Review Article

Feasibility of Anti-Idiotypic Aptamers to Detect Rickettsia Exposure

John G. Bruno

OTC Biotechnologies, LLC, 4100 NW Loop 410, Suite 230, San Antonio, TX 78229.

Abstract | Twenty-seven DNA aptamers were developed against the Fab fragments of murine antibodies developed to bind *Rickettsia rickettsii* and *Rickettsia typhi*. A feasibility study was conducted to generate anti-idiotypic aptamers against Fab fragments of these antibodies and to determine if the aptamers were capable of emulating rickettsial antigens to enable sensitive detection of rickettsia exposure in humans who have seroconverted. Such anti-idiotypic aptamer antigens might also serve as rickettsial vaccines, if fully developed. The top five highest affinity *R. rickettsii* and top five *R. typhi* anti-Fab aptamers as assessed by enzyme-linked aptamer sorbent assay (ELASA) screening were shown to specifically bind their cognate antibodies when captured by their Fc tails on the surface of Protein A-conjugated magnetic beads. While no clear similarities in the partial or complete primary DNA sequences or secondary stem-loop structures of these aptamers were observed, 3-dimensional computer models of these top ten anti-idiotypic aptamer candidates showed some topological similarities which suggest common binding motifs. Unfortunately, without knowledge of the hypervariable region amino acid sequences of the target antibodies, no docking simulations are possible to test this topological hypothesis, but the present work illustrates feasibility of the approach and paves the way for more sophisticated analysis that could lead to improved rickettsial serodiagnostics and aptamer-based vaccines.

Editor | Dr. Tarun Kumar Sharma, Centre for Biodesign and Diagnostics, NCR Biotech Science Cluster, 3rd Milestone, Faridabad-Gurgaon Expressway, Faridabad, India.
Received | January 20, 2016; **Accepted** | February 19, 2017; **Published** | February 21, 2017
***Correspondence** | John G. Bruno, Ph.D. OTC Biotechnologies, LLC, 4100 NW Loop 410, Suite 230, San Antonio, TX 78229; **Email:** brunobiotech@gmail.com
Citation | John G. Bruno. 2017. Feasibility of anti-idiotypic aptamers to detect rickettsia exposure. *Aptamers and Synthetic Antibodies,* 3(1): 64-73.
Keywords | 3-D Molecular Modeling, Antibody, Anti-idiotype, Fab, YASARA

Introduction

The genus *Rickettsia* consists of a variety of arthropod-borne exceptionally tiny obligate intracellular bacterial parasitic species which invade host cells to avoid the immune system. Due to their intracellular invasion, it is difficult to catch individual rickettsial bacteria outside of host cells in the blood or other body fluids so as to bind and detect them with antibodies or aptamers, although efforts have been made to enhance sensitivity to directly detect the rare extracellular organisms in vivo (Bruno et al., 2016). Current diagnosis of *Rickettsia* is primarily based on a variety of tests for detection of induced antibodies from host serum samples using rickettsial surface antigens, despite the availability of more expensive PCR-based assays and lower sensitivity or false negative results of serological tests especially prior to seroconversion (Chao et al., 2008; La Scola and Raoult, 1997; Kovacova and Kazar; 2000; Kowalczewska et al., 2012; Mahajan, 2012). In addition to the difficulties with rickettsial diagnostics, few effective vaccines for *Rickettisa* spp. exist (Mahajan, 2012). Research efforts have been undertaken to determine if newer aptamer technology might provide novel serodiagnostics to indicate rickettsial exposure and whether or not such anti-idiotypic aptamers might also serve as effective rickettsial vaccines in the future.

Several investigators have already demonstrated successful generation of anti-idiotypic aptamers (Hamm et al., 1997; Hu et al., 2013; Qin et al., 2014). However, one of the key difficulties with successful implementation of the anti-idiotypic aptamer approach is isolation of the correct purified hypervariable regions which bind the microbes to serve as targets for aptamer development. For this initial feasibility study, isolation of antibody Fab fragments was used to generate targets for anti-idiotypic development, although the author fully acknowledges that other constant regions of the antibody heavy and light chains are present and could lead to diagnostically useless aptamers which are included or even dominate the final aptamer pool. With this understanding of potential limitations, the following anti-Fab aptamer development and feasibility study was undertaken with the following results suggesting some convergence of the final aptamer structures against what might be the desired hypervariable regions.

Materials and Methods

Anti-Rickettsia antibodies and Fab-magnetic bead generation

Polyclonal murine anti-*R. rickettsii* and anti-*R. typhi* antisera were provided by Dr. Chien-Chung Chao of the Naval Medical Research Center (NMRC, Silver Spring, MD). Antisera were first purified using a Pierce™ antibody clean up kit employing Melon™ gel technology to remove serum albumins (Catalogue no. 44600, Thermo Fisher Scientific, Inc., Pittsburgh, PA) according to the manufacturer's instructions. Purified serum samples were then subjected to column-immobilized papain digestion and Fc removal with Protein A-column affinity chromatography to produce theoretically pure Fab fragments using a Pierce™ Fab Micro Preparation Kit (Thermo Fisher Catalogue no. 44685). The original serum samples and ~ 50 µg of each purified Fab fragment were electrophoresed at 100 V for 1 h in separate 4-20% polyacrylamide gels (Life Technologies, Inc., Carlsbad, CA). Gels were rinsed in 18 MΩ deionized water and stained with 50 ml GelCode™ Blue Safe Protein Stain (proprietary formulation of Coomassie Blue G-250) for 1 h with gentle mixing followed by destaining in several changes of 100 ml of deionized water. One ml of Dynal tosyl-M280 magnetic beads (~ 6.5 x 10^8 MBs; Thermo Fisher) in sterile phosphate buffered saline (PBS, pH 7.2) were added to 100 µg of each Fab fragment and gently mixed at 37°C for 2 h. Fab-conjugated MBs were then washed three times in 1 ml of PBS per wash by magnetic separation and resuspension. Fab-MBs were blocked in 10% ethanolamine in PBS at 37°C for 2 h and washed three times in 1 ml of PBS per wash as before. Fab-MB stock in 1 ml of PBS was stored at 4°C until needed in aptamer development.

