Aptamers ISSN 2514-3247 Aptamers, 2022, Vol 6, 1-9

RESEARCH ARTICLE

Isolation of DNA aptamers as potential tools for Leishmaniasis diagnosis

Juan Ospina*, María Osorio, Arley Caraballo and Miryan Sánchez

Instituto Colombiano de Medicina Tropical, Universidad CES, Sabaneta, CP 055450, Colombia

***Correspondence to**: Juan Ospina, Email: jospina@ces.edu.co, Tel: +574 4440555 ext 2522

Received: 30 August 2021 | **Revised:** 06 January 2022 | **Accepted:** 16 February 2022 | **Published:** 16 February 2022

This is an invited and peer-reviewed article, and was commissioned by Dr Victoria Calzada.

ABSTRACT

Leishmaniasis is a vector-borne tropical disease that, depending on the causative species, causes 3 different clinical manifestations: cutaneous, mucosal, and visceral leishmaniasis. In Colombia, *Leishmania (viannia) panamensis* is responsible for up to 79% of cases. Leishmaniasis diagnosis lacks a gold standard method, and the most sensitive tests can only be performed in laboratories with specialized equipment to perform qPCR, ELISA, and IIF. Accordingly, synthetic molecules such as aptamers have shown the potential to play a role in the development of accurate biosensors for the diagnosis and monitoring of leishmaniasis in endemic areas. In this work, sera samples from patients infected with cutaneous and mucosal leishmaniasis were used to select DNA aptamers that recognize a previously identified protein which was named *Leishmania panamensis* D protein (LpD) as a biomarker for mucosal leishmaniasis through Systematic Evolution of Ligands by Exponential Enrichment (SELEX). We present a list of unique NGS-identified aptamers with biomarker recognition for cutaneous and mucosal leishmaniasis evaluated by both ELASA and western blot assays. We propose that the interaction between PD1 aptamer and LpD recombinant protein is mediated by two hydrogen bonds. One of these bonds involves the Glu119 base and the G50 base which takes part in the predicted motif for LpD protein recognition. The aptamers PD1, PD2 and PD3 showed a low dissociation constant (Kd) value 51.83±8.725nM, 63.38±9.863nM and 84.85±15.87nM respectively, which makes them good specific biosensors candidates that will allow rapid and low-cost diagnosis of leishmaniasis in remote and low-resource endemic areas. In addition, we consider that a portable tool could be used in the field to further improve the sensitivity and specificity of this type of diagnostic test.

KEYWORDS: Aptamers, leishmania, diagnosis, ELASA, western blot

INTRODUCTION

Neglected tropical diseases (NTDs) are a group of diseases that occur in tropical and subtropical regions, generally poor (Engels and Zhou 2020). Together, NTDs affect more than 1 billion people and have devastating health, social and economic consequences (OMS, 2020). Unfortunately, NTDs are not a global priority, because they do not represent a good market for pharmaceutical companies and charitable foundations (Vélez et al, 2001).

Leishmaniasis is an NTD transmitted by vector sandfly mosquitoes of the genus *Lutzomyia*. The protozoan parasite of the genus *Leishmania* is injected by the vector into the human host. There are more than 20 species of *Leishmania* and, depending on the injected species, the infection can produce any of the three clinical manifestations: cutaneous, mucocutaneous, or visceral leishmaniasis. Cutaneous leishmaniasis is the most common of the three clinical presentations. Frequently, the clinical consequences include ulcers on the skin which leaves scars for life, disability, and/

or stigmatization of those who suffer from it. Mucocutaneous leishmaniasis is the consequence of parasite spread or untreated cutaneous leishmaniasis. It produces a partial or total destruction of mucosal membranes in the nose, mouth, and throat. The least common, but most deadly clinical presentation, is visceral leishmaniasis which, without proper treatment, can reach a mortality rate of 95% (OMS, 2020). *Leishmania (viannia) panamensis* is the most frequent species that causes leishmaniasis in Colombia, representing 79% percent of cases in the country (Ovalle et al, 2012). Ninety one percent of the Colombian population is at risk of suffering from leishmaniasis, making it a significant public health issue (Patiño-Londoño et al, 2017). Leishmania diagnosis is done by clinical examination, and direct (microscopy) and indirect (serological) laboratory tests.

Without a doubt, control, and prevention of leishmaniasis are the first and most efficient defense against this disease. However, the resources assigned to these programs are minimal, and health personnel in endemic zones lack the sufficient training to detect the real incidence of the disease. Diagnosis of leishmaniasis is essential to establish specific treatments and to limit progression of the disease, alleviate symptoms, and to improve the life quality of patients (Alvar et al, 2006).

Unfortunately, there is no gold standard for the diagnosis of leishmaniasis (Rodríguez-Cortés et al, 2010). The diagnostic tests that have shown high sensitivity are qPCR and definitive indirect tests, such as Indirect Immunofluorescence (IIF) and Enzyme-linked immunosorbent assay (ELISA). Unfortunately, these diagnostic tests can only be performed by specialized laboratories, located distantly from rural and rainforest areas where people are more likely to be exposed to the transmitting vector. In addition, only 1 in 10 infected people seek the appropriate treatment (Vélez et al, 2001).

Leishmaniasis rapid tests are commercially available and have proven to be reliable in different studies within Colombia. However, it has been demonstrated that the sensitivity and specificity of these tests depend enormously on the circulating strains in each population (Herrera et al, 2019). Therefore, it is necessary to create a portable, rapid, and efficient test to diagnose the disease. One approach to achieve this involves tests design with antigens of the circulating strains in each population to increase its diagnostic utility and implement biosensors that allow for the diagnosis of the disease based on stable molecules resistant to tropical temperatures.

Aptamers are small (70-120nt) single stranded nucleic acids that interact with molecules of biological or synthetic origin and have the potential to be developed as specific biosensors (Tuerk and Gold, 1990; Ellington and Szostak, 1990). These are also known as "*chemical antibodies*", and have considerable advantages over monoclonal antibodies, for example: (a) Their production does not require biological models and they are produced *in vitro* by a strategy called SELEX (**Se**lection of **L**igands by **Ex**ponential Enrichment), (b) their production is low cost, (c) they are scalabe without significant batch-to-batch variations, (d) they are resistant to extreme temperatures without losing function, which is ideal for tropical regions, and (e) they can recognize immunogenic and non-immunogenic targets (Ozalp et al, 2015; Zhu et al, 2015; Ospina-Villa et al, 2016; Ospina-Villa et al, 2018; Bayat et al, 2018; Ospina-Villa et al, 2020).

