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Development of specific scFv antibodies to detect neurocysticercosis antigens and potential applications in immunodiagnosis

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ABSTRACT

We have shown previously that detection of circulating antibodies against mimotopes selected by phage display were useful in neurocysticercosis diagnosis. However, circulating antigens may also be useful in patients' clinical follow-up. Therefore, we aimed to select novel combinatorial antibodies, single-chain variable fragment (scFv), which can be used for specific antigens with pre-defined affinity and specificity without prior immunization. A phage scFv antibody library was selected against *Taenia solium* mimotopes displayed on phages coupled in beads and total saline extract of *T. solium* metacestodes (S) immobilized on microtiter plate wells. After two rounds of selection, 96 phage clones were evolved and validated against each target by enzyme linked immunosorbent assay (ELISA), and dot-blot, and three specific antibodies (B6, G10 and A4) were further characterized by sequencing and indirect immunofluorescence (IFI) assays. IFI revealed tegument staining for the B6, while the others showed a non-uniform staining in the whole parasite. The selected scFvs were used to capture their antigen targets that were elucidated through mass spectrometry, and used for antibody detection in NC patients' sera by ELISA, which achieved sensitivities greater than 97% and specificities above 95%. We have successfully developed scFv antibodies against important mimotopes used in NC diagnosis, and can be further explored to detect circulating antigens for clinical follow-up of patients with NC. Our strategy also highlighted the possibility of using this combinatorial approach to select, capture and characterize specific antigens to better understand this intriguing parasite infection and disease evolution.

1. Introduction

Taenia solium taeniasis and cysticercosis are endemic parasitic infections in several African, Asian and South American countries [1]. Human neurocysticercosis (NC) is an important but neglected cause of epilepsy in developing countries where the parasite occurs [2].

The selection of antibodies from combinatorial libraries displayed on the surface of filamentous phage has become important for the generation of reagent, diagnostic, and therapeutic molecules and for the study of the natural immune response [3–5].

Recently, phage display has been shown to be a cost-effective technique for the production of antibody fragments such as a binding fragment (Fab) or a single-chain variable fragment (scFv) [6–8].

The variable fragment (Fv) of the immunoglobulin molecule is the region with antigen-binding function. A scFv combinatorial antibody consists of heavy (VH) and light (VL) chains of the variable region, which are joined by a flexible peptide linker, and can be functionally expressed in *Escherichia coli* to improve affinity and specificity without prior immunization [9].

ScFvs have many advantages and can mimic the maturation process of human antibody, so that it is possible to obtain a high affinity antibody from this selection. If an antibody library of human origin is used, the selected antibody is most suitable to human administration and is potentially applicable to clinical diagnosis and treatment of infectious diseases [10].

In this study we used a phage display technology to enable the direct isolation of scFv antibodies against (I) bead coupled mimotopes displayed in phages and (II) metacestode antigens by using solid-phase bound *T. solium* extract to screen out the phage display antibody that recognizes these antigens and proceeding immunofluorescence, and mass spectrometry to characterize antigens captured by each scFv clone selected. Potential of these antigens was evaluated to diagnose neurocysticercosis in serum samples.

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2. Materials and methods

2.1. Ethics statement

This study was conducted according to the ethical guidelines of the Brazilian Health Ministry after being approved by the Research Ethics Committee of Universidade Federal de Uberlândia (UFU), state of Minas Gerais, Brazil.

2.2. Antigens

T. solium metacestodes were collected from massively infected pig muscle and were carefully dissected from the host tissues, washed repeatedly using saline solution and stored at −20 °C. Total saline extract of *T. solium* metacestodes (S) was prepared as previously described [11].

Phage clones displaying specific peptides to NC were obtained according to Ribeiro [12].

2.3. scFv phage-display library

Antibodies against different peptides displayed in phages and total saline extract of *T. solium* were selected using a scFv phage display library constructed at the Laboratory of Nanobiotecnology (UFU) based on a pool of patients' peripheral blood mononuclear cells (PBMCs) with multiple diseases, with a library size of 2.3×10^6. Rescue of the library was performed as recommended [13].

2.4. Library screening

For selection three rounds of biopanning were performed using two peptides displayed in phages, previously described [12] or total saline extract of *T. solium* metacestodes, named herein as (a), (b) and (c) procedures.

Bead coupled phages $NC_2 2$ and $NC_4 1$ were used (10^{10} plaque forming unit (PFU)). Wild type M13 phage was used in a previous subtractive step of panning. Coupling of each phage to beads (Dynabeads® M-270 Epoxy, Invitrogen Life Sciences, USA) followed bead manufacturer's recommendations. To screen library and select scFv clones, bead coupled with wild type phage (20 μl) were put in a microtube and incubated with scFv library (50 μl) for 1 h, during this period those scFv clones that interacted with wild type phage were excluded. Next, supernatant was collected using a magnetic apparatus and transferred to a new tube containing beads coupled with the target mimotope displayed on phage ((a) $NC_2 2$ or (b) $NC_4 1$), and incubated for 1 h at 37 °C. Supernatant was discarded and specific scFv clones were eluted using 100 mM glycine–HCl, pH 2.2 for 10 min at room temperature (RT), neutralized with Tris base (2 M, pH 9.1) and then transferred to a XL1 Blue culture for amplification and titration [13].

