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Aptamer-nanobody based ELASA for specific detection of *Acinetobacter baumannii* isolates

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ABSTRACT

Acinetobacter baumannii has turned into an important threat in nosocomial outbreak infections and multidrug resistance leading to high mortality rates in the 21 st century. In recent years its mortality has increased by 15% which in part could be due to lack of a rapid and sensitive diagnostic test. In this work we introduced a new detection test for *A. baumannii* with two highly specific aptamer and nanobody molecules. High binding affinity DNA oligonucleotide aptamers toward *A. baumannii* were selected through 12 rounds of whole cell System Evolution of Ligands by EXponential enrichment process (SELEX). The SELEX procedures was monitored by flow cytometry. The dissociation constant and binding efficiency of the selected aptamer Aci49 was 7.547 ± 1:353 pM and 47.50%, respectively. A sandwich enzyme linked aptamer sorbent assay (ELASA) was designed with the biotinylated Aci49 aptamer and our previously developed nanobody against biofilm associated protein (Bap). The assay system was optimized with *A. baumannii* (ATCC 19606) and 47 clinical isolates of *A. baumannii* were tested. The threshold of detection in sandwich ELASA process was 10^3 CFU/ml. The sensitivity of test toward the clinical isolates was 95.47%. Our results reveal that the sandwich ELASA is sensitive and specific enough for the rapid detection of *A. baumannii* from clinical isolates.

1. Introduction

Recently, *Acinetobacter baumannii,* has become an important pathogen responsible for various nosocomial infections from bacteremia to lethal infections such as bloodstream infections, ventilator-associated pneumonia, meningitis, wound infections and urinary tract infections (Bruhn et al., 2015; Garnacho-Montero et al., 2015; Soo et al., 2013; Wenzler et al., 2015). Due to the prevalence of multidrug resistance, *A. baumannii* acquires the ability to spread among hospitalized patients and survive in the hospital equipment and environments (Kempf et al., 2012; Kempf and Rolain, 2012; Towner, 2009). The fatality rate related to *Acinetobacter baumannii* diseases has been reported as large as 61.6%, and hence considered as an important pathogen for healthcare units (Wenzler et al., 2015). In order to optimize antimicrobial treatment and decrease the mortality rate, prompt pathogen detection is important in nosocomial infections. Ordinary methods used for the detection of *A. baumannii* have limitations. These methods usually are prolonged and require specific media (Ajao et al., 2011). Recently developed nucleic acid based −test systems such as restriction enzyme analysis of 16SrRNA genes resulting from PCR amplification and 16S-23SrRNA gene intragenic spacer region also require costly facilities and instrumentations which are not always available in outbreak regions (Soo et al., 2013).

Aptamers are short single-stranded RNA or DNA of 35–100 nucleotides with the ability to form particular 3-dimensional structures with high affinity toward the specific target, from small chemical molecules to proteins and whole cells (Acquah et al., 2015; Darmostuk et al., 2015a; Gold, 1995). Recently attention has been paid by scientists towards aptamers as novel attractive targeting molecules for detection of pathogens. This is due to their low cost, facile, pH and heat tolerance, minimum batch to batch differences, greater specificity and binding affinity with cognate targets (Dorraj et al., 2015; Ilkhani et al., 2015; Kadioglu et al., 2015; Phizicky et al., 2003). SELEX has been used to separate out particular Aptamers, from the library with the highest affinity to the target (Acquah et al., 2015; Low et al., 2009; Shangguan et al., 2015; Wang et al., 2014). Different SELEX methodologies have been adopted to isolate aptamers against either cell surface proteins immobilized on a solid phase or using whole cells as targets (Dickey and Giangrande, 2015). Whole cell selection has clear advantages over selection performed using purified recombinant proteins (Dua et al., 2011). This is because of the difficulties in obtaining purified recombinant

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Table 1
Bacterial strains used in this research.

Bacterial cells	Collection center
A. baumannii ATCC 19606	obtained from Microbial Collection center of the Shahed University
Staphylococcus aureus ATCC 25923	
Haemophilus influenza type b (Hib) ATCC 10211	
Neisseria meningitidis type B ATCC 13090	
Streptococcus pneumonia ATCC 33400	
Escherichia coli ATCC 25922	
Pseudomonas aeruginosa ATCC 27853	
Bacillus cereus ATCC 1015	
Shigella sonnei ATCC 9290	
Vibrio cholorae ATCC 39315	
Moraxella catarrhalis ATCC 25240	from culture collection center of Iranian Biological Center
Acinetobacter lwoffii ATCC 15309	
Acinetobacter calcoaceticus ATCC 23055	from Culture Collection of Iranian Research Organization
Streptococcus agalactiae ATCC 27956	for Science and technology (Irost)

Table 2
Oligonucleotides used in SELEX and ELASA.