Currently, aptamer-based biosensors are being developed for the diagnosis of different parasitic infections, including trypanosomiasis, toxoplasmosis, trichomoniasis, malaria, and leishmaniasis with promising results (Ospina-Villa et al, 2020) and they have proven to be a real alternative to consider when creating specific biosensors. Using immunoproteomics, we previously identified potential biomarkers for *Leishmania (viannia) panamensis* that can be used in the diagnosis and treatment of leishmaniasis (Caraballo-Guzmán et al, 2021). These biomarkers were identified in circulating strains of afflicted patients in the Colombian territory, making them good candidates for the development of diagnostic tests. In addition, we collected sera samples from infected patients to identify new potential biomarkers presented in these samples. The objective of this work was to isolate DNA aptamers that can recognize purified biomarkers (recombinant proteins), and biomarkers present in the sera of patients infected with *L. panamensis* as a potential tool for developing a biosensor-type diagnostic test.

MATERIALS AND METHODS

Sample collection

Human sera samples were obtained from patients enrolled in a project carried out at the Instituto Colombiano de Medicina Tropical (Caraballo-Guzmán et al, 2021); An informed consent was obtained from all subject patients. A total of 20 patients participated with different NTDs manifestations and progressions: Thirteen patients with cutaneous leishmaniasis (CL), one patient with mucosal leishmaniasis (ML), three patients with Chagas diseases (CD), and three healthy subjects (HP). Leishmaniasis diagnosis was confirmed by direct identification of the parasite by microscopy of skin sample. Chagas disease diagnosis was confirmed by Indirect Immunofluorescence (IFI).

Sera proteins extraction

The sera of the patients in each group were mixed to make a pool. Total protein extraction was performed using the TRI reagent (Genbiotech), according to the manufacturer's protocol. Protein quantification was done-using the Bradford method and the integrity of the proteins was evaluated by SDS-PAGE.

Recombinant protein purification

Competent *E. coli* BL21 (DE3) pLysS bacteria and Lemo21 were transformed with the pRSET-A-LpD plasmid. The LpD was expressed with 1mM isopropyl beta-D-thiogalactopyranoside (IPTG) and purified by EconoFit Nuvia IMAC Column Ni-charged 1ml (BIORAD) using NGC Scout Plus 100 Chromatography System (BIORAD). The identity and integrity of the histidine-tagged LpD protein were confirmed by 10% (w/v) SDS-PAGE and western blot assays using anti-6×-His-tag antibodies (Roche) at a 1:5000 dilution and visualization of the reaction with (DAB) 3,3'-Diaminobenzidine (Sigma).

SELEX strategy

We used the protocol published by Wang et al, 2019. Briefly, we used library primers with a region of 40 random nucleotides flanked by two conserved sequences (5'- GTCTATATGATCTGTAACTC-N40-CCAGCAGTGAGTCATCAGAT-3'), a forward primer with a complementary region to the conserved 3' end of the library (5'-GTCTATATGATCTGTAACTC-3'), and a reverse primer with a complementary region to the conserved 5' end of the library (5'-ATCTGATGACTCACTGCTGG-3'). The library was amplified by PCR with forward and reverse primers with 2X PCR HotStart MasterMix with dye (ABM) as follows: 95°C for 2min; 95°C for 30sec, 58°C for 30sec, 72°C for 30sec; plus, a final extension step at 72°C for 10min during 6-12-18-24-40 cycles to identify the cycle with the higher amount of amplified without "*parasite DNA*" is obtained.

Then, 0.2nmol of the amplified library was heated to 95°C for 10min and then allowed to cool to room temperature to promote refolding. Then, was put in contact with previously sensitized proteins (0.1nmol) in 200µl of Selex buffer (pH 7.4 PBS containing 2.5mM $MgCl_{2,}$ Tween20 0.02% and Heparin 1mM in 1l of miliQ water) for 1hr at 37°C to promote interaction between ssDNA and proteins. Four washes were performed with Selex buffer. Bound ssDNA molecules were eluted with 50µl of water at 95°C by 5min, the resulting ssDNA fragments were PCR-amplified using forward and reverse primers with the protocol described above for 18 cycles. Ten rounds of selection (R10) were performed to select aptamers with an affinity for the LpD protein, and the pool of proteins extracted from sera of LC and LM patients.

SELEX negative rounds

After 10 rounds of selection by SELEX strategy, 3 rounds of negative selection were carried out for each group in duplicate, against 100µgm/ml of proteins extracted from sera of healthy patients, proteins extracted from patients with Chagas disease and against empty wells. In this case, the unbound molecules were recovered and then sent for sequencing.

Next-generation sequencing (NGS)

ssDNA pools from SELEX R10 were sent to be sequenced at Admera Health Biopharma Services by Illumina MiSeq 2x250 equipment. In brief, the workflow for the preparation of indexed libraries; barcodes are introduced at the ligation step using the commercial adaptors.

Sequencing data analysis

The analysis of NGS-data results can be divided into several stages: data pre-processing, primary enrichment analysis, library clustering, and a binding motif search. Obtained data were pre-processed using cutadapt 2.445 software to identify and cut the constant 5' and 3' SELEX primer binding regions. Every sequence had to consist of the 5' end constant region 'GTCTATATGATCTGTAACTC', a random region of 40nt, and the 3' end constant region 'CCAGCAGTGAGTCATCAGAT'. An error rate of 20% (≤4nt mismatches) within the constant regions was allowed. Sequences not fulfilling these conditions were discarded. Next, FastaQC was used to filter sequences by quality score, discarding all sequences with an overall Phred quality score ≤ of 30. Fastaptamer was also used to determine the abundance of each sequence in the population (Fastaptamer_count) and fold-enrichment to select unique sequences for each group (Fastaptamer_enrichment). GLAM2 software was used to search motifs in the top 5 sequences for each group and, finally, RNAFold online software was used to predict 2D structures of selected aptamers.

Library Biotin labelling

ssDNA resulting from round 10 of SELEX were labelled with biotin in the 3' end for 30min at 37°C using Biotin 3' End DNA Labelling Kit (PIERCE) according to the manufacturer's instructions.