To select scFv antibodies against total saline extract of *T. solium* metacestodes (c) microplates (Maxisorp™, Thermo Scientific, USA) were coated overnight with 1 μg/well of target antigen at 4 °C in carbonate buffer (0.1 M, pH 9.6), blocked with bovine serum albumin (BSA 3% in Tris-buffered saline (TBS), 37 °C, 1 h) and washed six times (TBS containing Tween®-20 (T) 0.1%). Phage scFv library (10^9 PFU) was added and incubated (1 h, RT) under gentle agitation. Plates were washed ten times with TBS-T 0.1%. Bound phages were eluted, amplified and titrated as follows.

For amplification the culture was grown until optical density (OD) at 600 nm reached 1.0 and after was infected with VCS-M13 helper phage (10^{12} PFU, Stratagene, USA). Amplified phages were submitted to another round of panning. Input and output phages were titrated on Luria Bertani (LB)/carbenicillin plates. Preliminary data showed that two selection rounds resulted in a high recovery of antigen-binding phage.

2.5. Production of soluble single-chain antibodies

Antibody fragments were produced by electroporating phagemid DNA (20 ng) into *E. coli* (One Shot® TOP10 Electrocomp™ *E. coli*, Invitrogen, USA), and plating the transformed cells on LB/carbenicillin plates. The bacterial culture was incubated under rotation (37 °C, 250 rpm) in Super broth (SB) agar/carbenicillin containing glucose 2% (5 h, OD600 = 1.0), induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 2 mM, Sigma, USA), and further incubated overnight (30 °C, 250 rpm). Supernatant containing antibody was harvested after centrifugation (5000 × *g*, 4 °C, 15 min), further clarified through a 0.45 mm filter (Millipore, USA) and His-tagged scFv fragments were purified by immobilized-metal (Ni) affinity chromatography (HisTrap™ HP, GE Healthcare, USA). ScFv clones had histidine (His) and hemagglutinin (HA) tags and purified fractions were concentrated and checked for the presence of antibody fragments by immunoblotting. Each selected scFv clone was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 16%, transferred to a nitrocellulose membrane, blocked with (w/v) skimmed milk 5% in PBS and then sequentially probed with anti-HA peroxidase conjugated (1:1000) (Roche, Switzerland) overnight 4 °C. All washes were done in PBS-T 0.05%. Following appropriate color development using the 3,3′-diaminobenzidine (DAB) substrate system (Sigma, USA), the membrane was washed extensively in distilled water, dried, and imaged on a flatbed scanner. To verify the integrity of each selected scFv clone, purified antibodies were analyzed on SDS PAGE 16% gel under non reducing conditions, gel was stained with silver nitrate [14].

2.6. Selection of phage antibodies

2.6.1. Phage ELISA

We used phage ELISA to verify expression of the scFvs. Briefly, supernatants containing soluble selected scFvs antibodies were incubated in ELISA microplates. After overnight incubation at 4 °C plates were blocked with PBS-BSA 3% (1 h, 37 °C) followed by two washes with PBS and three washes with PBS-T 0.05%. After, plates were incubated with anti-HA horseradish peroxidase conjugate (1:2000 in PBS-BSA 3%, Roche, Switzerland). Plates were washed again as described earlier. Orthophenylenediamine (OPD), peroxidase substrate was added, and the absorbance was read at 492 nm, using an ELISA reader (TP Reader, China).

Those clones positive for scFv expression were tested to verify antigen recognition, immuno 96 Micro-Well™ plates (Sigma, USA) were coated with each antigen: (a) $NC_2 2$ (10^{10} PFU/well), (b) $NC_4 1$ (10^{10} PFU/well) and (c) total saline extract (1 μg/well) and tested by ELISA for binding to each target as described above, using scFvs at a 1:250 dilution in blocking solution (PBS-BSA 3%).

2.7. Characterization of single-chain antibodies

2.7.1. Dot blot

Specificity tests were done by dot blot. Briefly, 10^{10} PFU of each target phage: $NC_2 2$, $NC_4 1$ and its negative control (wild type M13) and 1 μg from total saline extract using an on related antigen as negative control were blotted on nitrocellulose membranes. After, membranes were blocked by using PBS-BSA 3%, washed with TBS-T 0.05% and selected scFv antibodies were added at 1:150 in blocking solution and incubated for 1 h at room temperature, then anti-HA peroxidase conjugated 1:1000 in blocking solution was added and

incubated for 1.5 h, after three washings, reaction was developed by adding DAB (Sigma, USA).

2.7.2. ELISA

To confirm reactivity and specificity from those scFv clones positive in ELISA, plates were coated with each antigen: (a) $NC_2$2 (10^{10} PFU/well), (b) $NC_4$1 (10^{10} PFU/well) and (c) total saline extract (1 μg/well). As negative control wild type M13 phage (10^{10} PFU/well) was used for procedures (a) and (b), and a non-related antigen for procedure (c). ELISA was performed as described above.

2.7.3. DNA sequencing

Clones from the second round of panning were randomly picked from the output plates of the last selection round. Each colony was grown in SB/carbenicillin broth, and cultures were incubated overnight at 37 °C, 250 rpm. Plasmids were extracted from the bacterial pellets using the Miniprep kit (Qiagen, USA), analyzed on agarose 0.8% gels and sequenced using an automatic capillary sequencer, MegaBACE 1000 Genetic Analyzer (Amersham Biosciences, USA) with −96gIII and primer (5′-CCC TCA TAG TTA GCG TAA CG-3′), corresponding to the vector sequence downstream of the scFv gene. After, the amino acid sequences were translated and analyzed by using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/).