Name	Oligonucleotides
Aptamer library	5′-GCCTGTTGTGAGCCTCCTAAC(N38)CATGCTTATTCTTGTCTCC-3′
Forward −primer	5′-GCCTGTTGTGAGCCTCCTAAC-3′
Reverse −primer	5′- GGAGACAAGAATAAGCATG-3′
FITC-forward primer	5′-FITC- GCCTGTTGTGAGCCTCCTAAC 3′
P-reverse primer	5′-phosphate-GGAGACAAGAATAAGCATG-3′
Biotin-forward primer	5′-Biotin- GCCTGTTGTGAGCCTCCTAAC 3′

proteins for selection. Aptamers against recombinant targets have often failed post selection due to complex glycosylation patterns and structural conformation problems (Darmostuk et al., 2015b). Various aptamer sequences have been isolated for gram negative and positive bacteria, for instance, *Escherichia coli, Campylobacter jejuni, Salmonella typhimurium, Listeria monocytogenes* and *Pseudomonas aeruginosa* using whole cell as a target (Dwivedi et al., 2010, 2013; Kim et al., 2013; Suh and Jaykus, 2013; Tang et al., 2013b). In this research, in the down-selected pools, a specific DNA aptamer with high binding affinity toward to *A. baumannii* using whole cell-SELEX was obtained. We had previously developed a nanobody against biofilm associated protein (Bap) of *A. baumannii* (Payandeh et al., 2014). Therefore, we considered developing a new hybrid detection system utilizing two specific binding molecules, an aptamer and nanobody, recognizing *A. baumannii* in combinatorial detection assay based on an ELASA system.

2. Material and methods

2.1. Microorganisms, cultures and incubation conditions

A. baumannii (ATCC 19606) was used as a target for the SELEX procedure. The other bacterial strains which were used to achieve specificity of selection in counter-SELEX and cross-reactivity tests are shown in Table 1. Except for *Neisseria meningitidis* and *Haemophilus influenza* that require Chocolate Agar, all strains were cultured under aerobic conditions in Brain Heart Infusion broth at 37 °C. All species were harvested at log phase (OD_{600} = 0.4). One ml from each culture was harvested by centrifugation at 6000*g*/10 min, washed 3 times in wash buffer (1X phosphate-buffered saline (PBS) plus 0.02% tween20). Cell pellets containing about10^7–10^8 CFU/ml were suspended in 200 μl of binding buffer (wash buffer containing 1% bovine serum albumin (BSA)) and used for each round of SELEX.

2.2. Amplification and preparation of ssDNA aptamer library

A random DNA oligonucleotides library was synthesized by Metabion (Steinkirchen, Germany). Primers were synthesized at TAG Copenhagen A/S (Frederiksberg, Denmark). The sequences of DNA library, simple and labeled primers are listed in Table 2. One μl of aptamer library with initial concentration of 10 μM was amplified in a 25 μl PCR reaction by a three-step thermal protocol consist of initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30-s, 62 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 5 min. The amplified DNA library pools were degraded to single-stranded with Lambda exonuclease (Thermo SCIENTIFIC Company, USA) (Avci-Adali et al., 2010; Citartan et al., 2010). Five μg of purified double strand DNA was incubated with 5U λ exonuclease in a total reaction volume of 30 μl at 37 °C for 20 min. The reaction was terminated by inactivating λ exonuclease at 80 °C for 10 min, with subsequent flash cooling at 0 °C for 12 min to allow intra strand base pairing. The oligonucleotides concentration were measured with Pico200 (Picodrop, Hinxton, United Kingdom).

2.3. SELEX procedure

The ssDNA aptamers (1 nmol) in 250 μl of binding buffer were incubated with 10^8 CFU/ml of *A. baumannii* cells at room temperature (RT) with gentle agitation. After 1 h of incubation, the cells were precipitated and unbound aptamers or poor binders were removed along with supernatant. Cells were washed 3 times with 1 ml washing buffer and collected by centrifugation at 6000*g* for 10 min. The washing frequency increased through SELEX processes from 2 to7 times with ascending concentration of tween20 from 0.02% to 0.06%.The incubation time was gradually decreased from 60 min in the first SELEX to 15 min in final SELEX. In order to remove and recover the bound aptamers, cells were added with 100 μl of 1 x PCR Reaction Buffer and heated at 95 °C for 10 min then transferred to ice bath for 10 min. The supernatant was collected and used as a template to amplify the aptamer sequences for next round of selection. PCR reaction was carried out to amplify all cell-bound aptamers using the following conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 58.5 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 7 min. In order to eliminate the nonspecific binders and to divert the aptamers specifically to our target, counter-SELEX was carried out on 3rd, 4th and 8th round of SELEXes. To prepare bacterial cocktail, one ml aliquots (10^8 CFU/ml) of *Staphylococcus aureus*, *Haemophilus influenza type b*, *N. meningitidis type B*, *Streptococcus pneumonia*, *Escherichia coli*, *P. aeruginosa*,

