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An innovative approach in the detection of *Toxocara canis* excretory/secretory antigens using specific nanobodies

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ABSTRACT

Human toxocariasis is a zoonosis resulting from the migration of larval stages of the dog parasite *Toxocara canis* into the human paratenic host. Despite its well-known limitations, serology remains the most important tool to diagnose the disease. Our objective was to employ camelid single domain antibody fragments also known as nanobodies (Nbs) for a specific and sensitive detection of *Toxocara canis* excretory/secretory (TES) antigens. From an alpaca immune Nb library, we retrieved different Nbs with specificity for TES antigens. Based on ELISA experiments, these Nbs did not show any cross-reactivity with *Ascaris lumbricoides*, *Ascaris suum*, *Pseudoterranova decipiens*, *Anisakis simplex* and *Angiostrongylus cantonensis* larval antigens. Western blot and immunocapturing revealed that Nbs 1TCE39, 1TCE52 and 2TCE49 recognise shared epitopes on different components of TES antigen. The presence of disulphide bonds in the target antigen seems to be essential for recognition of the epitopes by these three Nbs. Three separate sandwich ELISA formats, using monovalent and bivalent Nbs, were assessed to maximise the detection of TES antigens in solution. The combination of biotinylated, bivalent Nb 2TCE49 on a streptavidin pre-coated plate to capture TES antigens, and Nb 1TCE39 chemically coupled to horseradish peroxidase for detection of the captured TES antigens, yielded the most sensitive ELISA with a limit of detection of 0.650 ng/ml of TES antigen, spiked in serum. Moreover, the assay was able to detect TES antigens in sera from mice, taken 3 days after the animals were experimentally infected with *T. canis*. The specific characteristics of Nbs make this ELISA not only a promising tool for the detection of TES antigens in clinical samples, but also for a detailed structural and functional study of TES antigens.

1. Introduction

Human toxocariasis (HT) is a zoonotic disease caused by the larval stage of the roundworm parasite *Toxocara canis* in the human host (Glickman and Schantz, 1981). *Toxocara canis* adult stages naturally infect the lumen of the gut in dogs, which serve as the final hosts in the life cycle of the parasite. Following infection, eggs are released via the faeces into the environment, where they remain viable for long periods of time. Upon accidental ingestion of *Toxocara* eggs by humans, the parasites do not develop to the adult stage, but they migrate as L_3 through the circulatory system towards the brain, eye, lung or liver. This can result in two variants of the disease: (i) the disseminated form including covert toxocariasis (CT) and visceral larva migrans (VLM), or (ii) the compartmentalised form represented by ocular larva migrans (OLM) and neurotoxocariasis (Rubinsky-Elefant et al., 2010).

CT is the most common and benign form of the disease. Its clinical presentation includes weakness, wheezing, abdominal pain together with mild eosinophilia. VLM is the result of massive ingestion of larvae. It is a severe disease that courses with massive eosinophilia accompanied by acute hepatic and pulmonary symptomatology (Beaver et al., 1952). OLM is an infection circumscribed to one single eye without any signs of systemic disease. Neurotoxocariasis is a rare syndrome, mostly associated with myelitis.

In the disseminated forms of HT, the larvae of the parasite release a variable amount of *T. canis* excretory–secretory (TES)

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antigens into the tissues of the human host. TES antigens can also be harvested in vitro through collection and hatching of *T. canis* eggs (de Savigny, 1975). The amount of TES secreted by a larva in vitro can vary from 0.1 (de Savigny, 1975) to 2 (Bowman et al., 1987) or even 8 ng (Badley et al., 1987) of protein/larva/day. The amount of TES antigen in vivo depends on the number of eggs ingested. An inoculum of 100–200 eggs is presumed to be enough to produce CT (Smith and Beaver, 1953; Chaudhuri and Saha, 1959). Based on these estimations, the amount of TES antigen available for detection in serum samples with presumptive diagnoses of CT is probably in the low pg per ml range. Higher amounts of TES antigen are expected in VLM as the inoculum for this form of the disease is very high.

TES antigen comprises at least 50 different components (Kennedy, 2006) with TES-120 as the most abundant fraction. The characteristic TES-120 band is composed of a set of three proteins translated from three different mRNAs (muc1, 2 and 3) (Loukas et al., 2000a). These proteins are rich in Serine and Threonine residues, providing attachment sites for *O*-glycosylation (Page and Maizels, 1992). Glycosides account for more than 50% of the molecular weight of the TES-120 (Page and Maizels, 1992). The TES-70 and TES-32 proteins are secreted C-type *N*-glycosylated lectins implicated in immune evasion of the parasite by a host-mimicking mechanism (Loukas et al., 2000b; Maizels et al., 2006). TES-120 makes up the shedding "fuzzy coat" of the parasite together with the TES-70 fraction, whereas TES-32 is a component of the cuticle (Page et al., 1992b). While the complex interactions of these proteins are not completely understood, it is known that they share a core structure that includes a signal peptide, followed by the Serine/Threonine-rich region and a Cysteine-rich domain that allows protein–protein interactions (Loukas et al., 2000a). The TES-400 proteoglycan is the biggest component of TES antigens (Page and Maizels, 1992; Maizels and Loukas, 2001). TES-26 is a homologue of mammalian phosphatidylethanolamine (PE)-binding protein without known function (Maizels, 2013). TES-55 is also a lectin, with a similar structure to TES-32, however it is less well established compared with the other TES proteins (Page et al., 1992b; Maizels and Loukas, 2001).

Currently, the laboratory diagnosis of HT relies on the detection of TES antigen-specific host antibodies in sera using native TES antigens (de Savigny et al., 1979; Fillaux and Magnaval, 2013; Jin et al., 2013) or less commonly using recombinant proteins for capture (Mohamad et al., 2009; Zahabiun et al., 2015). The first anti-TES antibody ELISA was developed in 1979 (de Savigny et al., 1979). A commercial serological test was developed and validated in 1991, reporting a sensitivity of 91% and specificity of 86% (Jacquier et al., 1991). Subsequently, other commercial kits have shown similar levels of performance (Smith and Noordin, 2005). More recently, the development of high-throughput analysis with multiple recombinant antigens has scaled sensitivity and specificity up to 99% and 94%, respectively, for VLM but a sensitivity of only 64% for OLM (Anderson et al., 2015). While serological tests remain the least invasive and most sensitive approach to diagnose HT, they exhibit two important shortcomings that have not yet been solved. The first one is the lack of specificity due to cross-reactivity with other parasitic antigens such as *Ascaris lumbricoides*. This is especially problematic in regions where polyparasitism is prevalent (Lynch et al., 1988; Fillaux and Magnaval, 2013). Immunodiagnosis has been significantly improved since the development of a western blot (WB) for the detection of IgG with specificity to *T. canis* (Magnaval et al., 1991). Initially, a characteristic pattern of high (200, 147 and 132 kDa) and low (35, 30, 28 and 24 kDa) molecular weight bands was described in sera from patients who were suspected to be infected with *T. canis*, and from experimentally infected animals. Combined with data yielded by the testing of sera from other helminthiases, these results suggested that the low molecular weight bands were specific for toxocariasis (Magnaval et al., 1991). Subsequently, this conclusion was confirmed by Roldán and Espinoza (2009). The second drawback of serology is its inability to distinguish current and past infections, which has important implications for clinical diagnosis as well as for treatment follow up. A logical approach to tackle this problem is to aim for a direct detection of larval TES antigens in body fluids, instead of detecting the host antigen-specific antibodies. Unfortunately, to date attempts have been largely unsuccessful due to a lack of sensitivity or specificity (Gillespie et al., 1993; Iddawela et al., 2007; Ishiyamna et al., 2009).

