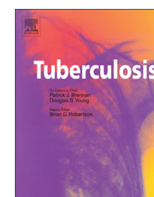
Tuberculosis 104 (2017) 70–78

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



Selection, characterization, and application of DNA aptamers for detection of *Mycobacterium tuberculosis* secreted protein MPT64

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form
5 February 2017
Accepted 14 March 2017

Keywords:
Mycobacterium tuberculosis
Diagnostics
MPT64
Aptamer
SELEX

ABSTRACT

Rapid detection of *Mycobacterium tuberculosis (Mtb)*, an etiological agent of tuberculosis (TB), is important for global control of this disease. Aptamers have emerged as a potential rival for antibodies in therapeutics, diagnostics and biosensing due to their inherent characteristics. The aim of the current study was to select and characterize single-stranded DNA aptamers against MPT64 protein, one of the predominant secreted proteins of *Mtb* pathogen. Aptamers specific to MPT64 protein were selected *in vitro* using systematic evolution of ligands through exponential enrichment (SELEX) method. The selection was started with a pool of ssDNA library with randomized 40-nucleotide region. A total of 10 cycles were performed and seventeen aptamers with unique sequences were identified by sequencing. Dot Blot analysis was performed to monitor the SELEX process and to conduct the preliminary tests on the affinity and specificity of aptamers. Enzyme linked oligonucleotide assay (ELONA) showed that most of the aptamers were specific to the MPT64 protein with a linear correlation of $R^2 = 0.94$ for the most selective. Using Surface Plasmon Resonance (SPR), dissociation equilibrium constant K_D of 8.92 nM was obtained. Bioinformatics analysis of the most specific aptamers revealed the existence of a conserved as well as distinct sequences and possible binding site on MPT64. The specificity was determined by testing non-target ESAT-6 and CFP-10. Negligible cross-reactivity confirmed the high specificity of the selected aptamer. The selected aptamer was further tested on clinical sputum samples using ELONA and had sensitivity and specificity of 91.3% and 90%, respectively. Microscopy, culture positivity and nucleotide amplification methods were used as reference standards. The aptamers studied could be further used for the development of medical diagnostic tools and detection assays for *Mtb*.

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1. Introduction

Tuberculosis (TB) remains one of the main causes of human death around the globe. According to the World Health Organization (WHO), 2015 recorded the highest TB cases than in previous years. 9.6 million people were infected, and 1.5 million people died of TB in 2014 [1]. The mortality rate for patients infected with active TB goes beyond 50% when not diagnosed, hence, rapid and accurate diagnostics coupled with further prompt treatment of the disease is the cornerstone for controlling TB outbreaks. An early detection of TB would allow fast recovery, therefore, reducing the number of fatal cases. However, TB detection remains a significant healthcare issue in the developing countries owing to a number of challenges. Firstly, mycobacterium is a slow-growing bacterium, it takes 4–8 weeks to cultivate on traditional solid media, and 10–14 days even with rapid liquid culture [2]. Secondly, pulmonary TB causes low clinical symptoms early in the course of disease, leading to a delay in seeking patient care. Thirdly, active pulmonary TB may present a low bacillary burden at the early stage, which often leads to low sensitivity for sputum smear microscopy commonly used in the developing countries [3]. Therefore, it is important to develop a fast

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http://dx.doi.org/10.1016/j.tube.2017.03.004

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and accurate detection tool specific for TB. Despite the existing technologies and advances over the last decades in biosensing, the development of simple point-of-care tests in the near future is still challenging.

The most promising approach for developing such biosensor could be based on the detection of antigens or *Mtb* DNA specific to TB. Antigen specific for TB diagnostic is based on the detection of abundantly secreted proteins with high levels of immunogenicity [4]. The predominant immunogenic secreted proteins of *Mtb* are 23 kDa MPT64, 6 kDa early secreted antigenic targets (ESAT-6) and 10 kDa culture filtrate protein (CFP-10). These proteins were used as antigens in skin patch test [5], immunochromatography assays [6] and immunohistochemical analysis [7] and were detected based on antibody detection methods. The sensitivity of such diagnostic tools in clinical specimens could be high; however, the use of antibodies as recognition elements has some limitations [8].

An alternative to antibodies are aptamers, which are single stranded oligonucleotides (both DNA and RNA) that have abilities to specifically bind to a broad range of targets [9–11] with high affinity and specificity [12]. Aptamers are synthesized *in vitro* using a method called systematic evolution of ligands by exponential enrichment (SELEX) [9]. This method includes three main stages: incubation of a random library with the target; separation of bound aptamers from unbound ones; amplification of bound sequences using PCR [12]. Aptamer technology has interesting advantages over antibodies. For example, aptamers are synthesized *in vitro* from a library that contains large numbers of random sequences; thus, alleviating the use of animals during selection process compared to antibodies. The properties of aptamers can be tuned on demand and, therefore, be selected to bind specific targets in non-physiological conditions or targets that are non-immunogenic and toxic to animals. The production of selected aptamers is performed using chemical synthesis, optimizing their purity while reducing eventual batch-to-batch variations. Furthermore, once the selection process is over, they can be massively synthesized lowering their cost compared to antibody production. Aptamers made from nucleic acids are more stable than antibodies, and they can be stored at ambient temperatures. In addition, they are smaller in size, and their affinities are not affected by labelling [13–15]. Due to these properties, aptamers are used in areas such as functional studies of proteins, therapeutics, diagnostics and biosensing [12,16]. For biosensing, aptamers are very promising because they can be selected and used under any pre-defined conditions, and they can be easily immobilized to obtain a custom-made surface.

Taking into consideration advantages of aptamers in diagnostics, aptamers have been selected against TB related antibodies [19–21] and predominant secreted antigens ESAT-6, CFP-10 [17] MPT64 [18]. Aptamer selected against antigens ESAT-6 and CFP-10 [17] showed that aptamer CSIR 2.11 had sensitivity and specificity of 100% and 68.75%, respectively. Selected aptamer was tested on clinical sputum samples with K_D values being at nM range indicating a strong binding. Aptamers selected against antigen MPT64 were 35 nucleotides long and had 86.3% sensitivity and 88.5% specificity [22]. The selection was based on immobilising the protein on ELISA wells and consequent aptamer binding, elution and amplification. Aptamers in this study were not tested on clinical sputum samples as in previous study with ESAT-6 and CFP-10 but tested on diluted bacterial culture filtrates. There was neither information presented on cross reactivity with other relevant proteins that are abundant in culture filtrates nor the information on K_D values.