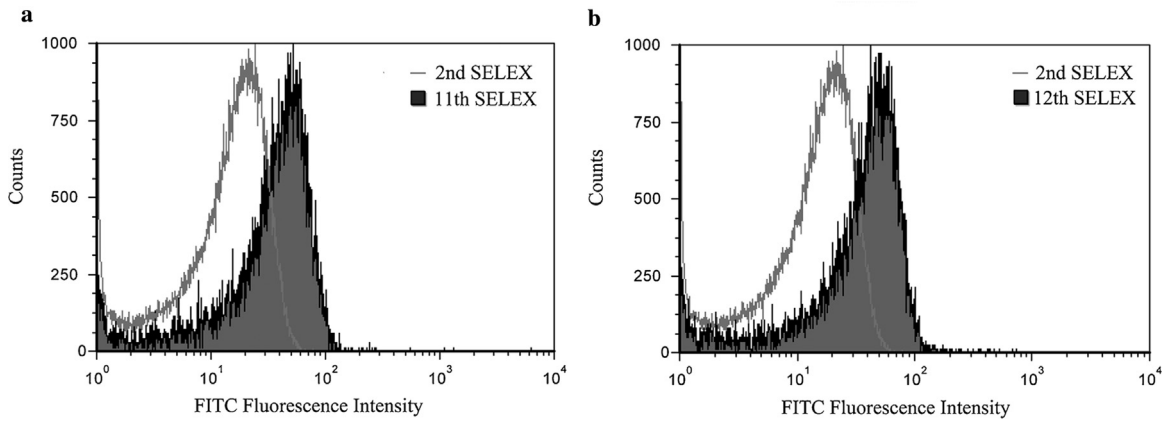

Fig. 1. Flow cytometry results of 2nd, 11th and 12th SELEX rounds. The binding efficiency of 11th (a) and 12th (b) SELEX rounds has been increased compared to that of the second SELEX.

Table 3
Selected aptamers with high affinity to *A.baumannii* and lowest affinity to physiologically related bacteria.

Aptamer name	*A. baumannii*	*Bacterial cocktail*	*A. calcoaceticus*	*A. lwoffii*	*M. catarrhalis*	*V. cholorae*
	Fluorescent intensity efficiency					
Aci7	24.21%	0.83%	–	–	–	–
Aci52	25.92%	0.53%	–	–	–	–
Aci31	26.02%	0.5%	–	–	–	–
Aci18	28.12%	0.43%	–	–	–	–
Aci39	28.92%	0.3%	–	–	–	–
Aci3	34.14%	0.3%	–	–	–	–
Aci1	36.11%	0.29%	–	–	–	–
Aci5	41.87%	0.23%	0	0	0.01%	0.75%
Aci55	46.39%	0	0	0	0	0.61%
Aci49	47.50%	0	0	0	0.24%	0.48%

Binding efficiency of top ten aptamers toward *A. baumannii* estimated by Flow cytometry.

Table 4
The variable sequences of highest binding affinity of aptamers.

Aptamer No.	Sequence (without primers)	dG value[a] (kcal/mol)
Aci49	TACATGGTCAACCAAATTCTTGCAAATTCTGCATTCCTACTGT	−10.01
Aci55	GTTACATGGTCAACCAAATTCTTGCAAATTCTGCATTCCT	−6.14

[a] Gibb's free energy of each sequences were calculated by UNAFold for predicted secondary structure.

Bacillus cereus, *Moraxella catarrhalis*, *Acinetobacter lwoffii*, *Streptococcus agalactiae* and *Shigella sonnei* suspensions were pooled, and washed in wash buffer 3 times and suspended in 10 ml of PBS. One ml of this bacterial cocktail was taken and used in the counter-SELEX. The single-stranded DNA pools were incubated with the bacterial cocktail at RT. After 1 h, the supernatant was collected and used for next round of selection.

2.4. Identification of specific and high binding aptamers

To evaluate binding efficiency of aptamer pool, selection enrichment was monitored with flow cytometry using FITC-labeled primers. In brief, aptamers of 2nd, 6th, 9th, 10th, 11th and 12th round of SELEX were amplified with 5′-FITC-forward primer and fluorescence intensity was estimated using SysmexPartec GmbH (Görlitz, Germany). Binding affinity assay was carried out by incubating 50 pM of FITC labeled aptamer with 10^8 CFU/ml of target cells in binding buffer, followed by incubating at RT for 15 min. The mixture was washed with washing buffer, and the pellet resuspended in 1 ml binding buffer. The intensity was measured by flow cytometer. Enriched aptamers pool was amplified by earlier standardized PCR reaction using labeled-free primers and were cloned into pTG19-T vector (Vivantis, Selangor, Malaysia). The ligated vectors were transformed into DH5α competent cells (Thermo Fisher Scientific, Waltham, MA USA) and plated on Luria-Bertani agar containing 50 μg ml^{-1} ampicillin. Colonies were verified by colony PCR using aptamer-specific primers. Positive clones were amplified with FITC-labeled primers and the fluorescence intensity was estimated individually. The top ten aptamer candidates with the highest binding affinities were selected for further characterization.

2.5. Specify binding exclusivity and affinity of aptamer

To examine the cross-reactivity of selected aptamers, 10^8 CFU/ml of the bacterial cocktail, were treated with 50 pM of the highest binding affinity fluorescently labeled aptamers amplified by colony PCR. Individual samples of *M. catarrhalis*, *A. lwoffii*, *A. calcoaceticus* and *Vibrio cholorae* were also checked with the same procedure for cross-reactivity. The florescent intensity of the samples was estimated with flow cytometry. The aptamers showing highest affinity to *A. baumannii* and minimum affinity to other related bacteria, were selected and send for sequencing (Bioneer, Daejeon, South Korea). The aptamer sequences were analyzed using CLC Sequence Viewer 6. In order to determine the equilibrium dissociation constant (K_d), varying concentrations of aptamers (0, 50, 100,150, 200 and 250 pM) were added to