2.7.4. Immunofluorescence (IF)

Prior to staining, metacestodes were included in tissue freezing medium (Sakura, Netherlands) and frozen at −70 °C. Thick sections were obtained (2 μm), placed on glass slides and air dried for 30 min. The scFv preparations (10 μg) were diluted in PBS and incubated for 40 min at 37 °C. After 2 washes with PBS, sections were incubated for 40 min at 37 °C with fluorescein isothiocyanate (FITC) labeled anti-HA (6 μg/ml) and counterstained with Evans blue. After two PBS washes, slides were mounted using buffered glycerin and cover slides. Negative controls were made using a non related scFv. Binding was detected by fluorescence microscopy (LSM 510 Meta, Carl Zeiss, Germany).

2.8. Affinity purification of the corresponding native antigen

To characterize the corresponding native antigens for each scFv obtained, selected clones were coupled to Ni sepharose resin. Briefly, resin (Ni Sepharose 6 Fast Flow – GE Healthcare Life Sciences) was prepared according to the manufacturer's recommendation, with modifications. A pre-wash with 5 resin volumes of distilled water and two with washing/binding buffer (20 mM Na_2HPO_4, 0.15 M NaCl, pH 7.0) was made. After, clones (135 μg each) were added (each clone on a separate tube) and incubated under gentle shaking for 1 h at room temperature. After two washings using washing/binding buffer, total saline extract was added (540 μg), incubated and then resin was washed as described. To elute native antigens captured a buffer containing imidazole (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) was used, and after a 5 min incubation supernatant was collected.

Eluted fractions were analyzed for protein content according to Bradford [15]. Proteins were visualized in SDS-PAGE 16% by silver staining [14]. Gel analysis was performed using a graphical method to generate protein lane profile plot of each antigenic fraction, by Image J version 1.44 software (National Institutes of Health, EUA); and then peaks referred to bands were compared with protein standard marker (Real Biotech, RECOM™ Blue Wide Range Prestain Marker, Taiwan) to estimate their relative molecular weight.

2.9. Determination of native antigens

Eluted proteins were precipitated out of solution using the ProteoExtract® kit (Calbiochem, Germany) and the protein pellet was left to dry overnight in a sterile fume hood. The pellet was then resuspended in ammonium bicarbonate (50 mM, pH 8.0) and subjected to an in-solution tryptic digestion (Mike Myers, Cold Spring Harbor modified by Brett S. Phinney, UC Davis Proteomics Core). Digested peptides were then de-salted using aspire tips (RP30 Tips, Thermo-Fisher Scientific, USA) before being resuspended in the loading buffer.

2.9.1. LC–MS/MS methods

Digested peptides were analyzed using a LTQ-FT (Thermo-Fisher Scientific, USA) coupled with a MG4 paradigm HPLC (Michrom, USA). The samples were loaded onto a Michrom cap trap (0.5 mm × 2 mm). The peptides were then separated using a Michrom Magic C18AQ (200 μm × 150 mm) reversed-phase column and eluted using a gradient with a duration of 60 min. Collision induced dissociation was applied to the peptide samples and data was acquired with an isolation width of 1, a normalized collision energy of 35 and a resolution of 50,000. The spray voltage on the Michrom captive spray was set to 1.8 kV with a heated transfer capillary temperature of 200 °C.

2.9.2. Data analysis

Raw data was analyzed using X!Tandem and visualized using Scaffold (Proteome Software, version 3.01). Samples were searched on Uniprot *T. solium* (304 protein sequences) databases appended with the cRAP (commonly found laboratory contaminants) and the reverse decoy databases.

2.10. ELISA to detect IgG using antigens captured by scFv clones

To verify the diagnostic potential of captured antigens related to each scFv clone obtained, ELISA tests were made.

2.10.1. Serum samples

Serum samples were collected from 90 subjects attended at the Laboratory of Clinical Analysis at the Clinical Hospital (Groups 1 and 2) and from the Laboratory of Parasitology (Group 3) of the Universidade Federal de Uberlândia in the State of Minas Gerais, Brazil. Group 1 (G1) consisted of 30 patients with definitive diagnosis of NC, as follows: (a) all patients presented at least one type of clinical manifestation suggestive of NCC such as: epilepsy, cephalea, dizziness, dementia, faintness, hydrocephalus, and no signs or symptoms of cysticercosis in other organs; (b) all patients came from or lived in an area where cysticercosis is endemic; (c) for immunodiagnosis, serum samples were positive in an ELISA test for the detection of IgG anti-metacestodes of *T. solium*; (d) they presented evidence of parasite in neuroimaging. Group 2 (G2) consisted of 30 patients who harbored other parasites: *Ascaris lumbricoides* ($n = 4$), *Enterobius vermicularis* ($n = 4$), hookworm ($n = 5$); *Hymenolepis nana* ($n = 3$), *Schistosoma mansoni* ($n = 3$), *Strongyloides stercoralis* ($n = 4$), *Taenia* sp. ($n = 4$) and *Trichuris trichiura* ($n = 3$). Group 3 (G3) consisted of 30 healthy volunteers based on their clinical profile. Although they came from an area where cysticercosis is endemic, all volunteers from this group did not present evidence of household contact with *T. solium* infection or a history of taeniasis or cysticercosis. In addition, these individuals had three fecal samples that tested negatively by the parasitological methods [16,17].