Direct ELASA

The proteins extracted from the sera of the patients (CL, ML, CD and NP) was immobilized in 96-well microtiter plates (NUNC) diluted in 0.1M carbonate buffer at a pH of 9.6 (Na_2CO_3 0.159 gm/100ml; $NaHCO_3$, 0.293gm/100 ml) and 100µl was added per well. The proteins were incubated overnight at 4°C as previously reported (Rotherham et al, 2012; Luo et al, 2013)**.** The plate was washed with Selex buffer, and wells were blocked with 3% (w/v) skim milk. Then, 0.2nmol of biotinylated aptamers R10 (100µL) were added and incubated for 1hr at 37ºC. After washing, streptavidin-HRP-conjugate (Jackson ImmunoResearch, 1µg/ml) 1:1000 was added and incubated for 60min. After washing the plate three-times using Selex buffer containing 0.02% (w/v) Tween 20, UV emission was performed using Quantablue Fluorogenic Peroxidase as a substrate and stop solution according to manufacturer's protocol (Thermo Scientific, USA), The measure of relative fluorescence units (RFU) absorbance was carried out at 325nm and 420nm using xMark Microplate Spectrophotometer (BioRAD, USA).

Western blotting

Proteins extracted from the serum of the different groups of patients and recombinant D protein were loaded in a 12% (w/v) SDS-PAGE. Then proteins were transferred to nitrocellulose membranes using Towbin buffer (trizma base and glycine) by semi-dry transfer method. Subsequently, the membranes were washed three times for 5min in 1x TBS with Tween-20 pH 7.4 (TBST) and finally blocked with TBS plus 5% (w/v) skim milk at 4°C overnight. To identify immunoreactive proteins, the membranes were cut into 4mm strips. The strips were incubated for 60min at room temperature with biotinylated aptamers from R10. After incubation, the strips were washed three times for 5min with TBST and incubated with a 1:2000 dilution of the secondary streptavidin-HRP-anti-human IgG antibody produced in rabbits (Sigma-Aldrich, USA) in TBS buffer with skim milk 1% (w/v) at room temperature for 1hr. The strips were then washed three times for 5min with TBST. Quantablue Fluorogenic Peroxidase was used as substrate (Thermo Scientific, USA) for 5-10min until the reaction changed color. Finally, the stop solution was added per the manufacturer's protocol.

Kd value determination

Each well was saturated with 100nM of LpD in a total volume of 100µl in bicarbonate Buffer overnight. Then, dif-

ferent concentrations (0nM, 10nM, 20nM, 50nM, 100nM, 200nM, 500nM and 1000nM) of each of the biotinylated aptamers (PD1, PD2, PD3) were added, and the interaction was allowed at room temperature in SELEX buffer for 1hr. 3 washes were carried out with Wash Buffer, and streptavidin-HRP-conjugate (Jackson ImmunoResearch, 1µg/ml) 1:1000 was added and incubated for 60min, 3 more washes were carried out and the reaction was revealed using Quantablue Fluorogenic Peroxidase as a substrate and stop solution according to manufacturer's protocol (Thermo Scientific, USA), The measure of relative fluorescence units (RFU) absorbance was carried out at 325nm and 420nm using xMark Microplate Spectrophotometer (BioRAD, USA).

Protein-aptamer Docking

3D structure of LpD protein was predicted using online software I-Tasser (Zhang et al, 2008; Roy et al, 2010; Yang et al, 2015). Top 1 ssDNA 3D structure from the PD group was predicted. First, ssRNA 2D structure was obtained using RNAFold WebServer (Gruber et al, 2008; Lorenz et al, 2011). Then, ssRNA 3D structure was obtained using RNAComposer web server (Popenda et al, 2012; Antczak et al, 2016;). Next, we used Web 3DNA 2.0 (Li et al, 2019) to exchange uracil bases for thymine in the PDB file. We also replaced the ribose sugar backbone with deoxyribose using the AutoPSF VMD plugin (Humphrey et al, 1996). Finally, we performed an energy minimization using the AutoIMD VMD plugin to enhance the final ssDNA 3D structure (Humphrey et al, 1996).

Once the PDB files of the LpD protein and the selected aptamer were obtained, the protein-ssDNA docking was performed in the HDOCK online software with the parameters recommended by default (Yan et al, 2017). The model with the highest ranking and lowest interaction energy was analyzed with the Chimera 1.15 software (Pettersen et al, 2004) to identify the amino acids of the protein and the ssDNA bases involved in the interaction. The possible formation of hydrogen bonds with the FindHBond intra-molecule functionality was also searched.

RESULTS

Protein purification

Proteins from sera samples for the each distinct group were purified and the concentrations obtained were: 650,25µg/ml of CL group, 656,56µg/ml of ML group, 580,80µg/ml of CD group and 814,39µg/ml of HP group. From the LpD recombinant protein, 119µg/ml was obtained from 100ml of initial culture.

Proteins were separated in 10% (w/v) SDS-PAGE to verify their integrity (Figure 1A, 1C). The chromatogram of the recombinant protein shows a peak of pure protein (Figure 1B) which was previously confirmed by electrophoresis (Figure 1C) and western blotting (Figure 1D).

SELEX strategy

100µg/ml of each group of proteins (CL, ML and PD) were sensitized in 96-well plates in duplicate and the SELEX strategy was carried out as explained above. Non-bound molecules (NBM), bound molecules (elution) and subsequently amplified by PCR molecules (PCR) were monitored by quantification (Nanodrop) through the different rounds of SELEX strategy. Figure 2A, 2B, 2C show how the bound and amplified molecules remain stable between 600-1000ng/µl. As expected, the unbound molecules decrease as the SELEX strategy advances, and the bound molecules (elution) progressively increase, giving a considerable peak from cycle 7 for CL and PD, and from cycle 8 for ML. This increase in bound molecules was used as an indicator to determine the end of the SELEX strategy when the value remains stable. 10 rounds of SELEX strategy were performed for each group: CL (Figure 2A), ML (Figure 2B), and PD (Figure 2C). A schematic representation of SELEX strategy and the monitoring that was carried out is illustrated in Figure 2D.