A nanobody (Nb) is the recombinant variable domain of heavy chain-only antibodies circulating in the blood of camelids (Muyldermans, 2013). Despite their small size (~15 kDa), autonomous Nbs are fully functional in antigen binding via interactions between the three hypervariable loops of the Nb with the cognate antigen. In contrast to the flat or concave antigen-binding site of classical antibodies, the architecture of the Nb paratope is convex and ideal to reach hidden epitopes in cavities on the protein surface, which are normally cryptic for conventional antibodies (De Genst et al., 2006). Despite their structural peculiarities, Nbs are very stable and easy to clone, express and purify through standard procedures (Muyldermans, 2013). Moreover, in comparison to 'regular' monoclonal antibodies, Nbs provide an exceptional modular entity allowing easy bioconjugation, labelling and generation of multivalent constructs. These 'plug and play' engineering possibilities generate a much higher versatility in designing next generation tools, with no interference in the capacity of the Nb entity to recognise its target. This feature can be extensively exploited in the construction of a wide variety of immunoassay formats for sensitive and specific detection of antigens in biological samples (Helma et al., 2015; Dmitriev et al., 2016)

In this paper, we identified a panel of three Nbs able to specifically recognise TES components of *Toxocara canis*. The complex 'carbohydrate-peptide' epitopes on TES antigens recognised by these Nbs were characterised via immunoblots. Subsequently, these Nbs were engineered to develop a specific sandwich ELISA for detection and quantification of TES antigens, spiked in PBS or serum and in sera of mice experimentally infected with *T. canis* embryonated eggs.

2. Materials and methods

2.1. Production of antigens

TES antigen was produced by the method of Savigny (1975), dialyzed against four times diluted PBS pH 7.4, lyophilised, weighted and stored at −20 °C. Antigens were divided into separate lots according to larval batches. Reconstitution of antigens was carried out in sterile Milli-Q water. Antigen concentrations were determined in triplicate by UV spectrophotometry (Nanodrop™), (OD_{280nm} of 1.0 corresponds to an estimated concentration of 1 mg/ml). All sandwich ELISAs were performed with TES antigen lot-14, whereas immunoblots were carried out with TES antigen lot-15.

2.2. Immunisation of an alpaca and construction of a Nb library

An alpaca was immunised by s.c. injection of 125 μg of TES antigen mixed with Gerbu Adjuvant, six times at weekly intervals. Four days after the last immunisation, 50 ml of blood were taken and peripheral blood lymphocytes (PBLs) were extracted with Lymphoprep (Nycomed, Switzerland). A Nb library was constructed as described previously (Conrath et al., 2001). Briefly, total mRNA isolated from PBLs was used as a template to synthesise cDNA using

oligo dT primers. The single-stranded cDNA was subsequently amplified with a variable domain heavy chain (VHH) leader-specific primer CALL001 (5′-GTC CTG GCT GCT CTT CTA CAA GG-3′) and a CH2-specific primer CALL002 (5′-GGT ACG TGC TGT TGA ACT GTT CC-3′) to amplify the heavy chain antibody gene fragments from the variable region to the CH2 region (Conrath et al., 2001). The resulting PCR products containing the VHHs (i.e. the PCR products with sizes of approximately 700 bp) were cut out of the gel and amplified with the nested VHH-For primer PMCF (5′-CTA GTG CGG CCG CTG AGG AGA CGG TGA CCT GGG T-3′) and VHH-Back primer A6E (5′-GAT GTG CAG CTG CAG GAG TCT GGR GGA GG-3′), containing *Not*I and *Pst*I restriction enzyme sites, respectively (Conrath et al., 2001). The resulting amplicons were ligated in the phagemid vector pMECS (Vincke et al., 2012) in frame with a hemagglutinin (HA)-tag and a His_6-tag, and transformed in electro competent *Escherichia coli* TG1 cells.

2.3. Isolation and screening of TES-specific Nbs

Two rounds of panning were performed to enrich for TES-specific Nbs, according to our in-house protocol (Conrath et al., 2001). First, a representative aliquot of the library with the complete repertoire of Nbs was expressed on phage after infection with M13K07 helper phages. Phage particles were then recovered through precipitation with polyethylene glycol and subjected to two rounds of panning on solid-phase coated antigen (20 μg/well). The antigen-bound phage particles were released by a pH shock, neutralised and used to infect fresh *E. coli* TG1 cells. A fraction of the infected cells was grown and used for the subsequent round of panning and part of it was plated on agar with selective medium. Individual colonies were picked, cultured and expression of the Nb protein was induced overnight with 1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, United States). After extraction, the periplasmic extracts containing the Nbs were added to wells of a microtiter plate coated with TES antigens (1 μg/well). The presence of antigen-specific Nbs was detected in an ELISA using a mouse anti-HA antibody and an alkaline phosphatase conjugated goat anti-mouse antibody (Biolegend, United States). The DNA inserts in pMECS of colonies scoring positive in ELISA were sequenced and analysed. The pMECS vector containing unique antigen-specific Nbs were then transformed in the non-suppressor *E. coli* WK6 strain. Nbs were subsequently produced and purified by immobilised metal ion affinity chromatography (IMAC) and size-exclusion chromatography (SEC) under standard conditions (Conrath et al., 2001). Each purified Nb was tested by indirect ELISA in 96-wells ELISA plates coated with 100 μl of 5 μg/ml of TES antigen, diluted in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, 0.1% NaN_3, pH 9.2) and incubated overnight at room temperature. Then, the plates were blocked for 2 h with 200 μl of 2% non-fat dried milk in PBS. In the next step, 100 μl of each Nb (5 μg/ml in PBS) were added and incubated for 1 h at room temperature. In the control wells, only PBS was added. Detection was carried out with 100 μl of a mouse anti-HA or anti-His (AbD Serotec, United Kingdom) monoclonal antibody, followed by a 2000-fold diluted goat anti-mouse antibody conjugated to alkaline phosphatase (Biolegend), incubated for 1 h each time. After each step, plates were washed five times with PBS containing 0.05% Tween-20 (PBST20). The ELISAs were developed with 4-phenyl phosphate disodium (Sigma-Aldrich) (2 mg/ml) and read at 405 nm after 45 min of reaction.

2.4. Plasmids and constructs

Nbs 1TCE39, 1TCE52 and 2TCE49 in a pMECS vector were subcloned into the expression vector pHEN6c with a His_6-tag downstream of the Nb (Conrath et al., 2001) or in pBAD-17 containing a C-terminal AVI-tag (Beckett et al., 2008) preceded by the structural upper hinge of human IgA (amino acid sequence: PSTPPTPSPS). After PCR amplification of the *Nb* genes, restriction enzyme digestions of the amplicon with *Pst*I and *Eco*91I (ThermoFisher Scientific, United States) were ligated in the appropriate vectors and transformed into electro competent WK6 cells. Bivalent Nb constructs were generated by PCR using primers SS097 FW (5′-A TTC CTG CAG CTG CAC CTG ACT ACC GCC GCC TCC GGA ACC TCC ACC GCC GGA TCC GCC TCC GCC TGA GGA GAC CGT GAC CTG GGT BCC-3′) and SS106 RW (5′-TAG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTT CAG GAG TCY GGR GGA G-3′). Plasmid pHEN6c with a Nb insert was used as a template. In this way, the PCR product encodes the Nb with a $(G_4S)_3$ linker, which is inserted via *Nco*I and *Pst*I restriction enzyme sites upstream of the original VHH in the pHEN6c or pBAD-17 vectors.