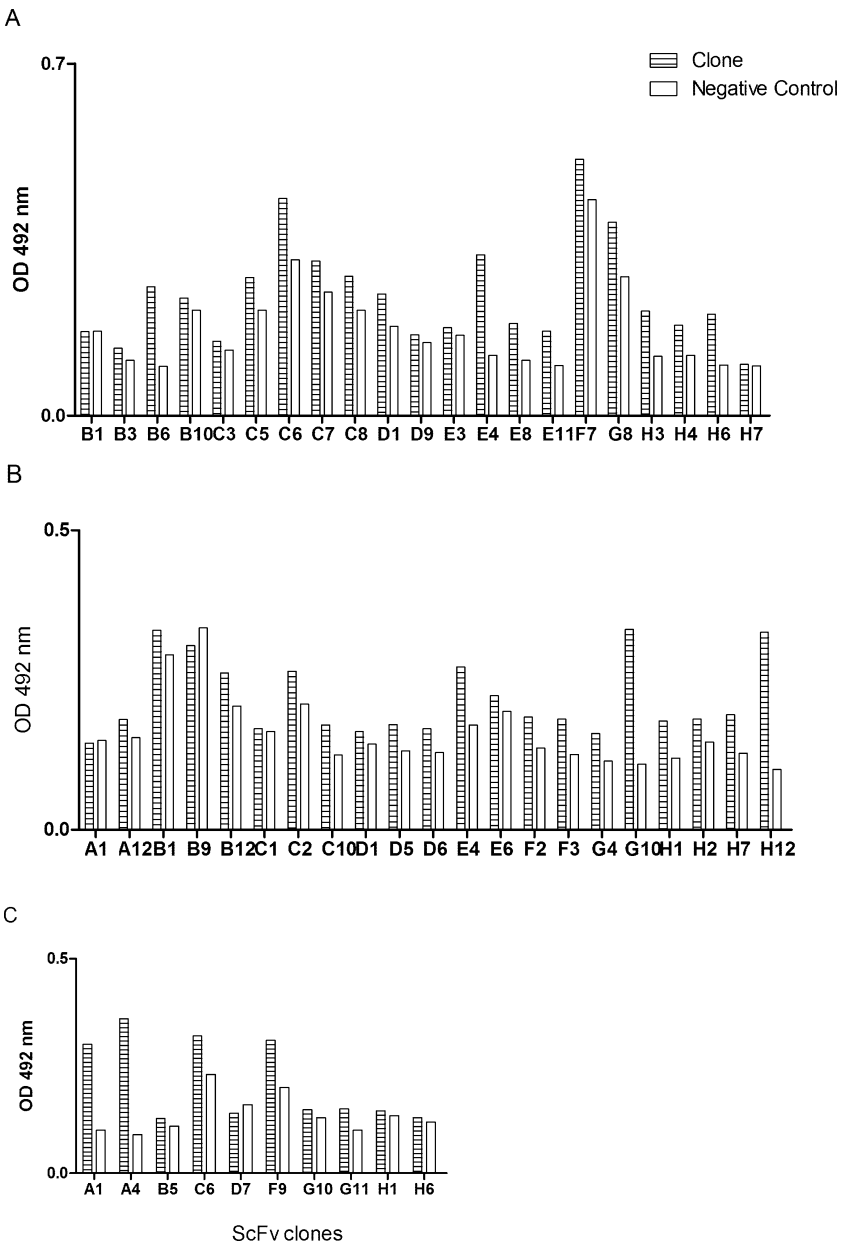

Fig. 1. Binding specificity of scFv from pannings a, b and c. ELISA absorbance at 492 nm for selected phage clones. Dashed bar shows scFv reactivity against the targets $NC_2$2 (A), $NC_4$1 (B) and total saline extract of *T. solium* metacestodes (C) by ELISA absorbance. Open bar shows negative controls: wild type M13 phage (A and B) and non-related antigen (C).

2.10.2. Antibody detection by ELISA

Preliminary experiments were carried out to determine the optimal conditions for ELISA, through block titration of reagents (antigens, sera and conjugate).

ELISA was carried out as previously described [18] with modifications. Polystyrene microplates (Interlab, Brazil) were coated with each antigen (total saline extract and antigens captured by each scFv clone) 5 μg/ml in carbonate bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. Microplates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Then serum samples were diluted 1:200 in PBS-T and incubated for 60 min at 37 °C. After another step of washing, the immunoenzymatic conjugate (peroxidase-goat anti-human IgG, Fc specific; Sigma) was added diluted 1:2000 in PBS-T and incubated for 60 min at 37 °C. The assay was developed after an additional washing procedure by adding the enzyme substrate consisting of hydrogen peroxide and OPD in 0.1 M citrate phosphate Na_2HPO_4 buffer pH 5.5 for 15 min. The reaction was interrupted by the addition of 25 μl per well of 2 N H_2SO_4. Optical densities (OD) were determined at 492 nm in an ELISA reader (Titertek Plus, Flow Laboratories, USA).

