

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 20 Mar 2006		2. REPORT TYPE Journal Article		3. DATES COVERED (From - To) Feb 2003 - Feb 2007	
4. TITLE AND SUBTITLE Anti-Francisella Tularensis DNA Aptamers Detect Tularemia Antigen from Different Subspecies by Aptamer-Linked Immobilized Sorbent Assay				5a. CONTRACT NUMBER F41624-03-D-7000	
				5b. GRANT NUMBER N/A	
				5c. PROGRAM ELEMENT NUMBER 63231F	
				5d. PROJECT NUMBER 5020	
6. AUTHOR(S) Jeevalatha Vivekananda and Johnathan L. Kiel				5e. TASK NUMBER P4	
				5f. WORK UNIT NUMBER 01	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Air Force Materiel Command Biosciences and Performance Division Air Force Research Laboratory Biobehavior, Bioassessment & Biosurveillance Branch 711 Human Performance Wing 2486 Gillingham Dr Human Effectiveness Directorate Brooks City-Base, TX 78235				8. PERFORMING ORGANIZATION REPORT NUMBER N/A	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Air Force Materiel Command Biobehavior, Bioassessment & Biosurveillance Branch Air Force Research Laboratory Brooks City-Base, TX 78235 711 Human Performance Wing Human Effectiveness Directorate Biosciences and Performance Division				10. SPONSOR/MONITOR'S ACRONYM(S) 711 HPW/RHPF; 711 HPW/RHPFB	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) AFRL-HE-BR-JA-2005-0019	
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution A. Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES This journal article was submitted under the previous organization name of the Biosciences and Protection Division, Counterproliferation Branch (AFRL/HEPC). Division and Branch names and acronym(s) listed above reflect new organizational changes.					
14. ABSTRACT Aptamers are powerful candidates for molecular detection of targets due to their unique recognition properties. These affinity probes can be used to recognize and bind to their targets in the various types of assays that are currently used to detect and capture molecules of interest. They are short single-stranded (ss) oligonucleotides composed of DNA or RNA sequences that are selected in vitro based on their affinity and specificity for the target. Using combinatorial oligonucleotide libraries, we have selected ssDNA aptamers that bind to Francisella tularensis subspecies (subsp) japonica bacterial antigen. F. tularensis is an intracellular, nonmotile, nonsporulating, Gram-negative bacterial pathogen that causes tularemia in man and animals. Just as antibodies have been used to detect specific targets in varying formats, it is possible that nucleic acid-binding species or aptamers could be used to specifically detect biomolecules. Aptamers offer advantages over antibody-based affinity molecules in production, regeneration and stability due to their unique chemical properties. We have successfully isolated a set of 25 unique DNA sequences that specifically bind to F. tularensis subspecies japonica. When tested in a sandwich Aptamer-Linked Immobilized Sorbent Assay (ALISA) and dot blot analysis, the aptamer cocktail exhibited specificity in its ability to bind only to tularemia bacterial antigen from subspecies japonica, holarctica (also known as palacartica) and tularensis but not to Bartonella henselae. Moreover, there is no binding observed either to pure chicken albumin or chicken lysozyme. Thus, it appears that this novel antitularemia aptamer cocktail may find application as a detection reagent for a potential biological warfare agent like F. tularensis.					
15. SUBJECT TERMS DNA aptamers; Francisella tulatensis; SELEX; ALISA; ELISA; dot blot; bioterrorism					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON Jeevalatha Vivekananda
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (Include area code)

Anti-*Francisella tularensis* DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay

Jeevalatha Vivekananda and Johnathan L Kiel

Air Force Research Laboratory, HEPC, Brooks City-Base, TX, USA

Aptamers are powerful candidates for molecular detection of targets due to their unique recognition properties. These affinity probes can be used to recognize and bind to their targets in the various types of assays that are currently used to detect and capture molecules of interest. They are short single-stranded (ss) oligonucleotides composed of DNA or RNA sequences that are selected *in vitro* based on their affinity and specificity for the target. Using combinatorial oligonucleotide libraries, we have selected ssDNA aptamers that bind to *Francisella tularensis* subspecies (subsp) *japonica* bacterial antigen. *F. tularensis* is an intracellular, nonmotile, nonsporulating, Gram-negative bacterial pathogen that causes tularemia in man and animals. Just as antibodies have been used to detect specific targets in varying formats, it is possible that nucleic acid-binding species or aptamers could be used to specifically detect biomolecules. Aptamers offer advantages over antibody-based affinity molecules in production, regeneration and stability due to their unique chemical properties. We have successfully isolated a set of 25 unique DNA sequences that specifically bind to *F. tularensis* subspecies *japonica*. When tested in a sandwich Aptamer-Linked Immobilized Sorbent Assay (ALISA) and dot blot analysis, the aptamer cocktail exhibited specificity in its ability to bind only to tularemia bacterial antigen from subspecies *japonica*, *holarctica* (also known as *palaeartica*) and *tularensis* but not to *Bartonella henselae*. Moreover, there is no binding observed either to pure chicken albumin or chicken lysozyme. Thus, it appears that this novel antitularemia aptamer cocktail may find application as a detection reagent for a potential biological warfare agent like *F. tularensis*.

Laboratory Investigation (2006) 86, 610–618. doi:10.1038/labinvest.3700417; published online 20 March 2006

Keywords: DNA aptamers; *Francisella tularensis*; SELEX; ALISA; ELISA; dot blot; bioterrorism

Aptamers are single-stranded oligonucleotides with a length of tens of nucleotides, obtained by systemic evolution of ligands by exponential enrichment (SELEX) technology, exhibiting high affinity and specificity towards any given target molecule.^{1,2} These single-stranded (ss) nucleic acid molecules have highly defined tertiary structures, which allow them to form stable and specific complexes with a range of different targets including small molecules such as amino acids to highly complex proteins and whole viruses.^{3–7} For example, DNA-binding species have been selected that can interact with thrombin⁸ and RNA aptamers have been selected that recog-

nize a variety of cytokines.⁹ These specialized molecules are analogs to antibodies in specificity and affinity with an apparent advantage of being reproduced by chemical synthesis and more easily labeled with fluorescent or other reporters during their synthesis. Comparisons of various ligand-binding aptamers with proteins that bind to their targets have shown that both nucleic acids and proteins use similar strategies for the formation of well-defined binding patterns.^{10,11} Structural studies with aptamer-target complexes have demonstrated insights into molecular diversity associated with nucleic acid architecture and molecular recognition.¹² Aptamers frequently form complexes that have dissociation constants in the nanomolar range and can clearly distinguish between even closely related protein targets.^{13,14} The degree of molecular distinction achieved by aptamers may surpass that of antibodies with a remarkable diversity in structure and function.¹⁵

Correspondence: Dr J Vivekananda, PhD, HEPC, Air Force Research Laboratory, 2486 Gillingham Dr, Bldg 175 E, Brooks City Base, TX 78235, USA.