NGS analysis

After filtering the sequences as mentioned above with the cutadapt software, there were 804932 reads from the CL files, 648455 reads from ML and 657949 reads from the PD

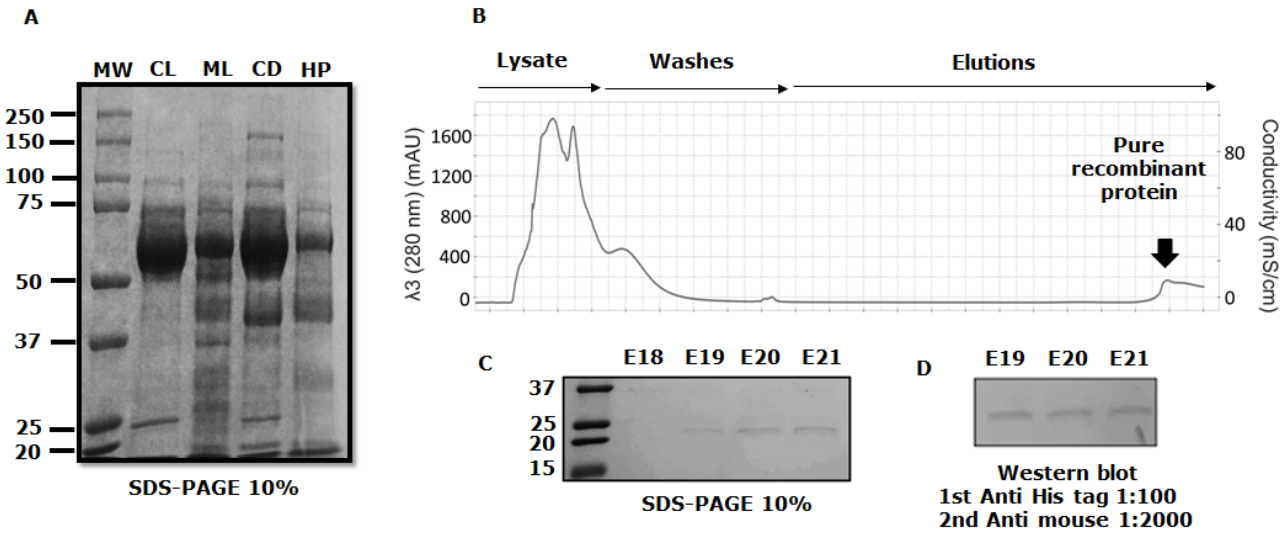


Figure 1. Protein purification. (**A**) Proteins extracted from sera samples were visualized in 10% (w/v) SDS-PAGE. (**B**) chromatogram diagram of pRSET-A-LpD recombinant protein. (**C**) Verification of pRSET-A-LpD recombinant protein of the expected size on a 10% (w/v) SDS-PAGE. (**D**) Western blot assay to confirm pRSET-A-LpD recombinant protein expression.

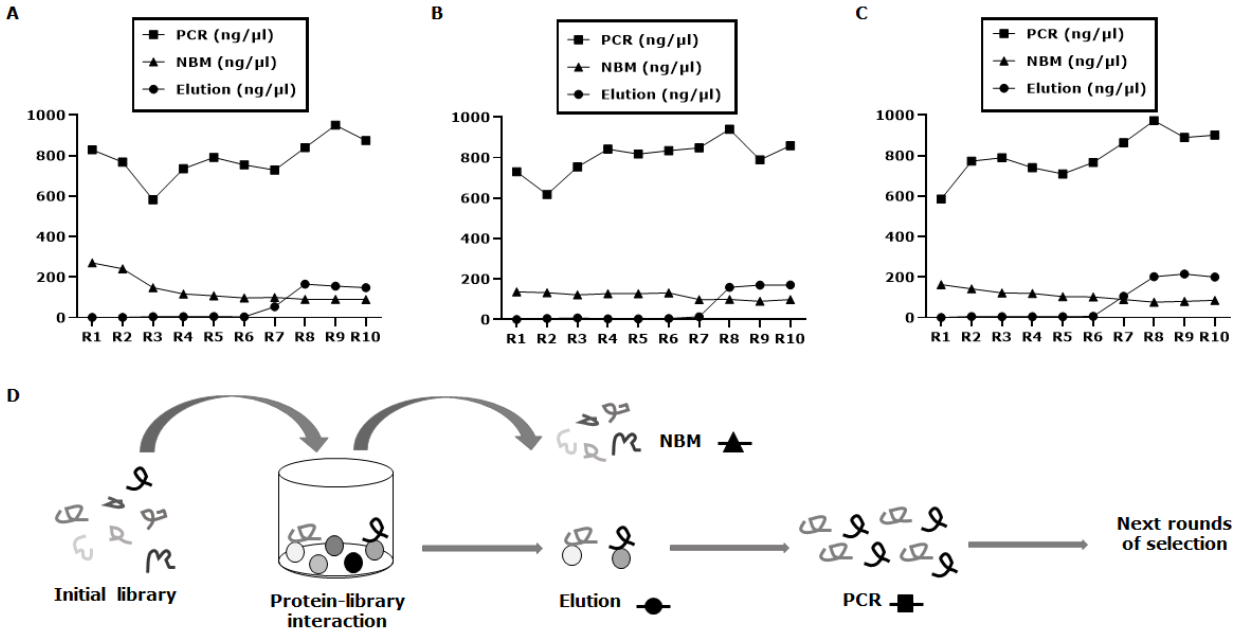


Figure 2. SELEX strategy. Values were obtained from non-bound molecules (NBM), bound molecules (elution) and amplified molecules (PCR) after each round of the SELEX strategy for the CL (**A**), ML (**B**) and PD (**C**) groups. (**D**) Schematic representation of the SELEX strategy used in this study.

files. The frequency of each sequence in each groups were determined using the fastaptamer_count script. The fold-enrichment values for each sequence in each group was then calculated with the fastaptamer_enrich script. Next, a file was created containing the unique sequences for each of the groups. The five most frequent sequences were chosen according to the RPM value.

In Figure 3, we show the top 5 sequences chosen for each group: CL (Figure 3A), ML (Figure 3E), and PD (Figure 3I), as well as the ranking, the number of reads, and RPM for each sequence are displayed there. Then, uing GLAM2 software, a search for motifs was performed allowing gaps that were highlighted in each of the sequences and illustrated in Figure 3B (CL), Figure 3F (ML), and Figure 3J (PD). The 2D and 3D structure of the most abundant sequence in each group was predicted using w3DNA 2.0 online software.

ELASA

The sequences obtained from round 10 of SELEX were biotinylated and subsequently used as recognition molecules in an ELASA-type assay in which the specific proteins for each group were previously sensitized. After the interaction between aptamers and proteins, streptavidin HRP (Horseradish Peroxidase) was added, and the reaction was revealed. Experiments were performed in triplicate to validate the results. Figure 4A shows the development of the ELASA test by means of a UV emitter at 320nm. CL biotinylated aptamers specifically recognize CL proteins and 1 to 13 sera from patients with previously confirmed CL with different intensities and different optical density values. However, they were all positive as they exceed the set cut-off value.

