Original article

Innovative approaches to improve serodiagnosis of *Strongyloides stercoralis* infection

Marcelo Arantes Levenhagen [a,*], *Patrícia Tiemi Fujimura* [b],
Fabiana de Almeida Araújo Santos [b], *Lara Vecchi* [b], *Renata Pereira Alves-Balvedi* [c],
Ana Graci Brito Madurro [c], *João Marcos Madurro* [c], *Julia Maria Costa-Cruz* [a],
Luiz Ricardo Goulart [b,d]

[a] *Universidade Federal de Uberlândia, Instituto de Ciências Biomédicas, Laboratório de Diagnóstico de Parasitoses, Uberlândia, MG, Brazil*
[b] *Universidade Federal de Uberlândia, Instituto de Genética e Bioquímica, Laboratório de Nanobiotecnologia, Uberlândia, MG, Brazil*
[c] *Universidade Federal de Uberlândia, Laboratório de Filmes Poliméricos e Nanotecnologia, Instituto de Química, Uberlândia, MG, Brazil*
[d] *Department of Medical Microbiology and Immunology, University of California, Davis, CA, USA*

ARTICLE INFO

ABSTRACT

Strongyloidiasis is a helminthiasis of neglected condition that has no gold standard parasitological diagnosis due to the intermittent release of larvae in feces. This study aimed to use an scFv (single chain variable fragment) *obtained by Phage Display*, previously validated to detect immune complexes in serum samples from individuals infected with *Strongyloides stercoralis* by enzyme-linked immunosorbent assay (ELISA). Now the ability of scFv to detect the immune complexes was verified by immunofluorescence, flow cytometry using magnetic beads and surface plasmon resonance (SPR). As ELISA, the SPR, immunofluorescence and flow cytometry demonstrated the ability of scFv to detect immune complexes in sera from individuals with strongyloidiasis and discriminate them from sera of individuals with other parasitic diseases and healthy individuals. Besides de conventional ELISA, the novel approaches can also be promptly applied as auxiliary diagnostic tools to the existing parasitological method for accurate diagnosis of human strongyloidiasis.

Introduction

Human strongyloidiasis is a helminth infection of worldwide distribution. Considering only acute infections, strongyloidiasis is one of the major intestinal infections in humans. An important feature of this parasitic disease occurs by the possibility of autoinfection in which large amount of infective larvae present in the intestine spread to other organs. This process is more frequent in infected individuals under immunosuppression.[1–3]

Several parasitological, immunological and molecular biology methods have been described for diagnosis of human strongyloidiasis. Parasitological methods are often used to

---

identify rhabditoid larvae, but due to small and irregular release of larvae in the feces these methods have low sensitivity. Immunological methods have the advantage of showing high sensitivity compared to parasitological methods, besides being useful in the evaluation of host immune response and in seroepidemiological surveys.[4,5]

Due to the large number of potentially exposed people with subclinical infection or misdiagnosed, this parasitosis is presented as a neglected condition. Considering the magnitude of strongyloidiasis and the importance of early diagnosis, there is an urgent need to develop diagnostic methodologies that are innovative, because of the possibility of chronicity, hyperinfection and dissemination, which can be fatal in immunosuppressed individuals.[6–8]

The aim of this study was to further improve the ELISA assay for strongyloidiasis diagnosis, as a reference method, which is based on the detection of an immune complex with an scFv (single chain variable fragment). The scFv binds to a specific protein from *Strongyloides* sp., HSP60, and it was used to detect immunocomplexes in human sera. This serodiagnosis demonstrated 97.5% sensitivity and 98.81% specificity to *Strongyloides stercoralis*.[9] Now three novel biotechnological approaches were introduced, surface plasmon resonance, immunofluorescence and flow cytometry that are discussed herein.

Material and methods

Ethical approval

All experimental procedures were performed in accordance with the ethical guidelines of the Brazilian Health Ministry, and approved in 2013 (protocol number 307.605) by the Research Ethics Committee from the Federal University of Uberlândia, Minas Gerais state, Brazil.