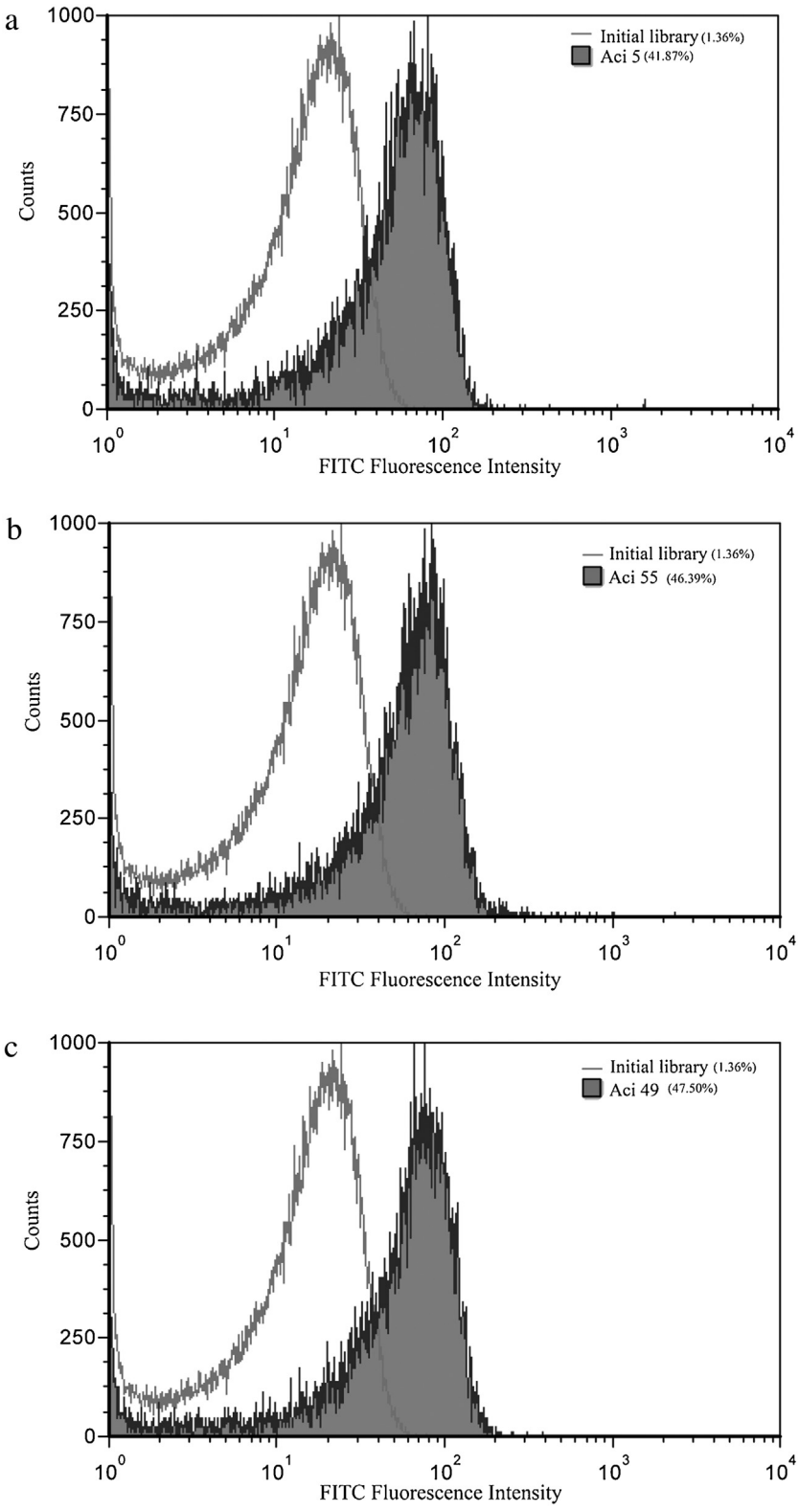

Fig. 2. Flow cytometric analysis of three aptamer candidates (5, 55, 49) with the highest affinity to *A.baumannii*. Each of the three aptamer sequences named as Aci5 aptamer with 41.87% (a), Aci55 with 46.39% (b) and Aci49 with 47.50% (c) affinities compared to initial library toward *A. baumannii*.

10^8 CFU ml^{-1} of *A. baumannii* in binding buffer and incubated at RT for 45 min with gentle rotation. The mixtures were washed and analyzed by flow cytometry (Duan et al., 2013; Kim et al., 2013; Mondal et al., 2015). The saturation curve was plotted and K_d of aptamer was calculated with non-linear regression analysis by Sigma Plot 12.0.

2.6. Secondary structure prediction

The online software UNA-Fold Tool (http://eu.idtdna.com/calc/analyzer) was used for the prediction of the secondary structures and determination of free energy of selected aptamer sequences (Zuker, 1989, 2003).

2.7. ELASA based detection of A. baumannii

ELASA assay was carried out for detection of *A. baumannii (ATCC19606)* and 47 clinical isolates obtained from Mostafa Khomeini hospital of Tehran, using single-domain antibody fragments (VHHs) against biofilm associated protein (Bap) of *A. baumannii* as a capture and biotinylated aptamer Aci49 as a detector. *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *M. catarrhalis* ATCC 25240, *A. lwoffii* ATCC 15309, *A. calcoaceticus* ATCC 23055, *and S. agalactiae* ATCC 27956 were used for evaluation of any possible cross-reactivity. All the reactions were repeated four times. The clinical isolates of *A. baumannii* confirmed by PCR using 16srRNA sequence as a target (unpublished data).

