Review Article

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Aptamers: Trending perspective in the diagnosis of leishmaniasis

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Leishmaniasis is one of the neglected tropical diseases responsible for the high mortality and morbidity ratio in middle and low-income countries. Accurate diagnosis and proper chemotherapy are crucial to alleviate the symptoms and limit the disease progression. Aptamers are single-stranded DNA or RNA sequences that can bind to the different target proteins by forming a unique three-dimensional conformation. In this present review, we represent the insights gleaned from the published literature on the relevance of aptamers in diagnosing leishmaniasis. Various experimental studies using systematic evolution of ligands by exponential enrichment (SELEX) approach to identify promising aptamers libraries against the different proteins of the *Leishmania* parasite have been elaborated. These selected aptamers represent invaluable molecular and cellular probes for unraveling novel strategies in diagnosing leishmaniasis.

Keywords: Aptamers, Diagnosis, ELONA, Leishmaniasis, SELEX

Introduction

Infectious diseases caused by protozoan parasites are one of the major health concerns and affect millions of people living in tropical and subtropical regions of the world. Many protozoan diseases affect humans and animals, including African trypanosomiasis, Amoebic dysentery, Chagas disease, leishmaniasis, malaria, and toxoplasmosis.[1] Leishmaniasis is a primary tropical vector-borne parasitic protozoan disease caused by obligatory parasites belonging to the genus *Leishmania*. It is mainly prevalent in Asia, Africa, and Central America. *Leishmania* parasites are carried by the female sandfly vectors belonging to the genus Phlebotomus and Lutzomyia. These sandflies inject promastigotes as an infected agent into the blood. The saliva of sandflies contains anti-clotting factors and some vasodilatory factors, which play a crucial role in causing the infection. The host of parasites includes humans, along with 70 other animal species. Studies suggest that around 350 million people are on the verge of getting infected, and annually 0.7-1.2 million new leishmaniasis cases are reported.[2,3] The disease mainly has three clinical manifestations: cutaneous, mucocutaneous, and visceral leishmaniasis (Figure 1). Cutaneous infection is the most general form, and its main symptoms include lesions and ulcers on uncovered body parts. People suffering from this have few or many sores on their skin, which vary in size and appearance with time. These sores leave lifelong scars. It may also cause severe and significant disability.[4] Mucocutaneous infection damages mucosal or soft tissues of the mouth, nose, and throat. Symptoms include epistaxis, running nose, and cases of shortness of breath. Visceral infection is the most severe and fatal type of leishmaniasis. Clinical signs include fever, loss of weight, spleen and liver enlargement, and symptoms of anemia. Swollen gland and abnormal blood test like low blood count, low platelets count are noticed in some cases. When HIV co-exists with visceral infection, it becomes a significant opportunistic infection.[4,5]

To date, leishmaniasis chemotherapy mainly relies upon pentavalent antimonials, liposomal Amp B, miltefosine, and paromomycin. As such, no effective vaccination is available to treat the disease. Thus, globally effective and safe treatment for the disease is still challenging due to prolonged hospitalization, toxic side effects, lack of oral drugs, and the emergence of drug resistance.[6] However, to limit the progression of the disease, an appropriate and timely diagnosis can play a crucial role in controlling disease exacerbation. Various diagnostic methods for leishmaniasis are parasitological (direct microscopy, histopathology, and culturing), immunological (direct agglutination test, enzyme-linked immunosorbent assay (ELISA), and chromatographic strip test), and PCR-based molecular methods.[7,8] However, an idealistic approach for accurate diagnosis is still elusive due to the broad spectrum of clinical manifestations and