The main goal of our research was to select and characterize ssDNA aptamers that specifically bind to MPT64 protein of *Mtb* using SELEX technology based on capturing the aptamer-protein complex on a nitrocellulose membrane and consequent elution and amplification. The results revealed that one aptamer had the highest binding affinity when evaluated and characterized with SPR (surface plasmon resonance) and ELONA (Enzyme Linked Oligonucleotide Assay). The selected aptamer is 40 nucleotides long and has different sequence from previously published work [22]. It can distinguish MPT64 protein among other mycobacterial secreted proteins, such as ESAT-6 and CFP-10 and also has improved sensitivity and specificity (91.3% and 90%, respectively). The selected aptamers could be used for the TB diagnosis in a multiplexed assay along with other aptamers that have been previously selected against other TB antigens such as ESAT-6 and CFP-10.

2. Materials and methods

Fig. 1 illustrates the principles and concepts of selection and characterization of MPT64 aptamers, and the five major steps of the experiments: *in vitro* selection of aptamer candidates against MPT64 using SELEX method; dot blot analysis of affinity of the pool of aptamers from selected SELEX rounds and aptamers with known sequences; cloning, sequencing, and bioinformatics analysis of aptamers; and detection of MPT64 in buffer and clinical sputum samples using ELONA. The methods are described below.

2.1. Proteins

Target immunogenic protein MPT64 (46 kDa) (Rv1980c) (Gene ID: 581375; Accession#: CAA53143) of *Mtb* H37Rv strain with a concentration of 1 mg/ml was obtained from EnoGene Biotech Co Ltd (Nanjing, China). His and Trx tagged MPT64 protein was purified from recombinant *Escherichia coli* strain by affinity chromatography with >90% purity. According to manufacture, the protein is suitable for use in multiple immunoassay formats, including ELISA, Western Blot and Rapid Tests. Non-target ESAT-6 (p463-1) and CFP-10 (p460-1) proteins were purchased from Sunny lab (USA).

2.2. DNA library, primers

The oligonucleotide template was synthesized as a single-stranded 80-mer with the following sequence: 5′- TCA CTT CAA ATG TGC GCT TC – N40 – CGT CAA AAC AGG GGG TAG AA - 3′ where the central 40 nucleotides represent random oligonucleotides based on equal incorporation of A, T, G and C at each position. The dsDNAs were obtained by PCR amplification using Forward 5′- TCA CTT CAA ATG TGC GCT TC-3′ and Reverse 5′- TTC TAC CCC CTG TTT TGA CG -3′ primers. Biotinylated forward primer for Dot-blot analysis and phosphorylated reverse primers to obtain ssDNAs by lambda exonuclease digestion were of the same sequences as forward and reverse primers. Both the library and primers were synthesized and PAGE purified by Integrated DNA Technologies (Coralville, IA, USA).

2.3. In vitro selection of aptamers

Aptamer candidates against MPT64 were selected using the SELEX protocol based on [23] with slight modifications. SELEX is an oligonucleotide-based combinatorial library approach which has been extensively used to isolate aptamers against various targets including proteins, ions, cells and surface epitopes [11,24]. This method includes three main stages: incubation of a random library with the target; separation of bound aptamers from unbound ones; amplification of bound sequences using PCR [12]

(Fig. 1). The DNA was passed three times prior to the selection cycle through a pre-wetted nitrocellulose acetate membrane (0.45 μm HAWP filter, Millipore, MA, USA) in a filter holder

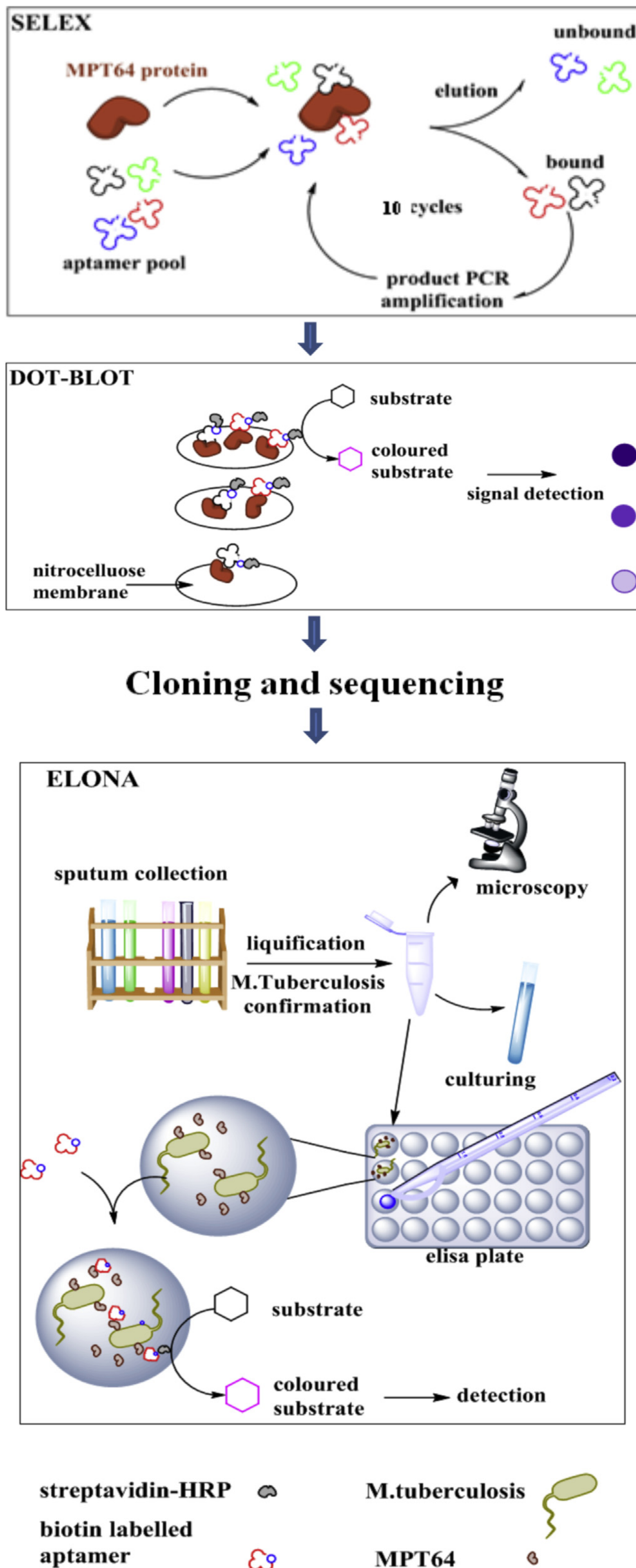

Fig. 1. Schematic representation of principles and concepts of selection and characterization of MPT64 aptamers using SELEX, dot blot, cloning, sequencing, and ELONA techniques.