Serum samples

Serum samples were the same used previously,[10] comprising 124 human sera which were pooled (10 μL of each serum sample) and divided into three groups: Group 1 (P1) – 40 serum samples from individuals infected only with strongyloidiasis; Group 2 (P2) – 44 serum samples from individuals positive by single infection of other intestinal parasitic diseases including *Ascaris lumbricoides* ($n = 8$), hookworm ($n = 7$), *Enterobius vermicularis* ($n = 5$), *Trichuris trichiura* ($n = 5$), *Schistosoma mansoni* ($n = 4$), *Hymenolepis nana* ($n = 4$), *Taenia* sp. ($n = 6$) and *Giardia lamblia* ($n = 5$); Group 3 (P3) – 40 serum samples from healthy individuals, based on their clinical observation, without contact or previous infection by *Strongyloides* and three fecal samples tested negative.

Sandwich enzyme-linked immunosorbent assay

High affinity microtiter plates (Nunc MaxiSorp™) were incubated with 50 μL of scFv (10 μg/mL) in carbonate bicarbonate buffer (0.06 M, pH 9.6), overnight at 4 °C. Plates were blocked with PBS/5%BSA. Pooled serum samples were diluted 1:50 in PBS/0.05% Tween 20 (PBST) (v/v) and 5% BSA (w/v) and added

to the wells. Subsequently it was added a mouse anti-human IgG peroxidase conjugated diluted in PBST (1:10,000). In the steps of blocking, sera and conjugated were incubated for 45 min at 37 °C and between each step it was performed three washes with PBST for 5 min. The reaction was revealed by adding orthophenylenediamine (OPD) diluted in 0.1 M citrate-phosphate (pH 5.0) and 30% H_2O_2. Plates were incubated for 15 min at room temperature, and reactions were stopped by adding 2 N H_2SO_4. The optical densities (OD) were determined at 492 nm in an ELISA reader (Titertek Plus, Flow Laboratories, USA).[10]

Surface plasmon resonance

A gold electrode was prepared by using a cleaning solution, comprising of 3:1 H_2SO_4 PA/H_2O_2 PA, to remove all possible contaminants. All steps were performed using a surface plasmon resonance equipment (Metrohm, AUTOLAB B.V., Utrecht, Netherlands). The electrode was coupled to the equipment, and after two washes in 0.1 M phosphate buffer (pH 7.4), 100 μL of scFv (10 μg/mL) was added. The electrode was blocked with 1% casein and pooled sera (1:10) was added. The whole process was carried out at 35 °C. The three steps: scFv incorporation, blocking, and sera addition, were performed for 30 min; and in between all steps, two washes were performed in 0.1 M phosphate buffer (pH 7.4) followed by 30 min in the same buffer until obtaining the baseline.

Immunofluorescence and flow cytometry

Magnetic epoxy beads (Dynabeads M270 Epoxy, Invitrogen, Carlsbad, CA, USA) were washed three times in 0.1 M phosphate buffer (pH 7.4) using a magnetic apparatus DynaMag-2. One hundred microliters of scFv (750 μg/mL) and 3:1 ammonium sulphate solution 3 M/1.2 M phosphate buffer (pH 7.4) were added to 10 μL of magnetic beads (2×10^7 beads), and further incubated overnight at 4 °C under stirring. Beads were blocked with PBS/5%BSA. One hundred microliters of pooled sera (1:50) were added and then 100 μL of anti-human IgG/FITC (1:200) both diluted in PBS/5%BSA. In the steps of blocking sera and conjugated were incubated for 45 min at 18 °C and between each step it was performed three washes with PBST for 5 min. Beads were analyzed in a fluorescence microscope (AMG EVOS fl, Cell Imaging System, Thermo Fisher Scientific Inc., Waltham, MA, USA) followed by flow cytometry (10,000 events/sample) (Accury™ C6, BD Biosciences, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Analysis of variance (ANOVA) followed by the Tukey post hoc test was used to determine differences among groups. Values were considered statistically significant when $P < 0.05$.
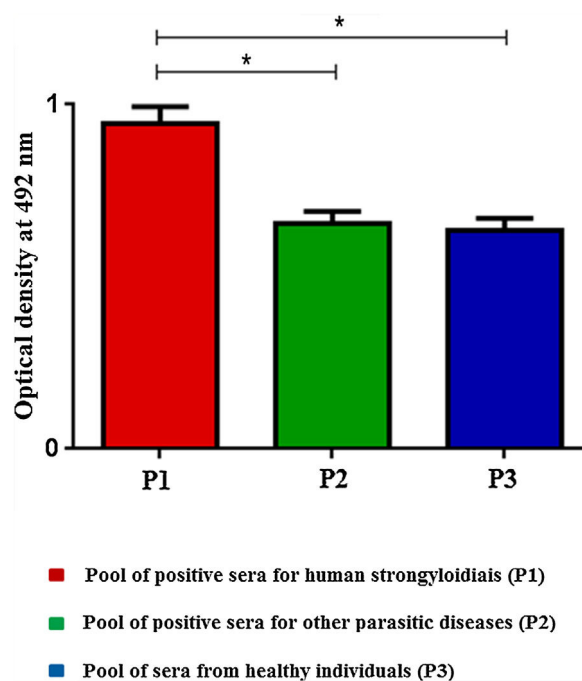
Results

Enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and immunofluorescence/flow cytom-

Table 1 – General features of methods to verify the ability of scFv to detect immune complexes in human sera from individuals infected by *Strongyloides stercoralis*.

	ELISA	IF/flow cytometry	SPR
Material for assay	Microtiter plates	Magnetic beads	Gold electrode
scFv immobilization	18 h	18 h	30 min
Blocking	45 min	45 min	30 min
Serum samples	45 min	45 min	30 min
Detection antibody	45 min	45 min	–
Total time for washing	1 h	1 h	1 h 30 min
Total time without immobilization	3 h 15 min	3 h 15 min	2 h 30 min

ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; SPR, surface plasmon resonance.

■ Pool of positive sera for human strongyloidiais (P1)

■ Pool of positive sera for other parasitic diseases (P2)

■ Pool of sera from healthy individuals (P3)

Fig. 1 – ELISA using scFv to detect immune complexes in pooled human sera. The reaction was developed with anti-human IgG/peroxidase. Data are expressed as mean ± standard deviation (*n* = 2) and are representative of two independent experiments with similar results. *P < 0.05.

etry methods were conducted in order to verify the ability of scFv to detect immune complexes in sera from positive individuals with strongyloidiasis and to discriminate from those positive with other parasitic diseases and from healthy individuals. Similar to ELISA, SPR, immunofluorescence (IF)/flow cytometry (FC) approaches showed to be highly effective to detect *S. stercoralis* infection by using scFv and human sera. General features of the methods are shown in Table 1. The SPR technique demanded less time to be done and the turnaround time (2 h and 30 min) compared to the IF/FC and ELISA (3 h and 15 min). Furthermore, it is important to emphasize that both methods require the immobilization of the scFv for 18 h prior to assays.

ELISA was performed by scFv immobilization in microtiter plates to detect immune complexes and differences on optical densities (492 nm) were obtained according to the pool of sera (Fig. 1). The SPR showed high efficiency during the whole process; from immobilization of the scFv to the gold electrode (1), blocking (2), incorporation of sera (3), and washings (*) steps

(Fig. 2A). The end result of the process demonstrated the significant difference in the SPR response in accordance with the pool of sera added (Fig. 2B).

Flow cytometry was preceded by immunofluorescence, which showed that beads were fluorescent after immobilization of scFv in the beads and according to the pool of sera used: positive for strongyloidiasis (Fig. 3A), positive for other parasites (Fig. 3B) and healthy individuals (Fig. 3C). As a negative control beads were coupled with scFv and added anti-human IgG/FITC to the beads (Fig. 3D). Subsequently, this result could be seen, confirmed and counted by flow cytometry (Fig. 4A). It was observed significant differences in the fluorescence intensity of the beads according to the pool of sera (Fig. 4B).