The aptamers do not recognize the ML, PD, HPS (Healthy Patient Serum), HPP (Healthy Patient Proteins), or BSA (Bovine Serum Albumin) proteins, neither the patients with Chagas disease 14-17 nor the patient with ML (18), yielding optical density values below the established cut-off. ML biotinylated aptamers specifically recognize ML proteins and the patient with mucosal leishmaniasis (18). The same results were obtained with PD biotinylated aptamers.

Western bloting

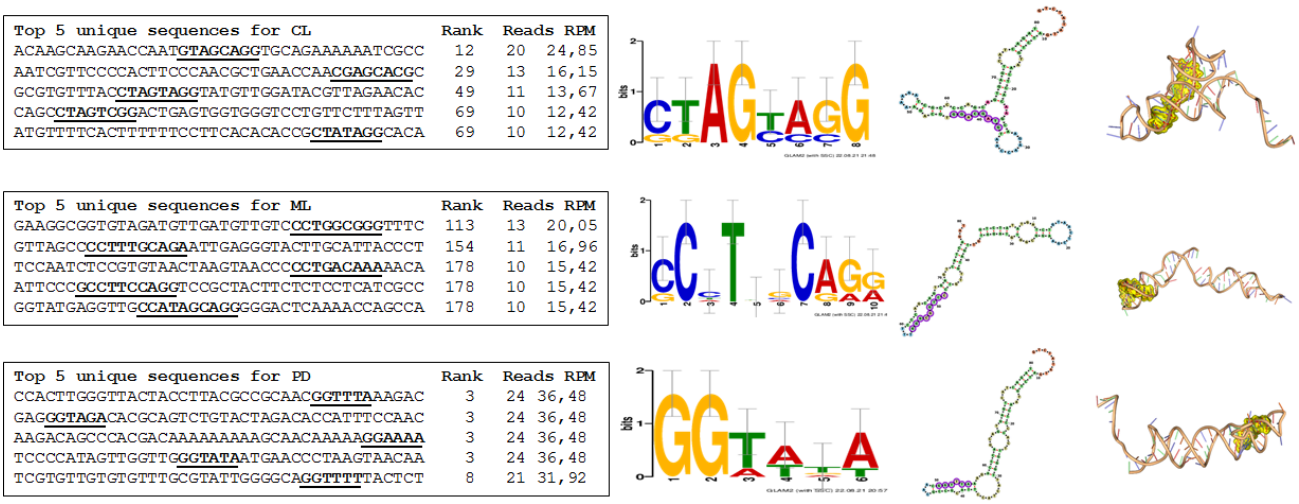
In the western blot assay (Figure 4B), 3 bands recognized by CL biotinylated aptamers are observed around at 37kDa, 75kDa and 25kDa. ML biotinylated aptamers recognize 4 bands around at 25kDa, 50kDa, 75kDa and 250kDa. Biotinylated PD aptamers recognize a single band of 18kDa that corresponds to the expected molecular weight for recombinant protein D.

Kd values

The Kd values obtained were; 51.83±8.725nM, 63.38±9.863nM and 84.85±15.87nM for aptamers PD1, PD2 and PD3, respectively (Figure 5).

Protein-aptamer Docking

Results obtained from the HDOCK server show how the LpD protein interacts with the PD-1 aptamer in the variable region (40nt) (Figure 6A). Analysis performed in Chimera 1.15 software shows the formation of 2 hydrogen bonds, between the C48 base and the Gly86 (2,584Å) and between Glu119 and the G50 base (2,784Å) (Figure 6B). The G50 base is part of the previously identified motif among the aptamers with the highest amount of RPM, which confirms the importance of this motif for the recognition of LpD.



Top 5 unique sequences for CL	Rank	Reads	RPM
ACAAGCAAGAACCAATGTAGCAGGTGCAGAAAAAATCGCC	12	20	24,85
AATCGTTCCCCACTTCCCAACGCTGAACCAACGAGCACGC	29	13	16,15
GCGTGTTTACCTAGTAGGTATGTTGGATACGTTAGAACAC	49	11	13,67
CAGCCTAGTCGGACTGAGTGGTGGGTCCTGTTCTTTAGTT	69	10	12,42
ATGTTTTCACTTTTTTCCTTCACACACCGCTATAGGCACA	69	10	12,42

Top 5 unique sequences for ML	Rank	Reads	RPM
GAAGGCGGTGTAGATGTTGATGTTGTCCCTGGCGGGTTTC	113	13	20,05
GTTAGCCCCTTTGCAGAATTGAGGGTACTTGCATTACCCT	154	11	16,96
TCCAATCTCCGTGTAACTAAGTAACCCCCTGACAAAAACA	178	10	15,42
ATTCCCGCCTTCCAGGTCCGCTACTTCTCTCCTCATCGCC	178	10	15,42
GGTATGAGGTTGCCATAGCAGGGGGACTCAAAACCAGCCA	178	10	15,42

Top 5 unique sequences for PD	Rank	Reads	RPM
CCACTTGGGTTACTACCTTACGCCGCAACGGTTTAAAGAC	3	24	36,48
GAGGGTAGACACGCAGTCTGTACTAGACACCATTTCCAAC	3	24	36,48
AAGACAGCCCACGACAAAAAAAAAGCAACAAAAAGGAAAA	3	24	36,48
TCCCCATAGTTGGTTGGGTATAATGAACCCTAAGTAACAA	3	24	36,48
TCGTGTTGTGTGTTTGCGTATTGGGGCAGGTTTTTACTCT	8	21	31,92

Figure 3. SELEX Libraries sequencing results, motifs and 2D and 3D structure prediction of DNA aptamers. (**A**) Top 5 of unique sequences for each group (**B**) A common motif for each group (**C**) 2D structure of more abundant unique sequence of each group (**D**) 3D structure of more abundant unique sequence of each group.