("pop-top", diameter 13 mm, Millipore) in order to exclude filter-binding ssDNA sequences from the library. To initiate *in vitro* selection, ssDNA library was denatured at 95 °C for 10 min in a thermocycler (Mastercycler gradient, Eppendorf) and was allowed to be cooled down approximately to 34–38 °C inside the thermocycler. Denatured DNAs were then incubated with the target protein at 15 rpm on a variable speed rotor (Grant Bio PTR-30, Keison) for 1 h and 45 min at room temperature (for the first cycle). This reaction mixture was then filtered over a HAWP filter and washed with binding buffer (50 mM Tris-HCl; 25 mM NaCl; 5 mM $MgCl_2$; 10 mM DTT; pH 7.5). ssDNA that was retained with the protein on a filter were eluted with elution buffer (0.4 M sodium acetate, 5 mM EDTA, and 7 M urea, pH 5.5) at 70–80 °C for 5 min twice. Afterwards, the eluted DNA was diluted with an equal volume of dH_2O and was precipitated with ethanol and ammonium acetate and incubated for 1 h at −80 °C. After centrifugation at 13,000 rpm at 4 °C for 1 h, supernatant was discarded, and pellet was washed twice with 75% ethanol solution and resuspended in dH_2O for further PCR. Amplification conditions were as follow: initial denaturation at 95 °C for 5 min and final extension at 72 °C for 10 min; and 30 cycles of denaturation at 95 °C for 30 s; annealing at 58 °C for 45 s and extension at 72 °C for 45 s. Amplified products were analyzed on non-denaturing 6% Tris-borate EDTA (TBE)-polyacrylamide gels (Invitrogen, CA, USA) at 200 V for 20 min after binding with SYBR Green 1 (Invitrogen, CA, USA). ssDNA was obtained by digestion of PCR products amplified with phosphorylated reverse primers with 5 units of lambda exonuclease (New England Biolabs, Ipswich, MA, USA) per 50 μl reaction. Reaction was done for 1 h at 37 °C and then heat inactivated for 10 min at 72 °C. Digested products were precipitated as above (resuspension of pellet in binding buffer) and used for the next round of SELEX. 10 iterations of SELEX were performed. For additional rounds of selection, the amount of protein was reduced twice in each cycle: from 4.35 μg to 0.07 μg in a binding buffer (total volume of 150 μl). Incubation time was in range from 1 h 45 min for the first cycle and 40 min for the last. Incubation conditions as well as concentrations of target protein and ssDNA are listed in Table 1. Concentration of ssDNA was measured using NanoDrop 1000 Spectrophotometer (ThermoScientific, DE, USA). All procedures (where applicable) were performed in a biosafety level 2 cabinet (Purifier Delta Series Class II, Type A2 Biological Safety Cabinet, Labconco Corporation, MO, USA).

2.4. Dot blot analysis

Dot blot is a technique that enables identification and analysis of proteins of interest where sample proteins are spotted onto membrane and hybridized with a probe. The successful interaction results in colored spots (Fig. 1). Dot blot in this study was used for a

Table 1
Concentrations of ssDNA and target MPT64 used in *in vitro* selection of aptamers.

SELEX cycle	Target MPT64 (μg)	ssDNA pool (μg)	Incubation time (min)
1	4.35	35	105
2	2.17	~30	90
3	1.08	~30	60
4	0.54	~30	60
5	0.27	2.9	60
6	0.13	7.1	50
7	0.07	~7	40
8	0.04	10	40
9	0.03	10	40
10	0.03	10	40

rapid analysis of affinity of the pool of aptamers from selected SELEX rounds and aptamers with known sequences. Selected SELEX DNA products were amplified using forward biotin-labeled primers and phosphorylated reverse primers with further digestion using lambda exonuclease to obtain biotinylated ssDNA. MPT64 protein was applied onto a nitrocellulose membrane (BA85 Protran, 0.45 μm, Whatman, USA) in different concentrations (87, 8.7, and 0.87 μg/ml) for dot blot analysis of SELEX products. For dot blot analysis with known aptamer sequences, 500, 250, 100, 50, 25, 10, 5, and 1 μg/ml of MPT64 protein was applied onto the nitrocellulose membrane. After immobilization on the membrane, blocking buffer (casein 12.5 g; NaCl 4.5 g; Tris 605 mg; Thimerosal 100 mg) was applied for 45–60 min, and 475 μl of 817 ng biotinylated aptamers from selected cycles were left to incubate for 30 min. After washing three times with 1 × KPL (KPL, Gaithersburg, Maryland) washing solution (0.002 M imidazole, 0.02% Tween20, 0.5 mM EDTA, 160 mM NaCl) the membrane reacted with streptavidin-alkaline phosphatase diluted to 1:500 for 30 min. The membrane was then coated in BCIP/NBT (5-Bromo-4-chloro-3-indoxyl-phosphate and nitroblue tetrazolium) (KPL, MD, USA) substrate for 15 min in the dark after washing. The results were determined by observation of violet stained spots on the membrane (Fig. 2b, c). *Escherichia coli* antibodies labeled with biotin served as a positive control (diluted at 1:500) (Meridian Life sciences, ME, USA), and deionized water served as a negative control.