Discussion

Human strongyloidiasis is a neglected condition because it has underestimated prevalence data mainly due to the para-
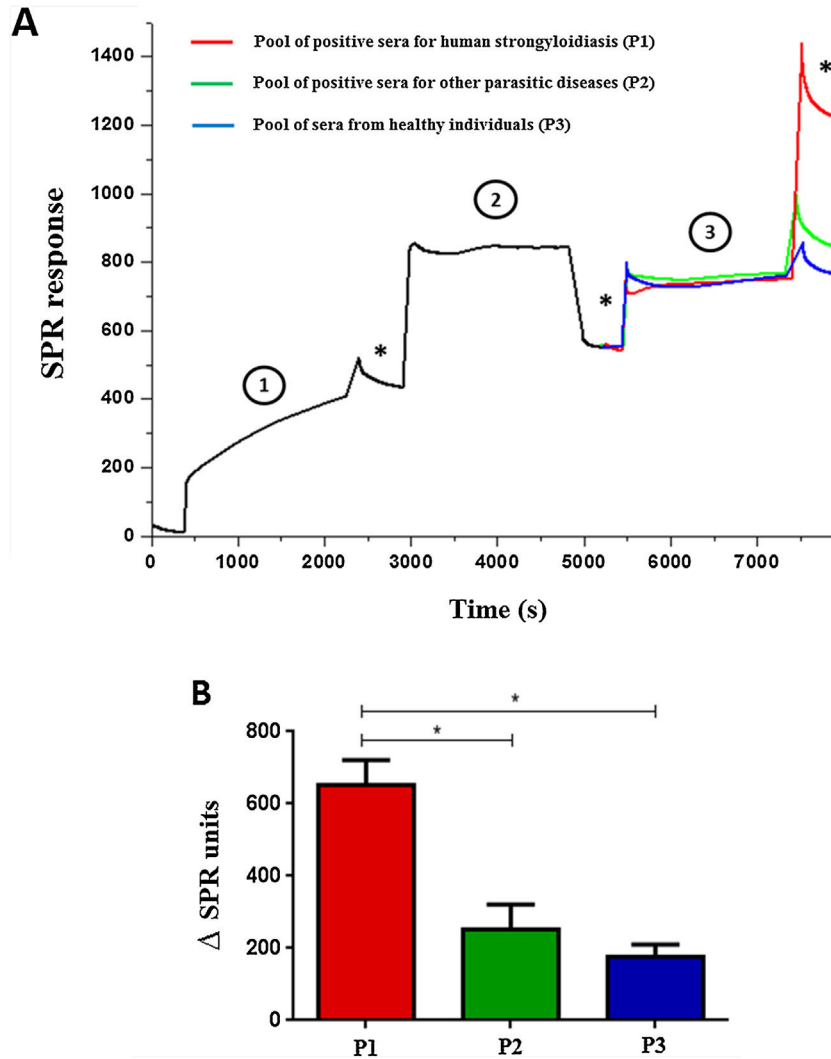
Fig. 2 – Surface Plasmon Resonance using scFv to detect immune complexes in pooled human sera (A) and variation between the end result and early immobilization of each pool of sera (B). The gold electrode was added of scFv molecules (1) blocked with 1% casein (2) and sera pool samples were added (3). (*) Washing steps in phosphate buffer. Data are expressed as mean ± standard deviation (*n* = 2) and are representative of two independent experiments with similar results. *P < 0.05.

sitological methods frequently used to detect larvae.[5] Among immunological methods routinely applied, the ELISA technique has great advantage over other immunological methods due to the possibility of detecting circulating immune complexes, which is indicative of active infection.[10–14]

The development of functional probes, such as an scFv (single-chain variable fragments), has allowed the selection of high affinity molecules through Phage Display against various antigens of interest. These antibody fragments may represent an effective tool in the development of more specific diagnostic methods.[15] In this study, we have used a previously selected specific scFv against the heat shock protein 60 (HSP60 antigen) from S. *venezuelensis*, and successfully applied in the immunodiagnosis of human strongyloidiasis.

The ability of the scFv to detect immune complexes (HSP60 + IgG) in sera from individuals with strongyloidiasis was analyzed by surface plasmon resonance (SPR), Immunofluorescence/flow cytometry and compared to ELISA.

In both new methods, the scFv molecule was able to detect immune complexes in a pooled sera of positive individuals for strongyloidiasis, and discriminated from individuals that were positive for other parasitic diseases and from healthy individuals.