2.11. Statistical analyses of ELISA data

Analyses were performed using GraphPad software package 5.0 (GraphPad Software Inc., San Diego, USA). Optimum point for each condition of ELISA reaction and cut-off points were established using a two-graph receiver operating characteristic curve (TG-ROC) [19] based on ELISA results from patients positive for neurocysticercosis (positive controls) and individuals from the other two groups (other parasites and healthy). ELISA reactivity index (RI) was obtained by the ratio between OD and cut-off. Values of RI greater than the optimum point of reaction for each extract were considered positive (RI > 1). Sensitivity (Se) and specificity (Sp) were calculated accordingly to the formulas: $Se = a/(a + b)$ and

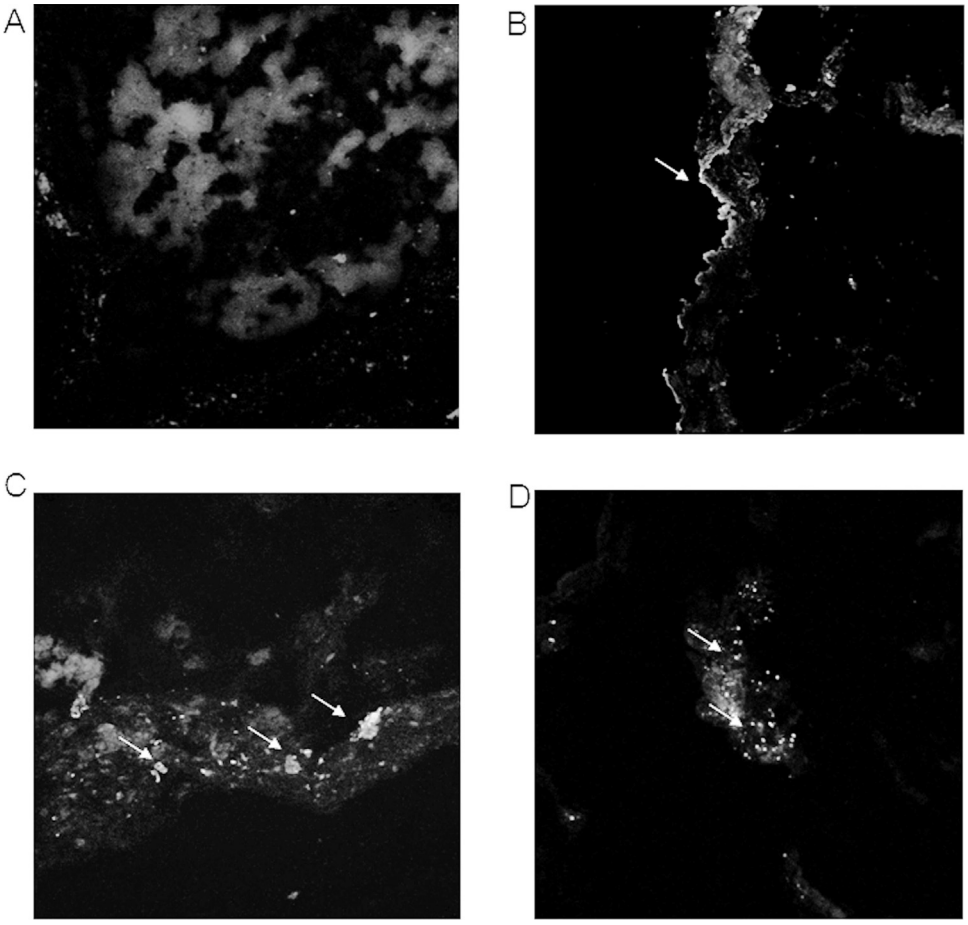

Fig. 2. Immunofluorescence of *T. solium* metacestodes using scFvs. Metacestodes were incubated with 10 μg of soluble scFv for 40 min. After washing, bound scFv was labeled with FITC/anti HA antibody and detected by fluorescence microscopy. (A) Negative control, (B) B6, (C) G10 and (D) A4.

Sp = $d/(c+d)$ where a = is true positive, b = false positive, c = false negative and d = true negative [20].

Receiver operating characteristic curves (ROC) were built to describe the tests [21]. The area under ROC curve (AUC), an overall index of diagnostic accuracy, was calculated, values close to 1 indicate an informative test; and close to 0.5 indicate an uninformative test [22]. Likelihood ratio (LR), an efficiency diagnostic parameter independent of prevalence [19] were calculated, as follows: LR+ = Se/(1 − Sp), which indicates how likely patients with neurocysticercosis are to have a positive test result compared with other patients. Probability (P) values of <0.05 were regarded as significant and 95% confidence intervals (CI) were provided to Se, Sp and AUC statistical calculations.

3. Results

3.1. Analysis of eluted phage displaying scFv antibodies

The phage output increased at the second round indicating that binder clones were isolated and amplified successfully. The observation of specific binders amplification was also evident from the phage ELISA whereby as the stringency of the selection conditions increased, more phage displaying scFvs with better binding capabilities were successfully isolated.

HisTrap™ HP column eluted fractions of scFvs were subjected to SDS-PAGE and Western blot to identify the purity and molecular mass, the purified antibodies showed only one protein band with the expected size of approximately 29 kDa. Purified scFv showed the expected band, without minor small molecular weight bands detected, indicating no degradation of the scFv during purification.