The expression and purification of the VHH was carried out as described previously (Payandeh et al., 2014). One hundred microliters of VHH (20 μg ml^{-1}) in carbonate–bicarbonate buffer (pH 9.5) was coated on 96-well microplate and incubated at 4 °C for overnight. Wells were washed four times with PBS-T (PBS + 0.05% Tween-20) and unbound sites were blocked with 200 μl of 1% BSA in PBS at 37 °C for 1 h and washed four times with PBS-T. The bacterial suspension containing 10^8CFU.ml^{-1} was added to the wells and incubated at 37 °C for 2 h. After washing with PBS, 100 μl of biotinylated aptamer Aci49 was allowed to interact with target at RT for 2 h. After incubation, wells washed and 100 μl of 1:1000 dilution of Horseradish peroxidase (HRP) conjugated with streptavidin (Biolegend, San Diego, USA) was added to each well and incubated at 37 °C for 90 min and then washed with PBS. Finally, 100 μl of tetramethylbenzidine (Bangalore Genei, India) was added as a substrate. The wells incubated at RT for 10 min without direct light exposure. The reaction was terminated by addition of 100 μl 3 N H_2SO_4 and the absorbance was determined at 450 nm using Infinite F50 microplate reader (TECAN, Mannedorf, Switzerland). In order to rule out the binding of aptamer and nanobody to other bacterial, negative control was carried out with the above mentioned bacteria CFU ml^{-1}. Finally, clinical samples were used at 10^4 CFU/ml too. To find out the limit of detection (LOD) of our ELASA method, the same experiment was carried out with different concentrations of aptamer (20, 30 and 50 pM) and different dilution ratios of bacteria ranging from 10 to 10^8 CFU/ml.

3. Results

3.1. Amplification of aptamer pool

Double strand DNA digestion reaction was best achieved at 25 min incubation time. The intensity of ssDNA band is reduced with further incubation of the reaction mixture. Therefore the optimum time selected for digestion was considered to be 25 min. Analysis of the PCR products on 2.5% agarose gel electrophoresis revealed the achievement of optimum product with annealing temperature of 58.5 °C in 30 cycles.

3.2. Binding efficiency of aptamers by flow cytometry

The fluorescent intensity recorded for the second round of SELEX (1.36%) increased to 22.77% and 23.80% of 11th and 12th SELEX rounds, respectively (Fig. 1). Since the fluorescent intensity of SELEX rounds 11 and 12 were approximately equal, therefore the SELEX procedures was terminated after 12 round of selection. The selected aptamer pools were cloned into plasmid and transfected into *E. coli DH5α*. Out of 110 clones, 10 aptamers showing the highest binding affinity were farther analyzed with bacterial cocktail used in counter-SELEX and with *A. calcoaceticus*, *M. catarrhalis*, *A. lwoffii* and *v. cholorae* individually (Table 3). Data indicating the fluorescent intensity of three aptamers with the highest binding affinity to *A. baumannii* is presented in Fig. 2. The two highest binding aptamers were named as Aci49 and Aci55 and were stored −80 °C for further studies.

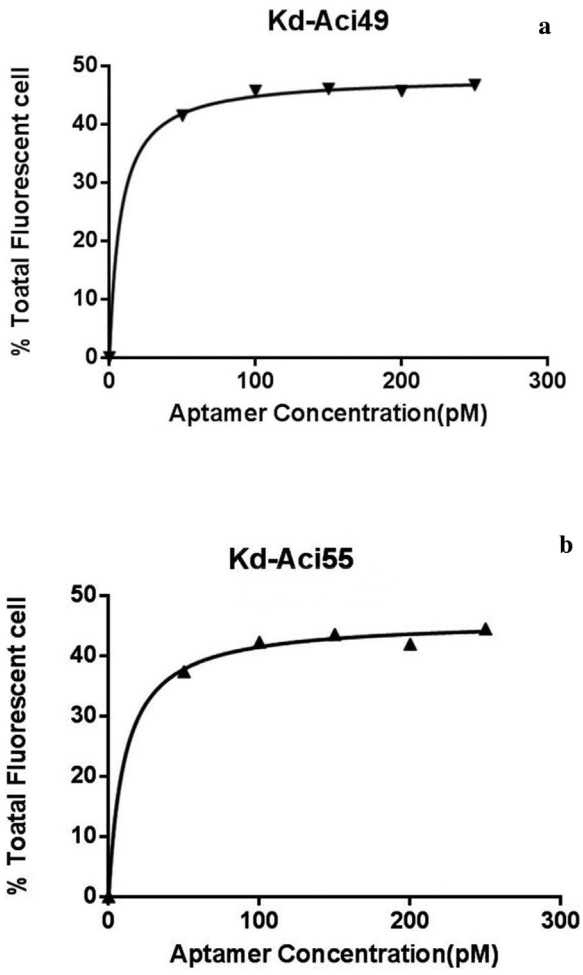

Fig. 3. Dissociation constant (K_d) curve of selected aptamer (Aci49 and Aci55). Aptamer concentrations ranging from 0 to 250 pM. The data were analyzed according to $y = B_{max} \times x/[k_d + x]$ equation by non-linear regression analysis, which yielded K_d values of (a) Aci49 7.5472 ± 1.3533 and K_d values of (b) Aci5510.76993 ± 2:561 pM.

