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Selection of Nucleic Acid Aptamers Specific for *Mycobacterium tuberculosis*

Erkan Mozioglu[1,2] · **Ozgur Gokmen**[3] · **Candan Tamerler**[1,4] · **Zuhtu Tanil Kocagoz**[5,6] · **Muslum Akgoz**[2]

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Abstract Tuberculosis (TB) remains to be a major global health problem, with about 9 million new cases and 1.4 million deaths in 2011. For the control of tuberculosis as well as other infectious diseases, WHO recommended "*ASSURED*" (*Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to the end user*) diagnostic tools that can easily be maintained and used in developing countries. Aptamers are promising tools for developing point-of-care diagnostic assays for TB. In this study, ssDNA aptamers that recognize *Mycobacterium tuberculosis* H37Ra were selected by systematic evolution of ligands by exponential enrichment (SELEX). For this purpose, two different selection protocols, ultrafiltration and centrifugation, were applied. A total of 21 TB specific aptamers were selected. These aptamers exhibited "G-rich" regions on the 3′ terminus of the aptamers, including a motif of "TGGGG," "GTGG," or

✉ Erkan Mozioglu
erkanmozioglu@yahoo.com; erkan.mozioglu@tubitak.gov.tr

Ozgur Gokmen
ozgurgokmen75@hotmail.com

Candan Tamerler
candan.tamerler@gmail.com; ctamerler@ku.edu

Zuhtu Tanil Kocagoz
tanilkocagoz@gmail.com; Tanil.Kocagoz@acibadem.edu.tr

Muslum Akgoz
muslum.akgoz@tubitak.gov.tr

1 Molecular Biology—Biotechnology & Genetics Research Center, Istanbul Technical University, Istanbul, Turkey

2 Bioanalysis Laboratory, TÜBİTAK UME (National Metrology Institute), Kocaeli, Turkey

3 Chemistry Department, Gebze Institute of Technology, Kocaeli, Turkey

4 Mechanical Engineering and Bioengineering Research Center, University of Kansas, Lawrence, KS, USA

5 Department of Microbiology and Clinical Microbiology, Acıbadem University, Istanbul, Turkey

6 Trends in Innovative Biotechnology Organization, Istanbul, Turkey

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"CTGG." Binding capability of selected aptamers were investigated by quantitative PCR and Mtb36 DNA aptamer was found the most specific aptamer to *M. tuberculosis* H37Ra. The dissociation constant (K_d) of Mtb36 aptamer was calculated as 5.09±1.43 nM in 95 % confidence interval. Relative binding ratio of Mtb36 aptamer to *M. tuberculosis* H37Ra over *Mycobacterium bovis* and *Escherichia coli* was also determined about 4 times and 70 times more, respectively. Mtb36 aptamer is highly selective for *M. tuberculosis*, and it can be used in an aptamer-based biosensor for the detection of *M. tuberculosis*.

Keywords Tuberculosis · Diagnosis · Oligonucleotide · Aptamer · Selection · SELEX · Biosensor

Introduction

Tuberculosis (TB) remains to be a major global health problem. It was estimated that 8.7 million new cases of TB and 1.4 million deaths occurred because of TB over the world in 2011 [1]. TB-causing agent *Mycobacterium tuberculosis* is transmitted by respiratory secretion aerosols originating from patients with pulmonary TB [1, 2]. Because BCG vaccination has low level of protection, the most important component of tuberculosis control is to identify early the patients who produce sputum containing *M. tuberculosis* bacilli and treat them efficiently before they transmit the disease to uninfected individuals [1, 2]. In TB high burden countries, the laboratory capabilities are limited for early diagnosis of TB [1, 2]. Diagnosis should be made in an efficient, timely manner, preferably at point-of-care (POC), using accurate, field-friendly tools [3].

For that reason, WHO recommends "ASSURED" (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to the end user) diagnostics for developing countries [4]. Point-of-care testing platforms [2] include smear microscopy [5–7], nucleic acid amplification tests [8, 9], antigen detection-based tests [10], and newer nucleic acid amplification-based methods [11]. In addition, aptamer-based techniques are also alternatives for point-of-care assays for the diagnosis of TB [4, 12–18].

Aptamers are DNA, RNA, and protein molecules selected by SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process which was first defined by Tuerk and Gold (1990). They expressed SELEX as "the beginning of evolution in a test tube" [19]. In this process, a single stranded DNA or RNA library is used for the selection of DNA/RNA aptamers. These nucleic acid libraries have molecules with sites of random sequences which are approximately 40 nucleotides in the center. In addition to this random site, these libraries include flanked region on either end, which are primer sites. So, each single stranded nucleic acid molecule has the same primer sequences but different random sequences in a library. An aptamer library is expected to have 10^{14}–10^{16} unique sequences theoretically. When an aptamer library is incubated with a target of interest, some ssDNAs/RNAs are able to bind to the target. Then, nucleic acids bound to the target are separated from the unbound by washing the latter. Target-bound sequences are then amplified by PCR and amplicons are used for the next round selection. These selection rounds are performed until the sequences, with high affinity for target, are obtained. These aptamers are cloned and sequenced [20, 21].