E-mail: jeevalatha.vivekananda@brooks.af.mil

Received 9 September 2005; revised and accepted 10 February 2006; published online 20 March 2006

The concept of ss oligonucleotides as affinity molecules for proteins or other ligands was first described by Turek and Gold¹ and Ellington and Szostak² wherein they explained the ability of short sequences to fold in the presence of a ligand into unique three-dimensional structures that bind a target with high affinity and specificity. Following the incubation of a target with a library of DNA sequences, typically ranging from 10¹⁴ to 10¹⁸ molecules in complexity, target-DNA complexes are isolated, the DNA is amplified and the process is repeated until the desired sample is enriched with sequences that display high affinity and specificity for the target of interest. Since the selection stringency for this *in vitro* process of evolution is high affinity for the target, aptamers with low pico- to nanomolar-binding constants are often obtained. Aptamers offer advantages over antibody-based affinity due to the nature of DNA/RNA, which provides for increased stability, easy regeneration (PCR or chemical synthesis) and simple modifications for detection and neutralization. On the contrary, antibodies in general are stable only under physiological conditions and any modifications often effect or decrease in affinity for targets.

Francisella tularensis is an encapsulated, small Gram-negative nonmotile Coccobacillus¹⁶ that causes tularemia, a severe disease of humans and other mammals including squirrels, cats and prairie dogs. Additionally, this organism has an enzootic cycle involving wild animals such as rodents and blood sucking insects.^{17,18} Humans can acquire tularemia through contact with infected animals.^{19,20} It is a facultative intracellular bacterium that has been reported to infect macrophages, liver and related cells.^{21,22} Most common infections in humans are caused by two main subspecies, *tularensis* and *holarctica*.²³ These organisms are highly infectious; fewer than 25 organisms are capable of causing disease when spread by aerosol.²⁴ Interest in *F. tularensis* has increased recently because of its potential use as an agent of biological warfare.²⁵

Transmission of this organism to the human population can occur through insect bites, contaminated water sources or handling of infected animals. In spite of its high infectivity, human cases of tularemia are rare. However, its extreme hardiness, infectivity and the ability to cause lethal disease by aerosol raise serious concern that this organism can be exploited by terrorists as a possible biological weapon. As a result, tularemia bacteria is one of the high ranking pathogens categorized by the Centers for Disease Control as a category A agent, meaning it could have the greatest impact on public health if it were used as a weapon of mass destruction.²⁶ Human tularemia manifests itself in a variety of syndromes, most of them depending on the portal of infection. The clinical appearance varies from skin lesions to multi-organ involvement. Furthermore, the severity depends on the dose and the virulence

of the pathogen. *F. tularensis* subspecies *holarctica* (type B), which is spread over the northern hemisphere, is less virulent than *F. tularensis* sub *tularensis* (type A), which is distributed mainly in North America and is associated with a severe and generally fatal form of tularemia. In particular, the typhoid and pneumonic forms are the most deadly with mortality that can exceed 30% if patients are left untreated.²⁶

Here, we report the selection of 25 unique DNA aptamers that are specific to *F. tularensis* subspecies *japonica* antigen that can be used as a screening tool for detection and identification of tularemia bacterial antigen with detection limits as low as 25 ng using optical detection. Further, we demonstrate the ability of our aptamers to bind to three different subspecies of *F.t.*; *japonica* and *holarctica*, which generally cause a milder form of tularemia; and *F. tularensis* (SCHU 4—type A), which causes a lethal form of tularemia.²⁷ The aptamers bind with high affinity and specificity, and we detail their application in Aptamer-Linked Immobilized Sorbent Assay (ALISA) and dot blot assays. Thus, aptamers may represent a new class of reagents that could be used in detection and diagnostic platforms that were dominated by antibodies previously.

Materials and methods

F. tularensis subspecies *japonica* killed bacterial antigen and its antiserum, trypticase soy broth (TSP), Brain heart infusion (Difco), the glycerol production and agglutination test kits were purchased from Becton Dickinson and Company, Sparks, MD, USA. *F. tularensis* subspecies *holarctica* live vaccine strain (type B) was obtained from the Centers for Disease Control and Prevention (CDC) (live vaccine strain Lot #11, NDBR 101 Lot 0200). *F. tularensis* subspecies *tularensis* (SCHU 4—type A) was received from Peter Emanuel of the Critical Reagent Collection of the Department of Defense Joint Program in Chemical and Biological Defense. *F. tularensis* type B (#38) was isolated from a cat in Houston, TX, USA. *Bartonella henselae* (ATCC Number 49882) was purchased from American Type Culture Collection, Manassas, VA, USA. Bovine serum albumin fraction V, chicken albumin, lysozyme from chicken egg and other chemicals were obtained from Sigma. A synthetic random DNA library, biotin labeled primers and other primers were from Sigma/Genosys, The Woodlands, TX, USA. Dot blot reagents and nitrocellulose membranes and biospin disposable chromatography columns were from Bio-Rad, Hercules, CA, USA. Reagents for PCR were purchased from PGC Scientific (Gaithersburg, MD, USA) and Applied Biosystems (Foster City, CA, USA). The TOPO cloning kit was from Invitrogen, Carlsbad, CA, USA. QIAprep spin columns were from Qiagen Inc., Valencia, CA,

USA. Streptavidin-conjugated alkaline phosphatase, streptavidin-conjugated horseradish peroxidase (HRP), goat anti-rabbit IgG-conjugated HRP, goat anti-rabbit IgG-conjugated alkaline phosphatase, immunopure streptavidin, carbonate buffer and Superblock Buffer were from Pierce Biotechnology, Rockford, IL, USA. Fluor-coated TLC plates for UV shadowing were purchased from Ambion, Austin, TX, USA.