2.5. In vivo biotinylation of Nb constructs

Monovalent Nbs 1TCE39, 1TCE52 and 2TCE49 and the bivalent Nb 2TCE49 in pBAD-17 constructs were co-transformed in *E. coli* WK6 cells with the pACYC184 plasmid (Avidity) containing $pBirA_{Cm}$, a wild-type biotin-protein ligase. Transformants were plated on Luria Bertani (LB)-Agar supplemented with ampicillin and chloramphenicol, and incubated overnight at 37 °C. One single colony was chosen to inoculate 15 ml of LB supplemented with 100 μg/ml of ampicillin and 75 μg/ml of chloramphenicol, and cultured overnight at 37 °C. Next, 1 ml of starter culture was used to inoculate 330 ml of Terrific Broth media supplemented with 100 μg/ml of ampicillin (Sigma-Aldrich) and 75 μg/ml of chloramphenicol (Sigma-Aldrich), and grown at 37 °C while shaking. Expression of Nb was induced overnight at 28 °C with 1 mM IPTG. Cells were harvested by centrifugation at 11,300*g* and pellets from 1 l of bacterial culture were resuspended in 17 ml of lysis buffer (50 mM Tris–HCl, 500 mM NaCl) supplemented with 17 μl of protease inhibitor mix [1 mg of Leupeptin (Sigma-Aldrich), 30 mg of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma-Aldrich) and 100 mM EDTA (Merck, United States) per ml of Milli-Q water)]. Cells were subjected to three freeze–thaw cycles to disrupt the membranes and allowing the cytoplasmic enzyme BirA to reach the Nbs exported to the periplasm. Subsequently, 17 μl of DNase I (Sigma-Aldrich) (1 mg/ml) were added and incubated for 1 h at 37 °C to cut DNA and reduce viscosity. D-Biotin (Acros Organics, United States) was added to a final concentration of 50 μM and the mixture was incubated 1 h at 37 °C. After centrifugation for 30 min at 39,000*g*, the supernatant was collected and dialyzed overnight at 4 °C against Milli-Q water. Purification of biotinylated Nbs was performed by affinity chromatography on a Streptavidin-Mutein Matrix (Roche, Switzerland) according to the instructions of the manufacturer. Eluted fractions were concentrated in a Vivaspin concentrator (5000 MWCO, Sartorius, Germany) and purified by size exclusion chromatography (SEC) on Äkta Explorer (GE Healthcare Life Sciences, United States) using a Superdex 75 HR 10/30 column (GE Healthcare Life Sciences) in PBS.

2.6. Immunocapturing of TES with Nb-loaded paramagnetic beads

Specific components of TES antigens were captured by Nbs and loaded via their His_6 tag on paramagnetic beads (QuickPick™ IMAC metal affinity kit; Bio-Nobile, Finland). Briefly, 25 μg of Nb 1TCE39, 1TCE52 or 2TCE49 expressed from pHEN6c were mixed with 25 μg of TES antigen in 200 μl of PBS and incubated for 1 h on a rotator. A magnetic rod was used to collect the protein complex captured on Ni^{2+}-loaded paramagnetic beads. After two washing steps, the protein complex was eluted from the beads in 20 μl of imidazole (0.5 M) (Sigma-Aldrich). Eluates were analysed by gel electrophoresis and silver stained (Schägger, 2006).

2.7. Deglycosylation of TES antigen

TES antigen was deglycosylated by PNGase F (New England Biolabs, United States) under non-denaturing conditions. Briefly, 20 μg of TES antigen were digested with 5 μl of PNGase F at 37 °C for 24 h in a volume of 20 μl. Fetuin (20 μg) (New England Biolabs) was deglycosylated under the same conditions as a control. Mobility shifts of TES and *N*-deglycosylated TES (dTES) proteins under reducing and non-reducing conditions were monitored in SDS–PAGE. The binding properties of the three Nbs for TES and dTES were assessed by western blot (WB). Band analysis and estimation of molecular weights were performed with Image Lab 5.2.1.

2.8. Western blot analysis

Gels were run in duplicate for WB and silver staining according to available protocols (Schägger, 2006). TES proteins (8 μg per lane) in the presence or absence of reducing agent (NuPAGE® Sample Reducing Agent 10X, ThermoFisher Scientific) were separated by gel electrophoresis on 10% Criterion™XT Bis-Tris gels (Biorad, United States). Protein transfer onto a nitrocellulose membrane (Amersham Protran 0.45 μm NC, GE Healthcare) was carried out in a Mini Trans Blot® Cell (Biorad) filled with transfer buffer (25 mM Trizma base, 0.2 M Glycine and 10% methanol). After protein transfer, the membrane was blocked for 2 h in 2% casein (Sigma-Aldrich) in PBS. Then, the membrane was embedded for 1 h in 10 ml of solution containing biotinylated Nb 1TCE39, 1TCE52 or 2TCE49 (5 μg/ml). After washing, the membrane was submerged in 10 ml of horseradish peroxidase (HRP)-streptavidin (Biolegend) and incubated for 1 h. The membrane was rinsed, and the signal was developed with 4-chloro-1-naphthol (Sigma-Aldrich).

2.9. Sandwich ELISA

Three sandwich ELISA formats were designed. The first one was used for screening optimal pairs of Nbs to capture and to detect TES antigens. In the second ELISA, we compared monovalent or bivalent Nb 2TCE49 as a capturing agent. For the third ELISA, we used biotinylated, bivalent Nb 2TCE49, captured on streptavidin-coated plates, as a TES antigen capturing agent. In all ELISA experiments, 96-well microtiter plates (Nunc, Immulon ThermoFisher Scientific) were coated overnight at 4 °C. Plates were incubated with 200 μl of blocking solution for 2 h. In the remaining steps, we used 100 μl per well and incubated for 1 h at room temperature. After each step, the plates were washed five times with PBST20. Plates were read with a Versamax™ Microplate reader equipped with 405 and 450 nm filters (Molecular Devices, United States).

For the first ELISA, plates were coated with Nb 1TCE39, 1TCE52 or 2TCE49 expressed solely with a His_6-tag (pHEN6c), at 5 μg/ml. Blocking solution contained 2% non-fat dried milk in PBS (pH 7.4). In the next step, TES antigens (5 μg/ml) in PBST20 were added to each well. Alternatively, to test cross-reactivity of the assay, TES antigen was substituted by the same amount of protein from a panel of other antigens, namely: L_3 antigens from *A. lumbricoides* and *A. suum*; somatic (adult) and larval excretory–secretory antigens from *Anisakis simplex* and *Pseudoterranova decipiens* (kindly provided by Prof. Pierre Dorny from the Institute of Tropical Medicine, Antwerp, Belgium); and somatic (adult) and ES antigens (sera of rats 42 days p.i. with the parasite) of *Angiostrongylus cantonensis* (kindly provided by Dr. Beatrice Nickel from the Swiss Institute of Tropical Medicine, Basel, Switzerland). Next, wells were filled with 100 μl of Nb 1TCE39, 1TCE52 or 2TCE49 expressed from a pMECS vector (5 μg/ml). Nbs expressed from the pMECS vector and containing a HA and a His_6-tag enable detection of the captured TES antigens, with a 2000-fold diluted monoclonal anti-HA (Biolegend). Detection was carried out with 2000-fold diluted goat anti-mouse antibody conjugated to alkaline phosphatase (Biolegend). The plates were developed with (2 mg/ml) 4-phenyl phosphate disodium (Sigma-Aldrich) and read at 405 nm after 45 min.