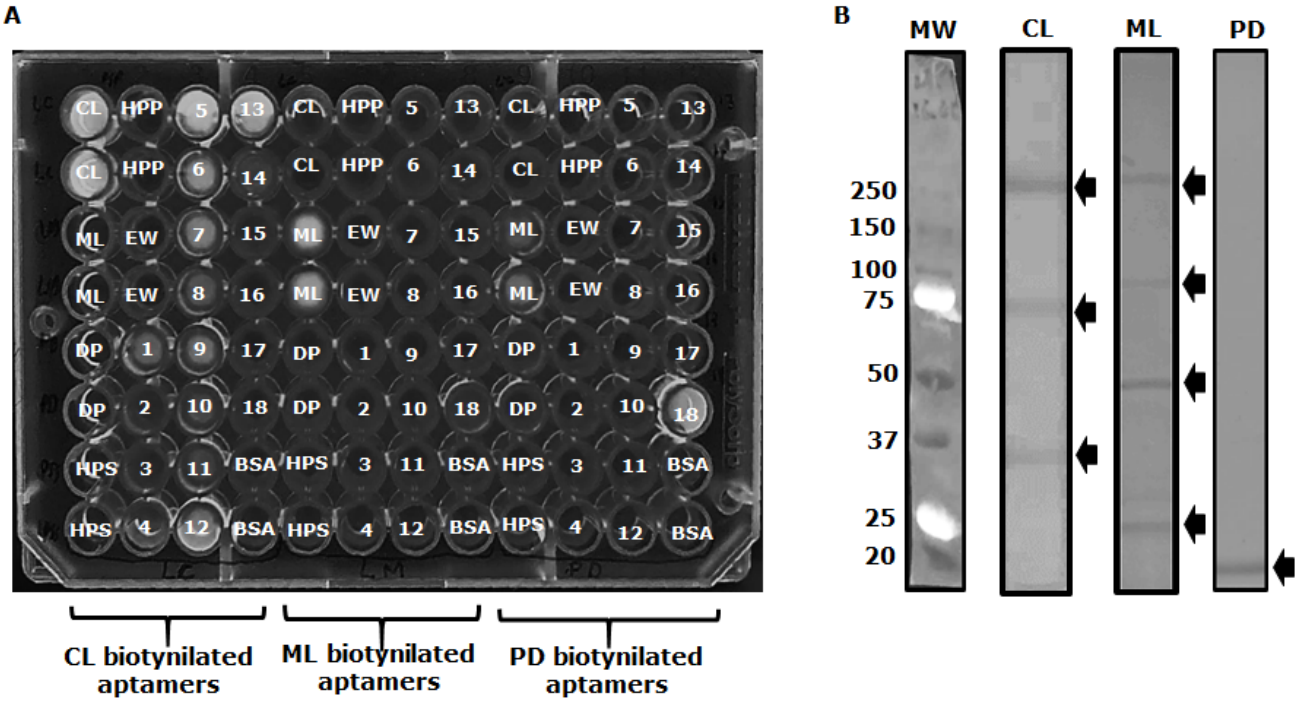


Figure 4. ELASA and western blot assay. (**A**) CL: cutaneous leishmaniasis proteins, ML: mucosal leishmaniasis proteins, DP: D protein, HPS: healthy patient serums, HPP: healthy patient proteins, EW: empty well, 1-13 sera of patients with cutaneous leishmaniasis confirmed, 14-17 sera of patients with chagas disease confirmed, 18 sera of a patient with mucosal leishmaniasis, BSA: Bovine serum albumin protein (**B**). MW: molecular weight, CL: cutaneous leishmaniasis proteins, ML: mucosal leishmaniasis proteins, DP: LpD protein. The black arrows show the more significant bands observed on each strip.

DISCUSSION

The results of this work show for the first time the isolation of DNA aptamers from sera samples of patients with cutaneous and mucosal leishmaniasis that identify specific biomarkers that could be parasite proteins or host proteins in response to infection. In addition, for the first time, DNA aptamers were isolated against the recombinant protein LpD previously identified as a potential biomarker for the diagnosis of mucosal leishmaniasis. (Caraballo-Guzmán et al, 2021).

Different studies led by Dr Victor M Gonzalez from the IRYCIS group have isolated aptamers against different targets of *Leishmania infantum*, such as the H3 protein (Frezza et

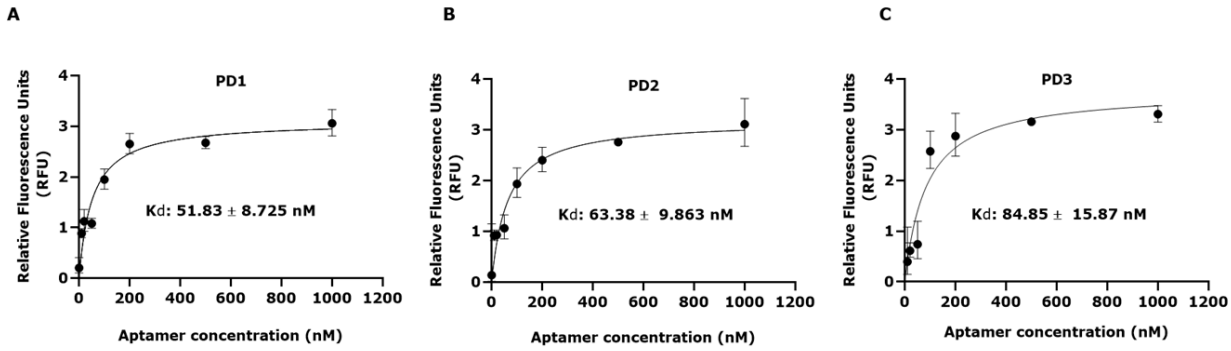


Figure 5. Kd values of LpD specific aptamers. (**A**) PD1 aptamer Kd value. (**B**) PD2 aptamer Kd value. (**C**) PD3 aptamer Kd value.