Bacterial Strains and Sample Preparation

F. tularensis subspecies *holarctica* live culture from CDC was grown in brain heart infusion media with 0.1% cystine. Tubes were incubated at 37°C with 5% CO₂. After 48 h growth samples were boiled for 1 h to heat kill viable organisms. Killed bacteria were centrifuged at 6000 rpm in a sorvall ss34 rotor for 30 min. The bacterial pellet was washed with phosphate-buffered saline (PBS) once and finally resuspended in PBS and stored at -80°C until further use. Formalin-killed *F. tularensis* subspecies *japonica* bacterial suspension was washed with PBS twice and resuspended in PBS for further use. To prepare total bacterial protein lysate, the suspension was frozen in liquid nitrogen and thawed on ice. The samples were freeze-thawed for two more times and vortexed for 1 min and used. An aliquot of the lysate was used to determine total protein concentration. All the lysates were stored at -80°C until further use. *F. tularensis* subspecies *tularensis* (SCHU 4) was supplied in PBS in the concentration of 10⁸ bacteria/ml. *B. henselae* was grown in tryptic soy broth (TSB) media at 37°C in a 5% CO₂ incubator without shaking. *F. tularensis* (type B) isolated from cat serum was grown in the same media as subspecies *holarctica* at 37°C.

Random Library and Primers

A 102-base ssDNA template containing 42 bases of random sequence flanked by defined primer-binding sites 5'-ACC CCT GCA GGA TCC TTT GCT GGT ACC NNNN (*N*=42) AGT ATC GCT AAT CAG TCT AGA GGG CCC CAG AAT-3' was synthesized by Sigma/Genosys. The pool was then amplified via polymerase chain reaction (PCR) using 5' biotin labeled and 3' primers and it was further purified by denaturing polyacrylamide gel electrophoresis. After UV shadowing, ssDNA bands were cut and eluted from the gel. Following ethanol precipitation, the ssDNA pool was recovered by centrifugation at 4°C and resuspended in Tris-EDTA (TE) buffer and finally used in the *in vitro* selection process.

In Vitro Selection Procedure

Iterative rounds of selection and amplification of ssDNA aptamers were performed as described previously by Vivekananda and Kiel.²⁸ In brief, to exclude filter binding ssDNA sequences from the

pool, the DNA was passed through a 0.45 µm HAWP filter (Millipore, Bedford, MA, USA) and washed with an equal volume of binding buffer containing 20 mM Tris-HCL, pH 7.5, 45 mM sodium chloride, 3 mM magnesium chloride, 1 mM ethylenediamine-tetra acetic acid (EDTA), 1 mM dithiothreitol (DTT). In the present study, ssDNA pools of 500 pmol for initial rounds and 200 pmol for later rounds were used in the selection process. ssDNA pools were heat-denatured at 94°C for 3 min, and then cooled immediately to 4°C in binding buffer. Selection was performed by incubating ssDNA pools with 100 µg of total bacterial protein (killed bacteria) at room temperature for 1 h in binding buffer by gentle rotation. After 1 h the aptamer-bacterial antigen complex was vacuum-filtered over a HAWP filter at 5 p.s.i. and washed three times with binding buffer. ssDNA that had been retained on the filter was eluted with 7 M urea, 100 mM MES (4-morpholine-ethansulfonic acid (pH 5.5)) and 3 mM EDTA by boiling. Eluted ssDNA was then precipitated with an equal volume of isopropyl alcohol. Selected ssDNAs were amplified by PCR and used for the next round of selection. After round 10, the pool was amplified by PCR and the product was cloned using a Topo TA cloning kit (Invitrogen, Carlsbad, CA, USA). The cloned sequences were transformed into *Escherichia coli*. In all, 35 colonies were picked randomly and the plasmid DNA was purified by mini-prep Qiagen kit and sequenced. In all, 25 unique sequences were obtained and used for further analyses.

Aptamer-Linked Immobilized Sorbent Assay

Sandwich ALISA assays were performed in 96-well microtiter plates (Corning, supplied by Fischer Scientific, Houston, TX, USA). The selected aptamer cocktail was coated with a concentration of 500 pmol/well and the non-specific sites were blocked with Superblock (Pierce Biotechnology). In brief, a cocktail of aptamer stock was made by reconstituting the lyophilized aptamers in sterile distilled water. The cocktail was then diluted to desired concentration in carbonate buffer pH 9.4 (Pierce Biotechnology). In all, 100 µl of the solution containing aptamers was added to each well in a 96-well clear plate and incubated for 2 h at room temperature. After the designated time, 100 µl of Superblock blocking buffer was then added on top of the aptamer solution. Plates were allowed to stand for an additional 30 min at room temperature. The contents were discarded from the plates and washed once with 200 µl of Superblock blocking buffer and dried.

Prior to the binding assay, the wells were further blocked with 1% BSA in PBS for 30 min at room temperature. Various quantities ranging from 25 ng to 2 µg of total bacterial antigen from *F. tularensis* subspecies *japonica*, were incubated for 1 h at room temperature by gentle shaking in 100 µl of binding

buffer containing 20 mM Tris-HCL, pH 7.5, 45 mM sodium chloride, 3 mM magnesium chloride, 1 mM EDTA, 1 mM dithiothreitol (DTT). After the designated time, unbound target was removed and the plates were washed twice with 0.1% Tween-20 in PBS (phosphate-buffered saline/Tween (PBS-T)). After the final wash, biotin-labeled aptamer cocktail was then added to the individual wells at a concentration of 500 pmol/well. The plates were further incubated at room temperature for another hour. Unbound biotin aptamers were removed and the plates washed twice with PBS-T. Finally 100 μ l of a 1:1000 dilution of a solution of streptavidin conjugated to HRP were added to the individual wells. Following 30-min incubation at room temperature on a shaking platform, wells were washed twice with PBS-T and developed using ABTS as substrate (Sigma) according to the manufacture's instructions, and absorbance was read at 405 nm using Synergy™ HT Multi-Detection Microplate Reader from BIO-TEK. Antigens from *F. tularensis* subspecies *holarctica* and *tularensis* (SCHU 4) were used to assess the specificity of the aptamers. *B. henselae*, chicken albumin and chicken lysozyme were used as negative controls.

