Cell-SELEX as an Approach to Develop Aptamer Molecules to Recognize *Leishmania mexicana*

Cárdenas David[1] and Marbel Torres Arias[1,2*]

[1]Departamento de Ciencias de la Vida y la Agricultura, Laboratorio de Inmunología y Virología, Maestría en Nanotecnología, Universidad de las Fuerzas Armadas ESPE

[2]Centro de Nanociencia y Nanotecnología, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Ecuador

ABSTRACT

The recognition of pathogen is a basic first step needed to move forward a treatment approach. Therefore, novel ways to identify parasites of *Leishmania mexicana* are needed to change the drawbacks of currents treatments. Aptamers are single stranded DNA or RNA molecules capable of target a specific protein or cell created during SELEX cycles. The aim of this investigation is to obtain DNA aptamers that recognize specifically *Leishmania mexicana* parasites using in silico approaches and Cell-SELEX technique for further use in the Leishmaniasis treatment. The cycles used to perform Cell-SELEX consist of exposure of the parasite culture to a DNA pool, binding, recovery and amplification of the bound sequences to the target through asymmetric PCR to recover back ssDNA pools to act as an aptamer for the parasite. Using variation of the SELEX conditions during every cycle created a robust aptamer pool able to recognize specifically *Leishmania mexicana*.

Keywords: Aptamers; Cell-SELEX; *Leishmania Mexicana*

[*]**Correspondence to**: Marbel Torres Arias, Laboratorio de Inmunología y Virología, CENCINAT, GISAH, Universidad de las Fuerzas Armadas, Sangolquí, Pichincha, Ecuador

Received: Sep 17, 2022; **Accepted:** Sep 23, 2022; **Published:** Sep 30, 2022

Citation: David C, Torres Arias M (2022) Cell-SELEX as an Approach to Develop Aptamer Molecules to Recognize Leishmania mexicana. J Nanomed Nanotech Nanomat. 3: 118.

INTRODUCTION

Aptamers are single stranded DNA (ssDNA) or RNA (ssRNA) molecules with a specific three dimensional structure capable to bind to a target molecule with high affinity [1]. These aptamer molecules can be used to create a bond with their target in a an antigen-antibody manner [2]. Aptamers have been developed through a specific technique called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) which consist of using a library of small ssDNA [3] or ssRNA [2] to recognize any specific molecule in several cycles refining its specificity [4].

The SELEX technique works with any known highly purified molecule and uses three steps in particular to be used [5].

The SELEX technique works with any known highly purified molecule and uses three steps in particular to be used [5]. First, the library and the target molecule are set in a favorable media to assist the binding. Second, only those ssDNA or ssRNA molecules that bind to the molecule are selected and captured. Third, the library is enriched of these molecules with the use of PCR reaction [6,7]. Before starting a SELEX cycle, *in silico* approaches

can be performed to determine a possible structure of the aptamer

molecule [8] or library pool to obtain aptamers for a specific target [9,10]. These approaches include docking, binding and spatial configuration of the molecules acting during the SELEX selection [11,12].

The SELEX technique has been improved to recognize a specific cell type using the exact same process but using complete cells instead of specific molecules [13]. The specific change of using complete cells instead of single molecules makes this technique faster and easier to apply to organisms causing any disease with unknown specific targeting sites [13,14]. Cell-SELEX is the variation of the SELEX technique to use it on specific organisms in order to target not a single molecule but the whole live cell [15].

Cell-SELEX has been used to produce specific aptamer molecules to recognize cancer cells [16,17], bacterial pathogens [18] and even parasitic infections [4,19]. This technique is not only used to recognize cells but also to study membrane capture of specific aptamers on any cell line through internalization mechanisms [20].

Leishmania is a known parasite from the class Kinetoplastea, in particular, causing a medical condition called as Leishmaniasis [21]. This disease spreads through sandflies in a zoonotic manner around the world more commonly in tropical areas Africa, Asia, America and Europe [22,23]. Leishmaniasis can be cutaneous, mucocutaneous and visceral depending on the organ the parasite affects creating ulcers in the area of infection [23], that can lead to severe damage at skin level or even dead for the visceral types [24].

A common study protein in Leishmania parasites is the surface protein GP63 which acts as the major surface protein that mediates the entrance of this parasite into macrophages [25,26]

In order to treat Leishmaniasis there are several drug models that have been proven to cure and kill the pathogen causing the disease [22,27]. Some of the treatments use a high dosage of antibiotics such as Paromomicyn Sulfate [23] and some Pentavalent antimonial compounds [27]. These antibiotic treatments have shown to produce several kidney and liver damage in the pacients [23]. For that reason, studies in Leishmaniasis have focused on alternative treatments and recognition methods of the parasite [27,28].