Unlike ELISA, in which scFv is coated in microtiter plates, SPR used an scFv-coated gold electrode whereas the immunofluorescence/flow cytometry used epoxy-functionalized beads. SPR provided real-time visualization and demonstrated the efficiency of all stages of the process, from the immobilization of the scFv at the gold electrode, blocking step, samples incorporation, washings, and final result. Additionally, different from other techniques, SPR required less time to achieve diagnosis, and no need to use secondary antibodies for detection, which may be called label-free. Thus, the SPR technique presented as advantages the shorter turnaround time and practicality. The drawbacks of this technology are the requirement of well-trained
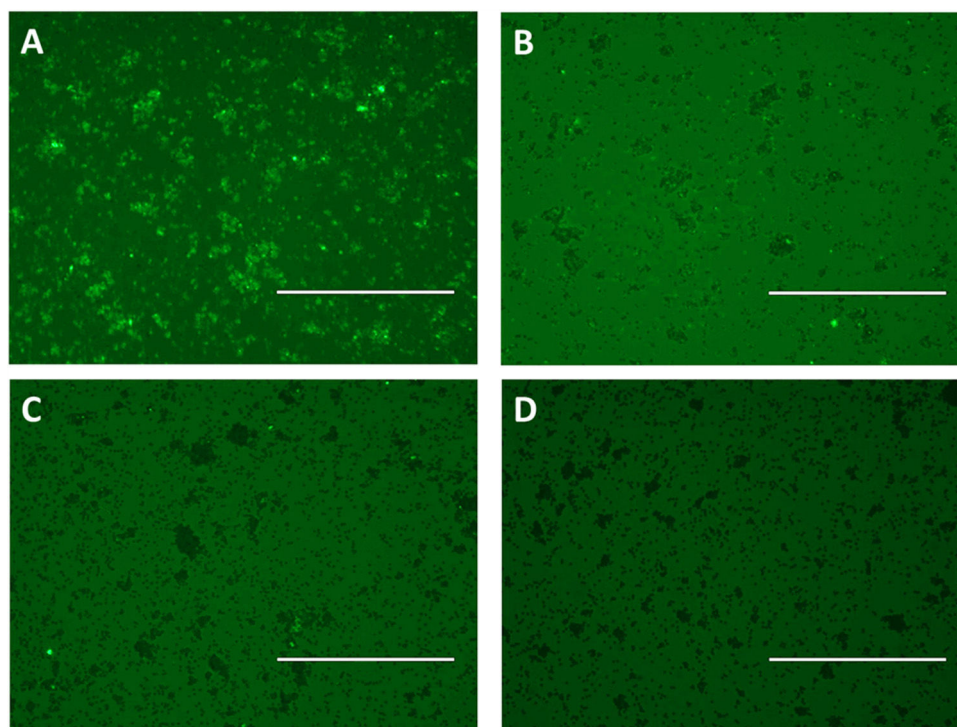
Fig. 3 – Immune complexes detection by immunofluorescence using epoxy beads coupled by scFv and pooled human sera positive for strongyloidiasis (A) and other parasites (B) and pooled sera from healthy individuals (C), labeled with anti-human IgG/FITC. As a negative control were used scFv coupled beads and added anti-human IgG/FITC (D). Bar: 200 μm.

human resource, medium to high cost equipment, and measurements can only be performed in multiple samples in high-end expensive equipment. However, this is the first demonstration that SPR can be used for human strongyloidiasis diagnosis. Only recently a study showed that this technique could be efficiently applied in serologic detection of IgM antibodies in dengue virus-infected individuals.[16]

There is no data in the literature that demonstrate the use of flow cytometry with epoxy beads to detect human strongyloidiasis. A method that uses beads coupled with protein A/G called LIPS (Luciferase Immunoprecipitation System), which analyzes the results in a luminometer has been applied in the detection of serum antibodies from individuals with strongyloidiasis and demonstrated high efficiency.[17,18] But, this is the first study that applied flow cytometry to detect immune complexes in the human strongyloidiasis, which is an important feature since immune complexes represents a serum biomarker of active infection. Our flow cytometry (FC) method was preceded by analysis of beads in fluorescence microscope, which provided a preview of the result, according to the pool of sera used, which may also be considered a qualitative approach.