Enzyme-Linked Immunosorbent Assay

To compare aptamer cocktail vs anti-tularemia antibodies, we developed a sandwich Enzyme-linked Immunosorbent Assay (ELISA) assay using commercially available *F. tularensis* rabbit antiserum (Becton-Dickinson and Company). Antiserum was diluted to 1:1000 with carbonate-bicarbonate buffer (pH 9.0). Ninety-six-well microtiter plates (Corning) were coated with 100 μ l of diluted antiserum for 1 h at 37°C. The wells were washed further with PBS-T (0.01%; pH 7.2) and blocked with Superblock for 30 min and dried at room temperature. Following further blocking with 1% BSA in PBS, *F. tularensis* subspecies *japonica* antigen was added to the wells in quantities ranging from 25 to 500 ng, and 1 to 2 μ g in PBS. After an incubation of 1 h at room temperature, wells were washed with PBS-T three times. After removal of unbound antigen, the wells were probed with anti-tularemia rabbit IgG at a dilution of 1:1000 for 1 h. After plates had been washed thrice with PBS-T, 100 μ l of horseradish peroxidase (Pierce Biotechnology, Rockford, IL, USA) conjugated goat anti-rabbit IgG at a dilution of 1:5000 was added to the wells and allowed to bind for 30 min. After three washes with PBS-T, the enzymatic reaction was started with 100 μ l of ABTS (Sigma) according to the manufacturer's recommendation. OD₄₀₅ values were determined using a microplate reader from BIO-TEK. As for the ALISA, antigens from subspecies *holarctica* and *tularensis* (SCHU 4) were used in the ELISA. *Bartonella henselae*, chicken albumin and chicken lysozyme were used as negative controls.

Dot Blot Analysis with Aptamers and Antibodies

Fifty μ g of bacterial lysate from *F. tularensis* subspecies *holarctica*, #38 isolate and *tularensis* (SCHU 4) were spotted onto nitrocellulose membrane discs and allowed to air dry. These samples were fixed under vacuum at 80°C for 1 h and blocked with 5% BSA in PBS. One batch was incubated with biotin-labeled aptamer cocktail at 500 pmol/disc in binding buffer (20 mM Tris-HCL, pH 7.5, 45 mM sodium chloride, 3 mM magnesium chloride, 1 mM EDTA, 1 mM DTT) for 1 h at room temperature with gentle rocking. The other batch was probed with anti-tularemia rabbit IgG (Becton-Dickinson and Company) in PBS at a dilution of 1:1000 for 1 h. After the designated time, both batches of membrane discs were washed with PBS-T three times and probed with streptavidin-conjugated alkaline phosphatase (Pierce Biotechnology, Rockford, IL, USA), and goat anti-rabbit IgG-conjugated alkaline phosphatase (Pierce Biotechnology, Rockford, IL, USA), respectively, for 30 min. Excess enzyme was removed by three subsequent washes with PBS-T. Finally, the membrane discs were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NTB)-BLUE (Sigma). *Bartonella henselae*, chicken albumin and chicken lysozyme were used as negative controls.

Results

Selection of Anti-Tularemia Aptamers from Combinatorial Library

DNA aptamers specific for *F. tularensis* subspecies *japonica* were selected from a library of ssDNA with a 42-nucleotide random region flanked by 27-nt 5' and 30-nt 3' fixed regions for PCR amplification. The initial library theoretically contained 10¹⁴–10¹⁶ different sequences. Amplification was performed with biotin labeled primer F (5') and nonbiotin-labeled primer R (3') during SELEX selection. For our application, we choose to use DNA rather than RNA sequences because, with and without terminal modifications, they have been shown to be effective in biological fluids and are generally nuclease resistant.^{8,29–31}

We used *F. tularensis* subspecies *japonica* antigen because this is the only type available for tularemia detection by the commercially available test kit. Prior to the selection process, the ssDNA pool was amplified to enrich the sequence pools. Upon mixing with the tularemia antigen, oligomers from the library may bind to the target DNA. During this process a bound candidate or candidates of the pool may be specific for the target and can be amplified with primers F and R. The number of PCR cycles was also optimized to avoid over-amplification. The stringency of the selection was controlled by adjusting the antigen concentrations, buffer conditions and the washes. During the process of binding

and amplification, there is a possibility that some of the sequences can bind nonspecifically to the membranes. To circumvent this problem, negative selection was performed after rounds 3, 6 and 9 by passing DNA pool through nitrocellulose filters. Biotinylated DNA sequences exhibiting affinity to the target were captured by agarose-conjugated streptavidin beads. The affinity selection was stopped after 10th round, and the candidates from the pool were cloned. In all 35, randomly picked clones were isolated and the segment of DNA exhibiting unique sequences was sequenced. Out of 35 sequences, 25 were unique and the remaining 10 were duplications.

Binding Affinity of DNA Aptamers to *F. tularensis* subspecies *japonica* by Aptamer-Linked Immobilized Sorbent Assay (ALISA)

To determine the affinity of the selected polyclonal DNA aptamer cocktail for *F. tularensis japonica* antigen we developed an Aptamer-Linked Immobilized Sorbent Assay. This assay provided a rapid assessment of the relative binding capabilities of this aptamer cocktail. Additionally, this enzymatic assay (ALISA) was used to provide information of specificity of these aptamers to tularemia antigen from other subspecies.

In this format, a nonbiotinylated aptamer cocktail was immobilized as a capture aptamer as described in the methods. Tularemia antigen from subspecies *japonica* was added to the wells with quantities ranging from 25 ng to 2 µg. Target molecule sensitivity was assessed for these aptamers by determining their binding affinity. Binding reaction was carried out as described in the earlier section. Employing a colorimetric detection system (ABTS) for horseradish peroxidase activity conjugated to streptavidin, we observed a significant signal over background, and data for four replicate samples ranged from 0.0451 (background) to 1.667 absorbance units. As seen in Figure 1, it is further confirmed that the antigen affinity to the aptamer cocktail is directly proportional to the quantity of antigen. It is evident from the data that aptamers exhibit impressive binding affinities for subspecies *japonica* achieving an unusually strong association even at 25 ng of total antigen. While it is clear that the detection limit depends primarily on the binding affinity of aptamers for the target, the formation of an affinity-based complex is also a crucial step in producing signal. In order to achieve an absolute assessment of target molecule sensitivity in terms of number of bacteria, an ALISA was performed with subspecies *holarctica*. Here, various concentrations of bacteria were prepared by serial dilution with PBS and used in the binding assay. The data presented in Figure 2 demonstrate that the sensitivity and detection of the target is attained as low as 1.7×10^3 /ml of bacteria. It is interesting and

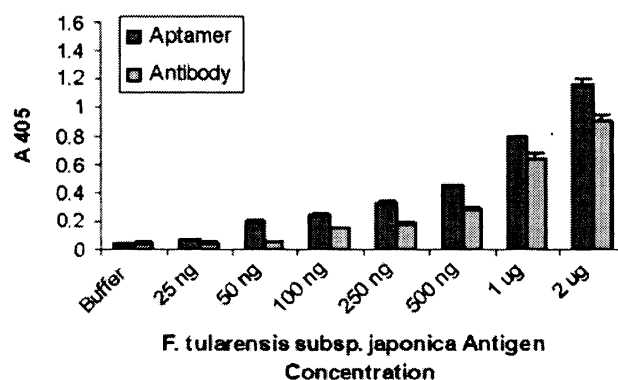


Figure 1 Sensitivity of anti-tularemia aptamer cocktail for *F. tularensis* subspecies *japonica* antigen and anti-tularemia antiserum as assessed by ALISA and ELISA. The assays were performed as described in 'Materials and methods'. The data are presented as OD at 405 nm vs antigen quantity. Averages of four replication measurements are shown in the figure.

