Research Article

Preliminary Development of an Enzyme-Linked Fluorescent DNA Aptamer-Magnetic Bead Sandwich Assay for Sensitive Detection of *Rickettsia* Cells

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Abstract | DNA aptamers were developed against *Rickettsia typhi* (*R. typhi*) whole cells and individual candidate aptamer sequences were ranked according to affinity by an ELISA-like microplate-screening assay (ELASA). Top three candidate aptamers were then paired in a matrix of all possible capture and reporter aptamer combinations and tested in a fluorescent peroxidase-linked Amplex® UltraRed (AUR; a resazurin-like substrate) version of a rapid (< 1 h) aptamer magnetic bead sandwich assay. The optimal sandwich aptamer combination utilized the same aptamer sequence (designated Rt-18R) for both capture and reporter functions while producing a signal to noise ratio of > 4.0 for detection of ~ 1,000, *R. typhi* cells. Titration experiments conducted in buffer revealed a limit of detection between 100 and 1,000 *R. typhi* cells per ml. The Rt-18R aptamer detected only one band at ~ 84 kD in *R. typhi* lysates on aptamer Western blots. While the homogeneous Rt-18R fluorescent sandwich aptamer-magnetic bead assay did not cross-react with comparable concentrations of *E. coli* or L929 murine host cells, the assay did cross-react strongly with members of the spotted fever and scrub typhus groups, making the assay specific only to the Rickettsiaceae family level (*Rickettsia* and *Orientia)*. When conducted in ≤ 100% human serum or a tick homogenate, the fluorescent aptamer assay maintained sensitive detection of *Rickettsia typhi* cells.

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Introduction

Although rarely fatal, *Rickettsia* and *Orientia* species from the spotted fever (SFG), murine typhus, and scrub typhus groups present a significant threat to U.S. military personnel in the field who are frequently subjected to tick and other arthropod bites. Rapid and correct diagnoses of spotted fevers and typhus are critical to selection of the proper antibiotics and treatment regimens (Kovacova and Kazar, 2000; La Scola and Raoult, 1997). However, rickettsial infections can present early signs and symptoms quite similar to a variety of other diseases which require different treatments. Traditional diagnosis of rickettsial

infections relies on serological tests which sometimes target antibodies developed by the patient (Kovacova and Kazar, 2000; La Scola and Raoult, 1997) instead of detecting low levels of the infectious agents themselves in body fluid samples early in disease progression prior to seroconversion when medical intervention could most help. Military health officials and researchers also wish to rapidly map the geographical distribution of *Rickettsia* and other pathogenic microbes in arthropod populations while in the field. As with many cryptic infectious diseases, nucleic acid detection using PCR or isothermal amplification has been employed, but DNA polymerase-based reactions are susceptible to inhibition from heme, collagen or other molecules in biological samples (Abolmaaty et al., 2007; Kim et al., 2000) and in the case of PCR, a typically heavy and bulky thermal cycler is required.

Therefore, we sought to develop a rapid, specific, and sensitive assay for detection and discrimination of at least one of the common *Rickettsia* species, which could be conducted easily in the field using a portable or hand-held fluorescence reader (Bruno et al., 2009a, 2015). Our experience with DNA aptamers for whole cell bacterial detection (Bruno et al., 2009a, 2010, 2012 and 2015) led us to hypothesize that high affinity and highly specific aptamers (Bruno et al., 2011) could be selected against whole rickettsial cells. Here we report the results of an initial *R. typhi* aptamer and assay development effort within our overall *Rickettsia* assay development program. We ultimately developed a system based on our previous ultrasensitive capture aptamer-magnetic bead (MB) and quantum dot (QD)-labeled reporter sandwich assay for *Campylobacter jejuni* (Bruno et al. 2009a) and similar aptamer-magnetic bead sandwich assays developed by other laboratories (Joshi et al., 2009). In the seven years since our *Campylobacter* assay development, and despite all the positive attributes of QDs, we have encountered some limitations of QDs such as loss of fluorescence intensity, "blinking" and "blue shift" when using some QDs in some biological matrices (Dwarakanath et al., 2004; Zhang et al., 2006) or when employing DNA conjugated to the QD surface (Riegler et al., 2008). Therefore, we decided to employ an enzyme (peroxidase)-linked version of our aptamer-MB sandwich assay similar to fluorescent enzyme-linked immunomagnetic assays reported by Bruno et al. (2015) and others (Wei et al. 2012; Yolken and Stopa, 1979) using a substrate such as Amplex® UltraRed (AUR; Molecular Probes/Life Technologies Corp.). A major advantage of AUR as a substrate is that it emits maximally at 581-585 nm, near the red region of the spectrum to avoid much of the blue-green autofluorescence background of biological samples. The use of an enzyme-linked assay system also produces bulk solution fluorescence to avoid potential self-quenching of fluorophores at high bacterial concentrations when bacteria are proximal to one another (i.e., numerous bacteria congregated on aptamer-coated magnetic beads) or absorption of fluorescent photons by magnetic beads and bacteria which can mask fluorophore emissions at very low bacterial concentrations and hinder sensitivity. The fluorescence data reported herein was acquired with a commercially available Quantifluor™ (Promega Corp.) handheld fluorometer (Bruno et al., 2009) which serves as a model of portable detection for military field use or potential civilian point of care applications. In recent years, our group has also developed a more sophisticated handheld fluorometer having an on board computer and graphical user interface (Bruno et al., 2015) for assessment of aptamer-MB fluorogenic assays in the field.