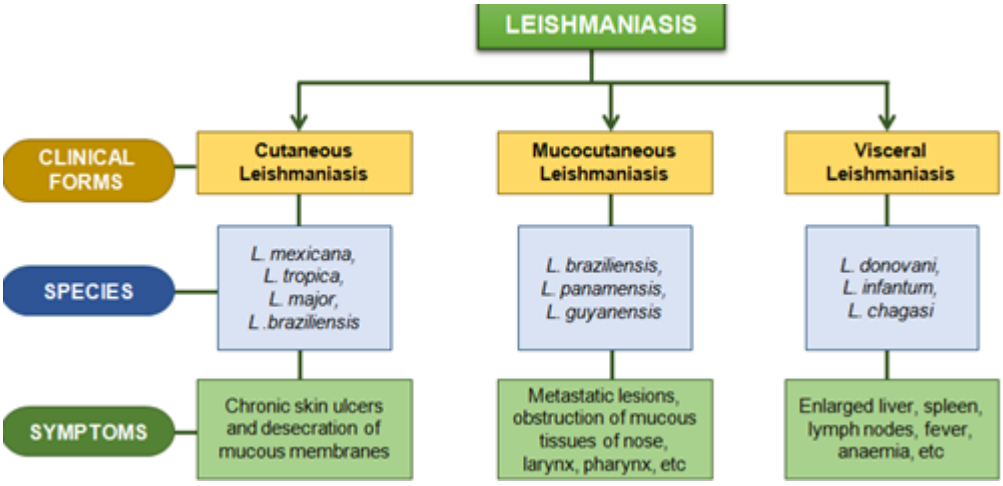

Figure 1: Clinical manifestations, species involved and symptoms related to the leishmaniasis disease.

involvement of several different parasitic species in infection establishment. Thus, to unravel novel diagnostic approaches, we have tried to explore the role of aptamers in diagnosing leishmaniasis in this review. Firstly, we have defined aptamers and then focused on the systematic evolution of ligands by exponential enrichment (SELEX) methodology for selecting appropriate aptamers. Next, we have elaborated on various published literature on the leishmanial diagnosis by aptamers libraries. Thus, this review represents the potential role of aptamers in unraveling new strategies for parasite diagnosis.

Aptamers and SELEX

The innovative invention of aptamers is an emerging tool for diagnostic and therapeutic goals. It is a cost-effective, sensitive technique that mainly focuses on the molecular diagnosis of infectious pathogens. Aptamers are artificially designed single-stranded (ss) DNA or RNA oligonucleotides that bind to the target site with high affinity. These are also called chemical antibodies, which are highly specific and target selective in nature.[9,10] Aptamers self-fold themselves into stable and compact three-dimensional structures upon binding to the target molecule. These nucleic acid-based aptamers adopt 3-D structures such as hairpin loop, quadruplex (side by side or cross over), and kissing complex.[11] These structures allow aptamers to bind to any target with high affinity and specificity through non-covalent interactions like hydrogen bonding, electrostatic and van der Waals interactions, etc.[12] Aptamers are analogous to monoclonal antibodies. They both have excellent properties of binding to the target molecule. However, the nature of the target protein varies. Aptamers bind to immunogenic and non-immunogenic targets, whereas monoclonal antibodies bind only to immunogenic targets. Aptamers are preferred over antibodies as these oligonucleotides can easily refold themselves if denatured. They are low-cost and have mighty tolerance power against surfactants.[11] They can be chemically modified by introducing 5' and 3' polyethylene glycol and biotin, which are biologically inert. These chemical modifications increase aptamers' efficacy, solubility, and stability by resisting their renal clearance, whereas any changes to monoclonal antibodies lead to reduced shelf-life. Small-sized aptamers penetrate smaller chambers or cells where antibodies fail to reach.[13] Aptamers provide exceptional coupling features with diverse nano-vehicles such as liposomes, micelles, serum albumin, etc. They can be coupled with different conjugation systems, such as B-linker, which consists of disulfide and peptide linkers. The purposeful designing of aptamers helps to deliver various anti-cancerous drugs, and cytotoxic drugs obstruct the target protein's function. This striking property of blocking protein function is extensively used in diagnosis and treatment of infectious diseases.[14,15]