3.2. Characterization of antibody binding specificity

The binding of clones expressing scFv was measured by using ELISA microtiter plates (we analyzed 21 clones from pannings a and b, and 10 from panning c). Positive binding was defined as an absorbance for immobilized antigen that was two-fold above negative controls (Fig. 1).

The antibodies obtained against $NC_2$2 (B6 and E4) and $NC_4$1 (H12 and G10) that were neither reactive against M13 wild type phage, and those against total saline extract (A1 and A4) without reactivity for negative control were expressed in large scale, purified and re-tested for specificity. After specificity tests using purified scFvs, clones B6, G10 and A4 were selected for further analyses.

In dot blot all selected scFvs showed to be specific for its targets. Each antibody showed a different amino acid sequence of immunoglobulin variable domains. Sequence analysis of the selected scFv clones revealed three unique sequences.

3.3. Immunofluorescence localization of the antigens recognized by the representative scFvs

To confirm reactivity and determine the recognition patterns, soluble scFvs were separately incubated with *T. solium* metacestode and the bound scFv was visualized by indirect fluorescence. No fluorescence was obtained with the negative controls. The patchy

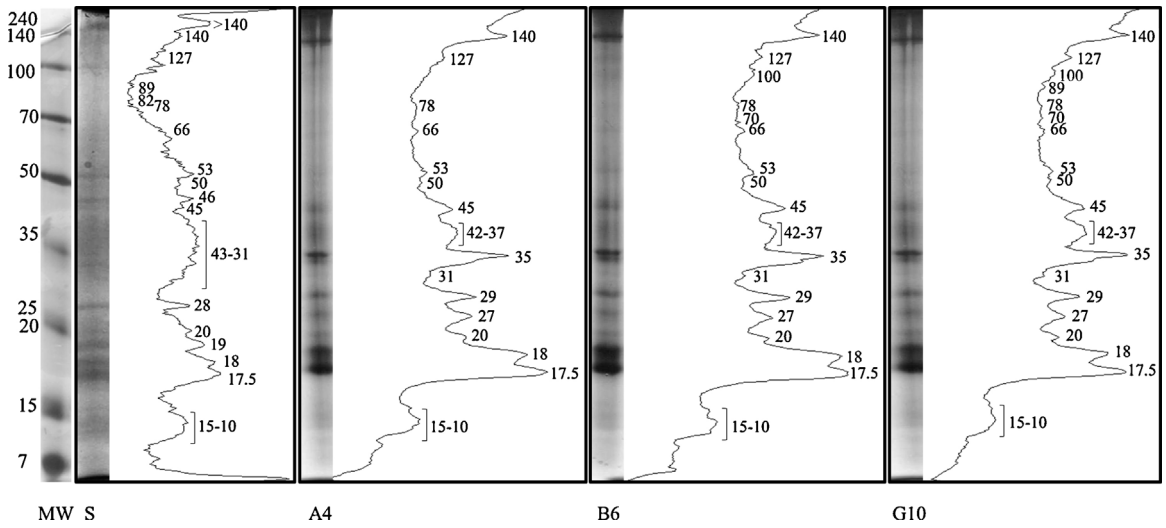

Fig. 3. Electrophoretical profiles of total extract and native antigens. Total saline extract of *T. solium* (S) and obtained fractions from affinity purification of the corresponding native antigens for each scFv clone (A4, B6 and G10) and Image J software analysis, in 16% SDS-PAGE, silver stained are shown. MW, molecular weight standard in kilodaltons (kDA).

Table 1
Native antigens revealed through mass spectrometry.

Clone	Accession number	Description	Gene	MW (kDa)
A4	G3GE64	Ts18 variant 2	Ts18v2	9455.10
B6	P68555	Actin	ACT1	41,727.50
G10	D2U5C3	Phosphoenolpyruvate carboxykinase	pepck	54,088.40

MW, molecular weight in kilodaltons.

staining observed to A4 and G10 clones was not uniform, but rather distributed in small, interspersed textural patches with no apparent regularity. ScFvs produced intermittent patches of bright fluorescence, clearly distinct (Fig. 2). Only B6 showed a belted pattern of staining at the tegument.

3.4. Determination of native antigen for each scFv clone

The analysis of electrophoretical profiles of antigens captured by each clone showed similar profiles. Polypeptide defined bands or intervals ranging from 10 to >140 kDa were detected on the total saline extract (Fig. 3). Clones specifically recognized antigens from 10–15, 17.5, 18, 20, 27, 29, 31, 35, 37–42, 45, 50, 53, 66, 78, 127 and 140. Although minor differences in band peaks can be observed, mass spectrometry revealed different proteins captured for each scFv clone (Table 1). The corresponding native antigen represents the most abundant protein revealed by LC–MS/MS procedure.