Materials and Methods

Rickettsia samples, DNA template and primers

All Rickettsia samples were obtained from Dr. Chien-Chung Chao at the Naval Medical Research Center (NMRC) in Silver Spring, MD or from Dr. Ricardo Carrion of the Texas Biomedical Research Institute (TBRI), in San Antonio, TX. *Rickettsia* or *Orientia* species were cultured in murine L929 cell line host cells, purified on density gradients and washed in buffer prior to freezing. *Rickettsia* cell stocks were kept frozen at -80°C until thawing for aptamer or assay development and testing. In some aptamer "Western" blot experiments, protein extracts of *Rickettsia* were used. These extracts were made by vortex mixing and storage of rickettsial cells in 1.5M $MgCl_2$ at 4°C overnight.

All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The degenerate template sequence was: 5'-ATCCGTCACACCTGCTCT-N_{36}-TGGTGTTGGCTCCCGTAT-3', where N_{36} represents the randomized 36-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. The random library was reconstituted in 500 µl of sterile nuclease-free water.

Whole cell-SELEX DNA Aptamer Development, Cloning and Sequencing

To begin the process of whole rickettsial cell SELEX (Systematic Evolution of Ligands by EXponential enrichment) aptamer development, 160 µg of template (DNA library) in sterile nuclease-free deionized water was heated at 95°C for 5 min to ensure interaction of rickettsial cells with the 72 base single-stranded DNA library free of concatamers. The hot DNA was added to 10 µg of whole rickettsial cells (~10^6 cells) with 200 µl of 2XBB (1 M NaCl, 20 mM Tris-HCl and 2 mM $MgCl_2$ in sterile nuclease-free deionized water at a final pH of 7.5-7.6). The cell suspension was gently mixed for 1 h at ~25°C. The suspension was then centrifuged in a 150 kD MWCO spin column (Millipore #UFC7PCR50) at 2,000 x G (5,500 rpm) for 20 min in an Eppendorf MiniSpin® microentrifuge. Next, 500 µl of 1XBB was added to the spin column which was spun again at 2,000 X G for 20 min to wash the rickettsial cells. Using a fresh collection tube, the 150 kD MWCO spin column was inverted and 100 µl of nuclease-free water was added to back flush the column by spinning at 7,000 x G for 3 min. Finally, 100 µl of sterile deionized nuclease-free water was added to the column and it was centrifuged again at 7,000 x G for 3 min to remove any remaining protein or DNA that may be adhering to the filter. The eluate, including first round aptamers, was heated at 95°C for 5 min to release them from bound proteins or other cellular materials. The hot eluate was then added to a Pall Nanosep 30K spin column (#OD030C34) and centrifuged at 12,000 x G for 3 min. The absorbance of the filtrate was then assessed at 260 nm using a UV spectrophotometer to estimate the DNA concentration.

Fifty ng of the filtrate in 150 µl of sterile nuclease-free water was heated at 95°C for 5 min. The hot supernatant was collected and 5 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 94°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. This constituted the first of 10 rounds of whole cell-SELEX.

PCR amplicons were verified to be 72 bp in length after each round of SELEX by electrophoresis in 2% TAE (Tris-Acetate EDTA) agarose gels with ethidium bromide staining. If more than one band emerged, the 72 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 72 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. For the negative selection rounds, following the 1 h mixing at 25°C, 2.5 µg of *R. bellii*, *R. conorii*, *R. parkeri*, and *R. rickettsii* whole cell samples were added and the sample was centrifuged in a 30K (30 kD molecular weight cut off) Pall Corp. spin column without heating or washing and the filtrate was used as the template for PCR amplification. 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 rickettsial cells to ensure that a single-stranded DNA pool was interacting with the cells.