The establishment of a conventional ELISA to detect immune complexes for human strongyloidiasis diagnosis is of great public health importance worldwide, due to the possibility of disease chronicity and hyperinfection that are potentially fatal to humans. The development of complementary innovative approaches also offer new possibilities to improve *Strongyloides* sp. detection, and the description
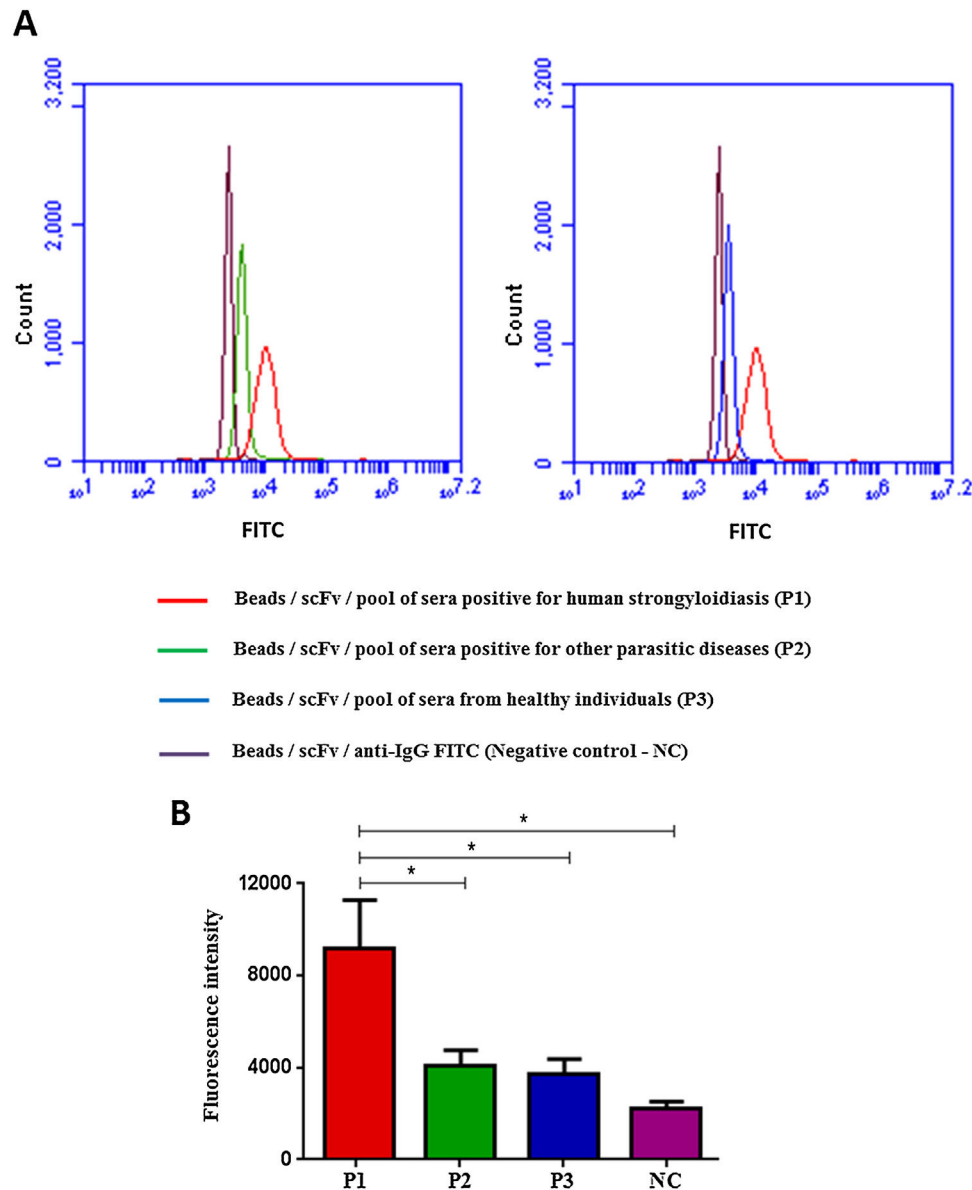
of three methods in this investigation; SPR, immunofluorescence and FC may become diagnostic choices that can be applied according to specific demand and resources settings. According to the present study, the immunofluorescence technique using magnetic beads may be more appropriate in low-resources laboratories, but an immunofluorescence microscope is still needed. In these places the dipstick technology may represent the best method to be used in the field. However, there is still no methodology that allows the detection of antigens or immune complexes such as the developed in the present study. All of them are used for IgG detection that may not be an indicative of active infection.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

A

Beads / scFv / pool of sera positive for human strongyloidiasis (P1)

Beads / scFv / pool of sera positive for other parasitic diseases (P2)

Beads / scFv / pool of sera from healthy individuals (P3)

Beads / scFv / anti-IgG FITC (Negative control - NC)