2.5. Cloning

The aptamer pools from SELEX rounds were purified on either 6% TBE gel (Invitrogen) or using Qiaquick PCR Purification Kit (Qiagen, Germany), ligated to pGEM®-T Vector System II and transformed into JM109 High Efficiency Competent Cells according to the manufacturer's manual (Promega, USA) and then incubated at 37 °C overnight. White colonies were picked and streaked on a plate. Individual colonies of the transformed cells were propagated in 5 ml of LB broth with ampicillin (50 μg/ml) for 15 h at 37 °C. The cells were harvested by centrifugation at 8000 rpm for 5 min, and plasmid DNA was extracted using the QIAprepMiniprep kit (Qiagen, Germany). Ampicillin and IPTG used in cloning were purchased from Calbiochem (San Diego, CA, USA); X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and S.O.C medium were purchased from Invitrogen. The purity of purified plasmids was analyzed by NanoDrop 1000 Spectrophotometer, and, then, the plasmids were sent for sequencing.

2.6. Sequencing and bioinformatics analysis of aptamers

Sequencing of plasmid DNA of the selected transformants was done by automated DNA sequencing using ABI 3130xl analyzer Big dye 3.1 diemistry (ABI 7300 Sequence Detector, Foster City, CA). Analysis of aptamer sequences nucleotide content, repeats and inverse complementary repeats were performed. For every selected aptamer, we computed all sites having mutually reverse complementary sequences and formed by three and more nucleotides. Sequence repeats within and between selected aptamers were computed as well. The secondary structures of sequenced aptamers were predicted by web-based OligoAnalyzer 3.1 tool from IDT (Integrated DNA Technologies, https://www.idtdna.com/calc/analyzer). Analysis of putative G quadruplex structures were carried out on a web-based server for predicting G-quadruplexes in nucleotide sequences (http://bioinformatics.ramapo.edu/QGRS/analyze.php). The analysis of surface charges for MPT64 protein was based on data obtained from protein database (RCSB Protein data bank). PDB database contained only one record 2HHI for *Mtb* MPT64 protein, resolved by solution NMR [25]. Electrostatic surface potential of 2HHI models were computed by DelPhi Web Server (http://compbio.clemson.edu/sapp/delphi_webserver/) using AMBER parameters and Linear solver MPT64 2HHI model structure and accessible surface area and residues for potential binding site were analyzed.

2.7. Detection of protein with an aptamer based sandwich enzyme linked oligonucleotide assay (ELONA)

ELONA is a plate-based assay designed for detection and quantification of complexes, where a protein is immobilized to a solid surface and, then, complexed with biotinylated aptamer [26]. The complex is assessed by the absorbance change upon addition of a substrate (Fig. 1). Seventeen identified aptamer sequences were further tested for their binding to target MPT64 using ELONA based on protocol [17] with modifications. Non-target CFP-10 and ESAT-6 proteins were used as controls. Each biotinylated ssDNA used in the assay was custom synthesized (Sigma). Ninety-six well plates (Bioster, A947978) were coated with 500 ng of protein in 100 μl of 100 mM Na_2CO_3 buffer overnight at 4 °C. After blocking with 1% BSA (Fisher Scientific, BPE1600-100) for 1 h at 4 °C the wells were washed 4 times with 1 × TBS buffer (24.2 g Tris Base, 80 g NaCl, pH 7.6), and biotin labeled ssDNA aptamers (500 nM) were added to each well and incubated for 2 h at 37 °C. The wells were then washed 4 times with 1 × TBS buffer (24.2 g Tris Base, 80 g NaCl, pH 7.6 for 1 L). Streptavidin-horseradish peroxidase conjugate (Thermo Scientific, NE170004) was diluted in 1:10000 in TBS buffer and 100 μl was applied and incubated for 30 min at 37 °C. Then, 50 μl of Turbo-3,3′5,5′-tetramethylbenzene (TMB, Sigma T8665) was added to each well and incubated for 15 min at 37 °C. The reaction was quenched by adding 50 μl of 1 M H_2SO_4,

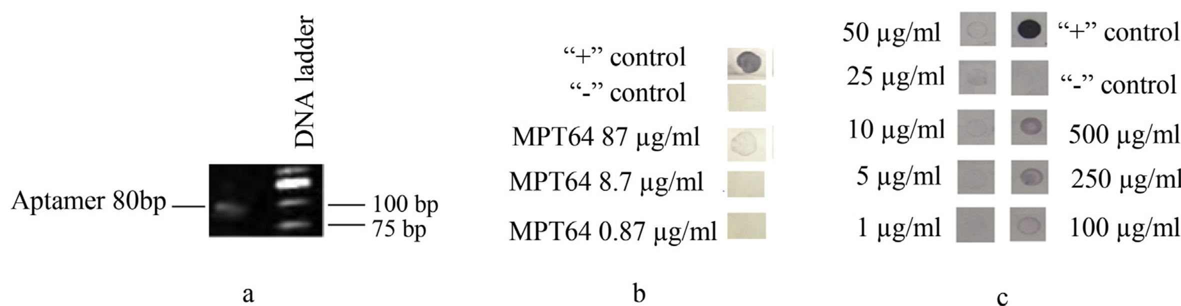

Fig. 2. **(a)** 6% TBE-PAGE analysis of PCR products from cycle 6 of *in vitro* selection of aptamers against target MPT64 by SELEX method stained with SYBR Green I: Lane 1. PCR product of SELEX cycle 6, Lane 2. 25 bp DNA ladder (Invitrogen); **(b)** Dot blot analysis of affinity binding between aptamer pools from the SELEX cycle 5 and target MPT64, and two controls; **(c)** Affinity evaluation of the ssDNA aptamer sequence (4) using dot blot analysis method. "+" control *Escherichia coli* antibodies labeled with biotin (diluted at 1:500), "-" control - deionized water.

and protein-aptamer complex was quantified by determining the absorbance at 450 nm using MultiScan FC (Thermo Scientific) (Fig. 1). In the background control, MPT64, CFP-10 and ESAT-6 were coated on the wells but none of the ssDNA aptamers were added. For each sample the optical density at 450 nm (OD) of background was subtracted from the OD value of the experimental sample.