SELEX DNA aptamer development, cloning and sequencing

A 200 base SELEX template having the following sequence was obtained from Integrated DNA Technologies, Inc. (Coralville, IA): 5'-ATCCGTCACACCTGCTCT-N_{164}-TGGTGTTGGCTCCCGTAT-3', where N_{164} represents the randomized 164-base region of the DNA template. Primer sequences were: 5'-ATACGGGAGCCAACACCA-3' (designated forward or F) and 5'-ATCCGTCACACCTGCTCT-3' (designated reverse or R) to prime the template and nascent strands, respectively. To begin the process of SELEX (Systematic Evolution of Ligands by EXponential enrichment) aptamer development against murine Fab fragments, 160 nmoles of template (randomized DNA library) in 100 µl of sterile nuclease-free deionized water was heated at 95°C for 5 min to ensure interaction of a linearized single-stranded 200 base DNA library free of concatamers with Fab-MB targets. The heated DNA was added to 100 µl of 1:10 diluted Fab-MB stock in 1 ml of PBS for 2 h at room temperature (RT ~ 25°C). The aptamer-Fab-MB complexes were magnetically separated and the supernate was carefully removed. One-hundred µl of sterile nuclease-free deionized water was added to the separated MBs which were heated at 95°C for 5 min to heat-elute bound DNA from the Fab-MBs. The 100 µl of hot supernatant was collected and 50 µl aliquots of eluted DNA were PCR-amplified in 100 µl reaction volumes using a SpeedStar® (hot start) PCR kit (Takara Bio Inc., Shiga, Japan). PCR was conducted as follows: an initial 95°C phase for 5 min, followed by at least 20 cycles of 30 sec at 94°C, 30 sec at 60°C, and 15 sec at 72°C followed by a 72°C completion stage for 5 min, and refrigeration at 4°C.

Aptamer PCR amplicons were verified to be ~ 200 bp in length after each round of SELEX by electrophoresis in 2% agarose TAE (Tris-Acetate EDTA) gels with ethidium bromide staining. If more than one band emerged, the ~ 200 bp band was excised on a UV transilluminator with a sterile razor blade. Aptamers from the gel slice were eluted into 50 µl of Qiagen elution buffer using a Qiagen Gel Purification spin column (Germantown, MD). If the aptamer amplicon

was faint or not visible in the gel, the number of PCR rounds was increased until a clearly visible ~ 200 bp band emerged on a subsequent electrophoresis gel. Negative control PCR reactions without the SELEX template were run to ensure that nonspecific DNA was not amplified. Following PCR amplification at the end of each round of SELEX, the double-stranded aptamer amplicons were again heated to 95°C for 5 min prior to adding the selected DNA pool to a new aliquot of 100 µl of 1:10 Fab-MBs for 2 h with gentle mixing at ambient temperature. This constituted the first of 9 rounds of MB-based SELEX.

Following round 9 of MB-SELEX, aptamers were cloned into chemically competent *E. coli* using a Lucigen GC cloning kit (Middleton, WI) according to the manufacturer's protocol and clones were sent to Sequetech, Inc. (Mountain View, CA) for proprietary GC-rich DNA sequencing. Plasmids from the *E. coli* clones were both forward- and reverse-primed to yield aptamers and their cDNAs which can potentially be useful aptamers in some cases. Aptamer DNA sequence names are coded throughout, indicating that particular aptamer sequences were developed against mouse (ms) antibodies which bind *R. rickettsii* (Rr) or *R. typhi* (Rt) along with F for forward-primed and R for reverse-primed (Table 1).

ELASA screening and ranking of candidate aptamer relative affinities

To evaluate and rank relative affinity for each of the candidate anti-Fab aptamers, an ELISA-like enzyme-linked aptamer sorbent microplate assay (ELASA) was conducted essentially as previously reported (Bruno et al., 2016) by first immobilizing 1 µg of each Fab fragment in 100 µl of 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4°C in covered flat-bottom polystyrene 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). The plates were decanted and washed 3 times with gentle mixing for 5 min per wash using 200 µl of PBS per well. Wells were then blocked with 150 µl of 2% ethanolamine in 0.1 M $NaHCO_3$ for 1 h at 37°C followed by 3 more washes with 200 µl of PBS as before. Ethanolamine was used instead of conventional protein blocking agents because it is a small molecule and less likely to hinder aptamer binding like serum albumins or other large proteins would on or around the Fab fragments.

A total of 27 different sequenced anti-mouse Fab aptamer candidates (Table 1) from the final selected pool were synthesized in a dry state by Integrated DNA Technologies and rehydrated in 100 µl of PBS for 1 h with gentle mixing on a rotary mixer and applied to their corresponding microplate wells at 1 nanomole per well for 1 h at RT with gentle mixing. The plates were decanted and washed 3 times in 200 µl of PBS for at least 5 min per wash with gentle mixing. One hundred µl of a 1:5,000 dilution of streptavidin-peroxidase from a 1 mg/ml stock solution (Thermo Scientific, Inc., Product No. 21126) in PBS was added per well for 30 min at RT with gentle mixing. The plates were decanted and washed 3 more times with 200 µl of PBS per well as before. One hundred µl of One-Component® ABTS substrate (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD) which had been equilibrated to RT was added to each well and incubated for 15 min at RT. Reactions were halted by addition of 100 µl of 1% SDS as the strongest reactions approached an absorbance of 1.0 at 405 nm using a Thermo Electron MultiSkan™ microplate reader (Thermo Fisher Scientific; Waltham, MA).

Aptamer secondary and tertiary structural analyses

Secondary stem-loop structures were determined by web-based Vienna RNA software available at: http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi using DNA parameters at 25°C. Three-dimensional (3-D) aptamer models were developed using the most probable (minimal free energy or most negative ΔG) dot-bracket notations from Vienna RNA which were submitted to web-based RNA Composer software (http://rnacomposer.cs.put.poznan.pl/) to generate PDB files for 3-D visualization using YASARA (http://www.yasara.org/) as previously described by Bruno (2017).

Fluorescent Enzyme-Linked Aptamer-Magnetic Bead Sandwich Assay for Fab Binding

Using a protocol similar to that of Bruno et al. (2016), murine *R. rickett*sii and *R. typ*hi antibodies in 50 µg of antisera were captured by their Fc tails on Dynal Protein A M280 (2.8 micron) magnetic beads (~ 4 x 10^7 MBs) in a total volume of 500 µl of PBS in ten separate microfuge tubes. Tubes were mixed gently on an orbital shaker for 30 min at RT and collected by magnetic separation for 2 min. The 500 µl supernatant was carefully aspirated and discarded and antibody-Protein A-MBs were washed twice in 1 ml of PBS with magnetic separation. Five hundred picomoles of each of the top 10 anti-*Rickketsia* Fab 5'-biotinylated aptamers (bolded in Table 1 and shown on

Table 1: *DNA Aptamer sequences developed against murine Rickettsia Fab fragments.*