Aptamers can be selected for a wide range of targets, from purified target molecules to whole living cells. These can include small molecules such as ATP [22], more complex molecules such as proteins or cell components [23, 24], and whole-living cells such as bacteria

[25]. SELEX process received a lot of interest and many studies have been done since Tuerk and Gold [19] published the first study.

In this study, we obtained ssDNA aptamers against *M. tuberculosis* H37Ra by SELEX. For this purpose, we used two different methods: ultrafiltration and centrifugation. Specificity of our aptamers to *M. tuberculosis* H37Ra was confirmed by exposure to *Mycobacterium bovis*, which belongs to tuberculosis complex and is also used in BCG vaccines, and *Escherichia coli*, which is a phylogenetically distant species to mycobacteria.

Materials and Methods

Microorganisms

E. coli was obtained from THSK (Turkish Public Health Agency, Turkey), and *M. tuberculosis* H37Ra and *M. bovis* from ATCC (The American Type Culture Collection, USA). *E. coli* were grown in tryptic soy agar (Merck, Germany) at 37 °C for 18 h; *M. tuberculosis* H37Ra and *M. bovis* were cultured in LJ Medium (Salubris Inc., Turkey) at 37 °C for 21 days.

Designing and Obtaining DNA Aptamer Library

We designed an aptamer library of random 40-nucleotide sequence, flanked with constant regions of primer sequences. The oligonucleotides in the library had the following sequences: 5′-CCGTCAGCCGAGGACCACAC-N40-TTGGGTGCAATGAAT-3′, where 40N is a variable 40-nucleotide site. Primer sequences for PCR were 5′-CCGTCAGCCGAGGACCACAC-3′ and 5′-ATTCATTGCACCCAA-3′. The aptamer library was synthesized by MerMade192 instrument (BioAutomation, USA) using 1-μmol columns. Oligomers were desalted using sephadex G25 (Sigma, Germany) column. Primer annealing temperature for the aptamer library was determined by gradient PCR (Applied Biosystems, USA). In order to obtain ssDNA pools, eluted aptamers were amplified using both regular PCR and asymmetric PCR [23], and amplicons were purified using ethanol precipitation [26] before use. While each primer was used in equal amounts in regular PCR, in asymmetric PCR, the concentration ratio for forward to reverse primers was 100 to 1.

Preparing BSA Coated Microcentrifuge Tubes

To prevent non-specific binding of aptamers to tubes, they were coated with bovine serum albumin (BSA). For this purpose, a method which was used in the coating of 96-well plates was performed [23]. Briefly, 1400 μl of 100 mM $NaHCO_3$ (pH 9.5) was added in microcentrifuge tubes (Axygen, USA) and tubes were agitated at 200 rpm for 1 h. Then, the tubes were similarly treated four times by PBST (0.05 % Tween 20 in PBS, pH 7.4). Finally, 1400 μl PBST-BSA (3 % BSA in PBST) was added into the tubes which were agitated at room temperature at 900 rpm for 1 h. The solution was discarded and the tubes were stored at 4 °C until use. It was determined by quantitative PCR (qPCR). All binding and selection procedures were performed in these BSA-treated tubes, where DNA binding was found negligible (data not shown).

Preparing Bacteria for SELEX

Bacteria were harvested from LJ Media (Salubris Inc., Turkey) and resuspended in glass-bead suspension tubes (Salubris Inc., Turkey) including PBS.

SELEX Cycles

We performed SELEX using two different methods, centrifugation and ultrafiltration. In ultrafiltration method, ultrafiltration filters were used to remove unbound ssDNA molecules from the ones bound to mycobacteria. In centrifugation method, unbound ssDNA molecules were removed by washing after sedimentation of aptamers bound to mycobacteria, by centrifugation. Different buffers, for binding and washing, were used in each method; PBS [26] (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and PBST (0.05 % Tween 20 in PBS) buffers were used as binding and washing buffers in centrifugation method [25], while HEPES buffer [27] (20 mM HEPES, 120 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and HEPES-T (0.05 % Tween 20 in HEPES buffer) were used in ultrafiltration method [28], as described previously in literature. Before each binding and selection protocol, the aptamer library was heated to 95 °C for 15 min and snap-cooled on ice for 10 min. *M. tuberculosis* H37Ra was used for SELEX cycles, while *M. bovis* was used for counter-SELEX.