2.8. Determination of the dissociation constant (K_D) of aptamers

BIAcore X100 SPR instrument with SA chips (GE Healthcare, Austria, Vienna) was used to determine the affinity of the ssDNA aptamer sequence (17) to MPT64 protein in kinetic studies. All experiments were conducted at 25 °C with flow rate of 10 μl/min. HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) was used as the running buffer and 12 mM NaOH (10 μl) was used as a regeneration buffer to remove the bound target. Selected biotinylated aptamers were immobilized onto the surface of the streptavidin coated SA chip at a concentration of 1 μM for 10 min and followed by injecting 10 μM of biotin for 5 min and ethanolamine-HCl (1 M, pH 8.5) as blocking reagents. The flow cell with ethanolamine-HCl blocking, but without aptamer immobilization, was used as a control to subtract non-specific binding. MPT64 proteins with different concentrations (2.5, 5, 10, 20, and 40 μg/ml) were injected over the sensor chip, and the affinity binding was monitored for 2 min followed by washing with running buffer. The evaluation was done using BiaEvaluation software (Biacore) by 1:1 [Langmuir] fitting model.

2.9. MPT64 antigen detection in sputum samples

Case controlled study was performed to determine the specificity of aptamers with the highest binding capacity to MPT64. We obtained 43 sputum samples, 20 from patients with definite presence of *Mtb* and 23 from non-tuberculous controls. Clinical samples were obtained from regional Tuberculosis hospital (Astana, Kazakhstan). Patients were diagnosed with *Mtb* by phthisiatrician based on the criteria of Kazakhstani preventive measures on tuberculosis. The sputum samples from the hospital confirmed the presence of *M. tuberculosis* in infected patient samples using Ziehl–Neelsen stain and culturing at 37 °C in Lowenstein-Jensen medium and Bactec MGIT for mycobacteria testing. Method of sputum liquefaction was same as in Ref. [27] with modifications. Briefly, 0.4 M NaOH solutions was added into equal volume of human sputum samples. The suspension was vortexed briefly. Subsequently, suspension from each tube was transferred to an empty tube, and 1 M HEPES buffer was added to each tube to neutralize the NaOH. The samples were then introduced onto the ninety-six well plates and left overnight at 4 °C. ELONA was performed to determine whether aptamer was able to detect the presence of MPT64 in sputum samples. The surface of microtiter plates was blocked using 1% BSA solution for 1 h at 4 °C. The wells were then washed 4 times with 1 × TBS. Biotinylated aptamer sequences (16) and (17) in concentration of 500 nM were added to each well and incubated for 2 h at 37 °C. In the background, control sputum samples were coated on the wells but none of the ssDNA aptamers were added. For each sample the optical density at 450 nm (OD) of background was subtracted from the OD value of the experimental sample. ELONA results were then used for specificity and sensitivity comparison using 43 sputum samples, where each sputum sample was tested in triplicate. The results were averaged and the standard deviation was calculated for each sputum sample.

2.10. Statistical analysis

All statistical analyses were performed using Graphpad Prism 6 (Graphpad Software, Inc). The diagnostic value of the assay using aptamers was evaluated using Receiver Operating Characteristics (ROC) curve analysis (Graphpad Prism 6, Graphpad Software, Inc). OD values for ELONA assay were plotted and the area under the curves (AUC) and 95% confidence intervals (95% CIs) were calculated. The optimal cut-points were determined based on maximum value of Youden's index (YI = specificity + sensitivity − 1).

3. Results

3.1. In vitro selection of aptamers

A random ssDNA library of about 10^{14} aptamers was screened for their efficiency to bind target MPT64. A commercially available MPT64 protein (Rv1980c) of *Mtb* H37Rv strain was used as the target. Following incubation, the bound oligonucleotides were separated from unbound ones via nitrocellulose filtration as described in the Methods section. Target-bound aptamers were eluted from the filters, and enriched at each selection round by amplification using PCR. After each round of selection the obtained DNAs were quantified by spectrophotometer and analyzed by PAGE gel. Fig. 2a shows the PAGE gel of the 6th round of selection with an expected 80bp DNA size. A total of ten rounds of repeated separation-amplification cycles were completed in order to receive high affinity and specificity of MPT64 DNA aptamers.

Obtaining pure ssDNA is an important step during *in vitro* selection. We chose to use lambda exonuclease as it was shown to improve the SELEX efficiency and final purity of the ssDNA solutions, compared with the methods involving streptavidin-coated magnetic beads and further alkaline treatment [28].

As a rapid assessment, dot blot analysis was performed after SELEX rounds for evaluation of binding affinity to the target MPT64. In this regard, target MPT64 was spotted onto the nitrocellulose membrane in different concentrations, air-dried at room temperature, and then incubated with biotinylated aptamer pool from the 5th cycle. *E. coli* antibodies labeled with biotin served as a positive control, and deionized water served as a negative control. The results of dot blot assay for the aptamer pool at the 5th cycle is presented in Fig. 2b. Violet stained spots on the nitrocellulose membrane (Fig. 2b) indicated the binding reaction between target protein and aptamer pool and developed a clear dot for the target MPT64 with a concentration of 87 μg/ml, while showed a faint dot for the concentration 8.7 μg/ml. With the increase of selection cycles, the selected aptamer pools showed stronger binding to the target with the increase of both dot size and intensity. Only after this confirmation, the next SELEX cycle was conducted. An affinity evaluation of obtained individual aptamers with known sequences was performed using the dot blot assay as well. Target protein in different concentrations was immobilized on the surface of the nitrocellulose membrane, which was further incubated with biotinylated aptamer sequence (4). The amount of enzyme-linked conjugate bound was assayed by incubating the strip with an appropriate chromogenic substrate leading to a color development. Therefore, ssDNA aptamers were deemed to react with target protein when the strips showed purple dots. Fig. 2c shows the dot blot affinity analysis for the aptamer sequence (4). The intensity of the violet stained spots increased with increasing concentrations of the selected aptamer (4), indicating that indeed the aptamer had an affinity to the target.