3.5. IgG detection

Analyzing Fig. 4 we can observe in G1 positivity rates of 80% (24/30) for total saline extract of *T. solium* (S). Considering captured antigens 100% (30/30) of samples were positive for the antigen captured by A4 and 97% (29/30) for antigens captured using B6 and G10 clones. In G2, 26.6% (8/30) were positive when using S and for captured antigens 3.3% (1/30) for A4 antigen, 6.6% (2/30) for B6, there

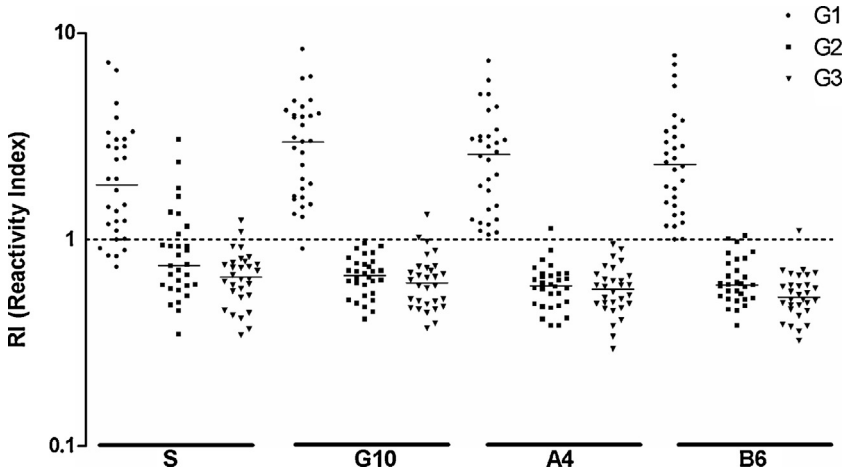

Fig. 4. IgG detection in serum samples. Samples from patients with NC ($n = 30$), other parasites ($n = 30$) and healthy individuals ($n = 30$) were tested using each antigen: saline extract of *T. solium* metacestodes and the native antigens correspondent to each scFv clone G10, A4 and B6.

was no cross reactivity when testing antigens captured by G10. In G3, positivity was 6.6% (2/30) for S and G10 target and 3.3% (1/30) for A4 and B6 targets.

Cross reactivity in G2 when using S was due to samples from patients harboring: *A. lumbricoides* (4/8), hookworm (2/8), *S. stercoralis* (1/8), *H. nana* (1/8) and *S. mansoni* (1/8). No samples were positive when using antigens captured by G10. For antigens captured by A4, reactivity was due to *E. vermicularis* (1/1), considering B6 target cross reactivity occurred with *A. lumbricoides* (1/2) and *S. mansoni* (1/2) samples.

Statistically significant differences were found considering median of RI, comparing antigens captured by G10 and A4 clones, the last one showed to be more specific ($p = 0.0004$). Analyzing ROC curve (Fig. 5), antigens captured by A4 showed to be the most specific to discriminate patients with NC from control groups (G2 and G3). Test performance, indicated by AUC for A4 target (0.998) almost reached the maximum value (1.00) of efficiency; LR conferred to this antigen a very high diagnostic value (60.0).

4. Discussion

Phage display antibody technology has become increasing popular for creating binding sites for use in all areas of research, and industrial applications. There are several examples of successful isolation of antibodies against various antigens from different phage displayed antibody libraries, including high throughput selection [23–26]. Phage bearing specific scFv fragments for the parasite antigen were selected successfully by panning, which allowed us to obtain, for the first time, this kind of recombinant antibodies that bind to epitopes on *T. solium* metacestode. These scFvs likely represent only a fraction of the parasite binding antibodies and we thus expect the scFv library to remain a useful resource for further studies of the host-exposed cisticerci epitopes.

Selection of scFv antibodies specific to phage displayed peptides is not conventional [27] the strategy used here was to select antibodies specific to peptides related to neurocysticercosis, $NC_2 2$ and $NC_4 1$, previously selected by Ribeiro et al. [12], and also to the total saline extract of *T. solium* to identify its targets. It was demonstrated previously [27] that mimotopes selected from phage display libraries could be used as targets to obtain scFv highly sensitive and reactive. It is important to emphasize that the option by direct selection against phage displayed peptides by a subtractive step using wild type M13 phage was made to avoid peptide synthesis and loss of the real identity and reactivity of mimotopes, due to a probable conformation with part of phage pIII. Our results demonstrate that selections were successful and highly efficient.

Three different, unique scFvs were selected from a scFv library for their ability to bind to both cisticerci extracts and phage clones displaying peptides. Dot-blot showed that the recombinant scFvs reacts with total saline extract of *T. solium* metacestodes. If these antibodies can be demonstrated to be *T. solium* specific, they can be good reagents for diagnosis of this parasite in serum samples from humans and animals. Importantly, it can be easily stored for long periods and can be cloned for gene encoding.

Immunofluorescence data show functionality of selected clones, once they were able to bind to its targets. This functionality allowed us to identify the immunolocation of target antigen at *T. solium* metacestode, and different staining profiles were achieved. When B6 clone was used it was observed tegument staining. In this region there are cells that actively produce proteins and other compounds