B

Fig. 4 – **Flow Cytometry with beads coupled with scFv and added of sera pool (A) and fluorescence intensity of each test (B), labeled with anti-human IgG/FITC. Data are expressed as mean ± standard deviation ($n = 2$) and are representative of two independent experiments with similar results. Count: 10,000 events; CN: negative control. $^*P < 0.05$.**

REFERENCES

1. Marcos LA, Terashima A, Canales M, Gotuzzo E. Update on strongyloidiasis in the immunocompromised host. Curr Infect Dis Rep. 2011;13:35–46.
2. Toledo R, Munoz-Antoli C, Esteban JG. Strongyloidiasis with emphasis on human infections and its different clinical forms. Adv Parasitol. 2015;88:165–241.
3. Barratt JLN, Lane M, Talundzic E, et al. A global genotyping survey of *Strongyloides stercoralis* and *Strongyloides fuelleborni* using deep amplicon sequencing. PLoS Negl Trop Dis. 2019;13:e0007609.
4. Levenhagen MA, Costa-Cruz JM. Update on immunologic and molecular diagnosis of human strongyloidiasis. Acta Trop. 2014;135:33–43.
5. Arifin N, Hanafiah KM, Ahmad H, Noordin R. Serodiagnosis and early detection of *Strongyloides stercoralis* infection. J Microbiol Immunol Infect. 2019;52:371–8.
6. Olsen A, Van Lieshout L, Marti H, et al. Strongyloidiasis – the most neglected of the neglected tropical diseases? Trans R Soc Trop Med Hyg. 2009;103:967–72.
7. Bisoffi Z, Buonfrate D, Montresor A, et al. *Strongyloides stercoralis*: a plea for action. PLoS Negl Trop Dis. 2013;9:e2514.
8. Buonfrate D, Gobbi F, Angheben A, Bisoffi Z. *Strongyloides stercoralis*: the need for accurate information. Lancet. 2018;391:2322–3.
9. Miguel CB, Levenhagen MA, Costa-Cruz JM, et al. scFv against HSP60 of *Strongyloides* sp. and its application in the evaluation of parasite frequency in the elderly. Dis Markers. 2020;2020:1–6.
10. Levenhagen MA, Santos FAA, Fujimura PT, Carneiro AP, Costa-Cruz JM, Goulart LR. Structural and functional

characterization of a novel scFv anti-HSP60 of *Strongyloides* sp. Sci Rep. 2015;5:10447.

11. Chaves-Borges FA, Souza MA, Silva DA, Kasper LH, Mineo JR. Detection of *Toxoplasma gondii* soluble antigen SAG-1 (p30), antibody and immune complex in the cerebrospinal fluid of HIV positive or negative individuals. Rev Inst Med Trop São Paulo. 1999;41:329–38.

12. Raja A, Uma Devi KR, Ramalingam B, Brennan PJ. Improved diagnosis of pulmonary tuberculosis by detection of free and immune complex-bound anti-30 kDa antibodies. Diag Microbiol Infect Dis. 2004;50:253–9.

13. de Carvalho CA, Partata AK, Hiramoto RM, et al. A simple immune complex dissociation ELISA for leishmaniasis: standardization of the assay in experimental models and preliminary results in canine and human samples. Acta Trop. 2013;125:128–36.

14. Emmerich P, Mika A, Schmitz H. Detection of serotype-specific antibodies to the four dengue viruses using an immune complex binding (ICB) ELISA. PLoS Negl Trop Dis. 2014;7:e2580.

15. Weisser NE, Hal C. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. Biotechnol Adv. 2009;27:502–20.

16. Jahanshani P, Zalnezhad E, Sekaran DS, Adikan FRM. Rapid immunoglobulin M-based dengue diagnostic test using surface plasmon resonance biosensor. Sci Rep. 2014;4:e3851.

17. Krolewiecki AJ, Ramanathan R, Fink V, et al. Improved diagnosis of *Strongyloides stercoralis* using recombinant antigen-based serologies in a community-wide study in Northern Argentina. Clin Vaccine Immunol. 2010;10:1624–30.

18. Ramanathan R, Burbelo PD, Groot S, Iadarola MJ, Neva FA, Nutman TB. A luciferase immunoprecipitation systems assay enhances the sensitivity and specificity of diagnosis of *Strongyloides stercoralis* infection. J Infect Dis. 2008;198: 444–51.