Aptamer Name	DNA Sequence 5' → 3'
Rr-ms-3F	ATACGGGAGCCAACACCATATGGGTAGGGTGCTGTAGCTGATAATGCTCATACAGAGCAT-TACACATGTTCTTTGTAATTTGCAGGTAGAGGCTTTGGATTAGTTTAATAAATACTAGT-GAGAAAATATGTACGTTGCACAAATAAAGCCAATAAATAAAAGTATATGGGAAGGGAGAG-GTAGAGCAGGTGTGACGGAT
Rr-ms-4R	ATCCGTCACACCTGCTCTAGTGAAGGTTTAGCGGTGGGGCAGAAATACGTGGATGGT-GAATTCGGTTGCGAAGGTGGCTATACGGTATTAGAATTGTGACTATAGGTTGGTGT-TCAGGTCTAGTCTTACATGAGCATCTCCCCTTTTCATGGAGCAGGCGCCGGCGCTTGTG-CAGCTTATGGTGGTGTTGGCTCCCGTAT
Rr-ms-5F	ATACGGGAGCCAACACCAGGATCGCCGAGCGTGCGGGCTGACTCGTACGGTGTGAT-CATCCGGGCTATACTTGCATGACTCACCATGTGTAGAGGACCTCAGTGGACTAGCTCT-CAAAAGACTAGGGAAGATACTAGGTCTCGCCTACAACCTAACCACACATCTTTAGCATTCT-TATGCCGAGAGCAGGTGTGACGGAT
Rr-ms-6R	ATCCGTCACACCTGCTCTCTCACACTTGAATGTAGACGTATATATTCTAGTGTTCTG-TACCTTCCGCTCTGTTAAAGTTACACTACATATTGTCTACACTTTTTGCGCTCGCTTGAT-TCGAGTGGCTTACTCCAACTTTTATATATTGTTTGGAAAATGAGAGGGTCTTCATCCG-TAGTTCCTGGTGTTGGCTCCCGTAT
Rr-ms-7F	ATACGGGAGCCAACACCACGTATTGTCGAGATCGAACGACTTGGGGACACGACATGTC-TACGTGAAACTGGATATCGTTGAGAACAGCGCGGACATGAGAAACTCACCACTAATGAAA-CAAAAGGCAGTGAAACTCCACACTTAAAATCTAAGGAGGTATTCATCTAAATTGGAGGT-TCTGTAGAGCAGGTGTGACGGAT
Rr-ms-8F	ATACGGGAGCCAACACCATTCGAGCTGCTCAGTTATGCCAAGATGGTCTCTAGCAG-CGCCACGCCTTACTGCAGTCCTGGAAGACAGATATTGCCTTAAGATAAAACAGATAAGT-TCCCCAAAAGAGCTGTGAAGACATCACATTGAGCCAGACCTTAAGATCACAGTACTCT-TTCAGAGCAGGTGTGACGGAT
Rr-ms-9F	ATACGGGAGCCAACACCATAGGCTTGCGGGGTCCGATAGTTGTCCCGAGTGCGTTAACA-CACCCGCCTCATGTGAGTCAGCCGTGTAACATACCAGCAACAATTGACGCCTTAAAACA-CAAAAAAACCCAACTATCTGATTAACCTCTCAAAAAGTCACCATACGAAATCTGTACTG-GGCTAGAGCAGGTGTGACGGAT
Rr-ms-10R	ATCCGTCACACCTGCTCTCCATGAAGTGCGAGTAGTTTACTATAACTGAGCTCCTCT-GGCTTTGTCTGCGCTCATTCTGTTATTTTCTTTTGCTCCCTGGCTGTCAACATTCCT-TCTCCTACCTGGCATGGATAAAAATTCTTTCACATACTCATTAATAACGCTTTTTTG-GAGATCACCGTGGGGGTGGGTCCCGGATA
Rr-ms-11R	ATCCGTCACACCTGCTCTGGTAGTGTTGACATATTGTCTTTGTCACCACACCTTCAT-TTCTTGGCTCTCATTATGTGTATTCACAAGTGGGTTTACTTTCTTTTCTAGTATGTCT-TTACGCGAATTTATTGTACTAGGCTATTATTTAGACATCATTAAGGAGATTAATGCCTGGTA-CATGGTGTTGGCTCCCGTAT
Rr-ms-12R	ATCCGTCACACCTGCTCTCTAGGATAAAGTGCGATAGTGCAAAGTGTTCCGTG-TATTGCCCTGAATATATTACAGGGGGTTTATCTAGTATCATGGGACGTAGAGCGAAAAT-TACTGACGTAGCGGTGACGTTTAGTTTCCAATGCAGCGAAGTTCCCAGTGGGGACTAT-TATATGTGATGATGGTGTTGGCTCCCGTAT
Rr-ms-13F	ATACGGGAGCCAACACCATCCCATTACCCAACCACCCCTCACAACCAACACCCATCACAAT-CACTACCCCAGCCTGCCCCATCCTGCGCTCCCATACTCCGCAGCGCGCTAATCGGTCCG-GATACGCTCATGGCTTGAACCGCACTTCTCCCCCAACTACACAACAAGTTTATCCTACAC-CAAGAGCAGGTGTGACGGAT
Rr-ms-15R	ATCCGTCACACCTGCTCTTGTAAACTCAGTCGTACGGGTTAAGTAATACATCTGCAGT-TCATGACTTTATGATATTTGTGCTGTAGGCGAGGACAATATATATTTAACTGTTTAAT-TTATTGGTCTAGTCTTCAGTTCATTTTTGTTTAAAGATGCTGTCAGCATTCTTATTCCGT-TATAGTGGTGTTGGCTCCCGTAT
Rt-ms-1R	ATCCGTCACACCTGCTCTTCGAAGAAATGTTTTCTAGTCGAAAACGTAATTGGTTTC-CGTTATTTCGTAAGTTGTGTACAACATACTGTACCTGTGTATATTCTTGTTAGTTGG-GTCTGCCTCGCTTTATTATTTGCTAGGATCTCGGAGGCTCATGGTAGATTGACAAGCTC-CAAGAATTGGTGTTGGCTCCCGTAT
Rt-ms-2F	ATACGGGAGCCAACACCACGAGCCGTCCCCCAGCGGGAACATCAAACTGCCGCCGAAA-TAATCTTTACCCCTAACTATTCATAAGAGCCACTCAGCAACGACGAAAACTCTGATGAAC-CGAAAAGGCGTTTCACAGAATTCTCCAGTTTTATCTACTAAGAATGGCACAATATTCATGA-CATAGAGCAGGTGTGACGGAT