The aim of this investigation is to obtain DNA aptamers that recognize specifically *Leishmania mexicana* parasites using *in silico* approaches and Cell-SELEX technique for further use in the Leishmaniasis treatment.

MATERIAL AND METHODS

Maintenance of the cell culture: A cell culture of *Leishmania mexicana* was kept in culture during the whole experiment in sterile conditions at room temperature (25°C) with Schneider's media supplemented with 15% of Fetal Bovine Serum (FBS) in a suspension culture.

***In silico* approach and definition of the library:** A full sequence genome analysis was performed with the information of the National Center of Biotechnology Information and the Kinetoplastid Genomics Resource in order to search for matches in the GP63 structure in at least seven species of parasites including Leishmania Mexicana.

These GP63 structures were modelled using MC-Fold and MC-Sym algorithms to obtain a 3D structure of the analogs, and the minimum energy analogs where used for the next steps.

A molecular complement that could act as an aptamer molecule for the GP63 protein was analyzed considering docking of the molecule to the protein information in databases using PatchDock web server and AutoDock4 algorithm.

A full set of aptamer candidates were obtained and using specific alignment algorithm from Molecular Evolutionary Genetics Analysis (MEGA) platform a potential library for the selection of the aptamers.

The designated pool was chemically synthetized and purchased with the following sequence as a result of the *in silico* analysis performed

5'-
GCTAATACGACTCACTATAGGGAGATCACTTACG
GCACC(NNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNN)CCAAGGCTCGGGACAGCG-
3'

A set of primers able to recognize both ends of the pool were also chemically synthetized and purchased with the following sequences: Upstream primer: 5'-GCTAATACGACTCACTATAGGGAGATCACTTACG GCACC-3' and downstream primer: 5'-CGCTGTCCCGAGCCTTGG-3'

Reagent setup: A washing buffer was prepared adding 4.5g of glucose and 5mL of 1M MgCl to a liter of Dulbecco Phosphate Saline Buffer (DPBS) that can be stored up to 3 months at 4°C.

A binding buffer was prepared adding 4.5g of glucose, 1g of Bovine Serum Albumin (BSA) and 5mL of 1 M MgCl to 1 liter of DPBS that can be stored at 4°C for 1 month.

The library pool was eluted in a 0.5% EDTA solution to reach a 0.5mM concentration of the pool and it was tested out with an electrophoretic run with agar at 3%. Just before starting the experiment 20 µL of the prepared library pool was mixed with 350 µL of binding buffer and heated at 95°C for 5 minutes. After this time, the pool was placed and kept in ice until use.

Cell preparation: The cell culture was counted, and an approximate of 10 million *Leishmania mexicana* parasites was poured in a 15mL centrifuge tube with a cell viability of 95%. The cell culture was centrifuged at 300g for 3 minutes at 4°C. The supernatant was removed and 3 mL of washing buffer was added. The cell line was mixed by pipetting and the washing protocol was repeated 2 more times.

Cell-SELEX: SELEX is a cyclic procedure. The first selection cycle is described next.

Once the cell culture was washed of any additional media the cell pellet was resuspended with 330 µL of binding buffer. All the 370 mL of the DNA pool with the binding buffer was also added to the cell suspension and it was incubated at 4°C in a rotatory shaker for 1 hour. After incubation, the cells were centrifuged at 300g for 3 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 3mL of washing buffer mixed by soft tube inversion movements and centrifuged again with the same conditions. The supernatant was removed carefully with a transfer pipette avoiding cell loss. Any residual buffer was spun down and removed with folded kimwipe. This pellet washing protocol was performed two additional times for a total of three washings.

To elute the bound sequences a total of 500 µL DNase-free water was added to the cell pellet and mixed by pipetting. This mixture was transferred to a 1.5mL microfuge tube. This cell mixture was heated at 95°C for 10 minutes and centrifuged at 13,100g for 5 minutes. The supernatant was collected and stored a -20°C

A PCR reaction was prepared using GoTaq® Colorless Master Mix (Promega) thawed at room temperature with the conditions shown in table 1 to perform an asymmetric PCR with three different concentrations of downstream primer in 3 PCR tubes for each concentration with a control tube with additional water instead of Eluted DNA.