The Centrifugation Method

Bacteria were harvested from LJ Media (Salubris Inc., Turkey) and resuspended in glass-bead suspension tubes (Salubris Inc., Turkey) including PBS. For the first cycle, 1 ml of 1 McFarland *M. tuberculosis* H37Ra suspension was transferred to low-DNA binding tubes (Eppendorf, USA) and centrifuged at 14,000×*g* for 5 min. Supernatants were discarded and pellets were resuspended with PBS. After centrifugation, supernatants were removed and pellets were resuspended with 100 μl of the aptamer library in PBS (final aptamer concentration was 50 μM). Tubes were incubated for 30 min at 37 °C by agitating at 1300 rpm in an incubator (Hangzhou, China) (at 15th minute, it was additionally mixed by pipetting). Then, tubes were centrifuged at 14,000×*g* for 5 min. The supernatant was discarded. Pellets were resuspended with 100 μl PBST followed by centrifugation. Supernatants were discarded and the washing step was repeated three more times. Then, pellets were resuspended in PBS. After centrifugation and discarding the supernatant, pellets were resuspended with PCR buffer [26] (50 mM KCl, 100 mM Tris–HCl, 15 mM $MgCl_2$, pH 8.3). For elution, tubes were boiled [29]. Briefly, tubes were then kept at 95 °C for 10 min and centrifuged at 14,000×*g* for 2 min. Supernatants were transferred to clean tubes and used as template to amplify aptamers by PCR (Fig. 1). For this purpose, first regular PCR, then asymmetric PCR was performed (Fig. 2). PCR products obtained by asymmetric PCR were mixed with 2× PBS in equal volume (50 μl/50 μl), kept at 95 °C for 15 min, and snap-cooled on ice for 10 min. These molecules were used as aptamer pools (100 μl) for the next selection period. For the second cycle, 1 ml of 1 McFarland *M. bovis* suspension was transferred to the aptamer pools (100 μl) and incubated for 30 min at 37 °C and agitated at 1300 rpm for binding (at 15th minute, it was additionally mixed by pipetting). And then, tubes were centrifuged at 14,000×*g* for 5 min. Supernatants were transferred to a clean tube and used for the next selection round for *M. tuberculosis* H37Ra. These selection rounds were repeated six more times. At the seventh round, binding

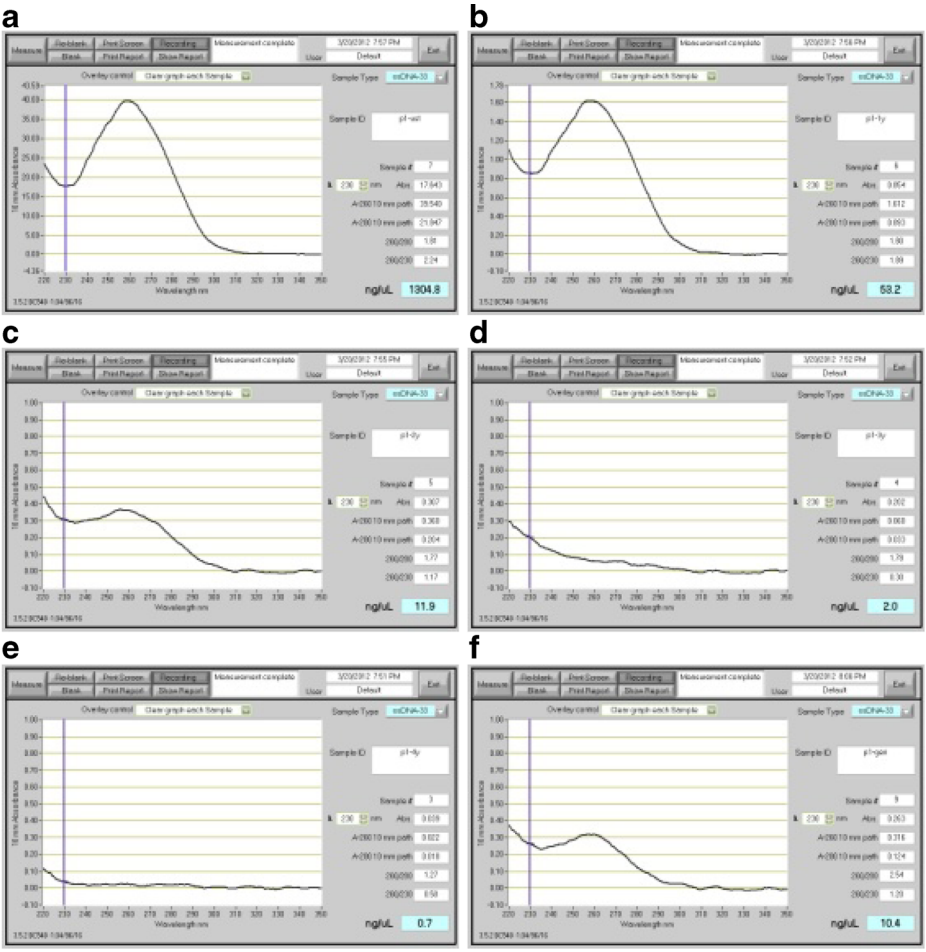

Fig. 1 Binding, washing, and elution profiles of aptamer selection to *M. tuberculosis* H37Ra bacteria. Nanodrop spectrums were recorded after binding to *M. tuberculosis* H37Ra (**a**), after washing steps of 1st (**b**), 2nd (**c**), 3rd (**d**), and 4th (**e**), and after elution step (**f**)

time for *M. tuberculosis* H37Ra was shortened to 15 min and washing step was performed ten times. Finally, DNA molecules obtained in this last round were amplified by PCR and cloned to a plasmid to determine DNA sequences.