Rt-ms-18F	ATACGGGAGCCAACACCATAATGACTATGCACAAAAACCATCGATGTGCCAGAAGAGT-TCAAGACCAAACTACAACATAGAACTAATAAATTTTTAAAAGTAACCAAATGTGGACT-TAATAAAGAACATAAATGTAAAGTTTCAATATGACCTAGCAAGATCCGATTTAACAGT-CAAAAGAAAGAGCAGGTGTGACGGAT
Rt-ms-19F	ATACGGGAGCCAACACCACATAGTCCACTTGGAGCCCCCACTTGATATTCACACTAACACG-GGCATGACAACACTTAGACAAGGCAAAAGTATAACTCACATATTCTAAAAAACGGATAGAA-GGGACAAATGTTGATAACATCCGTAGATTGAAAACTACCTTAAATTCCGCAAACCCAGAG-CAGGTGTGACGGAT
Rt-ms-21F	ATACGGGAGCCAACACCAACCCAGACATATAGCAAATTGGTCTGCCGGTGTGCTAT-TTCTCCATGTCAACCTAGACTATAGGGTGTTGTTCTTTTGTCTCGTAACGCAGTGAGT-GAACTAGAAGACCATGCAAACCCCTAGCAGGGACTCATTCATTATTAAAAAAGAGAGCTG-GATTGCCAAGAGCAGGTGTGACGGAT
Rt-ms-22F	ATACGGGAGCCAACACCATGATAGGCCTTGCCGGGCGCATCCTAAAAGATGCGG-CGTAGGCTGACTGAAAATTACTACGAATATCTTGAATTGTGTCAATAGGTCGTCGAT-TAAGATAAACAGTAATTGTTACATTTATTTGACATCATTCCACTTCAGACCAGCTG-TAAAAAACGCTAACAAGAGCAGGTGTGACGGAT
Rt-ms-23R	ATCCGTCACACCTGCTCTTGTAGGACAGCTGATGTTTAGGTTTGCTGAAGAAATTA-GACTTGTGACAGTGTCCTTTAGGATCAACGAGGAATAAATGGCCTTCTCGGGCTTTAT-GGTATGCATAATGAAGTGTTTCACTTGATATGACGTGGTGATGCGTGCAGTGGGAGGCG-GGTTTTGGTGTTGGCTCCCGTAT
Rt-ms-24R	ATCCGTCACACCTGCTCTGCCCGTGAGAGTATCGCAAATTGGGCTAGCGGAACTCAAG-TATTGTCACTTTGTCGGTGATAGTAAACGACATCCGCTTGTGAATGCCGTTGGAAGTAT-TCTACACTTTGTACTTCCTTTGATCGGTACTTGGTCTCTCGACAATCTCAGGAATACGCT-TTTGTGGTGTTGGCTCCCGTAT
Rt-ms-25R	ATCCGTCACACCTGCTCTGTCGAGTCTCATGCCTTTAGATATTGGTTCACTGGCTATAT-TACTGCGATTGGCGGTACCTTCTGTGTGTTCGTTGTAGTGGATTCACATGATGCTCT-GACTCTTCATTCACCCTGGCCCGGCCTGCAGGCTCGCGCTGCAAAGTTTCGCGCGCAG-CATGCCCATGGTGTTGGCTCCCGTAT
Rt-ms-26aR	ATCCGTCACACCTGCTCTCGCGGGGATAATAATACAAGCCCGGTTATTCATACGTAGTCTC-TATTTTGTTACAAGGGTCTTGTACCATTCCGTTCCAATATTATGGAACGTCGCACTTACTT-GACTATTCCCTGGGAGCTGCTTGCCGCGTGTCCCAGAACGGAATGTTGTCATATGGT-GTTGGCTCCCGTAT
Rt-ms-26bF	ATACGGGAGCCAACACCACCGCCGCAATTTGTAAATAGATAAGTTAGAAGTTACAAAAT-CAGTTCCTAGGAGTCATCGAACCGATGTTTGTATGAAAAGGCTGTTGGCAACTAAAACCT-TAAGATTCAGAACGAGTATTATTTTATACAAAGAGAGACGATTTCTGTGTATAAAACCCT-TAAGAGCAGGTGTGACGGAT
Rt-ms-27F	ATACGGGAGCCAACACCAAAAAGTACCGAGGTAACTAAAAAGCATTTGGATGAAAAC-TATAATTACTACAGTGGGTAATAAATGAACGGGTGTTAACAACGCATCCGCCAGGGG-CACGTCCCGCGAAAGGAAGAATGTGAATCTAAGGCCTGACCCATTATCTGCTGAGACAAT-TCATTGTAGAGCAGGTGTGACGGAT
Rt-ms-28R	ATCCGTCACACCTGCTCTCCAACTACTGCGAAAGCTTGTAGTAGCGCAATGCCATCGTAT-GGTAGCGTTCTAGCCTATTTTATGGGGATATTAGATATTGACTTACTCAAGCAACTCCGCT-TTCTGTTGGGTCATCTAAATGGGCCTTTTGTTGCTTCTTTAGGAGCTGAAACAGTGCAT-GTGGTGTTGGCTCCCGTAT
Rt-ms-30F	ATACGGGAGCCAACACCAACACCTGTCGAACGGGCGGTACGTCTCGTCTATTGCCCTAG-GAGTATATACAAGGGCTAAGCAGATTGAATACATTATCAATGAGGGTATAGAGCAG-CAATAATCTTACACACAGAATGTAAATTTGTAATTCAGGGAACGGACGAAAAGCAGAGAC-CATTACGAGAGCAGGTGTGACGGAT
Rt-ms-31R	ATCCGTCACACCTGCTCTAGACCCGCGTGCTACCGTCATACCGGGATCGTCTTTTGAAT-TAATGCTCATTCGTAGAGTGTTTTTAAAGATCGATGGTAACGGAGCGGACTTGTTGAA-CAATAATATGAACAGATCCAAGTCCATAGAAGTTCGCCTGATGGAGTTATTCCAGTTG-CGCCTACTGGTGTTGGCTCCCGTAT

Notes and abbreviations: Rr R: rickettsia; Rt R: typhi; ms: mouse antibody; F: forward-primed, and R; reverse-primed.

the X-axis in Figure 2) were added in 500 µl of PBS in the ten separate tubes and gently mixed again for 30 min at RT. MBs were again collected on a magnetic rack for 3 min. MBs were washed three times for 2 min per wash in 1 ml of PBS and MBs were resuspended in 500 µl of 0.25 µg/ml of streptavidin-horseradish peroxidase (Sav-HRP) in PBS per tube for 15 min at RT with gentle mixing. MBs were again collected using a magnetic rack for 2 min per sample and washed 3 times in 1 ml of PBS per wash as before.