The idea of nucleic acid binding affinity to target molecule emerged after examining the molecular mechanism of adenovirus and Human Immunodeficiency virus (HIV). Specific RNA molecules were found to cohere with viral proteins, and this concept was used in the SELEX technique. The generation of aptamers is entirely in vitro and reduces the chances of contamination by any pathogen. The most efficient procedure of generating aptamers through an in vitro method, which involves repeated rounds of selection and amplification, is defined as SELEX. It is a primary method of developing aptamers against any target, from small molecules to larger targets such as proteins or cells.[16] The standard SELEX method is initiated by incubating a target of interest with a pool of randomly sequenced single-stranded desired oligonucleotides. It consists of 40-100 nucleotides containing random sequences in the middle while definite sequences on the terminals. The next step involves positive selection, separating specific oligonucleotides that bind efficiently to the target from the pool of unbound oligonucleotides. The separation includes diverse techniques such as size exclusion, affinity chromatography, capillary electrophoresis, or nitrocellulose-based separation.[11] Another crucial and fundamental step that ensures

high affinity and specificity to oligonucleotides is negative selection. It is a process in which oligonucleotides that recognize any other target are discarded. In contrast, those molecules which specifically bind to the target of interest are selected, eluted, and then amplified by PCR.[13] The classical SELEX technique was mainly used to generate RNA aptamers. RNA aptamers self-folded into a more compact and stable three-dimensional structure than DNA aptamers, even with the same monomer number. An additional step is mandatory for developing RNA aptamers: ssDNA is first converted into dsDNA using the Klenow enzyme and then transcribed to ssRNA. Then the ssRNA pool is incubated with the target, and the obtained oligonucleotides are referred to as RNA aptamers after selection. Multiple chemical modifications are implemented to enhance the binding ability to a specific target.[17] A graphical representation of the general SELEX method for generating DNA and RNA aptamers is shown in Figure 2.

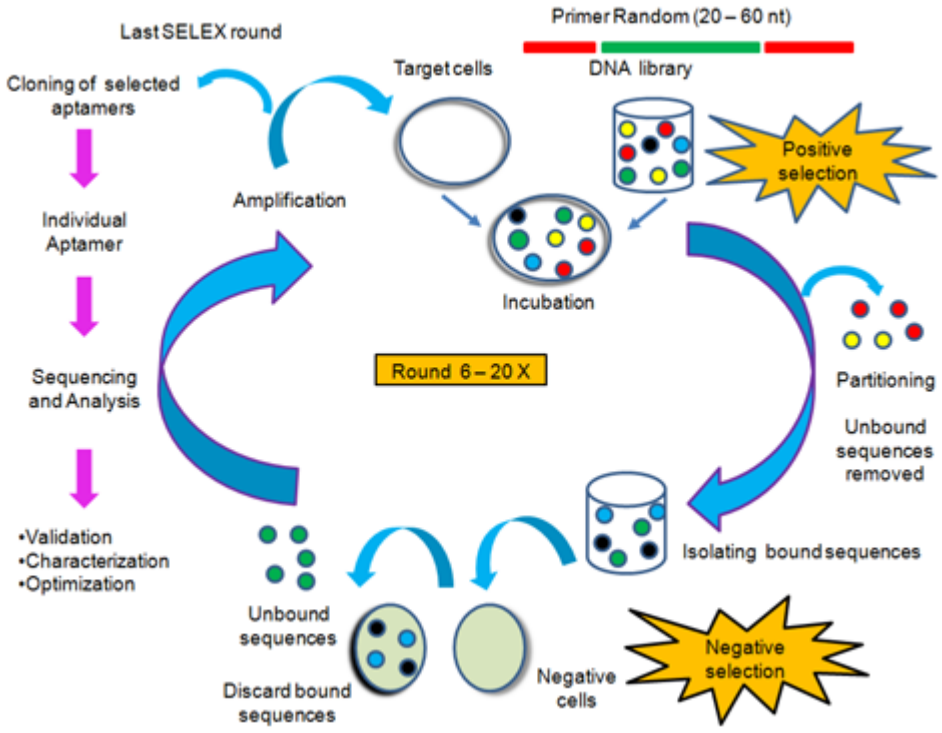

Figure 2: Diagrammatic illustration of SELEX technique.