The Ultrafiltration Method

For ultrafiltration method, ultrafiltration tubes (Amicon Ultra–Millipore 50K, USA) were used. Five hundred microliters of the aptamer library was added to 500 μl of 2× HEPES binding buffer. Bacteria were harvested from LJ Media and resuspended in glass-bead suspension tubes in HEPES binding buffer. For the first cycle, 500 μl of 0.5 McFarland *M. bovis* suspension was transferred to a microcentrifuge tube coated with BSA and 500 μl of aptamer library (prepared above) was added (final aptamer concentration was 20 μM). The mixture was incubated at 37 °C, 1300 rpm agitation for binding for 30 min (at 15th minute, it was

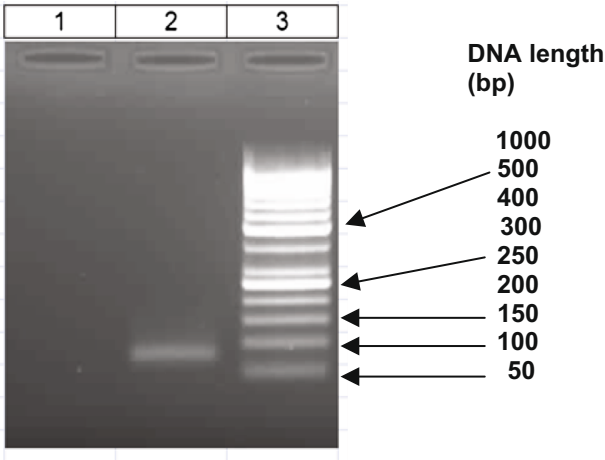

Fig. 2 PCR products of first cycle of aptamer selection to *M. tuberculosis* H37Ra bacteria. After the first cycle of selection, normal PCR amplification and secondly asymmetric PCR was performed. The PCR products were run in 2 % agarose gel. *Lane 1* negative control; *2* asymmetric PCR product; *3* 50-bp DNA ladder

additionally mixed by pipetting). Then, bacteria and aptamer mixture was transferred to the filters and tubes were centrifuged at 14,000×*g* for 5 min. Filter-through portions were used as an aptamer pool for *M. tuberculosis* H37Ra selection cycles. The filtered liquid was transferred to 1000 μl of 0.5 McFarland *M. tuberculosis* H37Ra suspension. It was incubated for 30 min at 37 °C agitating at 1300 rpm for binding (at 15th minute, it was pipetted). Then, the mixture was transferred to a new filter and the filter was centrifuged at 14,000×*g* for 5 min. The filters were washed three times by passing 500 μl of HEPES-T buffer and two times with HEPES binding buffer. Finally, 100 μl of PCR buffer was added and incubated for 1 min at room temperature for elution of the aptamers. Then, tubes were centrifuged at 14,000×*g* for 5 min, and eluted portions containing aptamers were collected into clean tubes and they were used as PCR template. To amplify DNA, two different PCR strategies were employed: regular PCR and asymmetric PCR. The first PCR products were obtained by performing regular PCR, and it was used as template for the asymmetric PCR. PCR products obtained by asymmetric PCR were mixed with 2× HEPES binding buffer in equal volume (50 μl/50 μl). And, the mixture was kept at 95 °C for 5 min and snap-cooled on ice for 10 min. Until the sixth SELEX cycle, binding and washing conditions were the same, but in the sixth cycle, washing step for *M. tuberculosis* H37Ra was performed five times with washing buffer and two times with binding buffer. In the eighth SELEX cycle, incubation time for *M. tuberculosis* H37Ra was decreased to 15 min and washing steps were performed nine times with washing buffer and once with binding buffer. In all these cycles, binding conditions for counter-SELEX of *M. bovis* were not changed. DNA molecules obtained in the last cycle were amplified by PCR and the PCR product was cloned to a plasmid to determine DNA sequences.

Cloning Experiments

After SELEX with both centrifuge and the ultrafiltration methods, DNA molecules obtained were amplified by PCR and amplicons were purified using ethanol precipitation method [26]. Briefly, 50 μl of each amplicon was transferred into a microcentrifuge tube and 10 μl of

precipitation solution (120 mM sodium acetate pH 5.2; 40 mM EDTA, 4 μg glycogen) and 120 μl of cold-ethanol (absolute) were added. The tubes were centrifuged at 4 °C, 14,000×*g* for 15 min. The supernatant was discarded. Precipitates were dissolved in 15 μl of distilled water and concentration of the DNA was determined by spectrophotometer (Nanodrop-1000, USA). DNA molecules were also run in 2 % agarose gel (Fig. 3).

Purified amplicons were cloned in a plasmid using InsTAclone PCR Cloning Kit (Fermentas, USA) according to the manufacturer's instructions. After transformation, colony screening was performed using PCR to identify colonies of bacteria which included DNA aptamers. PCR amplicons obtained from 50 positive colonies were purified using ethanol precipitation and DNA molecules were sequenced in both directions (Macrogen, Korea). A total of 21 aptamers were selected with the correct size.

Analysis of Selected Aptamer Candidates Using Quantitative PCR

Twenty-one different DNA aptamers were selected and their sequences are given in Fig. 4. Seven of the DNA aptamers were randomly selected and investigated to determine their relative binding capability to *M. tuberculosis* H37Ra over *M. bovis*. The number of bound aptamers per bacteria was calculated [30] according to formula 2(aptamers Ct−bacterial genomic DNA Ct) using qPCR. HPLC purified DNA aptamers (Metabion, Germany) were used for binding experiments. As negative control, a ssDNA molecule including the same aptamer primer sites, but a different random sequence in the middle, was used. Binding experiments were performed as biological repeats in three different days to be able to compare seven selected aptamers and a negative control. These aptamers were dissolved in binding buffer [27] (20 mM HEPES, pH 7.4; 120 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$) and their final concentrations were adjusted as 2.5 μM. qPCR experiments were performed in SYBR Green I Master Mix (Roche, Germany) at 0.7 μM primer concentration. Tubes were washed with PBST buffer and then with PBS for three times. Prior to each experiment, all aptamer solutions were kept at 95 °C for 5 min and snap-cooled on ice for 10 min. One hundred microliters of 0.5 McFarland bacteria (*M. bovis* or *M. tuberculosis* H37Ra) was prepared in binding buffer and they were transferred to different tubes. Then, 10 μl of 2.5 μM aptamer was added to these tubes. The mixture was mixed by pipetting. One hundred microliters of binding buffer was