Reagents	Reaction mixture (µL)
GoTaq® Colorless Master Mix, 2X	25
Upstream primer, 10 µM	5
Downstream primer, 10 µM	0.1, 0.5 or 1
Eluted DNA	5
Nuclease-Free Water to	50

Table 1: Asymmetric PCR conditions per tube.

The asymmetric PCR amplification with the primer set described before was performed using the PCR program shown in table 2.

These PCR amplicons are labeled as the first selected pool

In order to determine the optimum number of cycles for the preparative PCR the first selected pool was amplified under the conditions shown in table 1 with a control tube using a supernatant of pure cell lysate instead of the first selected pool. A total of 12 PCR tubes were used to vary the cycles between 3, 6, 9 and 12 and the same variation of downstream primer used before in a factor model fashion.

Step	Temperature (°C)	Time (s)
Initial denaturation	95	150
Amplification (10 cycles)		
Denaturation	95	30
Annealing	61	30
Extension	72	30
Final extension	72	180
Hold	4	

Table 2: PCR program.

After this PCR reaction using the conditions shown in table 2. A 3% agarose gel with SybrSafe was prepared and the lanes were charged 2 µL of BlueJuice loading Dye mixed with 8 µL of each sample. The electrophoretic conditions were 100V, 100mA for 1 hour.

The gel was revealed and the best setting were chosen from the results as 3 cycles at 0.5 µL of downstream primer.

With the selected conditions from the first selected pool a total of 100 µL was amplified with all the previous conditions shown as before but this time with exact 0.5 µL of downstream primer and 3 additional cycles in the amplification stage of the preparative PCR. The PCR product was cleaned with Wizard® SV Gel and PCR Clean-Up System for PCR products and measured its concentration by UV absorbance at 260nm in a Nanodrop 2000 spectrophotometer (Thermo Fisher). This pool was labeled as First Cycle.

For the second round of selection the whole setup was performed with a total of 5 million cells. Same washing steps described before. 200 µL of First Cycle DNA pool with 200 µL of cell suspension for incubation at room temperature this time. Last mixing with binding buffer was done for a total of 600 µL. And the asymmetric PCR conditions selected with 0.5 µL of downstream primer with 3 additional cycles in the preparative PCR.

This exact procedure was repeated three more times for a total of five complete cycles leaving with an aptamer pool able to identify Leishmania Mexicana that can be stored at -20°C for 6 months or lyophilized for further storage. The complete Cell-SELEX protocol can be seen in figure 1, as summarized graphic way.

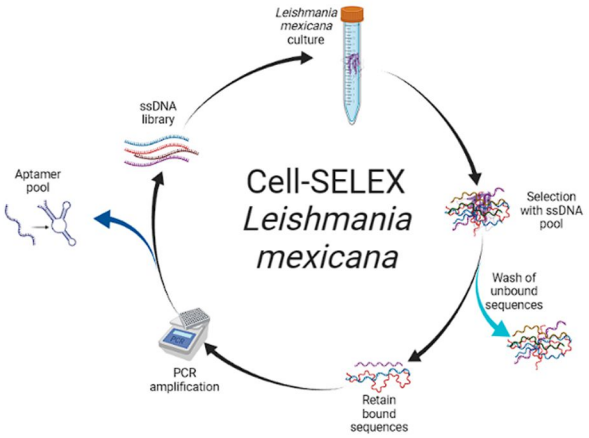

Figure 1: Graphical description of Cell SELEX protocol.

RESULTS AND DISCUSSION

The *in silico* approach derived several probable aptamers that could bind specifically with the surface glycoprotein GP63 which is the most common surface protein in Leishmania genus [29]. The probability of binding simulated and correlated with a protein simulation of GP63 giving a binding probability of 0.9051 with a total of 1344 nm^2 of contact with the protein at the lowest possible energy. Since the lowest energy model is more prone to derive in a real aptamer [8,10,30], this was the one selected to create a random DNA pool to search for it in live studies using a suspension culture of *Leishmania mexicana*.

During the first round of Cell-SELEX cycle a total of 10 million cells [14,31] were used in order to increase the binding sites for the ssDNA library pool and get the highest possible number of working aptamers attached to the cells [31]. The initial incubation temperature used for the first selection cycle of 4°C was performed to avoid the DNA sequences to internalize the cell membrane during the incubation process and keep the number of potential aptamers as high as possible [31]. During the next cycles, the number of cells was decreased and the temperature was increased in order to create a more stringent selection for aptamers more prone to bind in diverse binding conditions [13].