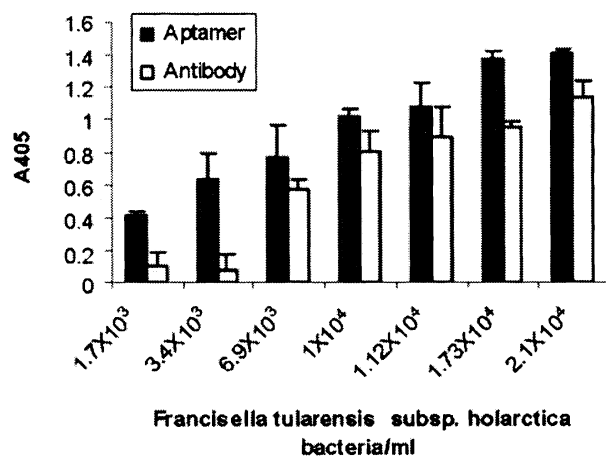


Figure 2 Tularemia bacterial antigen binding to anti-tularemia aptamer cocktail and anti-tularemia polyclonal antibodies as assessed by ALISA and ELISA using HRP activity. The assays were performed as described in 'Materials and methods'. The bacterial antigen used in the binding assay was prepared from *F. tularensis* subspecies *holarctica* (live vaccine strain). The data are plotted as OD at 405 nm vs number of bacteria/ml. Averages of triplicate measurements are shown in the figure.

perhaps paradoxical to note that these two different subspecies exhibited a similar binding affinity thereby indicating the detection capability of aptamers in this platform across the subspecies.

In order to verify the specificity of the aptamer cocktail to other *F. tularensis* subspecies, we tested the binding affinity of these aptamers against antigens from two other subspecies *holarctica*, *tularensis* (SCHU 4), and another isolate (#38) type B (Houston, TX, USA) which was positive for agglutination and negative for glycerol fermentation. Also the isolate #38 was further confirmed as type B by PCR amplification of IS*Ftu2* element using the CDC's primer sequences (data not shown).³² The

specificity of the aptamer cocktail was further evaluated by ALISA using antigens from three other subspecies listed earlier. As can be seen in Figure 3 the signal output is ranging from 1.256 for subspecies *tularensis* (SCHU 4) to 2.144 absorbance units for subspecies *holarctica*, indicating a substantial homology with common motifs for binding and cross reactivity between the subspecies. Finally, to rule out the nonspecificity of the aptamers, antigen from unrelated bacteria and other pure proteins were used in the assay. Sandwich ALISA and ELISA assays were carried out with total antigen from *B. henselae* a rod-shaped Gram-negative bacteria showed a very low affinity. However *B. henselae* exhibited some nonspecific binding with both aptamer and antibody. This most likely is due to some similar bacterial wall components because both of these bacteria are Gram negative. In contrast, pure proteins like chicken albumin and lysozyme showed no affinity with an optical density close to background.

Anti-Tularemia Polyclonal Antibody Specificity for Tularemia Antigen by ELISA

To compare aptamer specificity to that of antibodies, we performed a sandwich ELISA using commercial antiserum. The ELISA plates were coated with anti-tularemia rabbit antiserum as described in methods. Identical quantities of subspecies *japonica* total

antigen are used in the binding assay. The data presented in Figure 1 indicates that antibody is not as sensitive as aptamer cocktail. Furthermore, the detection limit for aptamer cocktail is 25 ng whereas the detection limit for antibody was 100 ng of antigen. Similar to aptamers, antibody exhibited concentration-dependent signal output in the ELISA. An attempt was made to assess the binding affinity of antibody to other related and unrelated antigens. To compare the detection capability of antibody in terms of bacterial cell number, similar to ALISA, whole *F. tularensis holarctica* antigen was used in the ELISA. In this assay, the diagnostic performance of the ELISA is inferior to the ALISA as depicted in Figure 2. While the aptamer cocktail can detect 1.7×10^3 bacteria/ml, (Absorbance units = 0.413), the threshold for antibody detection is 6.9×10^3 bacteria/ml (Absorbance units = 0.568). Further binding assays with identical quantities of total antigen from all the three subspecies *holarctica*, *japonica* and *tularensis* (SCHU 4) along with unrelated *B. henselae* and pure proteins from chicken were performed to compare the ELISA to the ALISA. As shown in Figure 3, the antibody detected the antigen from all the three subspecies of *F. tularensis* by generating positive signal in the enzymatic assay. As expected, there were negative signals for pure proteins from chicken. Additionally, we observed some nonspecific binding to *B. henlsee* (sic). This could be partly due to the fact that the