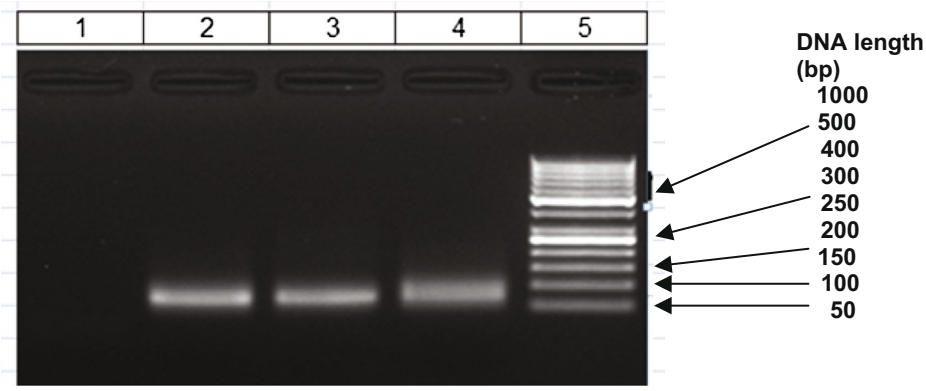

Fig. 3 PCR products of last cycle of aptamer elution from *M. tuberculosis* H37Ra bacteria. After last cycle of selection, normal PCR amplification was performed. The PCR products were run in 2 % agarose gel. *Lane 1* negative control; *2*, *3* ultrafiltration selection method; *4* centrifugation selection method; *5* 50-bp DNA ladder

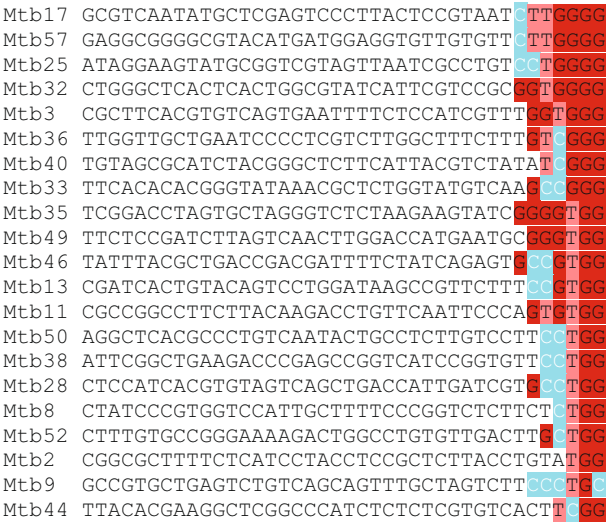

Fig. 4 DNA sequences of selected DNA aptamers to *M. tuberculosis* H37Ra. DNA nucleotides were colored *red* for "G," *blue* for "C," *pink* for "T," and *green* for "A"

also transferred to the tubes and the same amount of aptamer was added for negative controls of tube binding. The mixtures were incubated for 30 min at 37 °C and mixed at 1300 rpm for binding (at the 15th minute, it was pipetted). Tubes were centrifuged at room temperature (14,000×*g*) for 5 min. The supernatant was discarded and pellets were resuspended with 1000 μl of wash buffer (binding buffer including 0.05 % Tween 20). Tubes were centrifuged at room temperature, 14,000×*g* for 5 min. The washing steps were repeated seven times. Then, pellets were resuspended in 100 μl of binding buffer, and the suspensions were transferred to clean tubes. Tubes were centrifuged at room temperature (14,000×*g*) for 5 min. Pellets were resuspended with 50 μl PCR buffer and tubes were boiled for 10 min. After centrifugation, the supernatant from each tube was transferred to a clean tube and 5 μl was used for qPCR both with aptamer primers and primers [31] Tb11 (5′-ACCAACGATGGTGTGTCCAT) and Tb12 (5′-CTTGTCGAACCGCATACCCT) which are specific to *M. tuberculosis* DNA. Primer final concentrations in qPCR mixture were 0.7 μM.