The result of the complete first round of cell-SELEX was a 3% agarose gel image pictured in figure 2.

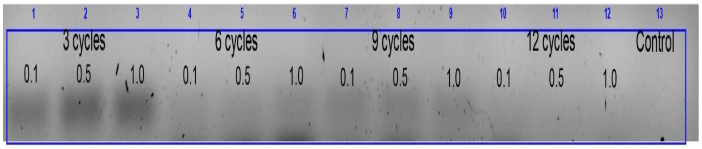

Figure 2: Agarose gel result from the first cycle of selection. First row 100bp DNA ladder. Then, each 3 next rows with varying concentrations of downstream primer with varying amplification cycles.

During the first selection phase, the results of the optimization protocol to obtain the best results varying the number of amplification cycles during the preparative PCR amplification shown that the best protocol is 0.5 µL of downstream primer and 3 additional cycles during the preparative PCR amplification. This protocol yielded an approximate 100 bases ssDNA pool which is the length expected from the initial pool from which the next step can be performed [31].

After the optimization of the first protocol the following cycles were performed and a triplicate result from each cycle is shown in figure 3 with a 3% agarose gel.

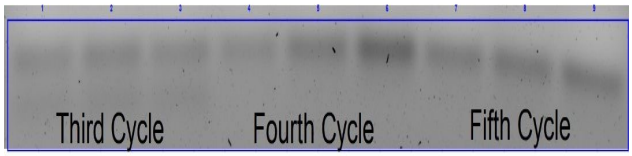

Figure 3: Agarose gel result from the third, fourth and fifth cycle of selection. First row 100bp DNA ladder. Then, each 3 next rows with replicates of each cycle.

The conditions of the cycles were changed to for the following cycles as stated before. This created some non-specific amplicons during the PCR process but during the next SELEX cycles under stringent conditions only one band appeared showing only specific amplicons [13].

The final result is an aptamer pool that can be used to specifically target *Leishmania mexicana* for further applications.

CONCLUSIONS

Modifying cell-SELEX conditions during the cycles it can create more robust and stable aptamers to use them in specific identification of any cell line under any specific condition.

With the present cell culture of *Leishmania mexicana* the conditions for an asymmetric PCR to yield aptamers are 0.5 µL of downstream primer during the PCR amplification cycles and 3 cycles during the preparative PCR cycles for the whole SELEX procedure.

REFERENCES

1. Zhou J, Rossi J (2017) Aptamers as targeted therapeutics: current potential and challenges. Nature reviews. Drug discovery 16: 181-202.
2. Zhou J, Rossi JJ (2014) Cell-type-specific, Aptamer-functionalized Agents for Targeted Disease Therapy. Molecular therapy Nucleic acids 3: e169.
3. Zhu G, Chen X (2018) Aptamer-based targeted therapy. Advanced drug delivery reviews. 134: 65-78.
4. Ramos E, Piñeiro D, Soto M, Abanades DR, Martín ME, et al. (2007) A DNA aptamer population specifically detects Leishmania infantum H2A antigen. A J tech met path 87: 409-416.
5. Sypabekova M, Bekmurzayeva A, Wang R, Li Y, Nogues C, et al. (2017) Selection, characterization, and application of DNA aptamers for detection of Mycobacterium tuberculosis secreted protein MPT64. Tuberculosis 104: 70-78.
6. Moore MD, Escudero-Abarca BI, Suh SH, Jaykus LA (2015) Generation and characterization of nucleic acid aptamers targeting the capsid P domain of a human norovirus GII.4 strain. Journal of biotechnology. 209: 41-49.
7. Pan W, Xin P, Clawson GA (2008) Minimal primer and primer-free SELEX protocols for selection of aptamers from random DNA libraries. BioTechniques 44: 351-360.
8. Hu WP, Kumar JV, Huang CJ, Chen WY (2015) Computational selection of RNA aptamer against angiopoietin-2 and experimental evaluation. BioMed Res Intern 2015: 658712.
9. Martín ME, García-Hernández M, García-Recio EM, Gómez-Chacón GF, Sánchez-López M, et al. (2013) DNA aptamers selectively target Leishmania infantum H2A protein. PloS one 8: e78886.
10. Kinghorn AB, Fraser LA, Lang S, Shiu S, Tanner JA, et al. (2017) Aptamer Bioinformatics. International journal of molecular sciences 18: 2516.
11. Rabal O, Pastor F, Villanueva H, Soldevilla MM, Hervas-Stubbs S, et al. (2016) In Silico Aptamer Docking Studies: From a Retrospective Validation to a Prospective Case Study-TIM3 Aptamers Binding. Molecular therapy Nucleic acids 5: e376.
12. Buglak AA, Samokhvalov AV, Zherdev AV, Dzantiev BB (2020) Methods and Applications of In Silico Aptamer Design and Modeling. International Journal of Molecular Sciences 21: 8420.
13. Li WM, Bing T, Wei JY, Chen ZZ, Shangguan DH, et al. (2014) Cell-SELEX-based selection of aptamers that recognize distinct targets on metastatic colorectal cancer cells. Biomaterials 35: 6998-7007.
14. Chen M, Yu Y, Jiang F, Zhou J, Li Y, et al. (2016) Development of Cell-SELEX Technology