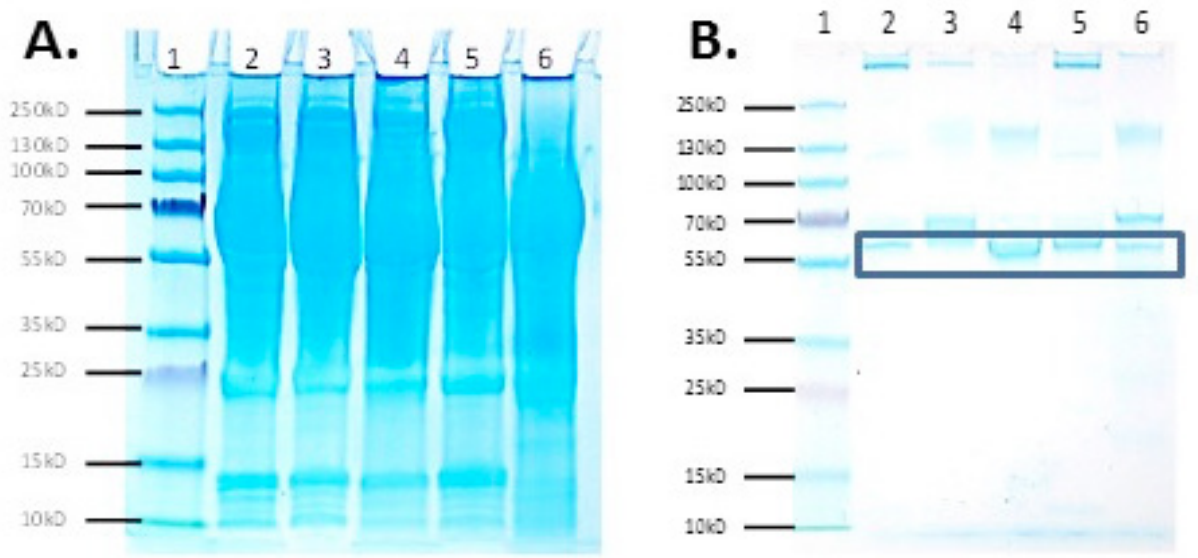

Figure 1: *A. Coomassie blue-stained 4-20% polyacrylamide electrophoresis gel showing the typical appearance of murine antisera developed against R. rickettsii and R. typhi. B. Appearance of the same samples following removal of serum albumins, papain digestion and passage through a Protein A affinity column to remove Fc fragments. The remaing bands should represent Fab fragments of IgG (boxed at ~ 55 kD) or other isotypes (e.g., IgA or IgM) in the 4-20% PAGE gel.*

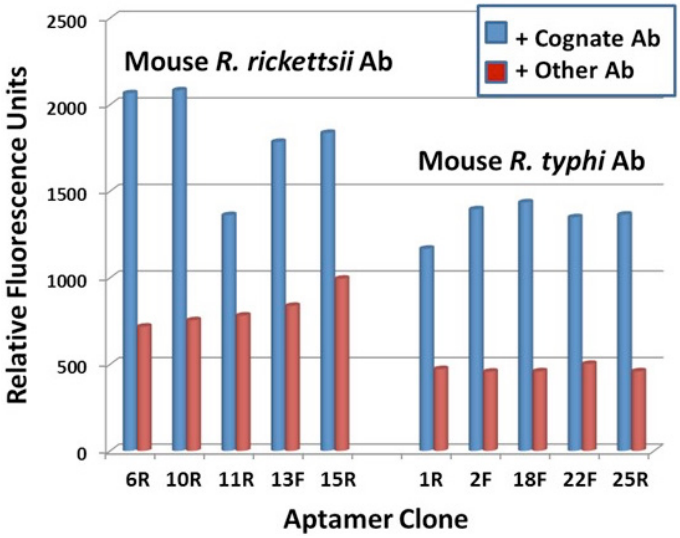

Figure 2: *Results of an Amplex® Ultra Red Protein A-magnetic bead capture plus aptamer-biotin-streptavidin-peroxidase fluorescence assay which immobilized IgG antibodies from 50 µg of murine antiserum against R. rickettsii or R. typhi. The top five aptamer candidates from ELASA screening against each type of antiserum bound with the greatest affinity to antibodies within their cognate antiserum (blue columns) versus antibodies from the other antiserum (red columns). Background fluorescence for deletion controls without aptamers averaged ~ 150 relative fluorescence units (data not shown).*

Amplex® Ultra Red (AUR; 1 mg, Life Technologies Inc.) was stored at -20°C, thawed just prior to use and dissolved in 100 µl of pure DMSO by brief vortex mixing. Stock AUR solution was diluted 1:1,000 in PBS prior to use along with 25 µl of 3% H_2O_2 per ml of diluted AUR. MBs were collected using the magnetic rack and resuspended in 1 ml of diluted AUR solution with 0.075% H_2O_2, vortex mixed for 5 sec and transferred to polystyrene cuvettes (Thermo Fisher Scientific No. 14-955-129) containing an additional 1 ml of diluted AUR plus 0.075% H_2O_2 solution. Fluorescence was assessed at 1 min of development using the green (rhodamine) channel of a Quantifluor™ handheld fluorometer (Promega Corp.) set to its highest sensitivity.

Results and Discussion

Table 1 gives the DNA sequences of the twenty-seven aptamer candidates developed against anti-*R. rickettsii* and anti-*R. typhi* Fab fragments with the top five highest affinity aptamers for each Fab target bolded in the table. Despite extensive study of the table, the author could not discern any full-length or significant partial length DNA sequences common to any of the aptamer candidates. This lack of aptamer sequence consensus or even partial homology could be a reflection of the complexity of the Fab targets having greater than one protein band (Figure 1) and probably multiple hypervariable or other regions to bind on each Fab fragment. More sequencing of the final aptamer library in the future might reveal full-length consensus DNA sequences or common partial sequences.

Figure 1A illustrates the appearance of typical complex raw murine antisera developed against *R. rickettsii* and *R. typhi* after polyacrylamide electrophoresis in a 4-20% gradient gel with Coomassie blue staining. By contrast, Figure 1B illustrates the appearance of the same antisera following removal of serum albumins, papain digestion and processing through an immobilized Protein A column to remove Fc tails and yield primarily Fab fragments in

the eluate. While not entirely pure samples, each lane reveals at least one band in the vicinity of 55 kD which probably corresponds to an Fab fragment of IgG (the intended target for aptamer development). Other bands may represent fragments from other isotypes (e.g., IgA, IgM, *etc.*) which co-elute with the Fab fragments of IgG. Such additional fragments may still contain useful hypervariable region targets