Analysis of Mtb36 Aptamer Binding Specificity to *M. tuberculosis* H37Ra

Selected Mtb36 DNA aptamer was subjected to three binding and washing cycles for *M. tuberculosis* H37Ra, *M. bovis*, and *E. coli* in different tubes. Before starting binding assays, aptamers were kept at 95 °C for 5 min and snap-cooled on ice for 10 min. One hundred microliters of 0.5 McFarland bacteria (*M. tuberculosis* H37Ra, *M. bovis*, or *E. coli*) prepared in binding buffer [25] [0.1 mg/ml tRNA, 1 % BSA, 1× PBS, 0.05 % (*v*/*v*) Tween 20, pH 7.4] was transferred to individual tubes, and 10 μl of 2.5 μM aptamers was added to these tubes and mixed by pipetting. For non-specific tube binding controls, 100 μl of binding buffer was also transferred to a tube and the same amount of aptamers was added to this tube. All of these tubes were incubated for 30 min at 37 °C agitating at 1300 rpm for binding. The tubes were centrifuged at 14,000×*g* for 5 min. Supernatant was discarded and pellet was resuspended with 1000 μl of washing buffer (PBST, pH 7.4). All tubes were centrifuged at room temperature at 14,000×*g* for 5 min. The washing step was repeated three times. Then, bacteria pellets were resuspended by 100 μl of binding buffer, and suspensions were transferred to clean tubes. The

tubes were centrifuged at room temperature at 14,000×*g* for 5 min. Pellets were resuspended with 50 μl of PCR buffer and the tubes were boiled for 30 min. After centrifugation, the supernatant was transferred to a clean tube and 5 μl of supernatant was analyzed by qPCR with both aptamer primers and bacterial genomic DNA primers, individually. Ct values and the number of DNA aptamers per bacterium were calculated.

Determination of the Dissociation Constant of Mtb36 Aptamer

The dissociation constant of Mtb36 aptamer was determined by using Sigma Plot (version 13.0). For this purpose, five different dilutions of the aptamer were prepared and were incubated with bacteria (*M. tuberculosis* H37Ra). After binding and washing steps, qPCR was used to determine the mean number of the aptamer per bacterium for each concentration. At the same time, the number of aptamers per tube was determined for each concentration by using tubes without bacteria in order to determine non-specific binding errors.

Results

For the purpose of determination binding, washing, and elution profiles of aptamer selection of *M. tuberculosis* H37Ra bacteria, Nanodrop spectrophotometers were used. After binding to bacteria, the concentration of aptamer was approximately 1300 ng/μl, whereas by increasing washing steps, the concentration decreased up to 0.7 ng/μl. After the elution step, recovered aptamer concentrations were found to be approximately 10 ng/μl. The profile showed that the washing step was effected to remove non-binding aptamers, and after serial washing steps, there were still aptamers which are bound to bacteria more strongly (Fig. 1). During the SELEX process, in order to obtain an aptamer pool, normal PCR was followed by asymmetric PCR. In Fig. 2, an agarose gel image was given for the purpose of showing PCR optimization that asymmetric PCR amplicons were not about non-specific amplifications such as primer dimers since negative control had no any amplification bands (lane 1) and the amplicons had a positive band as expected.

At the end of the SELEX process, eluted aptamers from both centrifugation and ultrafiltration methods were amplified using normal PCR (Fig. 3). It was clear that these PCR products were 75 bp as expected. Then these amplicons were purified, cloned, and sequenced in order to determine aptamer sequences.

Sequence Analysis of Selected DNA Aptamers Specific to *M. tuberculosis* H37Ra

After SELEX process, a total of 21 DNA aptamer clones were selected and DNA nucleotides were sequenced (Fig. 4). These sequences do not include primer sequences. It is clearly seen that "G-repeats" are found on the 3′ end and most of the selected aptamers which include the motifs of "TGGGG," "GTGG," and "CTGG" (Fig. 4).

Determining the Best DNA Aptamer that Binds *M. tuberculosis* H37Ra

At first, the selectivity of aptamers was evaluated by qPCR. For this purpose, seven different aptamers (named as Mtb 8, 13, 33, 35, 36, 50, and 57 in our study) were selected randomly and used to show binding capacities to both *M. tuberculosis* H37Ra and *M. bovis* as biological

repeats in three different days. These results indicated that Mtb36 aptamer (Fig. 5, it was predicted according to Mfold web server [32–35]) had the highest selectivity for *M. tuberculosis* H37Ra than *M. bovis* (data not shown). Therefore, Mtb36 aptamer was further assessed to show its binding specificity to *M. tuberculosis H37Ra* comparing binding ratios of Mtb36 DNA to *M. bovis* and *E. coli*. After binding and washing cycles, the number of bound Mtb36 DNA aptamer per the number of bacteria was calculated [30] by qPCR, and ratios were given (Fig. 6). According to these results, Mtb36 aptamer bound to *M. tuberculosis H37Ra* bacteria more than 4 times for *M. bovis* and 70 times for *E. coli* bacteria which were selected for the purpose of a representative of mycobacterium tuberculosis complex (MTC) and a representative of other than mycobacteria, respectively. Because of high binding selectivity of Mtb36 aptamer, it was further investigated in order to calculate its K_d.

Calculation of the Dissociation Constant of Mtb36 Aptamer

The dissociation constant of Mtb36 aptamer was determined by performing quantitative polymerase chain reaction. For this purpose, 500 μl of 0.5 McFarland *M. tuberculosis* H37Ra was incubated with Mtb36 aptamers in different concentrations (100, 50, 5, 0.5, 0.05 nM) in binding buffer for 30 min at 37 °C agitating at 1300 rpm for binding. After centrifugation at 14,000×*g* for 5 min, pellets were washed with 1000 μl of washing buffer (PBST, pH 7.4). It was repeated three times. After resuspension with 100 μl of binding buffer, suspensions were transferred to clean tubes and centrifuged at room temperature at 14,000×*g* for 5 min. Supernatants were discarded and pellets were resuspended with 50 μl of PCR buffer. After boiling for 30 min and centrifugation, supernatants were transferred to clean tubes and 5 μl of them was analyzed by qPCR to calculate the K_d value using Sigma Plot (version 13.0).