3.3. Dissociation constants and sequence analysis of selected aptamer

The percent binding efficiency as a function of aptamer concentration upon interaction with constant amount of *A. baumannii* cells (10^8 CFU ml^{-1}) was examined with increasing the ssDNA from 0 to 250 pM. The K_d values of Aci49 and Aci55 yielded to 7.547 ± 1.353 and 10.70 ± 2:561 pM respectively (Fig. 3). The sequence of the variable regions and predicted secondary structures at 21 °C with a minimum free energy of −10.01 kcal mol^{-1} for aptamer Aci49 and −6.14 kcal mol^{-1} for aptamer Aci55 are displayed in Table 4 and Fig. 4, respectively.

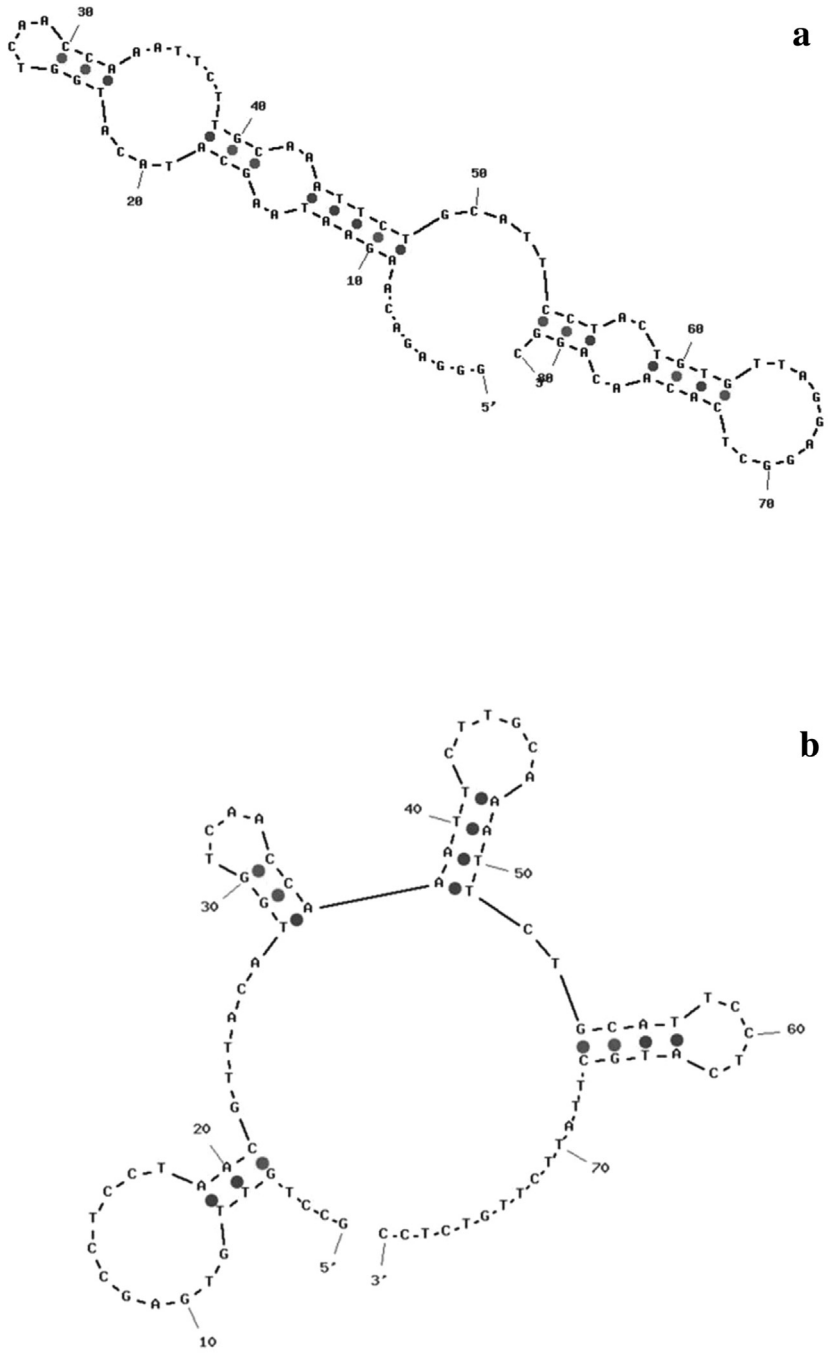

Fig. 4. The predicted secondary structure of aptamer Aci49and Aci55 using Oligo Analyzer 3.1 (UNAFold) online program based on a free energy minimization algorithm. The free energies were obtained in range of −6.69 to −10.01(kcal/mole) for (a) Aci49 and −4.4 to −6.14(kcal/mole) for (b) Aci55.The lowest free energy for each one is displayed.

3.4. Detection of A. baumannii *(ATCC 19606) and clinical isolates using aci49 aptamer in ELASA*

The readout values of ELASA for intended pentamerous complex (VHH-bacteria-biotinylated Aci49-streptavidinHRP conjugate and TMB) is displayed in Fig. 5. The OD_{450} value of the reaction containing 10^4 CFU ml^{-1} was 0.7, this bacterial concentration was selected as an optimum readout for further experiments. The results of cross-reactions carried out with seven different bacteria are shown in Table 5. There was a significant difference between the optical density obtained from *A. baumannii* ATCC19606 and these bacteria using at similar concentrations. The OD_{450} obtained for *A. baumannii* (10^4 CFU ml^{-1}) was 0.7 whereas the highest OD_{450} was 0.2 belonging to *A. calcoaceticus* (Table 5). The absorbance of background reactions without aptamer, bacteria or nanobody at 450 nm was always less than 0.2.