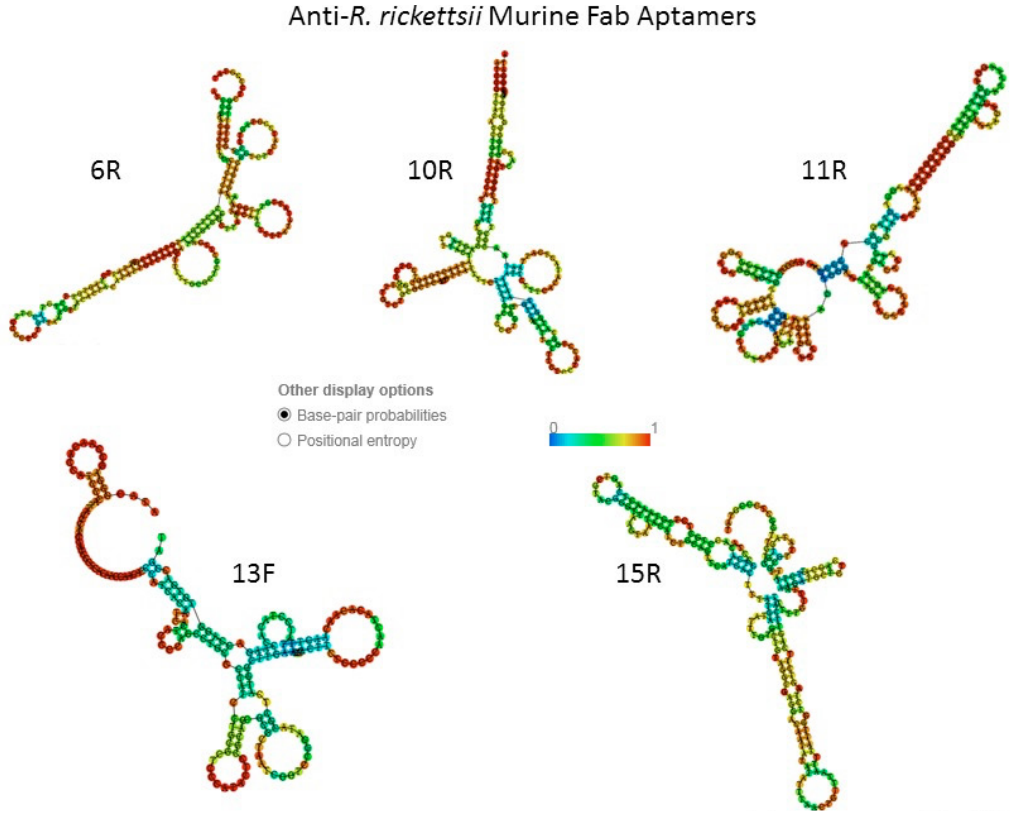

Figure 3: *Secondary stem-loop structures of the top five aptamer candidates against R. rickettsii antiserum as determined by Vienna RNA webserver using DNA parameters at RT. The inset shows the color code used to identify the probability of each base or base pair's placement in the secondary structure.*

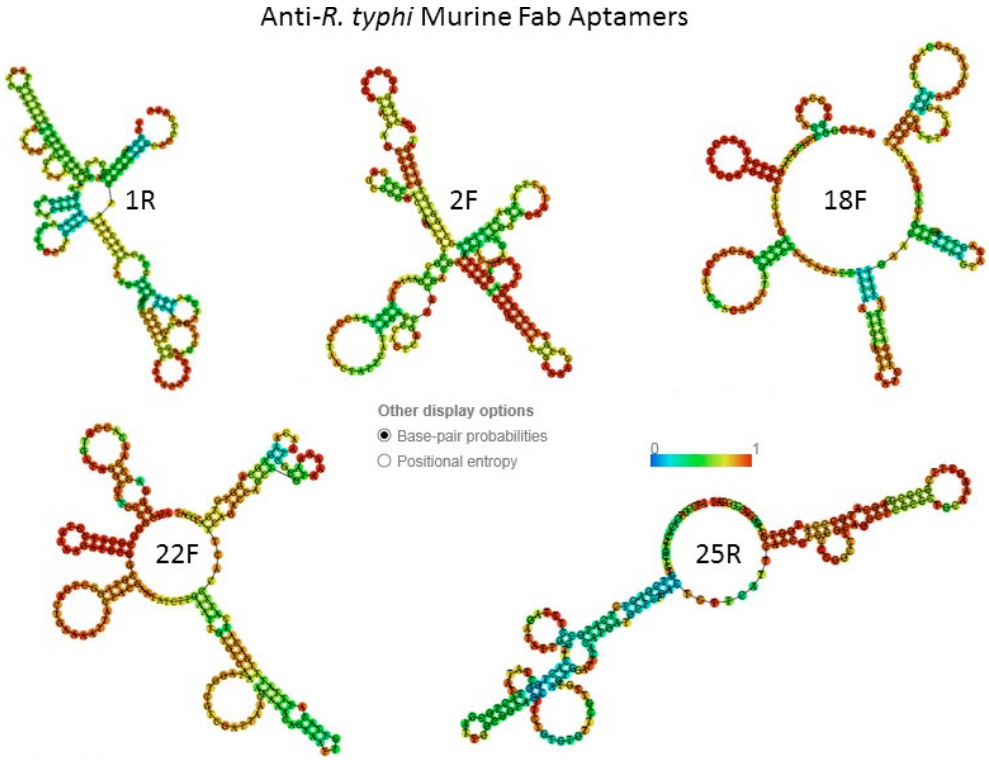

Figure 4: *Secondary stem-loop structures of the top five aptamer candidates against R. typhi Fab fragments as determined by Vienna RNA webserver using DNA parameters at RT. The inset shows the color code used to identify the probability of each base or base pair's placement in the secondary structure.*

for diagnosis of rickettsial exposure. The fluorescence cross-reactivity assay results in Figure 2 demonstrate a strong preference of the top five highest affinity aptamers from ELASA screening (data not shown) for their cognate antibody (Fab) targets. In other words, when the *R. rickettsii* Fab aptamers were tested with *R. rickettsii* antisera, a much stronger (blue column) fluorescence response was obtained (sometimes greater than two-fold fluorescence) versus when the other (*R. typhi*) antisera were tested (red columns) in Figure 2 (left side). A similar strong preference was obtained on the right half of Figure 2 when aptamers developed against *R. typhi* Fab fragments were tested against *R. typhi* antiserum (blue columns) versus the other (*R. rickettsii*) antisera (red columns), thus suggesting relative specificity of each aptamer for its cognate Fab fragment.

Figures 3 and 4 together illustrate the secondary stem-loop structures of the top five anti-*R. rickettsii* and anti-*R. typhi* Fab fragment aptamers. Vienna RNA software with DNA parameters were used to also illustrate the probability of the location of each base or base pair in the structures. As Figures 3 and 4 show a probability of 1 or 100% certainty of a given base or base pair's location within the aptamer secondary structure is coded red while relative uncertainty of a base or base pair's location is illustrated in blue with varying degrees of base location probability coded as different colors shown in the insets. This color coding underscores the flexible and potentially malleable nature of aptamer conformations and the reader should be mindful that the secondary and tertiary structures shown are the most probable structures (*i.e.*, lowest ΔG conformations), but are subject to change due to temperature or induced-fit with their targets. The most important point, however, to make about Figures 3 and 4 is that they do not seem to converge on any particular shapes whether in whole or in part. Interestingly, when studied as 3-dimensional structures