Following round 10, 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. Sequence names are coded throughout, indicating that particular sequences derived from selection against *R. typhi* whole cells along with F for forward-primed and R for reverse-primed designations (Table 1).

ELASA screening and ranking of candidate aptamers

To evaluate and rank affinity for each of the candidate aptamers, an ELISA-like enzyme-linked aptamer sorbent microplate assay (ELASA) was conducted essentially as previously reported (Bruno et al., 2009a, 2011 and 2015) by first immobilizing ~ 10^6 of whole *R. typhi* cells 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 1XBB 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 1XBB as before. Ethanolamine was used instead of conventional protein blocking agents because it is so small and less likely to hinder aptamer binding on or around rickettsial cells or their components. In previous experiments, blocking agents such as bovine serum albumin (BSA) have led to lower signal to noise ratios in ELASA experiments (data not shown), perhaps because aptamers can nonspecifically adhere to BSA and other proteins as well.

A total of 40 different sequenced 5'-biotinylated *R. typhi* whole cell aptamer candidates from the final selected pool (Table 1) were synthesized by Integrated DNA Technologies were rehydrated in 100 µl of 1XBB 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 room temperature (RT) with gentle mixing. The plates were decanted and washed 3 times in 200 µl of 1XBB 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 1XBB 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 1XBB 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 1.5 at 405 nm using a Thermo Electron MultiSkan™ microplate reader (Thermo Fisher Scientific; Waltham, MA).

Aptamer secondary structural analyses

Secondary stem-loop structures were determined using UNAFold software on Integrated DNA Technologies, Inc.'s website (http://www.idtdna.com/Unafold/). In particular, DNA parameters at 25°C, 0 mM $MgCl_2$, and 137 mM NaCl were used.

Aptamer "Western" blotting

Whole cell *Rickettsia* samples (22.5 µl) were added to 7.5 µl or 4X SDS-PAGE loading buffer containing beta-mercaptoethanol and heated for 5 minutes at 95°C. Thirty µL samples were loaded into a Tris-MOPS-SDS 4-20% gradient polyacrylamide gel and run at 90 mA for 1 h followed by 120 mA until the dye front reached the bottom of the gel. The gel was overlaid with a pre-wetted PVDF membrane in Tris transfer buffer containing 20% methanol and bands were transferred at 4 mA overnight in a cold room. The membrane was blocked with 10 ml of SuperBlock® (Thermo Scientific, No. 37515) for 1 h with gentle mixing followed by addition of 5 µl of 100 µM Rt-18R DNA aptamer -5'-biotin in SuperBlock® with an additional 1 h or gentle mixing at RT. The membrane was washed five times for 5 min per wash in 10 ml of PBS plus 0.1% Tween 20 (PBST). Next, a 1:10,0000 dilution of Streptavidin-Alkaline Phosphatase (Sav-AP, ~ 2 mg/ml stock) in 10 ml of PBST was added and the membrane was incubated for 1 h at RT with gentle mixing. The membrane was washed an additional five times for 5 min per wash in 10 ml of PBST and 3 times for 5 min per wash in 10 ml of PBS. The membrane was transferred to 10 ml of AP substrate (Immunostar-AP®, BioRad Laboratories) and incubated 5 min at RT. The wetted membrane was placed in an X-ray film cassette and developed for 10 min.

Fluorescent enzyme-linked aptamer-magnetic bead sandwich assay procedure

Fresh or lyophilized capture and reporter aptamer reagents containing 5% trehalose as an excipient or bulking agent (used to weigh down aptamer assay components during lyophilization and to enable stable assays with longer shelf life) were used to obtain the reported data. Twenty µl of 5'-biotinylated Rt-18R capture aptamer-streptavidin-Dynal/Life Technologies M280 (2.8 micron) magnetic beads (~ 4 x 10^7 aptamer-MBs) were added to 500 µl of phosphate buffered saline (PBS), ≤ 100% human serum (BioWhittaker, Lonza Corp), or local dog tick homogenate (5 medium-sized ticks ground in 2.5 ml of PBS in a GentleMACs™ tissue homogenizer, Miltenyi, GmBH, Germany) containing various concentrations of rickettsial cells in 1 ml of PBS except for blanks devoid of rickettsial cells (PBS only). Tubes were mixed gently on an orbital shaker or rotating mixer for 10 min at RT. A Dynal MPC-S® or comparable magnetic rack to collect MBs in microcentrifuge tubes for 1 min. The 500 µl supernatant without MBs was carefully aspirated and discarded in 5% Bleach solution. Five hundred picomoles of 5'-biotinylated reporter aptamer (Rt-18R) in PBS was added to each