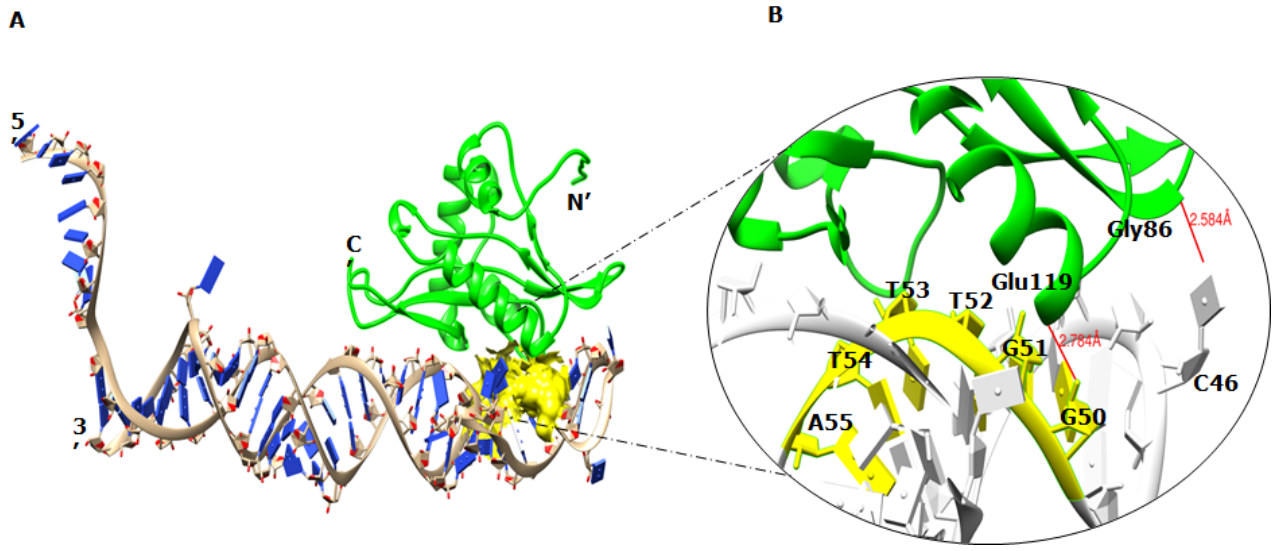


Figure 6. Docking protein-aptamer. (**A**) PDB structure obtained from LpD complex and PD-1 aptamer using HDOCK software**.** (**B**) Close-up of the interaction site where the formation of hydrogen bridges is illustrated (red color) with their respective distances C46-Gly86 (2,584 A) and G50-Glu119 (2,784A).

al, 2020), the KMP-11 membrane protein (Moreno et al, 2003), the H2A protein (Martín et al, 2013), and the LiPABP protein (Guerra-Pérez et al, 2015). All aptamers obtained have great utility for the specific detection of related proteins, and therefore have potential to be used as diagnostic tools for leishmaniasis.

The use of aptamers as a ligand for the detection of biomarkers has proven to be an efficient strategy for the diagnosis of other parasitic diseases such as Chagas disease caused by the protozoan parasite *Trypanosoma cruzi*. Fortes de Araujo et al, have reported an ELA assay to detect residual parasitemia in infected mice that can be very useful for diagnosis and to evaluate the efficiency of treatment with benznidazole (de Araujo et al, 2015). Similarly, aptamers have been obtained against malaria biomarkers (PfLDH and PvLDH) (Cheung et al, 2018), *Trichomonas vaginalis* (AP65) (Espiritu et al, 2018), and *Cryptosporidium parvum* (oocyst) (Iqbal et al, 2019) with great potential for the development of novel diagnostic tools.

The set of aptamers reported here can identify specific biomarkers (unidentified until now) from patients with cutaneous leishmaniasis and mucosa as shown in the ELASA assay (Figure 3A). Western blot analysis showed that at least 3 different targets were identified in the CL group and 4 in the ML group; the LpD protein was the only expected target (Figure 3B). The Kd values obtained for PD1, PD2 and PD3 aptamers are in a low nanomolar range, which makes them suitable for the development of a portable diagnostic test.

The information reported in this study is convenient for the scientific community interested in the development of aptamers and alternative diagnostic tools and biosensor for NTDs and other diseases. We are still studying the interactions of each of the reported aptamers to develop a device that allows easy use of this technology that can be taken into the field.

CONCLUSIONS

- We have identified a set of aptamers that recognized unknown biomarkers in serum samples from patients with cutaneous and mucosal leishmaniasis.

- These aptamers recognize at least 3 different biomarkers in the CL group and 4 in the ML group.
- Isolated aptamers against the LpD protein are a potential tool for the diagnosis of mucosal leishmaniasis
- The GG (T/A) (A/T) (T/A) (A/T) motif identified in the PD group aptamer set seems to be relevant for the interaction with the LpD protein.
- PD1, PD2 and PD3 aptamers could be used for the development of a diagnostic test.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from the Ministerio de Ciencia, Tecnología e innovación de Colombia. Convocatoria 848 (Programa de Estancias Postdoctorales en entidades del SNCT 2019. CT 80740–171–2020) and convocatoria 843 de 2019 (Puesta a punto de una plataforma de proteínas recombinantes para el desarrollo de pruebas diagnósticas. code: 325684368703).

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

NTD: **N**eglected **T**ropical **D**iseases
PCR: **P**olymerase **C**hain **R**eaction
ssDNA: **S**ingle-**S**tranded **D**eoxyribo**N**ucleic **A**cid
ssRNA: **S**ingle-**S**tranded **R**ibonucleic **A**cid
SELEX: **S**ystematic **E**volution of **L**igands by **Ex**ponential **E**nrichment
NGS: **N**ext-**G**eneration **S**equencing
IIF: **I**ndirect **I**mmuno**F**luorescence
ELASA: **E**nzyme-**L**inked **A**ptamer **S**orbent **A**ssay
NBM: **N**on-**B**ound **M**olecules

REFERENCES

Alvar J, Yactayo S and Bern C. 2006. Leishmaniasis and poverty. Trends Parasitol, 22, 552-557.

Antczak M, Popenda MM, Zok T, et al. 2016. New functionality of RNAComposer: an application to shape the axis of miR160 precursor structure, Acta Biochim Pol, 63, 737-744

Bayat P, Nosrati R, Alibolandi M, et al. 2018. SELEX methods on the road to protein targeting with nucleic acid aptamers. Biochimie, 154, 132-155.

Caraballo-Guzmán A, Ospina-Villa JD, Cuesta-Caicedo AP, and Sánchez-Jiménez MM. 2021. Immunoproteomics characterization of *Leishmania panamensis* proteins for potential clinical diagnosis of mucosal Leishmaniasis. Parasite Immunol, 43, e12824.

Cheung YW, Dirkzwager RM, Wong WC, Cardoso J, D'Arc Neves Costa J and Tanner JA. 2018. Aptamer-mediated Plasmodium-specific diagnosis of malaria. Biochimie, 145, 131-136.

de Araujo FF, Nagarkatti R, Gupta C, Marino AP and Debrabant A. 2015. Aptamer-based detection of disease biomarkers in mouse models for chagas drug discovery. PLoS Negl Trop Dis. 29, e3451.