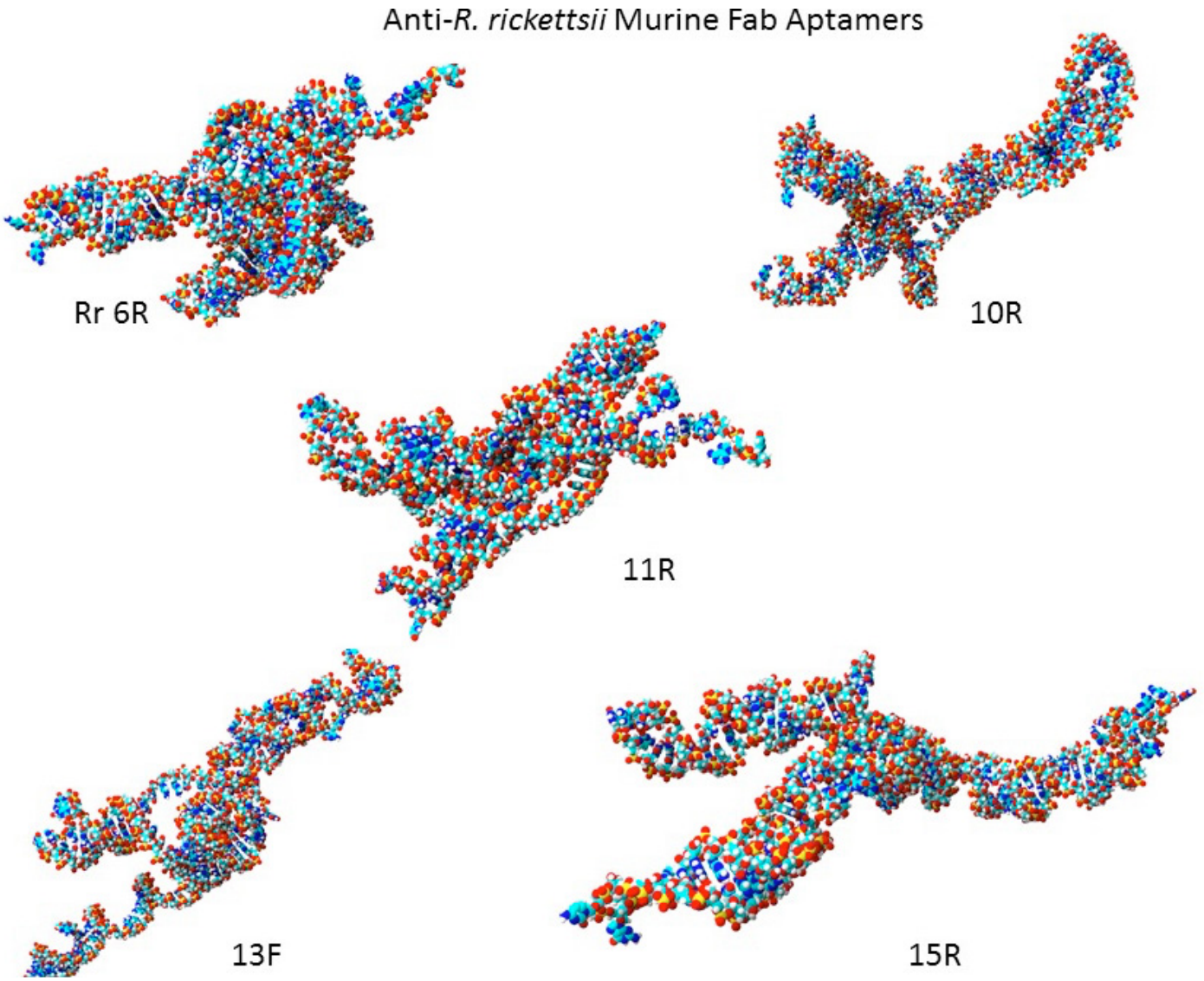

Figure 5: *Three-dimensional representations of the top five aptamer candidates against R. rickettsii Fab fragments as determined by YASARA. Note the tendency toward star-like structures with multiple spokes emanating from a central core in this family of aptamers.*

generated by YASARA, the anti-*R. rickettsii* Fab (Figure 5) and anti-*R. typhi* Fab (Figure 6) aptamer "families" of five aptamers each appear to share some overall surface topological features or motifs. The groups of five aptamers against each species of Fab fragments appear to cluster into similar shapes which may reflect the similar shapes of their cognate targets (different Fab fragments). For example, the 3-D structures in Figure 5 appear to have multiple spokes emanating from a central hub or nexus, while the structures in Figure 6 appear to show a more dimeric "subunit" nature in three dimensions.

A total of 27 aptamer sequences that probably bind the Fab regions of various murine anti-*Rickettsia* antibodies were developed and may be useful to detect exposure to *R rickettsii* or *R. typhi* for diagnostic assays. Based on the data reported here, development of aptamers against Fab regions appears possible, but is complicated by contaminating protein bands which are not in the expected 50-55 kD range and may represent fragments of other antibody isotypes (*e.g.*, IgA or IgM) which co-purify with the Fab fragments of IgG. Ideally, one should start the anti-idiotypic SELEX process with knowledge of the hypervariable region amino acid sequences and use highly purified peptides as hypervariable targets generated from that knowledge. Unfortunately, such knowledge is often lacking, especially for targets such as rickettsial antibodies.

No consensus or homology was seen in full or partial DNA sequences of the anti-murine Fab aptamers (primary structures from Table 1) or their secondary stem-loop structures (Figures 3 and 4), but 3-D models of these same aptamers appear to cluster into two more distinct families (Figure 5 versus Figure 6) which share some topological motifs within each family such as the star-like multiple spokes emanating from a central core area (Figure 5) or a dimeric "subunit" motif (Figure 6). Such 3-D similarities of