The evolution of SELEX provides an excellent opportunity to employ aptamers for multiple purposes, such as therapeutic, drug delivery, and diagnostic tools.[18,19] Thus, the fundamental SELEX protocol has recently been modified by involving various techniques to generate highly specific aptamers in less time. For example, during immunoprecipitation of SELEX, the target protein is exposed to the ssDNA pool and followed by cell lysis. The target protein is recovered by immunoprecipitation using beads coated with antibodies. It extensively generates high-affinity aptamers against target protein under physiological conditions. Similarly, the DNA library is immobilized during the capture SELEX technique instead of the target protein. It generates aptamers against soluble molecules. Cell-SELEX is a commonly used technique to generate parasite-specific aptamers. It is developed against the whole cell used as a target. No protein purification step is involved. This technique detects any post-translational modification on the cell surface. It is primarily used for cell-specific therapy, cell-targeted diagnosis, and drug discovery. Capillary SELEX is an analytical method that exploits the technique of electrophoresis. Aptamers are produced against ions based on electrophoretic mobility. M-SELEX adopts the SELEX protocol combined with a microfluidic system, efficiently managing small fluidic volumes and generating specific aptamers for minute molecules. Atomic force microscopy is utilized to create a 3D image of the target. Animal SELEX is an in vivo technique in which live animals such as infected cancer models are directly used as targets. Desired aptamers are introduced into an animal model, DNA sequencing is performed, and specific aptamers are amplified.[16,20]

Aptamers and leishmanial diagnosis

Appropriate disease diagnosis is critical in determining chemotherapy, and dosage regimen, leading to a reduction in the rising cases of any infection. Published literature retrieved from PubMed suggested that aptamers-based diagnoses for various parasitic diseases, including leishmaniasis, have a trending perspective. Thus, in the following sub-sections, aptamers developed for diagnosing different leishmanial proteins have been elaborated.

Aptamers against Kinesin

In a recent study, Bruno et al.[21] developed DNA aptamers against two synthetic peptides derived from leishmanial flagellar protein (kinesin) for their potential use in disease diagnosis. Several aptamer candidates were selected after performing ten rounds of SELEX against the kinesin peptides. Strong

binding and affinity for these aptamers were recognised using various approaches, including enzyme-linked aptamer sorbent assay (ELASA), aptamer-colloidal gold staining, confocal, and electron microscopy. Further, southwestern blotting results suggested that top hit aptamers could specifically detect the synthetic kinesin peptides. Out of all, aptamers (K2-13R) also successfully detected the kinesin protein band (~250 kDa) in the cell lysate of *L. major* parasites. In an interesting study by the same group[22] enzyme-linked fluorescence-based sandwich assay was developed using DNA aptamers for rapid and sensitive detection of *Leishmania* parasites in the sandflies. Using this approach, even ~100 ng of parasite cell extracts obtained from sand-fly homogenates were detectable.

Aptamers against Histones

Histone proteins have a crucial role in gene regulation and are highly conserved proteins evolutionarily. However, various characterization studies in trypanosomatids suggest that N- and C-terminal domains of histones in parasites have considerable sequence divergences. To explore these diverged histone sequences as diagnostic targets, Ramos et al.[23] performed the SELEX procedure to identify ssDNA aptamers against *L. infantum* H2A protein. Starting with a library of 1014-1016 different sequences, they identified the SELH2A aptamers population showing the highest binding affinity towards recombinant H2A protein of *L. infantum* (Figure 3). Next, experiments including enzyme-linked oligonucleotide assay (ELONA), binding kinetics and 8 of *L. infantum* H2A protein. A few years later, the same group,[24] using the SELH2A aptamer pool library, selected two clones (AptLiH2A#1 and AptLiH2A#2) out of 30 clones based on the highest binding affinities. As reported, AptLiH2A#1 and AptLiH2A#2 aptamers were able to detect H2A protein in a dose-dependent manner with dissociation constant (KD) 0.96 ± 0.17 nM and 1.16 ± 0.28 nM, respectively. To determine the sensitivity of these two aptamers to recognize endogenous H2A protein of *L. infantum* promastigotes, cell lysate from a varying number of parasites (1,000-10,000) was prepared. These two aptamers could easily detect LiH2A protein from the 7500 parasites count. Table 1 represents key outcomes from the various studies related to aptamers and leishmanial diagnosis.