In the second ELISA, plates were coated with either monovalent or bivalent 2TCE49 (5 μg/ml, expressed from a pHEN6c vector), and blocked with 2% casein in PBS (pH 7.4). Then, TES antigen was added in two-fold serial dilutions from 10 μg/ml onwards in PBST20 or in AB male human serum (Sigma-Aldrich). After washing, we added Nb 1TCE39 (5 μg/ml), as expressed from a pBAD-17 vector, followed by of 2000-fold diluted HRP-Streptavidin (Biolegend). Plates were developed with tetramethylbenzidine (TMB) substrate reagent (BD Biosciences, United States). The reaction was stopped with 0.5 M sulphuric acid and read at 450 nm after 18 min of reaction.

In the last ELISA format, plates were coated with 10 μg/ml of Streptavidin (ThermoFisher Scientific). Then, biotinylated bivalent Nb 2TCE49 (20 μg/ml), as expressed from pBAD-17, was added to the wells. Blocking was performed with Pierce™ Protein-Free (PBS) Blocking Buffer (ThermoFisher Scientific). Next, we added to the wells two-fold serial dilutions of TES antigen (from 1 μg/ml onwards) in PBST20 or in AB male human serum (Sigma-Aldrich). Conjugate, developing, stop and reading steps were performed as described in the previous ELISA format.

Optimisation of ELISA with bivalent and bivalent-biotinylated constructs was performed by chessboard titration testing various parameters including a range of concentrations of the bivalent capturing Nbs, the detection Nb conjugate and developing time before stopping the signal generation in plates.

2.10. Experimental infection of mice with T. canis eggs

All procedures were carried out based on Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament, 2010). Adult parasites were obtained from naturally infected dogs. Female worms were dissected and the eggs were kept at room temperature in H_2SO_4 solution (0.2 M), with constant artificial light and with brief daily manual agitation until mobile larvae were observed inside the eggs. The embryonated eggs were counted with a Neubauer chamber and calculated as the average of five aliquots of 50 μl of the egg suspension. Eight weeks old male BALB/c mice weighting between 18 and 20 g were supplied by the National Centre for the Production of Laboratory Animals (CENPALAB, Cuba). Five hundred embryonated eggs were administered orally to seven mice of the first group. Seven mice from the other group received only sterile 0.9% saline solution as controls. Before administration of embryonated eggs, each animal was weighted and 60 μl of blood were extracted from retro-orbital sinus into a non-heparinised capillary tube. The weighting and bleeding procedures were repeated on days 3, 6, 9, 12, 15, 22, 30 and 45p.i. Blood was centrifuged at 500 *g* and serum was stored at −20 °C. To detect the TES antigens, we diluted the sera of mice 50-fold in PBST20 and used the sandwich ELISA with bivalent biotinylated Nb 2TCE49 to capture TES antigens and 1TCE39HRP for TES antigens detection. The protocol was approved by the Institute of Tropical Medicine "Pedro Kourí", (Havana, Cuba) Ethics Committee (CEI-IPK 41-18).

2.11. Detection of anti-TES antibodies by indirect ELISA

For detection of specific antibodies against TES antigens, each well of a microtiter plate (Nunc-Maxisorp, ThermoFisher Scientific) was sensitised with TES antigens (5 μg/ml) in coating buffer and blocked with skimmed milk (2.5%) in PBST20. Then, 100 μl of the serum sample (1/500 in PBST20) were added to each well. After incubation for 1 h at 37 °C and three washes with PBST20, 100 μl

of commercial conjugate anti mouse IgG-horseradish peroxidase, diluted 1/2000 (Dako, Germany) were added. The plate was incubated again for 1 h at 37 °C and followed by three washes with PBST20. Thereafter, 100 µl of ortho-phenylenediamine (0.05%) (Sigma-Aldrich) and hydrogen peroxide (0.015%) in citrate buffer (pH 5.0) were dispensed into each well. The plate was incubated in the dark for 20 min at room temperature. The reaction was stopped with 50 µl of 2 N sulfuric acid and the absorbance was read at 490 nm in an ELISA reader (Dynatech, Slovakya).

2.12. Data analysis

Standard curves were constructed with linear and 5PL models in RStudio Version 1.0.136 – © 2009–2016 using the O.D. after blank subtraction. Values of 'blank' controls were calculated as the average of at least six different 'blank' wells containing only serum or PBST20 and the detection reagents, but no antigens. The limit of detection (LOD) was calculated as 3 S.D. plus the average of the blanks. All reactions were performed in triplicate.

3. Results

3.1. Construction of an immune Nb library and selection of TES-specific Nbs

An immune Nb library comprising $3x10^8$ individual transformants was constructed from blood of an alpaca (*Vicugna pacos*) immunised with TES antigens. A PCR on 30 individual colonies from this library indicated that 84% contained the vector with the right insert size (~360 bp) for a Nb. Two rounds of panning enriched the phage particles with TES antigen-specific Nbs. Ninety-five individual colonies from each round of panning were screened for the presence of Nbs specifically recognising TES antigens. From these colonies, 17 and 51 clones from the first and the second round of panning, respectively, were positive in ELISA. The cloned DNA inserts of 47 of these colonies were sequenced, resulting in 20 different Nb sequences. Based on their complementarity determining region-3 (CDR3), these 20 Nbs were grouped into 10 different families (Supplementary Fig. S1). The Nbs within one family share the same CDR3 sequence but might differ from each other by several point mutations, mainly spread in CDR1 and CDR2. We have demonstrated previously that Nbs within one family will recognise the same epitope, since the CDR3 of Nbs is the energetic hotspot for epitope association (De Genst et al., 2005). However, the equilibrium affinity constant and kinetic binding rate constants among the Nb members within a family might differ as a result of minor sequence differences within their CDR1 and CDR2 loops.

After expressing the Nb proteins with a C-terminal HA-tag and His_6-tag, each of the 20 Nbs was purified and tested by indirect ELISA on TES antigens (Supplementary Fig. S1). A clear signal was obtained for Nbs 1TCE39 and 2TCE18 (family 8); 1TCE49 and 1TCE52 (family 5); and 2TCE49 (family 10). Although the signal with 2TCE49 is low, a detailed screening of other TES antigens lots showed that a higher signal was obtained in the majority of the lots (data not shown). Despite this variability in signal among different lots, Nb 2TCE49 was kept for further experiments, together with Nbs 1TCE39 and1TCE52 as they represent different families of Nbs.

3.2. Immunocapturing of TES

Purified Nb 1TCE39 and 1TCE52 captured an identical set of proteins, while the proteins captured by Nb 2TCE49 revealed a similar band pattern with the exception of the band of ~27 kDa (see asterisk in Fig. 1). Additionally, in contrast to the two former

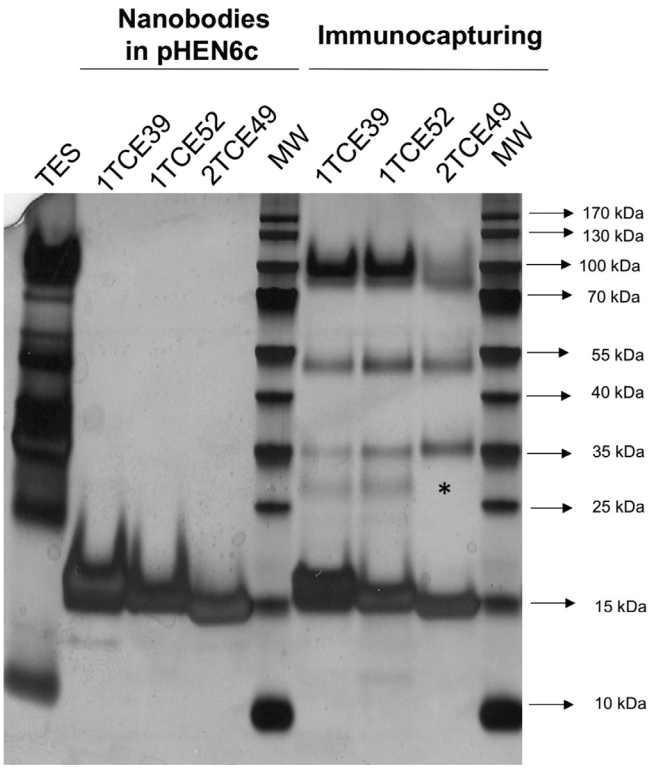

Fig. 1. SDS–PAGE under reducing conditions of nanobodies (Nbs) expressed from a pHEN6c plasmid and stained with silver. Also shown are samples showing *Toxocara canis* excretory/secretory antigens (TES) proteins immunocaptured with paramagnetic beads functionalised with Nbs 1TCE39, 1TCE52 or 2TCE49 expressed in pHEN6c. Asterisk indicates a band of ~27 kDa that is captured by Nb 1TCE39 and 1TCE52 but not by 2TCE49.