In order to confirm the feasibility of our detection system, 47 clinical isolates of *A. baumannii* were also tested. The results are given in Table 6. Out of 47 positive clinical samples examined by PCR based on 16rRNA gene, 45 were detected by our ELASA system corresponding to 95.74% sensitivity.

4. Discussion

According to the Centers for Diseases Control and Prevention, *A. baumannii* is responsible for 80% of all *Acinetobacter* infections.

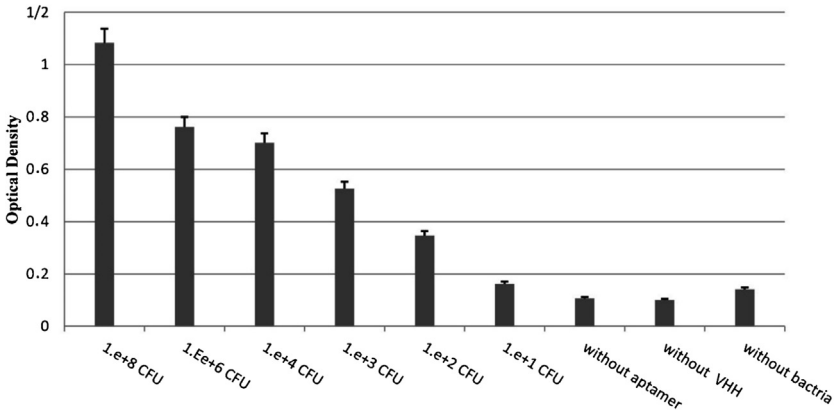

Fig. 5. Determination of the cut off value *A. baumannii* in ELASA test. Constant concentration (30 pM) of aptamer Aci49 with different concentration of bacteria (10 ~10^8 CFU ml^{-1}) were tested in ELASA. The horizontal axis shows bacterial concentration (CFU ml^{-1}). Three wells, one without bacteria, one without VHH and one without aptamer were used as a negative control. Error bars display the standard deviation of the OD_{450} mean of three experiments.

Table 5
ELASA results of *A. baumannii* and bacteria used to check cross reactivity. Results are presented as a OD_{450} mean ± standard deviation (n = 4).

Organisms(10^4)	Optical Density(450 nm)	Organisms(10^8)	Optical Density(450 nm)
S. aureus	0.1734 ± 0.03	*S. aureus*	0.226 ± 0.03
E.coli	0.1760 ± 0.05	*E.coli*	0.1868 ± 0.09
P. aeruginosa	0.1769 ± 0.07	*P. aeruginosa*	0.4206 ± 0.06
M. catarrhalis	0.1539 ± 0.09	*M. catarrhalis*	0.219275 ± 0.15
A. lwoffii	0.2329 ± 0.15	*A. lwoffii*	0.419125 ± 0.17
A. calcoaceticus	0.2415 ± 0.16	*A. calcoaceticus*	0.375275 ± 0.14
S. agalactiae	0.1759 ± 0.08	*S. agalactiae*	0.17245 ± 0.05
A.baumannii 19606	0.7018 ± 0.18	*A. baumannii 19606*	1.083325 ± 0.11
Negative control	0.1146 ± 0.16		

Table 6
ELASA results of *A.baumannii* and clinical isolates. Results are presented as a OD_{450} mean ± standard deviation (n = 4).

Clinical samples	PCR[a] results	ELASA results (OD_{450}mean)	Clinical samples	PCR results	ELASA results (OD_{450}mean)	Clinical samples	PCR results	ELASA results (OD_{450}mean)
1	+	0.704 ± 0.15	17	+	0.7324 ± 0.13	33	+	0.7449 ± 0.13
2	+	0.8561 ± 0.13	18	+	0.408 ± 0.14	34	+	0.7086 ± 0.13
3	+	0.8403 ± 0.12	19	+	0.834 ± 0.11	35	+	0.6991 ± 0.14
4	+	0.8668 ± 0.16	20	+	0.7322 ± 0.13	36	+	0.7128 ± 0.10
5	+	0.9276 ± 0.14	21	+	0.3417 ± 0.21	37	+	0.8148 ± 0.16
6	+	0.7724 ± 0.13	22	+	0.8254 ± 0.14	38	+	0.6834 ± 0.15
7	+	0.8526 ± 0.12	23	+	0.7759 ± 0.08	39	+	0.7365 ± 0.15
8	+	0.7869 ± 0.15	24	+	0.6804 ± 0.13	40	+	0.6211 ± 0.11
9	+	0.7412 ± 0.14	25	+	0.6647 ± 0.11	41	+	0.8728 ± 0.11
10	+	0.6341 ± 0.09	26	+	0.7706 ± 0.11	42	+	0.9834 ± 0.12
11	+	0.7314 ± 0.11	27	+	0.9482 ± 0.13	43	+	0.704 ± 0.12
12	+	0.7603 ± 0.12	28	+	0.8004 ± 0.18	44	+	0.6974 ± 0.16
13	+	0.6584 ± 0.0.14	29	+	0.7562 ± 0.14	45	+	0.8359 ± 0.13
14	+	0.6872 ± 0.14	30	+	0.5709 ± 0.10	46	+	0.7708 ± 0.14
15	+	0.6949 ± 0.08	31	+	0.8253 ± 0.11	47	+	0.6471 ± 0.17
16	+	0.8032 ± 0.14	32	+	0.6819 ± 0.10	Control[b]	+	0.7018 ± 0.09

[a] PCR method based on 16 S rRNA sequence.
[b] positive control with *A. baumannii* ATCC 19606.