Using similar experimental approaches, Ramos et al.[25] also identified ssDNA aptamers against the H3 protein of *L. infantum*, and the aptamers library was labeled as SELH3. Further, Frezza et al.[26] identified two individual aptamers (AptLiH3#4 and AptLiH3#10) against recombinant H3 protein of *L. infantum* from previously reported SELH3 aptamers population. Specificity and binding affinity for these two selected aptamers were confirmed using ELONA, slot blot, and immunoblotting approaches. Results suggest that these aptamers detect the H3 protein dose-dependent with a dissociation constant (KD) ranging from 0.35-0.55 nM. These aptamers also successfully detect the endogenous H3 protein from the *L. infantum* cell lysate. The detection limit from cell lysate of minimum *L. infantum* promastigotes count for AptLiH3#4 and AptLiH3#10 was reported to be 6000 and 9000 parasites, respectively. Next, to determine the mapping of H3-aptamers interactions, twelve peptides were synthesized, representing overlapped sequences of H3 protein. These aptamers showed the highest affinity towards peptides (3, 4, and 5), corresponding to the main antigenic region located in the amino-terminal domain (21-60 amino acids) of H3 protein. Thus, these studies confirm that DNA aptamers selected against histone proteins may be a valuable asset for diagnosing leishmaniasis.

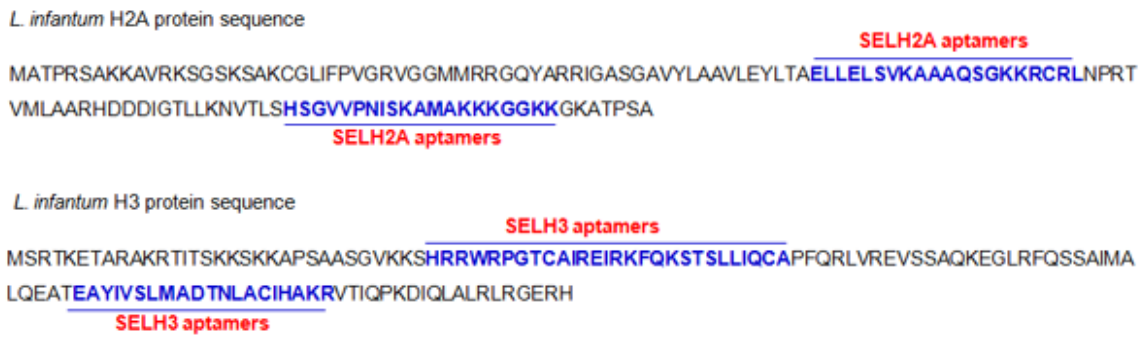

Figure 3: Protein sequences showing the high-affinity binding regions of selected aptamers population.

studies, slot blot, and immunoblotting analysis confirmed the binding capability and specificity of the SELH2A aptamers population only against H2A protein with no cross-reactivity towards other histone proteins of the *L. infantum* parasite. Further, for mapping the H2A-SELH2A interaction, nine different peptides of H2A protein were synthesized, and the ELONA experiment confirmed that SELH2A had the highest binding affinity towards peptides 5

Table 1: Various aptamers selected against different proteins of *Leishmania* parasites.

Target Protein	Selected Aptamers	Dissociation constant (Kd)	Diagnosis methods	Min. number of of detectable promastigotes	Ref.
H2A	AptLiH2A#1	0.96±0.17 nm	ELONA, slot blot, and immunoblotting	7500	23, 24
	AptLiH2A#2	1.16+0.28 nm			
H3	AptLiH3#4	0.52+0.05 nm	ELONA, slot blot, and immunoblotting	6000 9000	25, 26
	APtLiH3#10	0.37+0.05 nm			
KMP-11	SELK10	-	ELISA, dot blot, and immunoblotting	-	27
Kinesin	K2-13R	-	ELASA, southwestern blotting, confocal and electron microscopy	-	21
PABP	ApPABP#3	5.4+1.1 nm	ELONA, slot blot, immunoblotting poly-(A)- sepharose binding and pull-down assays	2500	31
	ApPABP#7	6.0+2.6 nm			
	ApPABP#11	10.8+2.7 nm			