Nbs, Nb 2TCE49 captured relatively less protein of ~100 kDa but more of the ~35 kDa protein. In any case, multiple TES proteins that were visible in the starting material were not captured in this assay. Conversely, the proteins that were captured might have been immunocaptured directly or indirectly by the Nbs. Alternatively, they could be bound immediately on the Ni^{2+} beads when histidine-rich stretches are exposed on their surface. In theory, this latter type of captured proteins would still associate with the beads when the Nb is omitted from the assay or using a non-TES antigen targeting Nb. However, we preferred to switch to a WB analysis (see Section 3.3) to obtain a clearer picture.

3.3. Western blot analysis to identify the TES target proteins

Native TES and dTES antigens were used to further investigate the epitopes of TES antigens recognised by our selected Nbs. The electrophoretic mobility of TES and dTES antigens is similar in the high molecular weight bands (>40 kDa). However, for TES proteins of lower MW (<40 kDa), the *N*-deglycosylation leads to the appearance of new bands (e.g. 31 kDa, asterisk in Fig. 2A), although the picture is complicated by the co-migration of PNGase in the same region.

WB experiments using biotinylated Nbs 1TCE39, 1TCE52 and 2TCE49 as probes served to reveal their target specificity on TES and dTES antigens separated by SDS–PAGE under reducing and non-reducing conditions (Fig. 2B). For all three Nbs, bands were only apparent in non-reduced TES antigen samples. Each Nb interacted with a unique pattern of non-reduced TES antigens, indicating that the reduction of disulphide bonds in the SXC region is detrimental for epitope recognition (Fig. 2B). Nevertheless, each Nb interacted with a unique pattern of non-reduced TES proteins,

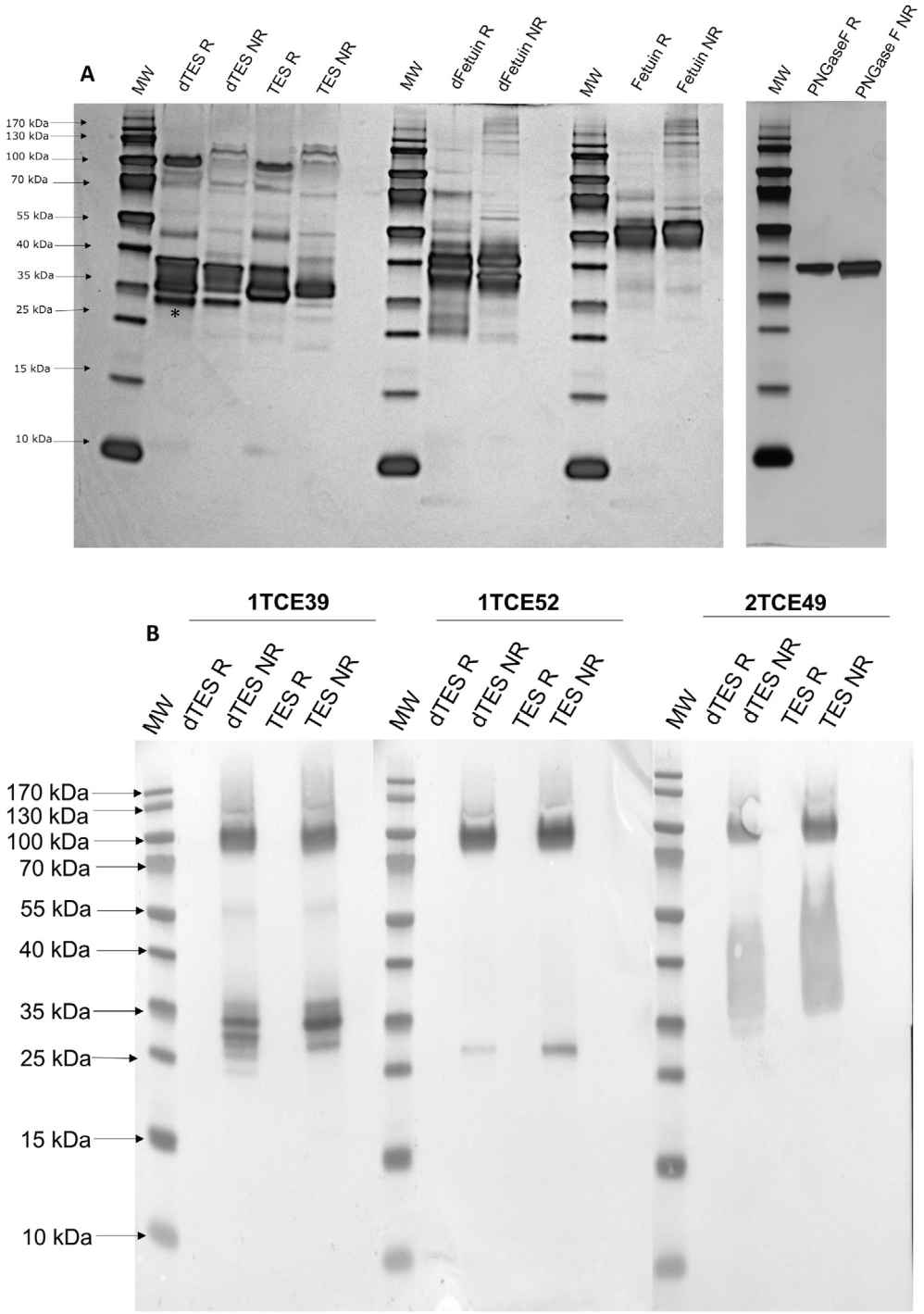

Fig. 2. *Toxocara canis* excretory/secretory (TES) antigen and *N*-deglycosylated TES (dTES) separated by SDS–PAGE under reducing (R) or non-reducing (NR) conditions and visualised after silver staining (A). The asterisk indicates a prominent band in dTES R that is absent in TES R. The next panel shows the control protein, fetuin, treated with PNGase F, causing a bandshift from ~52 to two bands of ~40 and 38 kDa. In the last panel, PNGase F is shown applied on gel, under reducing and non-reducing conditions. (B) Western blot (WB) of TES and dTES, separated under R and NR conditions using biotinylated nanobodies (Nbs) 1TCE39, 1TCE52 and 2TCE49 as probes. Bands are observed only under NR conditions in TES and dTES.

which remained unchanged after deglycosylation by PNGase F. All three Nbs recognised two bands with MW of ~120 and ~100 kDa in TES and dTES antigens. In addition, Nb 1TCE39 recognises a band of ~57 kDa (weakly) and a complex of six bands of low molecular weights ~34, ~32, ~28, ~27, ~25 and ~22 kDa in dTES antigens. Apart from the bands of ~120 and ~100 kDa, Nb 1TCE52 detects the ~27 kDa single band. The WB with Nb 2TCE49 exhibits a broad smear that extends from ~55 to ~25 kDa in TES and dTES antigens (Fig. 2B).