and Its Application in Cancer Diagnosis and Therapy. International Journal of Molecular Sciences 17: 2079.
15. Ohuchi S (2012) Cell-Selex technology. BioResearch Open Access 1: 265-272.
16. Jin C, Zheng J, Li C, Qiu L, Zhang X, et al. (2015) Aptamers Selected by Cell-SELEX for Molecular Imaging. Journal of Molecular Evolution 81: 162-171.
17. Park KS (2018) Nucleic acid aptamer-based methods for diagnosis of infections. Biosensors & Bioelectronics 102: 179-188.
18. Ospina-Villa JD, Zamorano-Carrillo A, Castañón-Sánchez CA, Ramírez-Moreno E, Marchat LA (2016) Aptamers as a promising approach for the control of parasitic diseases. The Brazilian journal of infectious diseases. An official publication of the Brazilian Society of Infectious Diseases 20: 610-618.
19. Wan LY, Yuan WF, Ai WB, Ai YW, Wang JJ, et al. (2019) An exploration of aptamer internalization mechanisms and their applications in drug delivery. Expert opinion on drug delivery 16: 207-218.
20. Moreira D, López-García P, Vickerman K (2004) An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. International journal of systematic and evolutionary microbiology 54: 1861-1875.
21. Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, et al. (2008) Spread of vector-borne diseases and neglect of Leishmaniasis. Europe Emerging infectious diseases 14: 1013-1018.
22. Volpedo G, Huston RH, Holcomb EA, Pacheco-Fernandez T, Gannavaram S, et al. (2021) From infection to vaccination: reviewing the global burden, history of vaccine development, and recurring challenges in global leishmaniasis protection. Expert review of vaccines 20: 1431-1446.
23. Taslimi Y, Zahedifard F, Rafati S (2018) Leishmaniasis and various immunotherapeutic approaches. Parasitology 145: 497-507.
24. Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT (2012) Leishmania virulence factors: focus on the metalloprotease GP63. Microbes and infection 14: 1377-1389.
25. Lieke T, Nylén S, Eidsmo L, McMaster WR, Mohammadi AM, et al. (2008) Leishmania surface protein gp63 binds directly to human natural killer cells and inhibits proliferation. Clinical and experimental immunology 153: 221-230.
26. Ghorbani M, Farhoudi R (2017) Leishmaniasis in humans: drug or vaccine therapy?. Drug design, development and therapy 12: 25-40.
27. Bruni N, Stella B, Giraudo L, Della Pepa C, Gastaldi D, et al. (2017) Nanostructured delivery systems with improved leishmanicidal activity: a critical review. International journal of nanomedicine 12: 5289-5311.
28. Kaye P, Scott P (2011) Leishmaniasis: complexity at the host-pathogen interface. Nature reviews Microbiology 9: 604-615.
29. Zulkeflee M, Azzar A, Mariam S, Zulkhairi M (2019) In-Silico Selection of Aptamer: A Review on the Revolutionary Approach to Understand the Aptamer Design and Interaction Through Computational Chemistry. Mater Today Proc 19: 1572-1581.
30. Sefah K, Shangguan D, Xiong X, O'Donoghue MB, et al. (2010) Development of DNA aptamers using Cell-SELEX. Nature protocols 5: 1169-1185.
31. Zhong Y, Zhao J, Li J, Liao X, Chen F, et al. (2020) Advances of aptamers screened by Cell-SELEX in selection procedure, cancer diagnostics and therapeutics. Analytical biochemistry 598: 113620.