3.2. Sequencing and bioinformatics analysis

Cloning and sequencing of aptamer pools from ten rounds of SELEX cycles revealed seventeen aptamers with unique sequences. We further analyzed the sequences of aptamers (16) and (17) since they were the most specific to MPT64 as it was shown in an ELONA assay (Fig. 3b). Computed GC/AT ratio (G + C)/(A + T) of all seventeen aptamers varies from 0.21 to 1.5, knowing that selected aptamer sequences (16) and (17) have GC/AT ratio of about 1.11. Aptamers (16) and (17) had the longest common TCCAGT sequence which was present once in each aptamer. Position of this sequence is similar in predicted secondary structures, based on Watson-Crick pairing A-T and G-C, for aptamers (16) and (17). In both aptamers this sequence is located within the biggest potential stem loop (hairpin), formed by four Watson-Crick base pairs in stem parts and having 6/4 bases in loop part for aptamer sequences (17) and (16), respectively. In both aptamers (16) and (17), the GT suffix of this sequence belongs to the stem of the loop. Selected aptamer sequences do not have known G quadruplex motifs as found in anti-thrombin aptamer [29]. The GC/AT ratio is one of the basic sequence characteristics in terms of nucleotide composition and in future studies, aptamer sequences could be modified and optimized taking into account this ratio.

Surface charge analysis for the target MPT64 model (PDB code 2hhi) showed positively charged hydrophilic amino acids, such as arginine (ARG85 and ARG117) and lysine (LYS118), were found to be grouped allowing a potential binding site for the negatively charged DNA backbone.

3.3. Evaluation and characterization of DNA aptamers

ELONA was used to determine the binding interaction between the selected aptamer sequences and the target MPT64. Non-target *M. tuberculosis* immunogenic secreted proteins ESAT-6 and CFP-10 with a concentration of 500 ng/ml was also tested to check any cross-reactivity. Experiments were conducted in six replicates. A general pattern shows that all aptamers have higher binding to the target MPT64 compared to non-target ESAT-6 and CFP-10 (Fig. 3a, supplem.mat/Fig. 1). As can be observed from Fig. 3a, specificity of aptamer sequence (17) is presented in red circles for target and non-target proteins, where OD value for both non-target proteins was significantly lower ($P < 0,0001$) in comparison to the target. ELONA analysis of the relative binding abilities of individual aptamer sequences (1–17) was further analyzed. This was done by subtracting the absorbance of MPT64-aptamer complex by the highest absorbance of either aptamer-ESAT-6 or aptamer-CFP-10 complexes (Fig. 3b). The negative value for aptamer sequence (10) shows that the oligonucleotide preferentially binds to either ESAT-6

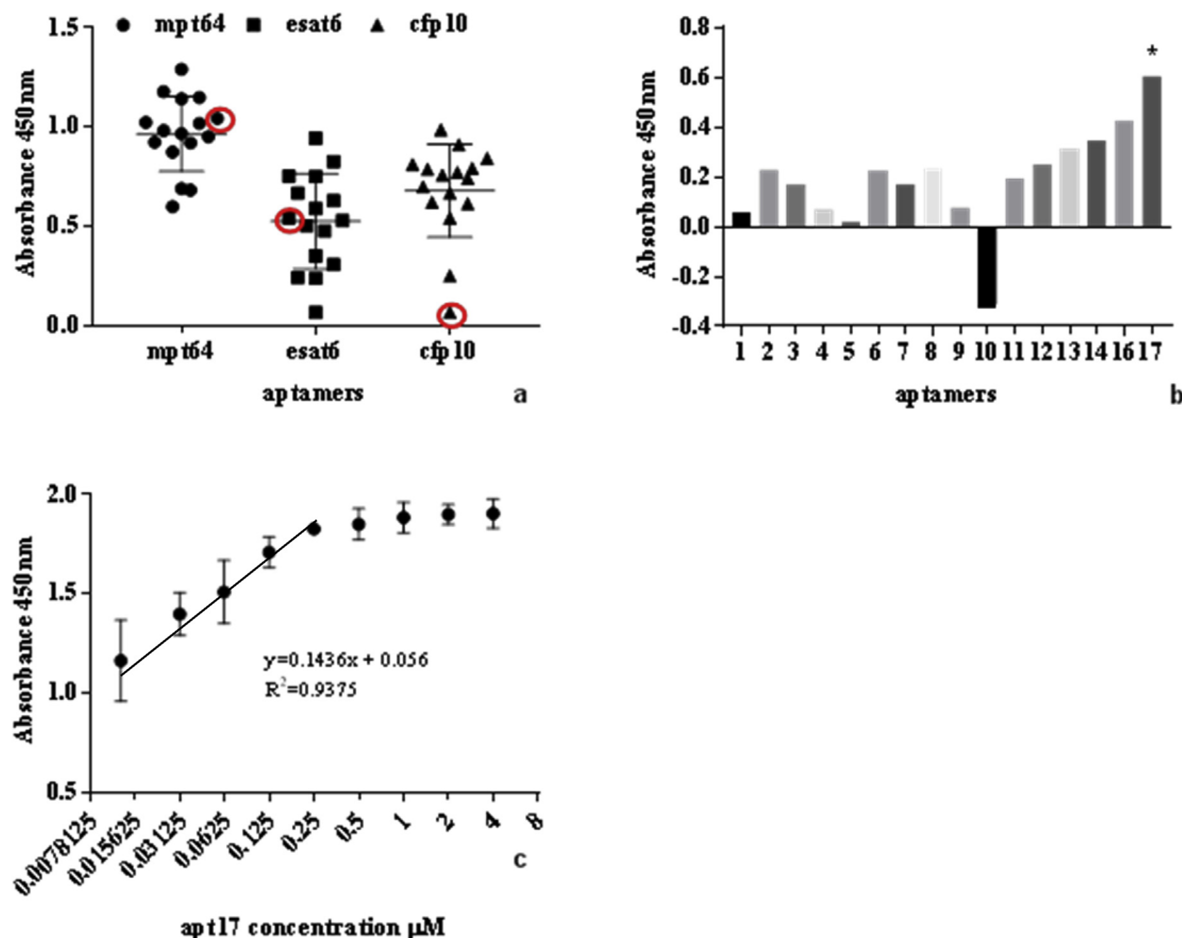

Fig. 3. Binding affinity of selected ssDNA aptamers to proteins MPT64, ESAT-6, CFP-10. Binding of biotinylated ssDNA aptamers to the target MPT64, and non-target ESAT-6 and CFP-10 was determined by an ELONA assay. Binding of the ssDNA aptamers to ESAT-6 and CFP-10 is shown for comparative purposes. **(a)** Individual aptamer sequences (1–17) (500 nM) were incubated in wells coated with target MPT64, and non-target ESAT-6 and CFP-10. Aptamers (17) are shown in circles. All data are shown as the means of ±SEMs (n = 6). **(b)** ELONA analysis of the relative binding abilities of individual aptamer sequences (1–17). **(c)** Binding of the aptamer sequence (17) to the target MPT64. All data are shown as the means of ±SEMs (n = 3).