Aptamers against Kinetoplastid membrane protein

11 kDa Kinetoplastid membrane protein (KMP-11) is a small immunogenic protein of the *Leishmania* parasite, present in both the human host phase (amastigotes) and the sandfly phase (promastigotes). It is localized on the cell surface, flagellum, flagellar pocket, and intracellular vesicles. KMP-11 has a crucial role during host-pathogen interactions as it assists the binding of the parasite to the host macrophage membrane during infection. Further, it also can induce both innate and adaptive host immune responses. Thus, to investigate the role of KMP-11 protein in leishmanial diagnosis, Moreno et al.[27] developed ssDNA aptamers against *L. infantum* KMP-11 protein using the SELEX approach. This study used a colloidal gold-based methodology to generate gold-labeled KMP-11 protein. Starting with ~1*1024 different sequences, the SELK10 aptamers pool was selected after ten rounds of PCR. Dot blot, ELISA, and immunoblotting studies were performed for binding affinity and specificity confirmation. The results suggest that the SELK10 aptamers pool specifically recognized the KMP-11 protein with no cross-reactivity, and these selected aptamers can be used as a powerful tool in diagnosing parasitic infection. A few years later, a very interesting study by the same group[28] was published regarding making aptamers-based electrochemical biosensor. Firstly, to standardize this procedure, horseradish peroxide (HRP)-conjugated gold nanoparticles were electrodeposited on the working electrodes, and then the electrochemical reduction of H_2O_2 was tested. Next, electrodeposition of gold-labeled KMP-11 protein was performed on screen-printed microelectrodes, and then KMP-11 protein was identified using aptamers population as the detection element. The electrochemical measurement using the biosensor device detected even a few micromolar concentrations of *L. infantum* KMP-11 protein. This study represents a unique approach to rapidly developing aptamer-based biosensors to detect *Leishmania* parasite infection.

Aptamers against Poly (A)-binding protein

Poly (A)-binding protein (PABP) plays a key role in different biological processes, including translational initiation and termination. It is also involved in transporting mRNA from the nucleus to the cytoplasm. Previously, PABP has been characterized in Trypanosoma brucei, T. cruzi, *L. amazonensis*, *L. major*, and more recently in *L. infantum*.[29,30] As reported, PABP, characterized in the trypanosomatidae family, has some sequence divergences compared to other eukaryotic organisms. Taking this into consideration, Guerra-Pérez et al.[31] identified ssDNA aptamers library (SELLiPABP) against recombinant PABP protein of *L. infantum* parasite using the SELEX approach. Next, using different experimental approaches, including ELONA, slot blot, and immunoblotting assays, three aptamers, ApPABP#3, ApPABP#7, and ApPABP#11, were shortlisted based upon binding affinity and specificity toward rLiPABP protein. Selected aptamers were able to detect rLiPABP protein in dose-dependent concentrations; KD values for ApPABP#3, ApPABP#7, and ApPABP#11 were reported to be 5.4 ± 1.1 nM, 6.0 ± 2.6 nM, and 10.8 ± 2.7 nM,

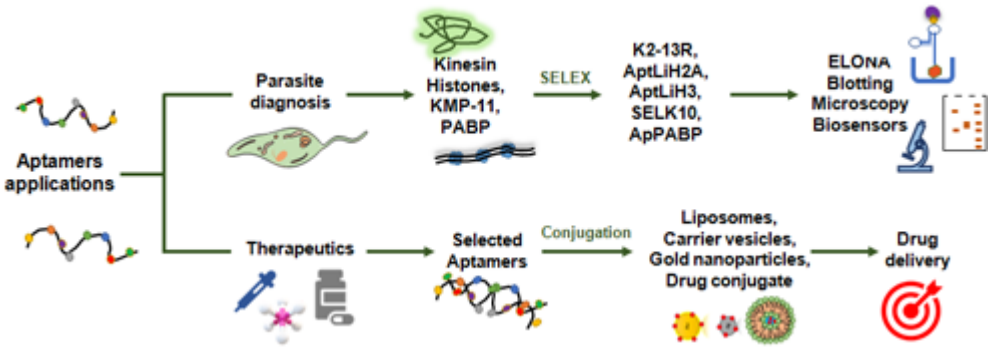

Figure 4: Schematic diagram showing potential applications of aptamers.