3.4. Identification of best pair of Nbs for sandwich ELISA

From all possible Nb pairs, the combination of 2TCE49-pHEN6c with 1TCE39-pMECS yielded the highest signal for TES antigens, followed by 1TCE39-pHEN6c with 2TCE49-pMECS and the 1TCE52-pHEN6c with 1TCE39-pMECS or the 2TCE49-pHEN6c with 1TCE52-pMECS combinations (Fig. 3). The signal for the two latter pairs was approximately half of the former Nb combinations. Importantly, the recognition of the TES antigens within our

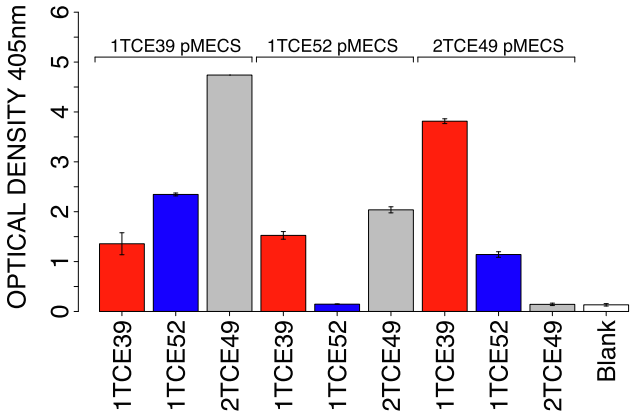

Fig. 3. Identification of best part of nanobodies (Nbs) for sandwich ELISA. Nb-based sandwich ELISA using Nbs 1TCE39, 1TCE52 and 2TCE49 in all possible combinations for *Toxocara canis* excretory/secretory antigen (TES) (5 μg/ml) detection. Capturing Nb (names on X-axis) expressed from pHEN6c were immobilised in wells by passive coating. Detection of captured TES was performed by the Nb expressed from a pMECS vector (as indicated). The sandwich reaction was revealed via mouse anti-haemagglutinin (HA) antibody and anti-mouse IgG antibody coupled to alkaline phosphatase.

sandwich ELISA was specific as no cross-reactivity for antigens from *A. lumbricoides*, *A. suum*, *P. decipiens or A. simplex* was observed in any combination (Supplementary Fig. S2). Although no absorbance was observed with somatic *A. cantonensis* antigens in our ELISA, a weak signal, particularly with Nb 1TCE52 as the capturing agent, was noticed with the ES antigens of *A. cantonensis* in infected rats, (Supplementary Fig. S2H).

In conclusion, it appeared that Nbs 2TCE49 and 1TCE39 form the best pair of Nbs to detect TES antigens in solution, preferably as the capturing and the detecting Nb, respectively. We further hypothesised that an increase in the valency of the Nbs could improve the sensitivity of the ELISA. This was demonstrated in a second sandwich ELISA whereby His_6-tagged 2TCE49 was passively coated, either as a monovalent or a bivalent construct. Biotinylated Nb 1TCE39 in combination with HRP-conjugated streptavidin was subsequently used to detect TES antigens. Dilution of TES antigens in PBST20 gave a lower background and a lower LOD compared with TES antigens diluted in serum. For TES antigens spiked in serum, which closely mimics clinical samples from patients, the LOD was 156 ng/ml and 78 ng/ml for the capturing monovalent and bivalent constructs, respectively. Thus, the passive-coated bivalent construct definitely exhibits an increased capacity to capture TES antigen from serum compared with the monovalent Nb (Table 1).

For the third sandwich ELISA format, we coated ELISA plates with streptavidin followed by biotinylated, bivalent Nb 2TCE49, so that both Nb entities within the bivalent construct would be in solution and available for antigen capturing. Detection of the captured TES antigens was performed with Nb 1TCE39 chemically coupled to HRP. The LOD of TES antigen in PBST20 or in serum was 0.32 and 1.6 ng/ml, respectively (Table 1). However, in comparison with the previous set-up, this format showed a 1.5-fold higher background and a lower O.D. signal at high concentrations of antigens (Table 1). The graphical representation of the results of the three sandwich ELISAs in PBST20 or serum is presented in Supplementary Fig. S3.

Considering that the concentration of TES antigens in clinical samples can theoretically be in the order of pg/ml, we further optimised the experimental parameters of our ELISA by chessboard titration of various components. This assay resulted in defining the optimal conditions as: 100 μl of streptavidin at 0.625 μg/ml in coating buffer; 100 μl of biotinylated bivalent 2TCE49 (0.78 μg/ml) in PBST20; 100 μl of Nb 1TCE39-HRP (0.16 μg/ml) in PBST20; and finally incubated the HRP substrate for 18 min before stopping the reaction with sulphuric acid. With these optimised conditions, we demonstrated for a two-fold serial dilution of TES antigens starting at 2.6 ng/ml that an LOD of 0.163 ng/ml or 0.650 ng/ml was obtained in PBST20 or serum, respectively (Fig. 4A). Cross-reactivity to *A. cantonensis* ES antigens was ruled out in this last format (Supplementary Fig. S4).

Finally, we assessed the variability among different, independent TES antigen lots with our optimised sandwich ELISA. The LOD of TES antigens from lots 14, 15 and 16 diluted in serum was 1.6 ng/ml, whereas TES antigen lot 19 showed an LOD of 8 ng/ml (Fig. 4B and C). The five-fold higher LOD for lot 19 compared with the other lots illustrates the uncertainty in measuring the exact concentration of TES proteins (Supplementary Table S1) and the variation in TES antigen composition across independent lots. The SDS–PAGE band patterns from the different lots of TES antigens, together with those from other antigens used for testing cross-reactivity, are shown in Supplementary Fig. S5. Lot 19 clearly lacks a band of ~26 kDa compared with the other lots (in particular, the higher MW bands are underrepresented), which obviously might result in a lower ELISA signal.

3.5. Detection of TES and anti-TES antigen antibodies in mice experimentally infected with T. canis eggs

No welfare problems associated with the inoculation procedures were observed in the animals. TES-specific antibodies were detectable 10 days p.i. and reached a plateau at 30 days p.i., which lasted until the end of the experiment (Fig. 5A). In contrast, the circulating antigen level increased immediately from the day of infection to reach a maximum between days 9 to 15. After a minor decline in signal, probably reflecting the clearance of *Toxocara* antigens by endogenous antibodies or its sequestration by the immune system, a steady state TES antigens titre of ~25 ng/ml was observed (Fig. 5B).

Table 1
Summary of sandwich ELISA in three different formats for detection of *Toxocara canis* excretory/secretory (TES) antigens. The O.D. cut-off value was calculated as 3 S.D. plus the O.D. value of the blank. Bivalent constructs coated passively or by streptavidin capturing showed an increased capacity to bind TES from solution.