or CFP-10 and not to target MPT64. Aptamer sequence (17) showed the highest binding to target MPT64 rather than to non-target proteins used in this assay (Fig. 3b). Therefore, aptamer sequence (17) was chosen for further assessment. The dose response analysis for the aptamer sequence (17) showed an increase in absorbance signal from concentration ranging from 12 nM to 250 nM. There was no further increase observed after 250 nM indicating that the surface was saturated above this concentration (Fig. 3c). A linear correlation was found between OD_{450} value and aptamer sequence (17) in a range of concentration (Fig. 3c). The regression equation for OD_{450} difference versus aptamer (17) concentration was $y = 0.1436x+0.056$ with $R^2 = 0.94$, where x is the concentration of aptamer sequence (17) in nM (Fig. 3c). The result showed that the selected aptamer could be used as a recognition ligand to detect target MPT64.

Surface plasmon resonance (SPR) is a technique used to follow the dynamics of interactions between a target molecule immobilized on a biochip and a ligand introduced in solution. It allows apparent kinetic constants to be estimated for the formation of a specific complex and a quantitative estimation of the affinity of the ligand for the target molecule to be calculated. In this study, SPR was used to identify the affinity of the selected aptamer sequence (17) against target MPT64. Immobilization of the aptamer was based on streptavidin-biotin interaction. SPR signal reached 1510.8 RU after injecting a biotinylated aptamer over a sensor chip coated with streptavidin, indicating immobilization of the aptamer on the sensor surface via biotin to streptavidin interactions. MPT64 protein at different concentrations (2.5, 5, 10, 20, 40 μg/ml) was injected on the sensor chip so the association and dissociation of the complex could be monitored as a function of time. The protein was injected at increasing concentrations over the aptamer coated surface and the reference was taken directly on the surface without aptamer. The analytical signal was computed as the difference between the signals (resonance units, RU) detected on the aptamer surface and on the reference. An overlay of the SPR signal from the interactions between aptamer (17) and the target MPT64 is shown in Fig. 4. With increasing MPT64 concentrations there was also an increase in RU. The calculated k_a (association rate constant) for aptamer sequence (17) was 9.93×10^{-4} (M^{-1} s^{-1}) and k_d (dissociation rate constant) was 8.85×10^{-4} (s^{-1}) and K_D (dissociation equilibrium constant) was 8.92 nM. The binding kinetics of the immobilized ssDNA aptamers to MPT64 protein was determined using BiaCore X100 surface plasmon resonance.

3.4. MPT64 antigen detection in sputum samples

Forty-three sputum samples were used to evaluate aptamer sequences (16) and (17) for detecting TB infection in case controlled study using ELONA. Aptamer (16) was the second in line for specific MPT64 binding, and it was used for comparison purposes. It was found that OD_{450} values were significantly higher in those patients with active TB than in healthy individuals (Fig. 5a, $p \leq 0.0001$). Twenty of sputum samples had a confirmed TB and twenty-three samples were obtained from healthy individuals. The specificity and sensitivity of the sputum sample analysis based on aptamer (17) by ELONA were calculated as 90% (96 CI: 68.3–98.7%) and 91.3% (95% CI: 71.96–98.93%), respectively, using Youden's index. The AUC was 94.78% (95% CI: 89–100%) from the ROC curve (Fig. 5b). The specificity and sensitivity for aptamer (16) was 75% (95% CI: 50.9–91.34%) and 100% (95% CI: 85.75–100%), respectively. The AUC for aptamer (16) was 95% (95% CI: 89.37–100%) (Fig. 5c).

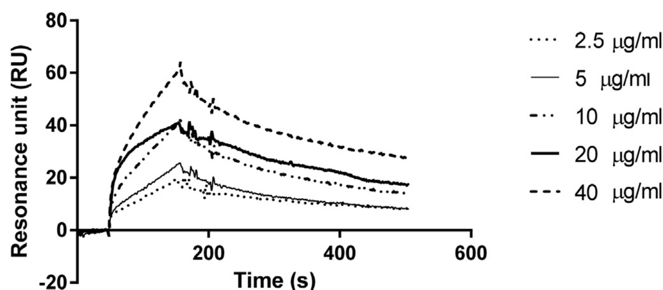

Fig. 4. An overlay of the SPR signals from the interaction between the target and aptamers (17). In each case, the protein was injected over immobilized aptamers (17) with an initial concentration of 2.5, followed by 5, 10, 20 and 40 μg/ml.

4. Discussion and conclusion

Theoretically, the DNA library used during this study could potentially contain up to 4^{40} or 10^{24} different sequences, since the diversity of an oligonucleotide library is 4^n, where n is the length of the oligonucleotides [11]. However, in practice 10^{16} sequences are usually used [30]. Nevertheless, this is still much higher than possible murine antibodies of 10^9 to 10^{11}. This large range of sequences gives an opportunity to select ligands with a very high affinity [11]. The stability of aptamers in various temperatures fluctuations offers another advantage, as they are tolerant to harsh chemicals and biological conditions as compared to antibodies. In addition to low cost production and variability by modifications, aptamer in several studies showed to be extremely specific and sensitive being able to detect down to pM and fM range [31–33]. To achieve this concentration range, an antifouling surface chemistry of aptamers on the surfaces needs to be carefully tuned and optimized for better detection. These parameters could greatly enhance both sensitivity and specificity of the aptasensor [32,33], where the detection limit could be lowered below the current clinical value detection.