Because of its remarkable resistance to an extensive range of antibiotics, treatment of these infections is very difficult (Ilkhani et al., 2015). Therefore rapid detection in early stages is critical particularly for transplant patients or those having immune deficiency (Moradi-Tabriz et al., 2010). Aptamers and recombinant antibodies have been widely used in different diagnostic systems such as ELASA, flow cytometry, immunofluorescence microscopy, immunocytochemistry, microarray and blotting assay (Bruno et al., 2012; Groff et al., 2015; Toh et al., 2015; Wandtke et al., 2015). In the present research, we have developed for the first time an accurate and specific ELASA system for detection of *A. baumannii* using an aptamer and antibody together both specifically developed against *A. baumannii*.

Whole cell SELEX is applied for the isolation of unique aptamer/aptamers from a large oligonucleotides pool against various bacteria previously (Chen et al., 2007; Hamula et al., 2011; Meng et al., 2015; Moon et al., 2013; Peng et al., 2014; Tang et al., 2013a; Yang et al., 2013). In whole cell SELEX technique, live cells are used as a target to isolate candidate aptamer without having any prior knowledge about the targets and their molecular properties. Moreover the aptamer binding functionality is largely dependent on the natural 3D structure of the target which is well maintained in live cells (Dickey and Giangrande, 2015). Although there are certain advantages to protein SELEX such as avoiding the process of counter SELEX, reducing nonspecific aptamer binding and easily controlling the reaction conditions, but the expression and purification stages

of the proteins often lead to altering the natural conformation of the protein (Dickey and Giangrande, 2015). The selected aptamers against a purified protein may not recognize the same target in its physiological condition (Payandeh et al., 2014).

The apparent dissociation constant (K_d) was 7.547 ± 1:353 pM, when the binding affinity is characterized after 12 rounds of SELEX. This indicates a higher binding affinity of the selected aptamer to *A. baumannii*. The resulting K_d is in consistent with our previous report for aptamer isolated against *Haemophilus influenzae* type b by whole SELEX (Bitaraf et al., 2016), but much better than the other studies where the reported K_ds were in μM and nM ranges (Hong and Sooter, 2015). The ultra-high affinity obtained can be attributed to suitable selection stringency and using bacterial strains with similar physiological properties to that of targets in counter-SELEX.

The affinity of the VHH against recombinant Bap protein used in ELASA was 3.8×10^{-8} M/L, characterized previously (Payandeh et al., 2014). In the sandwich ELASA, either aptamer or antibody is generally immobilized as a capture (Park et al., 2013; Toh et al., 2015; Vivekananda and Kiel, 2006). Our strategy was to immobilize the nanobody on the surface of the wells. The reason behind this strategy relates to the structure of aptamer. Having smaller size compared to antibodies, immobilizing aptamer as a capture reagent may alter the structural conformation of the aptamer which consequently affects its interaction with the target on the cell surface. Easier labeling without affecting their conformation is another advantage of aptamers making them suitable detectors compared to antibodies (Groff et al., 2015).

The hybrid VHH plus aptamer assay was evaluated in several steps to verify the efficiency and specificity of the method with different concentrations of aptamer and target cells. This was achieved using other bacteria with similar Bap sequence as a negative control to check any possible cross reactivity. We could detect 10^3 CFU ml^{-1} (OD_{450} = 0.526) with only 20 μg ml^{-1} of nanobody and 30 pM of biotinylated aptamer without any cross reactivity. In a similar experiment Chen et al. generated ssDNA aptamer for Hepatitis C virus Envelope. Chen et al. applied a poly clonal antibody as a capturing agent and aptamer as a detector in 250 nM concentration (Chen et al., 2009). Wang et al. have also characterized an aptamer against prion protein and developed a sandwich ELISA where aptamer was coated on the plate in 5 ng ml^{-1} concentration for capturing 2 ng ml^{-1} of prion protein. The protein was detected with antibodies using a common sandwich ELISA method (Wang et al., 2011).

Clinical isolates were also used to confirm the specificity and sensitivity of the assay system. Beside conventional methods, there are presently few PCR based methods for the detection of *A. baumannii*, each with its own limitations (Ajao et al., 2011; Wenzler et al., 2016; Zander et al., 2013). Diagnostic systems based on two different but specific agents are generally more reliable than a single parameter method. Our two specific binding agent method exhibited its high specificity by easily distinguishing *A. baumannii* from its homologous *A. calcoaceticus* and other physiologically related bacteria.

5. Conclusions

In conclusion, we have presented an easy, specific and high affinity detection system using a combination of anti-Bap nanobody and Aci49 aptamer without the need for any costly preparations or robotic equipment. Indeed, our ELASA system is reproducible, making it a useful method in laboratory detection. We believe that the configuration of the ELASA process is flexible enough to be adapted in other immunoassay-aptamer models for detection of a wide range of pathogens.

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