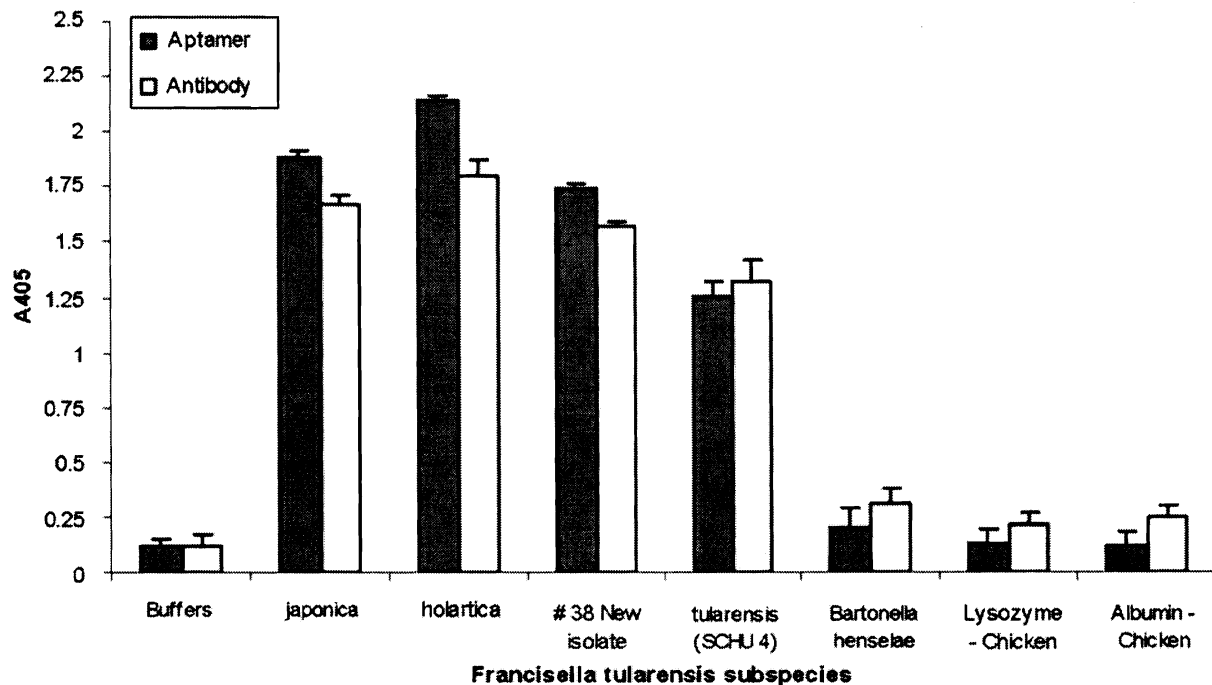


Figure 3 ALISA and ELISA to analyze the specificity of anti-tularemia aptamer cocktail. The assays were performed as described in 'Materials and methods'. Antigen from both type A and B of *F. tularensis* are used in the analysis. Subspecies include *holarctica*, #38 (type B) a new isolate from Houston, *tularensis* (SCHU 4) and an unrelated Gram-negative rod shaped bacteria *B. henselae*. Additional negative controls include chicken lysozyme and chicken albumin.

serum may have had some products binding non-specifically to the bacteria. The other notable observation under identical conditions is that the antibody exhibited a higher level of nonspecific binding affinity with albumin and lysozyme (Figure 3) compared to aptamer cocktail, indicating significant advantages of aptamers over their antibody counterparts. Although antibody-based detection systems are available, the presented data reinforce the superior performance of the aptamer-based detection/diagnostic system.

Comparisons of the Specificity of the Anti-Tularemia Aptamer Cocktail to Anti-Tularemia Polyclonal Antibody Using Dot Blot Analysis

The results of the ALISA and ELISA suggested that the anti-tularemia aptamer cocktail exhibited specificity for tularemia antigen irrespective of their source. In order to verify the specificity and to further investigate the potential uses of the aptamers, we performed a dot blot analysis (Figure 4). In all, 50 µg of total protein from the bacterial lysate from subspecies *holarctica*, #38, *tularensis* along with *B. henselae*, chicken lysozyme and chicken albumin were spotted onto nitrocellulose discs and air dried at room temperature. To test the exquisite binding specificity of aptamers compared to their counterpart antibodies, we also performed parallel immuno dot blot assay with anti-tularemia antiserum. The immobilized proteins were probed with biotin labeled aptamer cocktail and detected with streptavidin conjugated alkaline phosphatase. Parallel blots were treated with tularemia antiserum and developed with alkaline phosphatase conjugated goat anti-rabbit antibody. As shown in Figure 4, the signal/color produced by the aptamer cocktail and antibody showed strong affinity to tularemia

bacterial lysate but did not show any affinity to negative controls, thereby indicating their specificity towards the antigens of different subspecies of *F. tularensis*. Interestingly, in this particular technique, the performance of the aptamer cocktail was similar to that of antibody.

Discussion

In the present study, *F. tularensis* was selected as a target because of its high bioterrorism potential.²⁶ Monitoring of the environment for biological threats, such as *F. tularensis* requires molecular probes that bind to the agents and ensures their presence and detection in the field. Aptamer technology is based on the principle that biological agents whether bacterial cells or spores or viruses have unique surface markers that can bind to these molecular probes from diverse pools or libraries through screening and selection methods.³³ Nucleic acid aptamers would be effective because of their affinity and specificity for the binding pockets of the targets with proper folding.³⁴ These unique probes have been shown to discriminate closely related molecules from their targets and also exhibit chiral discrimination of target molecules.³⁵ The effectiveness of this emerging technology has been illustrated by development of diagnostic probes for various infectious agents including bacterial and viral targets.³⁶ In our present investigation, we selected DNA aptamers rather than RNA aptamers because of their stability in harsh environments and because they can be used easily in the field. It is also reasonable to assume that DNA aptamers without further modifications could perform better in detection and diagnostic assays in which the aptamers may come in contact with different biological