In this study, aptamers against *Mtb* secreted protein MPT64 were successfully selected and could be used as an alternative for detection and diagnosis of TB for in field or on-site application. The selected aptamer showed a good specificity and sensitivity values; therefore, they would likely be less prone to give false positive results. The selected aptamer could also be used in a multiplexed assay for TB diagnosis alongside with other aptamers selected against ESAT-6, CFP-10 and surface saccharides of *Mtb*. Previous study with ESAT-6 and CFP-10 showed that the binding of some aptamers could be abrogated by the presence of anti-MPT64 antibody by possibly competing to the same binding site [17]. In addition, due to the steric hindrance and the size difference between aptamer and antibody, the information obtained from ELONA i.e. absorbance value would be different as well. Therefore, current study did not compare the binding process of aptamer-MPT64 with that of antibody-MPT64. The comparison could be done after optimizing the surface chemistry for aptamer and measuring the values using different techniques, such as electrochemical impedance spectroscopy. In case of low signal, aptamers could be modified easily such as labeling with magnetic/gold nanoparticles or with fluorescent reporting molecules, which offer a great potential for the development of aptamer based detection technologies for rapid, cost effective and reliable detection method for TB diagnosis. Additional NMR, colorimetry studies and further bioinformatics analysis and aptamer manipulation could be conducted for improved biosensor applications. For future studies, the specificity and sensitivity test could be validated by testing sputum samples from chronic patients with no TB infection, such as asthmatic.

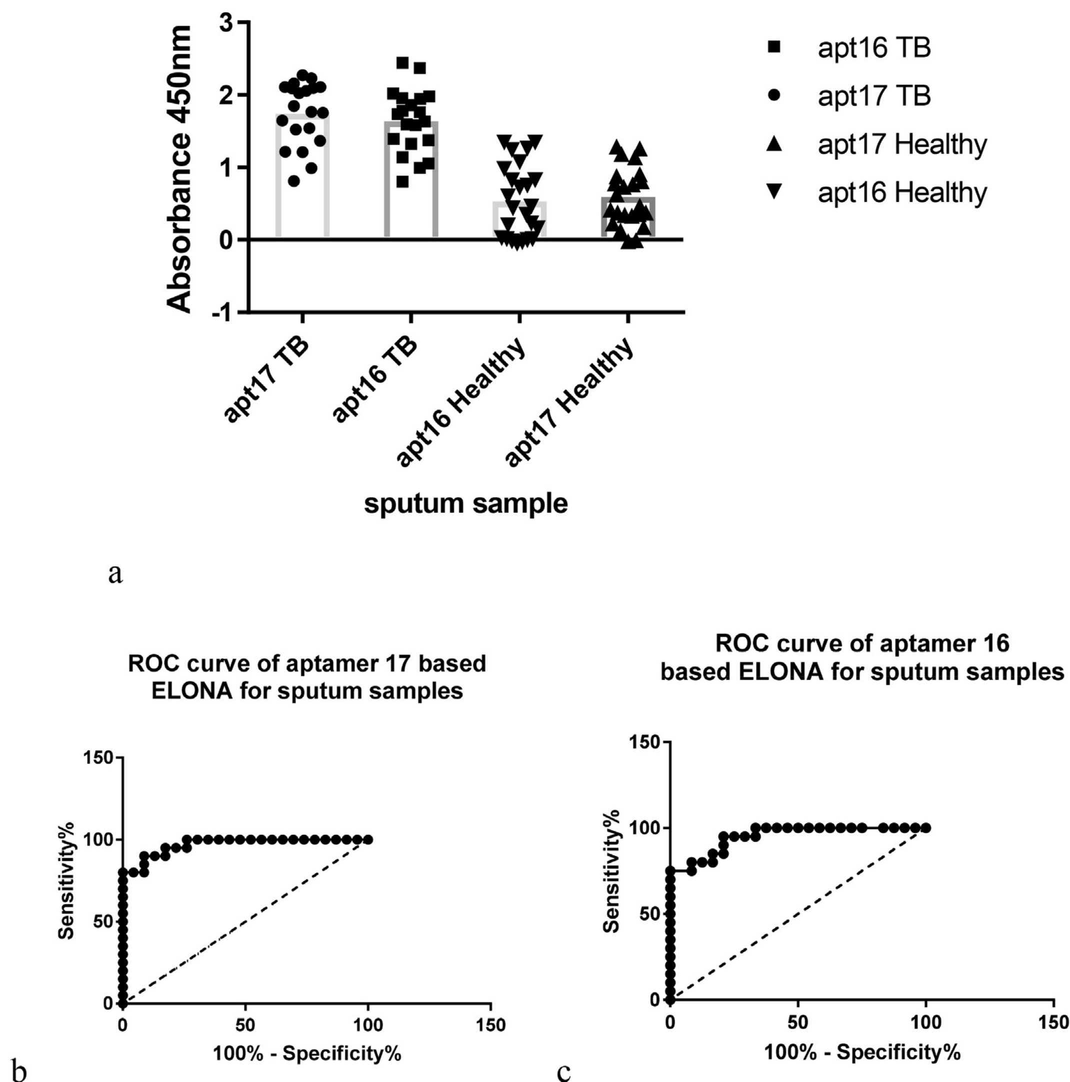

Fig. 5. Detection of MPT64 antigens in sputum samples. **(a)** ELONA based assay for the detection of MPT64 antigens in TB infected and healthy individuals using aptamer sequences (16) and (17); **(b, c)** The ROC curve of aptamer sequences (17) and (16), respectively, based on ELONA for sputum samples.

In conclusion, selected ssDNA aptamer bounded specifically to the target MPT64 using the SELEX technology. Selected aptamers further showed a strong binding affinity with high specificity against *Mtb* secreted protein MPT64. Future research will focus on optimizing the surface chemistry for the development of a sensor using the selected aptamers as bio-recognition elements for TB diagnosis.

Ethics statement

Approval for the use of sputum samples was obtained from the Nazarbayev University Research and Ethics committee. Written consents from patients whose sputum samples were used in this study were revived.

Funding

This work was supported by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan, state program #0115PK030040 and #0111PK00364, and grant #0115PK02453.

Acknowledgements

We would like to acknowledge Anna Tsepke, chief physician, and Akmaral Akisheva, head of the clinical diagnostic laboratory of the anti-Tuberculosis Dispensary of Astana city, Republic of Kazakhstan for providing TB patient sputum samples and working space in BSL-3 laboratory. In addition, we wish to thank Dr. Bakhyt Matkarimov for the bioinformatics analysis, and Dr. Jacob Lum for his help in SELEX experiments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2017.03.004.

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