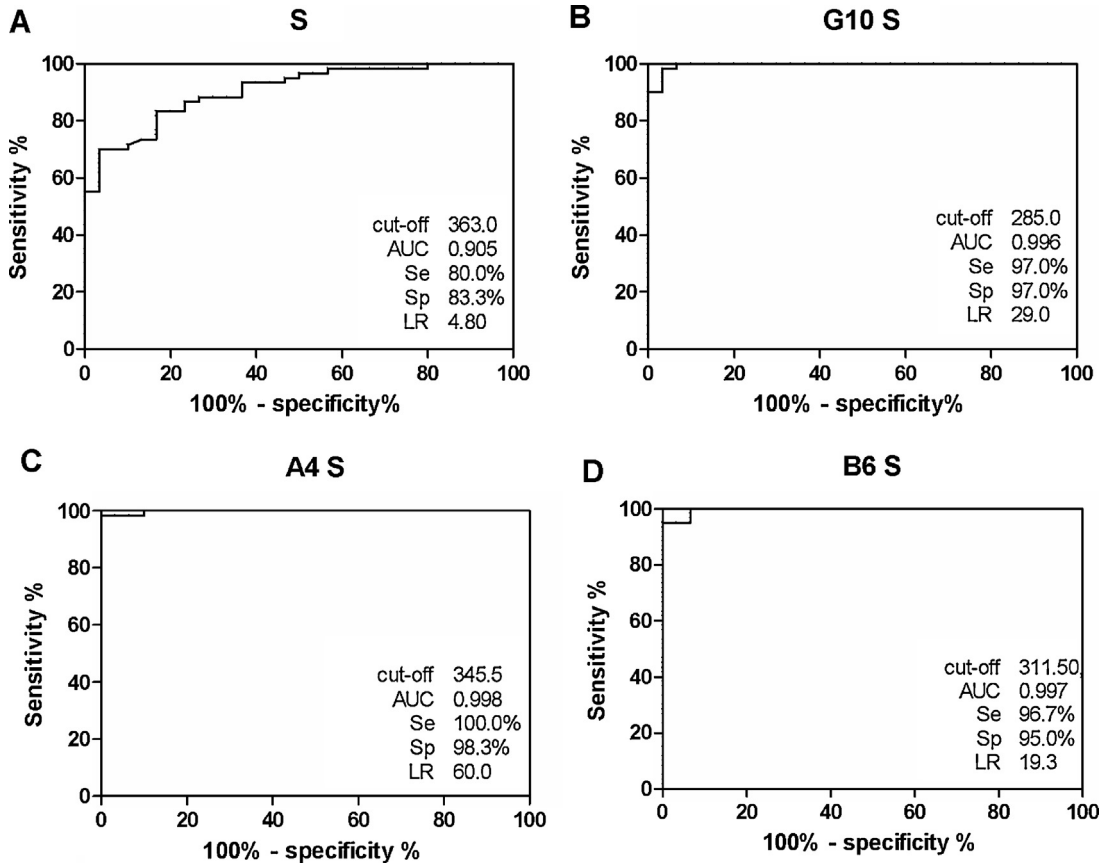

Fig. 5. ROC curve indicating optimum point of reaction. Sensitivity (Se), specificity (Sp), area under curve (AUC) and likelihood ratio (LR) are show for total saline extract of *T. solium* metacestodes (A) and the native antigens corresponding to clones G10 (B), A4 (C) and B6 (D) to detect IgG in serum.

that are transported to tegument. There are reports describing production of cytoskeleton proteins such as myosin type II and actin [28]. The staining profile reflects target identifications as revealed by LC–MS/MS where the most abundant protein was actin. Different amino acid sequences may be related to the different reactivity patterns.

Analyzing potential of captured antigens to detect IgG in serum samples from NC patients, ROC and TG-ROC curve provided an optimal cut-off point for each antigenic fraction studied. Information obtained from ELISA immunoassay showed a differential IgG detection profile for each fraction tested. Saline extract (S), and antigens captured by B6 and G10 diagnostic parameters were inferior if qualitatively compared with those from antigens captured by A4, which exhibited an increased sensitivity, specificity, and a higher AUC value. According to Jaeschke et al. [29] values of likelihood ratio LR greater than 10, practically confirm the diagnosis. Therefore, LR's showed that antigens captured by A4 had a remarkable diagnostic performance to detect IgG in patients with neurocysticercosis, reaching values of LR = 60.0.

Comparing our results with those from other authors using native, synthetic or recombinant antigens, fractions captured by scFv clones obtained here showed sensitivity and specificity values similar or superior to that previously reported [30–35]. Special attention should be made to the complete statistical analyses performed here that revealed very high diagnostic parameters reached by captured antigens. Our results reinforce the usefulness of mimotopes described previously for NC diagnosis [12], and presents the alternative use of selected scFvs on patients' follow-up.

Presented data show that was possible to select antibodies with appropriate reactivity leading to perspectives on its application to capture new antigens with potential diagnostic application. Selected clones can be excellent reagents in the search for promising vaccine targets, for dissecting the role of antibodies in *T. solium* immunity and for improved understanding of the host/parasite relationship.

The rationale of using the novel antibodies that target our previously described recombinant antigens (mimotopes) may be of great importance to monitor the evolution of the parasitic infection and the treatment efficacy. scFv antibodies selected here can be used for antigen identification in cerebrospinal fluid and serum samples from patients with NC, this usefulness and the economic advantage was previously described [36–39], suggesting that antigen identification may contribute as to the understanding of the disease, the characterization of the parasite's antigenic composition, besides the use for antigen and antibody detection in humans and animals for diagnostic purposes.

In conclusion, antibody fragments specific to *T. solium* antigens were successfully selected for the first time. The antibody phage display technology is a useful approach for the study of antigen–antibody interactions used to further characterize antigens related to mimotopes with potential use in immunodiagnostic tests.

Conflict of interest

The authors declare to have no conflict of interest.

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