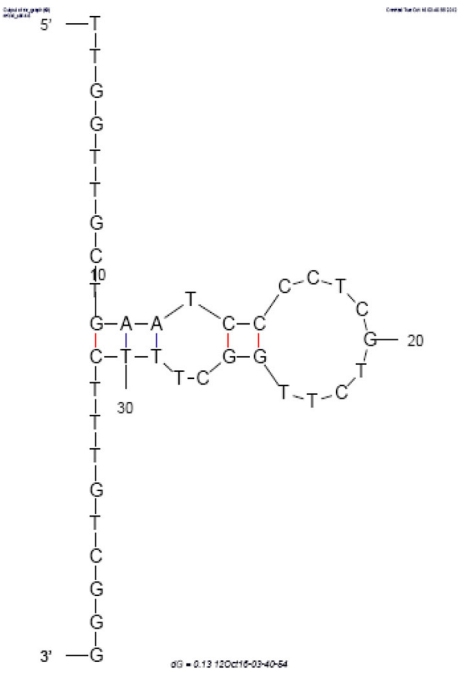

Fig. 5 Folding of the Mtb36 aptamer

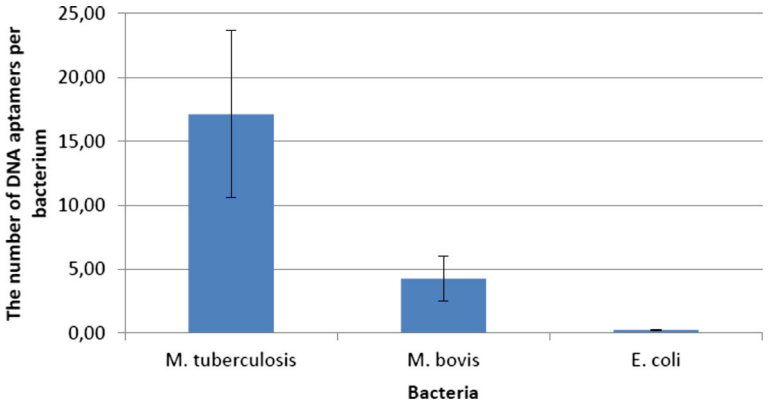

Fig. 6 Specificity of Mtb36 aptamer. Mtb36 aptamer was incubated with *M. tuberculosis* H37Ra, *M. bovis*, and *E. coli* bacteria; the bacteria was washed and the number of bound Mtb36 aptamers per bacteria was calculated using both aptamer and bacteria primers in different PCR tubes using 2Δ Ct method

Binding curves were plotted using aptamer concentrations against the number of DNA aptamers per bacterium. Results indicated that Mtb36 aptamer reached to saturation when its concentrations increased. The K_d value was calculated as 5.09±1.43 nM in 95 % confidence interval (Fig. 7).

Discussion

Tuberculosis continues to be a major health problem around the world. Rapid, easy-to-use, sensitive, and specific diagnostic tools which can be used at point-of-care are needed since laboratory capabilities are limited in developing countries [36, 37]. Aptamers are DNA molecules which have the capability to identify specific targets. Although several aptamer-based studies have been published to identify *M. tuberculosis* so far [4, 12], they did not lead to the development of a practical diagnostic tool yet. Further studies are needed to develop TB-specific aptamers and new biosensing methods using these aptamers.

SELEX can be used to identify aptamers binding specifically to several targets, from small molecules to proteins and even to whole bacterium. However, using whole bacterium as a target may create some problems. For example, Gram (−) bacteria like *Salmonella* have an outer membrane which includes negatively charged groups which may repel nucleic acid

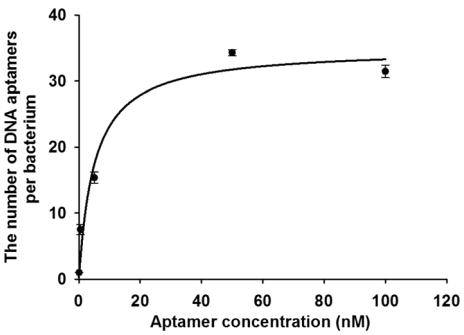

Fig. 7 The dissociation curve of Mtb36 aptamer as generated by Sigma Plot (version 13.0). In this graph, K_d=5.09±1.43 nM in 95 % confidence interval

molecules because of their negatively charged sugar-phosphate backbone [38]. In addition, the surface structure of bacteria can change quickly according to environmental conditions where bacteria grow, and aptamers selected with bacteria grown at a certain culture media may not be recognized by these aptamers when bacteria grow in a different environment [38]. Firstly, to determine and optimize aptamer binding conditions to a bacteria, previously reported *Salmonella* Typhimurium aptamer [39] binding to *Salmonella* Typhimurium bacteria were tested according to protocol [39]; however, a successful aptamer binding was not obtained and several optimization attempts also failed including different buffers, temperature, and aptamer concentrations (data not shown). Despite these problems, using whole bacterium as a target is important in selecting aptamers since the purpose of usage of these is to identify pathogenic bacteria in natural samples like clinical specimens from patients or food samples.