Sandwich ELISA format		Diluent	Blank[a]	S.D. Blank	O.D. Cut-off	LOD (ng/ml)
Capturing Nb	Detection Nb					
2TCE49	1TCE39 pBAD-17	PBST20	0.060	0.011	0.093	19.5
2TCE49	1TCE39 pBAD-17	Serum	0.069	0.013	0.108	156
Bivalent 2TCE49	1TCE39 pBAD-17	PBST20	0.070	0.013	0.108	4.8
Bivalent 2TCE49	1TCE39 pBAD-17	Serum	0.082	0.004	0.095	78
Streptavidin + Bivalent 2TCE49 pBAD-17	1TCE39HRP	PBST20	0.119	0.006	0.137	0.320
Streptavidin + Bivalent 2TCE49 pBAD-17	1TCE39HRP	Serum	0.129	0.003	0.139	1.6
After optimisation						
Streptavidin + Bivalent 2TCE49 pBAD-17	1TCE39-HRP	PBST20	0.090	0.006	0.109	0.163
Streptavidin + Bivalent 2TCE49 pBAD-17	1TCE39-HRP	Serum	0.079	0.004	0.091	0.650

[a] Average of six blanks where TES was omitted. LOD, limit of detection.

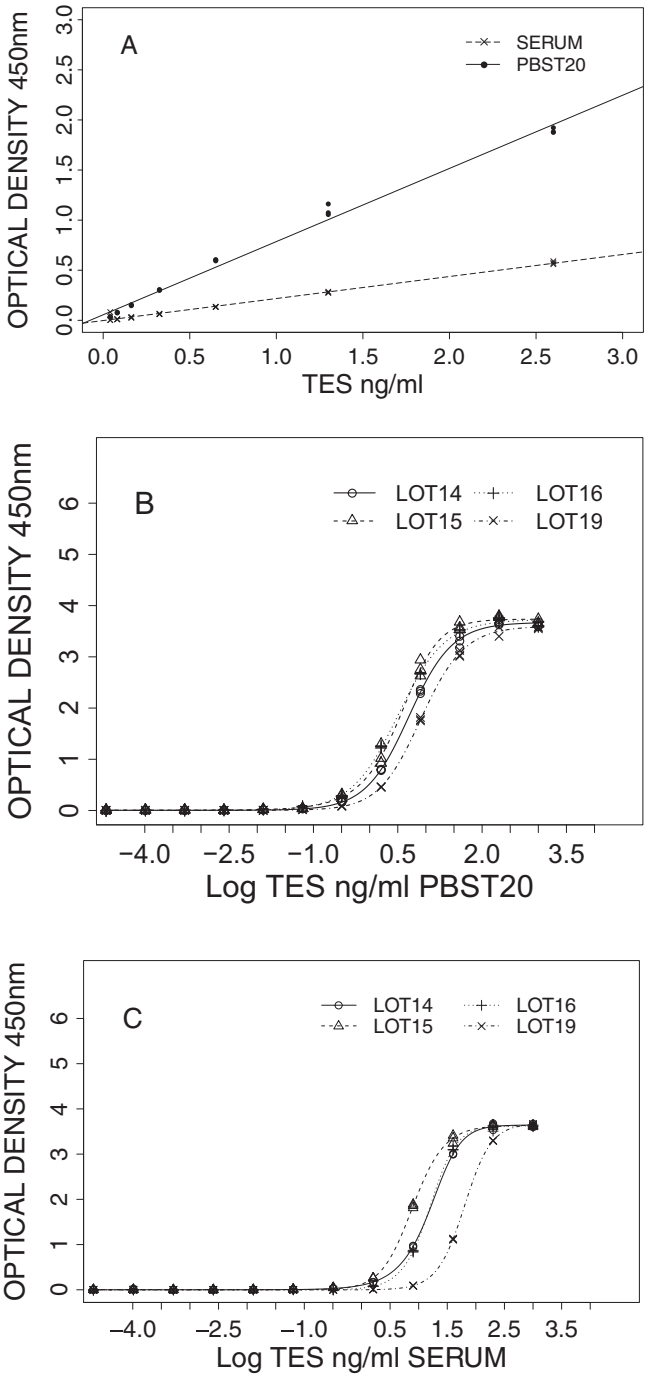

Fig. 4. Optimised, nanobody-based sandwich ELISA for *Toxocara canis* excretory/secretory (TES) antigen used in two-fold serial dilutions in PBST20 (R^2 = 0.99133) or serum (R^2 = 0.9979) using bivalent biotinylated Nb 2TCE49 (expressed from a pBAD-17 plasmid) and immobilised on streptavidin to capture TES antigens. The Nb 1TCE39 conjugated to horse radish peroxidase was used as an antigen detection agent. The limit of detection in serum and PBST20 is 0.650 ng/ml and 0.163 ng/ml, respectively (A). Comparison of sandwich ELISA with bivalent 2TCE49 pBAD-17 and 1TCE39-HRP in PBST20 (B) and serum (C) using four different lots of TES. The limit of detection of the assay for TES in PBST20 0.05% was 0.32 ng/ml in the four lots. In serum, the limit of detection was 1.6 ng/ml for lots 14, 15, 16 and 8 ng/ml for lot 19.

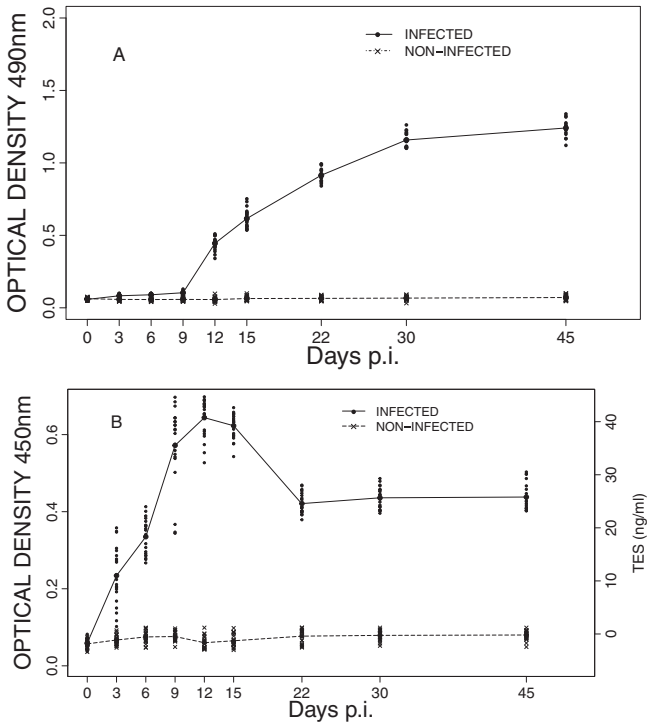

Fig. 5. Experimental infection of mice with *Toxocara canis* eggs. (A) Indirect ELISA for the detection of anti-*Toxocara canis* excretory/secretory antigen (TES) antibodies in sera from a group of seven mice infected with embryonated eggs of *T. canis* and analysed at regular time intervals. Black circles represent the average O.D. reading of seven infected animals (solid line) and non-infected (dashed line) animals. (B) TES detection in serum by nanobody (Nb) sandwich ELISA and the estimation of circulating TES antigen in ng/ml.

4. Discussion

TES antigen is a complex mixture of multiple proteins of various molecular weights. These polypeptide chains contain regions that are highly glycosylated. The N and C terminal regions of a roundworm's excretory and secretory proteins comprise 36 amino acid long sequences with six conserved cysteines in tandem repeats, referred to as six cysteine (SXC) domains (Loukas and Prociv, 2001). As described for *Caenorhabditis elegans* and *T. canis* (Blaxter, 1998), these SXC domains were initially identified as flanking regions in *muc* genes constituting TES-120 (Gems and Maizels, 1996), TES-26 (Gems et al., 1995), TES-32 (Loukas et al., 1999) and TES-70 (Loukas et al., 2000b). Although the function of SXC domains remains largely unknown, it has been proposed that they play a role in protein–protein interactions and in the modulation of the immune response exerted by TES antigens (Loukas et al., 2000a; Loukas and Prociv, 2001).