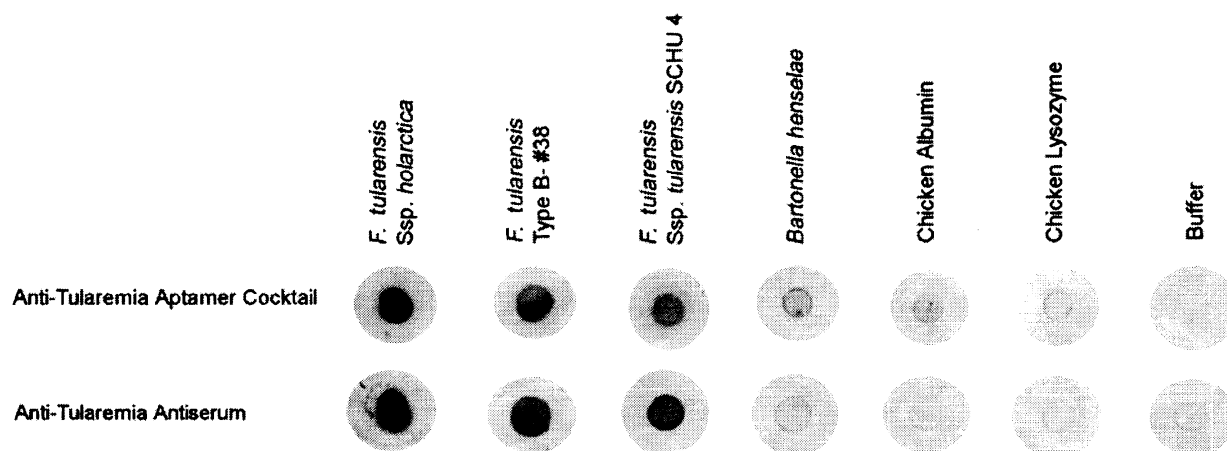


Figure 4 Specificity of anti-tularemia aptamer cocktail and anti-tularemia antiserum assessed by dot blot analysis. Fifty micrograms of total protein from *F. tularensis* subspecies *holarctica*, *F. tularensis* type B an isolate (#38) from Cat (from Houston, TX), *F. tularensis* subspecies *tularensis* (SCHU 4), *B. henselae*, chicken albumin and chicken lysozyme were spotted onto nitrocellulose membrane discs and probed with either aptamer cocktail or anti-tularemia rabbit antiserum and analyzed by alkaline phosphatase activity by using 5-bromo-4-chloro-3-indolylphosphate/Nitro Blue Tetrazolium (BCIP/NTB)-Blue as described in 'Materials and methods'.

samples for a brief period of time. Although other types of detection and diagnostic platforms utilizing peptides and antibodies are available, DNA-based technology may be better suited for the detection of bio-warfare agents and environmental monitoring, in which robust, nonperishable and inexpensive reagents capable of operating in severe and extreme environmental conditions are needed.

Enzyme linked assays provide a means of quickly evaluating the binding affinity. As early as 1996, Drolet *et al*³⁷ reported the first enzyme-linked aptamer assay (ELAA) consisting of a mixed ELISA/ELAA sandwich to detect human vascular endothelial growth factor on a microtiter plate. Later they demonstrated that the aptamers can be used to specifically detect isoforms of the same protein expressed due to an alternate splicing of the mRNA.³⁷ Since then, similar assays have been demonstrated using either labeled or immobilized nucleic acid molecules as capture or detecting agents. Aptamer conjugated immunoassays using other platforms to detect specific proteins were reported earlier by Rye *et al* and Yang *et al*.^{38,39} The most remarkable applications were the demonstration of fiber-optic microarray biosensor using aptamers by Lee and Walt⁴⁰ and an aptamer-based quartz crystal biosensor.⁴¹

Recently, Grunow *et al*⁴² reported the detection of *F. tularensis* in biological specimens using capture ELISA, whereas Porsch-Özcürümez *et al*⁴³ compared different detection methods including ELISA to identify *F. tularensis* from human samples. However, all of these technologies need more instrumentation involvement. Serology is the only test performed currently for the diagnosis of tularemia in humans but it is of limited value. Nonetheless, these tests do not differentiate between type A and B, as there is no antigenic difference between them.⁴⁴ None of these reports utilized nucleic acid-based enzymatic assays. In our study with aptamer sandwich assays, we are able to measure significant signals over very low background by using biotinylated aptamers to detect tularemia antigen in a 96-well microtiter plate format. Our findings suggest that in terms of detection test efficiency, ALISA is superior to ELISA. Very recently similar findings were reported by Baldrich *et al*⁴⁵ in a displacement enzyme linked aptamer assay using thrombin that demonstrated the superior performance of aptamers compared to antibodies.

Using aptamers in blot analysis is another means to study the affinity and specificity to the target. We have tested the aptamer cocktail for their binding affinity to tularemia antigen from different sub species along with unrelated Gram-negative bacteria and chicken albumin and chicken lysozyme by dot blot analysis with alkaline-phosphatase conjugated enzymatic assays. The aptamer cocktail showed no cross reactivity to either lysozyme or chicken albumin. Although the antibody detection system is the gold standard for protein detection and

identification, the data presented in this study reinforces the superior performance of the nucleic acid-based diagnostic system.

In summary, we have generated a subpopulation of oligonucleotide anti-ligands that bind with high specificity to *F. tularensis* antigen. These ssDNA molecules specifically recognize tularemia antigen from three different subspecies *japonica*, *holarctica* and *tularensis* (SCHU 4) and do not bind to other Gram-negative bacteria like *B. henselae*. The current work demonstrates that aptamers can be used in an ALISA format for detection of specific targets. Although antibodies have been the reagent of choice for detection and diagnostic assays, the increased specificity of aptamers might make these nucleic acids attractive alternatives to detect various targets. It is important to point out that aptamers have an unlimited potential to circumvent limitations associated with antibodies. In conclusion, it is reasonable to expect that the ALISA platform generated by aptamers will pave the way for future detection systems in which antibodies have previously dominated.

Acknowledgements

We would like to thank Ms Melanie D Woitaske, Ms S Kathryn Boyd, and Ms Shelly D Roper of Conceptual Mind Works Inc. for graphics, MSgt Mark S Wade and Ms Amy M Heathman for supplying bacterial antigen, and Dr Ramesh Ganapathy, Pierce Biotechnology for coating microtiter plates. This work was sponsored in part by the Department of Defense's Joint Service Technology Base Program in Chemical and Biological Defense and the Air Force Office of Scientific Research.

References

- 1 Turek C, Gold L. Systemic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990;249:505–510.
- 2 Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* 1990;346:818–822.
- 3 Famulok M. Oligonucleotide aptamers that recognize small molecules. *Curr Opin Struct Biol* 1999;9:324–329.
- 4 Osborne SE, Ellington AD. Nucleic acid selection and the challenge of combinatorial chemistry. *Chem Rev* 1997;97:349–370.
- 5 Famulok M. Molecular recognition of amino acid by NA-aptamers: An L-citrulline binding RNA motif and its evolution into an L-arginine binder. *J Am Chem* 1994;116:1698–1706.
- 6 Burgstaller P, Famulok PM. Isolation of RNA aptamers for biological co-factors by *in vitro* selection. *Angew Chem, Int Ed Engl* 1994;33:1084–1087.
- 7 Joshi PJ, Fisher TS, Prasad VS. Anti-HIV inhibitors based on nucleic acids: emergence of aptamers as