Ellington AD and Szostak JW. 1990. In vitro selection of RNA molecules that bind specific ligands. Nature. 30, 818-822.

Engels D and Zhou X. 2020. Neglected tropical diseases: an effective global response to local poverty-related disease priorities. Infect. Dis. Poverty, 28, 10.

Espiritu CAL, Justo CAC, Rubio MJ, et al. 2018. Aptamer Selection against a Trichomonas vaginalis Adhesion Protein for Diagnostic Applications. ACS Infect Dis. 4, 1306-1315.

Frezza V, Pinto-Díez C, Fernández G, et al. 2020 DNA aptamers targeting Leishmania infantum H3 protein as potential diagnostic tools. Anal Chim Acta, 22, 155-163.

Gruber AR, Lorenz R, Bernhart SH, Neuböck R, and Hofacker IL. 2008. The Vienna RNA Websuite. Nucleic Acids Res, 36, 70-74.

Guerra-Pérez N, Ramos E, García-Hernández M, et al. 2015. Molecular and Functional Characterization of ssDNA Aptamers that Specifically Bind Leishmania infantum PABP. PLoS One, 10, e0140048.

Herrera G, Castillo A, Ayala MS, Flórez C, Cantillo-Barraza O and Ramírez JD. 2019. Evaluation of four rapid diagnostic tests for canine and human visceral Leishmaniasis in Colombia. BMC Infect Dis, 19, 747.

Humphrey WW, Dalke A and Schulten K. 1996. "VMD - Visual Molecular Dynamics", J Molec Graphics, 14, 33-38.

Iqbal A, Liu J, Dixon B, Zargar B and Sattar SA. 2019. Development and application of DNA-aptamer-coupled magnetic beads and aptasensors for the detection of Cryptosporidium parvum oocysts in drinking and recreational water resources. Can J Microbiol, 65, 851-857.

Li S, Olson WK and Lu XJ. 2019. Web 3DNA 2.0 for the analysis, visualization, and modeling of 3D nucleic acid structures. Nucleic Acids Res, 47, 26-34.

Lorenz R, Bernhart, SH, Höner zu Siederdissen C, et al. 2011. "ViennaRNA Package 2.0". Algorithms Mol Biol, 6, 1- 26.

Martín ME, García-Hernández M, García-Recio EM, Gómez-Chacón GF, Sánchez-López M, and González VM. 2013. DNA aptamers selectively target Leishmania infantum H2A protein. PLoS One, 8, e78886.

Moreno M, Rincón E, Piñeiro D, et al. 2003. Selection of aptamers against KMP-11 using colloidal gold during the SELEX process. Biochem Biophys Res Commun, 308, 214-8.

Organización Mundial de la Salud (OMS). 2020. Poner fin a la desatención para alcanzar los Objetivos de Desarrollo Sostenible: una hoja de ruta para las enfermedades tropicales desatendidas 2021-2030.

Ospina-Villa JD, López-Camarillo C, Castañón-Sánchez CA, Soto-Sánchez J, Ramírez-Moreno E and Marchat LA. 2018. Advances on Aptamers against Protozoan Parasites. Genes (Basel), 28, 584.

Ospina-Villa JD, Zamorano-Carrillo A, Castañón-Sánchez CA, Ramírez-Moreno E and Marchat LA. 2016. Aptamers as a promising approach for the control of parasitic diseases. Braz J Infect Dis, 20, 610-618.

Ospina-Villa JD. Los aptámeros como novedosa herramienta diagnóstica y terapéutica y su potencial uso en parasitología. 2020. Biomedica, 40, 148-165.

Ospina-Villa, JD, Cisneros-Sarabia A, Sánchez-Jiménez MM and Marchat LA. 2020. Current Advances in the Development of Diagnostic Tests based on Aptamers in Parasitology: A Systematic Review. Pharmaceutics, 12, 1046.

Ovalle C, Porras L, Rey M, Ríos M and Camargo Y. 2012. Distribución geográfica de especies de Leishmania aisladas de pacientes consultantes al Instituto Nacional de Dermatología Federico Lleras Acosta, E.S.E., 1995-2005. Biomédica, 26, 145.

Ozalp VC, Kavruk M, Dilek O and Bayrac AT. 2015. Aptamers: molecular tools for medical diagnosis. Curr Top Med Chem, 15, 1125-1137.

Patiño-Londoño S, Salazar L, Tovar-Acero C, and Vélez-Bernal I. 2017. Aspectos socioepidemiológicos y culturales de la leishmaniasis cutánea: concepciones, actitudes y prácticas en las poblaciones de Tierralta y Valencia, (Córdoba, Colombia). Salud Colectiva, 13, 123.

Pettersen EF, Goddard TD, Huang CC, et al. 2004. UCSF Chimera-a visualization system for exploratory research and analysis. J Comput Chem, 25, 1605-1612.

Popenda M, Szachniuk M, Antczak M, et al. 2012. Automated 3D structure composition for large RNAs, . Nucleic Acids Res, 40, e112.

Rodríguez-Cortés A, Ojeda A, Francino O, López-Fuertes L, Timón M and Alberola J. 2010. Leishmania infection: laboratory diagnosing in the absence of a "gold standard". Am J Trop Med, 82, 251–256.

Roy A, Kucukural A and Zhang Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc, 5, 725-738.

Tuerk C and Gold L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science, 3; 505-510.

Vélez ID, Hendrick E, Robledo SM, et al. 2001. Leishmaniosis cutánea en Colombia y género. Cad Saude Publica, 17, 171-180.

Wang T, Yin W, AlShamaileh H, et al. 2019. A Detailed Protein-SELEX Protocol Allowing Visual Assessments of Individual Steps for a High Success Rate. Hum Gene Ther Methods, 30, 1-16.

Yan Y, Zhang D, Zhou P, Li B and Huang SY. 2017. HDOCK: a web server for protein-protein and protein-DNA/RNA docking based on a hybrid strategy. Nucleic Acids Res, 45, 365-373.

Yang J, Yan R, Roy A, Xu D, Poisson J and Zhang Y. 2015. The I-TASSER Suite: Protein structure and function prediction. Nat. Methods, 12, 7-8.

Zhang Y. I-TASSER server for protein 3D structure prediction. 2008. BMC Bioinformatics, 9, 40.

Zhu Q, Liu G and Kai M. 2015. DNA Aptamers in the Diagnosis and Treatment of Human Diseases. Molecules, 25, 20979-20997.