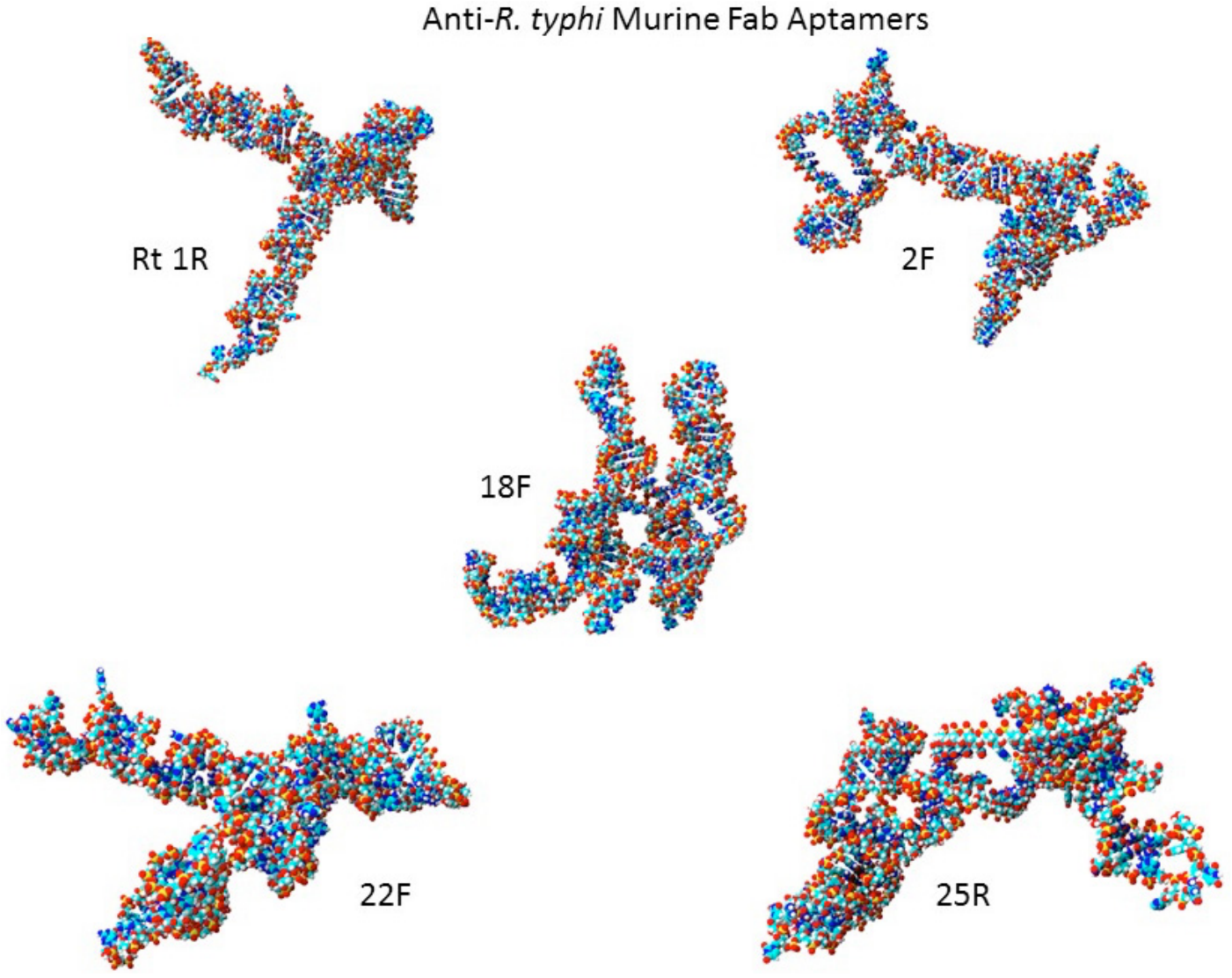

Figure 6: *Three-dimensional representations of the top five aptamer candidates against R. typhi Fab fragments as determined by YASARA. Note the tendency toward dimeric "subunit" appearance in this family of aptamers.*

otherwise seemingly divergent aptamers could be a reflection of shape complementarity selected by the SELEX process to bind common shapes in the 3-D conformations of the respective Fab fragments.

Conclusions

While the current work is only preliminary, it lays the methodologic foundation for future anti-idiotypic aptamer development and shows some promise especially at the 3-D level for discovery of useful anti-idiotypic aptamer antigens. And given the desire for more accurate, rapid and reliable serodiagnosis of rickettsial diseases and improved rickettsial vaccines, future research and development appears justified to enable these improvements. The best course forward appears to be amino acid sequencing of anti-rickettsial antibody hypervariable regions, followed by SELEX and primary, secondary and tertiary structural analysis as described here. Finally, 3-D molecular docking could be enabled, if the hypervariable region amino acid sequences are obtained and docked with candidate anti-idiotypic DNA or RNA aptamers using YASARA or other such molecular modeling programs to determine which anti-idiotypic aptamer candidates constitute the best antigens for serodiagnosis and vaccine development.

Acknowledgments

Funding was provided by U.S. Navy Contract No. Contract No. N32398-14-P-0340. The author is grateful to Dr. C.C. Chao or the Naval Medical Research Center (NMRC) in Silver Spring, MD for providing the anti-rickettsial antisera. The author also thanks Ms. Alicia Richarte for technical assistance in Fab purification and aptamer development, ELASA, and fluorescence assay screening.

Conflicts of Interest

The author declares that no conflict of interest exists.

Author Contributions

JGB conceived of and directed the project. JGB also analyzed all data and prepared the manuscript.

References

Bruno, J.G. 2017. Do it yourself 3-dimensional aptamer-ligand molecular modeling. J. Bionanosci. In Press. https://doi.org/10.1166/jbns.2017.1437

Bruno, J.G., Chao, C.C., Zhang, Z., Ching, W.M., Phillips, T., Edge, A., Sivils, J.C. 2016. Preliminary development of an enzyme-linked fluorescent DNA aptamer-magnetic bead sandwich assay for sensitive detection of *Rickettsia* cells. Aptamers Synth. Antibodies. 2: 1-12.

Chao,C.C.,Zhang,Z.,Wang,H.,Alkhalil,A.,Ching, W.M. 2008. Serological reactivity and biochemical characterization of methylated and unmethylated forms of a recombinant protein fragment derived from outer membrane protein B or *Rickettsia typhi*. Clin. Vaccine Immunol. 15: 684-690. https://doi.org/10.1128/CVI.00281-07

Hamm, J., Huber, J., Lührmann R. 1997. Anti-idiotype RNA selected with an anti-nuclear export signal antibody is actively transported in oocytes and inhibits Rev- and cap-dependent RNA export. Proc. Natl. Acad. Sci. USA 94: 12839-12844. https://doi.org/10.1073/pnas.94.24.12839

Hu, P., Liu, Z., Tian, R., Ren, H., Wang, X., Lin, C., Gong, S., Meng, X., Wang, G., Zhou, Y., Lu, S. 2013. Selection and identification of a DNA aptamer that mimics saxitoxin in antibody binding. J. Ag. Food Chem. 61, 3533-3541. https://doi.org/10.1021/jf400880r

Kovacova, E., Kazar, J. 2000. Rickettsial diseases and their serological diagnosis. Clin. Lab. 46: 239-245.

Kowalczewska, M., Vellaiswamy, M., Nappez, C., Vincentelli, R., La Scola B., Raoult, D. 2012. Protein candidates for the serodiagnosis of rickettsioses. FEMS Immunol. Med. Microbiol. 64: 130-133. https://doi.org/10.1111/j.1574-695X.2011.00912.x

La Scola, B., Raoult, D. 1997. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. J. Clin. Microbiol. 35: 2715-2727.

Mahajan, S.K. 2012. Rickettsial diseases. J. Assoc. Phys. India. 60, 37-44.

Qin, L.H., Liu, Z.H., Yang, H., Cai, J.L., Bai, W.J., Wang, J., Liu, J.M., Hu, Z.Y. 2014. Dynamic evolution and immunoreactivity of aptamers binding to polyclonal antibodies against MPT64 antigen of *Mycobacterium tuberculo*sis. Eur. J. Clin. Microbiol. Infect. Dis. 33, 1199-1209. https://doi.org/10.1007/s10096-014-2056-4