- potent antivirals. *Curr Drug Targets Infect Disord* 2003;4:383–400.
- 8 Bock LC, Griffin LC, Latham JA, *et al.* Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* 1992;335:564–566.
- 9 Green LS, Jellinek D, Bell C, *et al.* Nuclease-resistant nucleic acid ligands to vascular permeability factor/vascular endothelial growth factor. *Chem Biol* 1995; 10:683–695.
- 10 Hermann T, Patel DJ. Adaptive recognition by nucleic acid aptamers. *Science* 2000;287:820–825.
- 11 Marshall KA, Robertson MP, Ellington AD. A biopolymer by any other name would bind as well: a comparison of the ligand-binding pockets of nucleic acids and proteins. *Structure* 1997;5:729–734.
- 12 Gold L, Polisky B, Ublenbeck O, *et al.* Diversity of oligonucleotide functions. *Annu Rev Biochem* 1995; 64:763–797 (Review).
- 13 Uphoff KW, Bell SD, Ellington AD. *In vitro* selection of aptamers: the dearth of pure reasons. *Curr Opin Struct Biol* 1996;3:281–288 (Review).
- 14 Jenison RD, Gill SC, Pardi A, *et al.* High-resolution molecular discrimination by RNA. *Science* 1994; 263:1425–1429.
- 15 Gesteland RF, Atkins JF (eds). *The RNA World*. Plainview. Cold Spring Harbor Lab Press: NY, 1993.
- 16 Cross JT, Penn RL. *Francisella tularensis* (Tularemia). In: Bennet JE, Dolin R and Mandell GL (eds). *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases*. Philadelphia: Churchill Livingstone, 2002, pp 2393–2401.
- 17 Ellis JPC, Oyston M, Green M, *et al.* Tularemia. *Clin Microbiol Rev* 2002;15:631–646.
- 18 La Regina M, Lonigro J, Wallace M. *Francisella tularensis* infection in captive, wild caught prairie dogs. *Lab Anim Sci* 1986;36:78–80.
- 19 Magee JS, Steele RW, Kelly NR, *et al.* Tularemia transmitted by a squirrel bite. *Pediatr Infect Dis J* 1989; 8:123–125.
- 20 Quenzer RW, Mostow SR, Emerson JK. Cat-bite tularemia. *JAMA* 1977;238:1845.
- 21 Conlan JW, North RJ. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis* and *Salmonella typhimurium* involves lysis of infected hepatocytes by leucocytes. *Infect Immun* 1992; 60:5164.
- 22 Fortier AH, Green SJ, Polsinelli T, *et al.* Life and death of an intracellular pathogen: *Francisella tularensis* and macrophage. *Immunol Ser* 1994;60:349.
- 23 Ellis J, Oyston PCF, Green M, *et al.* Tularemia. *Clin Microbiol Rev* 2002;15:631–646.
- 24 Tarnvik A. Nature of protective immunity to *Francisella tularensis*. *Rev Infect Dis* 1989;11:440.
- 25 Dennis DT, Inglesby TV, Henderson DA, *et al.* Tularemia as a biological weapon: medical and public health management. *J Am Med Assoc* 2001;285:2763.
- 26 Rotz LD, Khan AS, Lillibridge SR, *et al.* Public health assessment of potential biological terrorism agents. *Emerg Infect Dis* 2002;8:225.
- 27 Isherwood KE, Titball RW, Davies DH, *et al.* Vaccination strategies for *Francisella tularensis*. *Adv Drug Delivery Rev* 2005;57:1403–1414.
- 28 Vivekananda J, Kiel JL. Methods and components for aptamers against anthrax. Patent 6,5696,30 B1 issued on May 27, 2003.
- 29 Tasset DM, Kubik MF, Steiner W. Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J Mol Biol* 1997;272:688–698.
- 30 Griffin LC, Toole JJ, Leung LLK. Discovery and characterization of a novel nucleotide-based thrombin inhibitor. *Gene* 1993;137:25–31.
- 31 Hicke BD, Watson SR, Koenig A, *et al.* DNA aptamers block L-selectin function *in vivo*. *J Clin Invest* 1996;98: 2688–2692.
- 32 Petersen JM, Schrieffer ME, Carter LG, *et al.* Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. *Emerging Infectious Diseases* 2004;10:419–425.
- 33 Ivnitski D, O'Neill DJ, Gattuso A, *et al.* Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents [Review]. *Biotechniques* 2003;35:862–869.
- 34 Huang DB, Vu D, Cassidy LA, *et al.* Crystal structure of N-kB (P50)2 complexed to a high-affinity RNA aptamer. *Proc Natl Acad Sci USA* 2003;100: 9268–9273.
- 35 Jenison RD, Gill SC, Pardi A, *et al.* High-resolution molecular discrimination by RNA. *Science* 1994;263: 1425–1429.
- 36 Petrenko VA, Sorokulova IB. Detection of biological threats. A challenge for directed molecular evolution [Review]. *J Microbial Methods* 2003;58:147–168.
- 37 Drolet DW, Moon-McDermott L, Romig TS. An enzyme-linked oligonucleotide assay. *Nat Biotechnol* 1996;8:1021–1025.
- 38 Rye PD, Nustad K. Immunomagnetic DNA aptamer assay. *Biotechniques* 2001;30:290–295.
- 39 Yang X, Li X, Prow TW, *et al.* Immunofluorescence assay and flow-cytometry selection of bead-bound aptamers. *Nucleic Acids Res* 2003;31:e54.
- 40 Lee M, Walt DR. A fiber-optic microarray biosensor using aptamers as receptors. *Anal Biochem* 2000;282: 142–146.
- 41 Liss M, Petersen B, Wolf H, *et al.* An aptamer-based quartz-crystal protein biosensor. *Anal Chem* 2002;74: 4488–4495.
- 42 Grunow R, Spletstoeser W, McDonald S, *et al.* Detection of *Francisella tularensis* in biological specimens using capture enzyme-linked immunosorbent assay, an immuno-chromatographic handheld assay and PCR. *Clin Diag Lab Immunol* 2000;7: 86–90.
- 43 Porsch-Özcürümez M, Kischel N, Priebe H, *et al.* Comparison of enzyme-linked immunosorbent assay, western blotting, microglutination, indirect immunofluorescence assay and flow cytometry for serological diagnosis of tularemia. *Clin Diag Invest Immunol* 2004; 11:1008–1015.
- 44 Mörner T, Sandström G. Tularemia. *Manual of Diagnostic Tests and vaccines for terrestrial animals*. World Organization for Animal Health (OIE) Part 2, Ch. 2.8.2. 2004.
- 45 Baldrich E, Acero JL, Reekmans G, *et al.* Displacement enzyme linked aptamer assay. *Anal Chem* 2005;77: 4774–4784.