclass for reactivity assessment could also improve the overall performance of our assays, as was previously reported by Mohamad (using *T. canis* recombinant antigens) and Noordin (using TES antigens) [25, 37]. This possibility must be explored in a phase II forthcoming study.

Analyzing the reactivity results obtained for the dog breeders serum samples (3.7% in ELISA-rCHITC0) and healthy random selected population (5.1% and 1.2 in ELISA-rCHITC0 and ELISA-rCHITC1 respectively), we observe a low proportion of reactivity (Table 4). Canine contact has been extensively reported as a factor for increased risk of *T. canis* L3 larvae infection [51, 68, 69, 70, 71]. Although we expected a higher reactivity in this group of samples, our statistical analysis showed no correlation between canine exposure and seropositivity with any of the different evaluated assays. Another explanation for this low reactivity could be the fact that canines to which the individuals under study were exposed were submitted to carefully processes in their feeding, cleaning and deworming, which might keep *T. canis* infection rates under control or absent, a phenomenon reported by Amaral and collogues in their research using canines [68]. Finally, reactivity in a randomly selected population is comparable to data reported in other studies using TES-ELISA. These studies developed in volunteer blood donors from the United Kingdom and Switzerland also reported low reactivity results (2, 6% and 4% respectively) [26, 32, 72]. Our findings with the chimeric antigens showed similar reactivity results to those reported in European healthy individuals with low parasitic prevalence. This might indicate that our recombinant chimeric-based assays, applied in people from tropical regions with higher infectious diseases prevalence, could perform, at least, with similar validity than the toxocariasis TES-ELISA, representing eligible candidates to enter into phase II studies.

## 5. Conclusions

Developing a more reliable human toxocariasis immunodiagnostic test to be applied in populations from tropical region is a complex technological challenge. However, the rCHITC0 and rCHITC1 chimeric antigens phase I study presented here, show encouraging results in the search of new and refined proteins that may behave as more specific antigens able to outperform the classic *T. canis* native TES assay. Evaluation of chimeric antigens in complementary phase II and phase III studies, would allow their final validation for diagnostic purposes.

Use of more specific recombinant rCHITC0 and rCHITC1 antigens in the ELISA and/or immunoblot formats, could represent an important new alternative to support presumptive human toxocariasis diagnostic with higher confidence than serology using TES antigen-based ELISA or Immunoblot assay. A positive result with one of these chimeric antigens, represents a very significant laboratory evidence in human toxocariasis, when it is combined with epidemiological evidence of exposition to *T. canis* L3 larvae and/or clinical findings compatible with an OLMS or VLMS.

The method proposed here for the detection of cross-reactive polypeptidic regions and development of chimeric proteins, allowed us to produce highly specific *T. canis* prototype chimeric antigens with potential application in human toxocariasis immunodiagnostic. This methodology could represent a new tool for controlled antigen development, especially for parasitic pathogens where immunological tests are paramount for diagnosis.

## Declarations

### *Author contribution statement*

Jairo A. Mesa-Arango: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ana M. Olave-Velandia: Performed the experiments.

Gisela M. García-Montoya: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Juan P. Isaza-Agudelo: Analyzed and interpreted the data.

Antonio Jiménez-Ruiz: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Juan F. Alzate: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data included in article/supplementary material/referenced in article.

### *Declaration of interest's statement*

The authors declare no conflict of interest.

### *Additional information*

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