In this study, for the selection of DNA aptamers specific to *M. tuberculosis* H37Ra, new binding and selection protocols were developed and optimized. *M. tuberculosis* H37Ra bacteria were used for selection, whereas *M. bovis* bacteria were used for counter-selection since this is one of the phylogenetically related species of mycobacteria. *M. bovis* bacteria are included in the group of mycobacterium tuberculosis complex together with *M. tuberculosis*. An attenuated strain of *M. bovis*, bacillus Calmette-Guerin (BCG), is used as a vaccine for tuberculosis [12].

One of the most important steps in SELEX is to obtain ssDNA pools. So, there are many strategies about it. One of them is to use some separation techniques after PCR such as use of in vitro transcription and treatment with DNAse I [4], whereas another one is to design some special primers in order to obtain unequal lengths of ssDNAs [23]. Besides these difficult applications, as a simpler one, asymmetric PCR can be used to obtain ssDNA pools [25]. In this study, the last one was preferred and results confirmed that the simpler strategy could be used for this purpose.

In literature, as conventional, two methods such as centrifugation and ultrafiltration can be used [25, 28]. In this study, in order to increase our efficiency, both methods were performed as parallel. Nine of the selected DNA aptamer sequences were obtained by using ultrafiltration method, whereas twelve of them were selected by using centrifuge method. Despite the use of two ways, similar motives were obtained and we did not see any big differences, excluding the application process. Since microcentrifuge tubes are always available in all molecular biology laboratories and they can be coated easily with BSA, their use is preferred than ultrafiltration tubes as a cheaper choice.

Coating of tubes with BSA can prevent non-specific binding. This technique is already used for coating of 96-well plates in SELEX procedure [25], but, according to our knowledge, it has not been tried for SELEX in tubes until now. In addition, it was observed that the use of tRNA, BSA, and Tween 20 in binding buffer [25] could eliminate non-specific binding and decrease binding uncertainty. So, it was shown that it could be used effectively for these purposes.

In SELEX application for whole bacterium, in order to calculate K_d, isothermal titration calorimetry (ITC) [12] and flow cytometry [25] are generally used. In this study, we tried to show that we could calculate K_d using qPCR for whole-bacterium SELEX process.

Chen and co-workers (2012), who studied whole-bacterium SELEX for *M. tuberculosis*, obtained 31±4 as the lowest K_d (nM) [40], whereas in this study, K_d of Mtb36 aptamer for *M. tuberculosis* H37Ra was found to be 5.09±1.43 nM. So, it is the lowest K_d value for whole-bacterium SELEX specific to *M. tuberculosis* until now. In this study, K_d value of Mtb36 was found less than from previous studies. It may be due to the use of a different aptamer library because each library gives us a new chance to be able to find more specific aptamer molecules.

In addition, the use of coated tubes and different buffer ingredients may have promoted in the selecting of aptamers only more specific binding. Therefore, the study has provided better results about a new aptamer such as Mtb36 for the literature. Therefore, Mtb36 aptamer can be used to detect *M. tuberculosis* as a part of several new biosensing methods using aptamers like electrochemical [21, 41–45], electronic-based [44, 46], fluorescence-based [47–54], chemiluminescence-based [55], and colorimetric methods [56, 57].

There are several studies in literature using aptamers for the diagnosis of tuberculosis. In one study, aptamers are used to determine the amount of IFN- release from T-lymphocytes, by stimulation TB-specific antigens, as an indicator for tuberculosis [13]. They developed an electrochemical biosensor to detect IFN- in sodium phosphate buffer and fetal bovine serum. Their results showed a low detection limit which was 100 fm with RNA aptamer in buffer and 10 pM with DNA aptamer in serum. Another group developed an amplified surface plasmon resonance immunosensor using interferon-gamma aptamers [16] for serological diagnosis. The detection limit for IFN- was found to be 33 pM using their DNA aptamer hairpin structure designing [16].

In another study, aptamers were also selected for *M. tuberculosis* based on the secreted bacterial proteins [15]. A sandwich ELISA assay with MPT64 antibody aptamers was used to detect MPT64 which is a protein secreted by *M. tuberculosis* complex. Their linear detection range was found as 10 to 800 mg/l. In addition, there are also some studies related to the use of aptamers in direct analysis of TB from sputum samples [4] or serum [58].

It has also been suggested that aptamers can be used as therapeutic agents [12, 40, 59, 60]. Chen and co-workers (2007, 2012, 2013) selected aptamers to virulent *M. tuberculosis* strains [12, 40, 59] to develop therapeutic agents. Shum and co-workers (2011) studied aptamer-mediated inhibition of *M. tuberculosis* polyphosphate kinase 2 using G-quadriplex aptamers [60].

Conclusions

All of these aptamer studies exhibit promising results; however, further studies are needed to use these aptamers in the diagnosis and treatment of tuberculosis. Aptamer Mtb36 that was developed in this study needs further binding assays to *M. tuberculosis* in different conditions such as in clinical specimens like sputum before being integrated into biosensing systems to become a rapid, sensitive, and specific diagnostic tool for tuberculosis.

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