respectively. Even 50 nM of these three shortlisted aptamers could detect a minimum (6.25 ng) of rLiPABP protein. Next, to determine the sensitivity against endogenous LiPABP protein, cell lysate from a varying number of parasites (0-5000) was prepared. All three shortlisted aptamers detected LiPABP protein from 2500 parasites count in a significant manner. Furthermore, in vitro functional analysis performed using poly-(A)-sepharose binding and PABP pull-down assays also revealed that ApPABP#11 significantly disrupted the binding of myc-LiPABP and endogenous LiPABP to poly-(A)-sepharose as compared to the other two aptamers. In this experiment, lysate obtained from HEK293T cells over-expressing myc-LiPABP protein was used. Thus, this study strongly suggests that aptamers inhibiting the binding of PABP to the poly (A) tail can unravel new strategies for leishmaniasis therapeutic and diagnosis.

Importance and application of aptamers in other disease diagnosis

The self-folded three-dimensional structure adopted by aptamers can be modified to employ it as a magnificent tool for developing biosensors for detecting and diagnosing diseases. Aptamers-based biosensors called Aptasensors are prepared using fluorescent tags. They have been extensively used for accurate and rapid diagnosis and treatment of various diseases, including cancer, tuberculosis, and viral infections (avian influenza, Ebola, HIV, Zika, hepatitis, and Rubella).[32-34] Cell proteome changes due to some morphological, molecular, and biochemical changes in cancer cells are used as biomarkers for early and rapid detection. Aptamers prepared by the cell-SELEX method have been used to detect cancer at the molecular level.[35] Despite the low molecular expression level, aptamers show higher sensitivity than conventional detection methods. Early diagnosis of HIV infection is crucial even in asymptomatic persons for early drug delivery, preventing viral multiplication and prevalence. Tat HIV protein, a regulatory element for viral replication, is a crucial target for aptasensors for the diagnosis of HIV.[36] Conventional tests such as skin and sputum tests are employed in diagnosing tuberculosis, but these are time-consuming, increasing the risk of infection. Aptamers-based detection methods developed against bacterial proteins such as ESAT-6, CFP-10, and MPT-64 have shown sensitivity and specificity >90%.[37]

Concluding remarks and way forward

In the last few years, nucleic acid-based approaches have gained massive attention from researchers around the globe. Aptamers have high specificity and efficacy toward the selected target; thus, they play a pivotal role in disease diagnosis and therapeutics. The research studies suggest that various aptamers libraries were selected against leishmanial proteins. The specificity and binding affinity of the selected aptamers were confirmed by multiple methods, including ELONA, dot blot, immunoblotting, microscopy, and pull-down assays (Figure 4). Recent studies have also highlighted the importance of a drug delivery approach using a variety of agents/molecules like aptamer-conjugated nanoparticles, siRNA, and drugs. These strategies will improve therapeutic efficacy and bioavailability and significantly reduce systemic toxicity and drug resistance issues. Other factors like high thermal stability, low cost, ease of scalability, and manipulation are also associated with the extensive applications of aptamers. Several bottleneck limitations of aptamers are resolved by coupling these molecules to nanoparticles and adopting some chemical modifications. This combination of aptamers and nanoparticles allows them to employ efficiently as a therapeutic drug. Aptamers are resistant to nucleases by modifying the 2' end with aminopyrimidine. Another problem of renal clearance of low molecular weight aptamers (5-15 kDa) has been solved by conjugating these molecules to polyethylene glycol. These modified aptamers have a long half-life and remain circulating in the blood. Thus, these aptamers represent a valuable tool for diagnosing and improving drug therapy, which can be critical in limiting disease progression.

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