The complex, heterogeneous, heavily glycosylated nature of TES antigen, in conjunction with its low levels in blood, seriously complicates its analysis and quantification. To tackle these challenges, we made use of Nbs as analytical and functional tools for TES antigen detection. We identified 20 Nbs, categorised in 10 families. Nbs of three different families gave high and specific signals for TES antigens in ELISA. The nature of the targeted epitope for these Nbs was investigated and a Nb-based sandwich ELISA to quantify TES antigens was developed.

Our three selected Nbs (2TCE49, 1TCE39 and 1TCE52) recognise shared (or common) epitopes, present on multiple polypeptide chains of TES antigens, as was noticed in the immunocapturing and immunoblot assays (Figs. 1 and 2). A similar cross-reactivity for multiple TES antigen polypeptides was also observed previously with monoclonal antibodies (Maizels et al., 1987). It is clear that the epitopes of our three Nbs are conserved, extensively distributed among several TES components and highly immunogenic within the immunised alpaca. Remarkably, in WB, our three Nbs detected TES proteins only when separated by SDS–PAGE under non-reducing conditions. This antigenicity is in sharp contrast with

that of monoclonal antibodies Tcn-2 and Tcn-8 (Page et al., 1992a), which detect TES antigens under reducing conditions in WB. The failure to recognise TES antigens under reducing conditions with our Nbs highlights the critical involvement of disulphide bonded SXC domains in the detection of TES antigens. It is noteworthy that although TES-70 also contains SXC domains, it was not recognised by any of our Nbs, neither in immunoblot nor in immunocapture experiments. This indicates that TES-70 probably possesses distinctive features that differentiate it from the rest of the proteins, conserving nevertheless the same global structure as TES-120, TES-55, TES-32 and TES-26.

Treatment of TES antigen with PNGase F demonstrates its susceptibility to *N*-linked deglycosylation. This is shown by the band shifts and weakening of TES antigen after PNGase F treatment, resolved in SDS–PAGE under reducing and non-reducing conditions. Moreover, we have proved that such changes affect not only previously described *N*-glycosylation sites at ~32 kDa (Zhan et al., 2015), but also mucins of high molecular weight that are known to be very rich in *O*-Glycosylation (Loukas et al., 2000a). Treatment of TES antigen with PNGase F did not affect the binding of the three Nbs to dTES antigen, which indicates that Nbs recognise conformational epitopes either in the protein backbone or in *O*-Glycosylation sites, resistant to PNGase F enzymatic digestion.

Additionally, each characterised Nb exhibits distinct features. Our immunocapturing experiments demonstrated that Nbs 1TCE39 and 1TCE52 recognise an identical set of bands of nominally ~100, ~85, ~48, ~35 and ~27 kDa, while Nb 2TCE49 binds to the same TES antigen components with the notable exception of TES-27. This study also clearly demonstrated that the epitope of Nb 2TCE49 on TES antigens differs from that of the other two Nbs. It is also likely that the interactions of Nb 1TCE39 and 1TCE52 with TES proteins are different. This conclusion is supported from the widely different signals obtained in the sandwich ELISA when these Nbs are used as capturing and detecting Nbs (Fig. 3).

Serology has so far been the most important laboratory tool to diagnose HT. Its use is hampered by cross-reactivity with other helminths and its inability to differentiate between active and past infections. The detection of TES antigens instead of TES antibodies in body fluids appears to be a better approach that overcomes these obstacles. Here, we introduce Nbs for this task. Since their discovery, a considerable body of research has been dedicated to introduce these single domain antibody fragments into diagnostic tests. Some examples include the detection of a specific *Trypanosoma congolense* glycosomal fructose-1,6 biphosphate aldolase in a sandwich ELISA format (Odongo et al., 2016) or a streptavidin–biotin-based directional sandwich ELISA to detect influenza H5N1 (Zhu et al., 2014).

The design of our Nb-based TES-ELISA is relying on two Nbs that recognise different epitopes within the TES antigen complex. All the reported systems developed for the diagnosis of HT to date have been based either on one single monoclonal antibody recognising a repeated epitope or on polyclonal antibodies (Robertson et al., 1988a; Ishiyamna et al., 2009; Rodríguez-Caballero et al., 2015). Hence, double recognition achieved by two different monoclonal Nbs recognising two distinct epitopes should provide extra specificity and reproducibility to the assay compared with other systems. This was confirmed by the lack of cross-reactivity with other helminths in all the possible combinations of our nanobody-based sandwich ELISA.

Our experiments with infected mice indicate that Nbs are able to detect TES antigens in vivo in sera of mice experimentally infected with *T. canis* eggs. The TES antigens are detected as from 3 to 6 days p.i., reaching a peak around day 12, the timepoint when the humoral immune response starts to be observed. Thereafter, TES antigen detection slightly decreases, probably as a result of immunocomplex formation, and remains stable until day 45. A similar trend has been described previously during infection with 50 or 100 *T. canis* eggs and larvae (Rodríguez-Caballero et al., 2017). The main difference between this report and our experiments is that our mouse serum samples were diluted 50-fold in PBST20 and our Nb-based antigen detection approach does not require dissociation of the immunocomplexes. This highlights the powerful analytical sensitivity of the Nb-based test.

When using a biotinylated bivalent Nb 2TCE49 to capture TES antigen in combination with Nb 1TCE39-HRP for detection, a LOD of 0.163 and 0.650 ng/ml was reached for TES antigen diluted in PBST20 and serum, respectively. Previous studies reported variable LOD values for TES antigen of 20 ng/ml (Robertson et al., 1988b), 78 ng/ml (Luo et al., 1999) and 4 ng/ml (Yokoi et al., 2002). However, it is difficult to compare the LOD of different assays because the methods used to quantify TES antigens might vary among laboratories. The classical method for TES antigen quantification used by Savigny (1975) is UV 280 spectroscopy. Assuming that in all these assays TES antigen was measured by UV 280 spectroscopy, our test has an analytical performance that is at least six times better. It is noteworthy that measuring TES antigens by colorimetric methods such as bicinchoninic acid (BCA) or Bradford assays, yields TES antigen concentrations that can be significantly different (Supplementary Table S1).

The LOD in our two formats of ELISA (i.e. using the passively coated bivalent or the streptavidin captured biotinylated bivalent Nbs) yielded a 50-fold higher sensitivity in detection of TES antigens in serum for the streptavidin-mediated attachment (Supplementary Fig. S3). An increased performance of bivalent Nbs over monovalent constructs to neutralise a given target has been shown previously (Hmila et al., 2010; Unger et al., 2015; Pinto Torres et al., 2018). Similarly, pentavalent and even decavalent constructs have been successfully developed as a way to increase avidity of low affinity binders (Zhang et al., 2004; Stone et al., 2007). Apart from the increase in avidity, the use of biotinylated bivalent Nbs on streptavidin-coated plates improves the capturing capacity of antigens (Supplementary Fig. S3). Indeed, according to this immobilisation strategy, both Nb monomers within the bivalent construct can participate in antigen capturing without steric hindrance from the plastic surface. This factor, together with the uniform and directional immobilisation provided by the streptavidin–biotin interaction, confer extra sensitivity to our assay.

The characterisation of different Nbs against TES antigens represents an important achievement in the study of *T. canis*. It opens the possibility for future research to investigate the detailed structure–function relationships of the various TES antigen components. In addition, the Nb-based sandwich ELISA provides a promising tool for sensitive and specific diagnosis of HT. Future studies should focus on conducting a dedicated validation of the assay using carefully selected TES antigen-positive and TES antigen-negative samples to deliver proof of clinical and epidemiological relevance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.03.004.

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