Table 1: *R. typhi Whole Cell Aptamer DNA Sequences*

Rt -1/9/17/24F ATACGGGAGCCAACACCAGTCCGTTATGACATGTCCGGACCCGTACGCGTGTCAAGAGCAGGTGTGACGGAT
Rt -1/9/17/24R ATCCGTCACACCTGCTCTTGACACGCGTACGGGTCCGGACATGTCATAACGGACTGGTGTTGGCTCCCGTAT
Rt - 2F (58) ATACGGGAGCCAACACCACCGCAACACACTATCCACGACCAGAGCAGGTGTGACGGAT
Rt - 2R (58) ATCCGTCACACCTGCTCTGGTCGTGGATAGTGTGTTGCGGTGGTGTTGGCTCCCGTAT
Rt - 3F ATACGGGAGCCAACACCACCGCCCGCCTCCTGGCGCCACACCCCCGCCGCAGCGAGAGCAGGTGTGACGGAT
Rt - 3R ATCCGTCACACCTGCTCTCGCTGCGGC**GGGGG**TGTGGCGCCAGGAGGCGGGCGGTGGTGTTGGCTCCCGTAT
Rt - 4F ATACGGGAGCCAACACCAAATACAGTGCCTAATAGGTATGAAAATTATAGTAATAGAGCAGGTGTGACGGAT
Rt - 4R ATCCGTCACACCTGCTCTATTACTATAATTTTCATACCTATTAGGCACTGTATTTGGTGTTGGCTCCCGTAT
Rt – 5/16F ATACGGGAGCCAACACCACACTACCGTCCCACCCCCTCCCAGCTCCTCCGGCCGAGAGCAGGTGTGACGGAT
Rt – 5/16R ATCCGTCACACCTGCTCTCGGCCGGAGGAGCTGGGA**GGGGG**TGGGACGGTAGTGTGGTGTTGGCTCCCGTAT
Rt - 6F ATACGGGAGCCAACACCACTAGTTATTTCATA**GGGG**AAATTAACAAATTTTGACAGAGCAGGTGTGACGGAT
Rt - 6R ATCCGTCACACCTGCTCTGTCAAAATTTGTTAATTTCCCCTATGAAATAACTAGTGGTGTTGGCTCCCGTAT
Rt - 7aF ATACGGGAGCCAACACCACGGACAATCTGGTAGTAGTAAACAATATATAAGTATAGAGCAGGTGTGACGGAT
Rt - 7aR ATCCGTCACACCTGCTCTATACTTATATATTGTTTACTACTACCAGATTGTCCGTGGTGTTGGCTCCCGTAT
Rt - 7bF ATACGGGAGCCAACACCAGTACTCGCTGTGGCAAAAGCAGCATTTCGTCTATCTAGAGCAGGTGTGACGGAT
Rt – 7bR ATCCGTCACACCTGCTCTAGATAGACGAAATGCTGCTTTTGCCACAGCGAGTACTGGTGTTGGCTCCCGTAT
Rt - 8F ATACGGGAGCCAACACCAAAGCTCCCCCCCTCATCCCTGGCATCTCCGCTAACCAGAGCAGGTGTGACGGAT
Rt - 8R ATCCGTCACACCTGCTCTGGTTAGCGGAGATGCCAGGGATGA**GGGGGGG**AGCTTTGGTGTTGGCTCCCGTAT
Rt - 10F ATACGGGAGCCAACACCATTAACGTCGCAATAGCGCTCATCTAACGTCAAGGGCAGAGCAGGTGTGACGGAT
Rt - 10R ATCCGTCACACCTGCTCTGCCCTTGACGTTAGATGAGCGCTATTGCGACGTTAATGGTGTTGGCTCCCGTAT
Rt - 11F ATACGGGAGCCAACACCAAAGTGTCGTAATTTAAGATGCATACGCATGCCGTTAAGAGCAGGTGTGACGGAT
Rt - 11R ATCCGTCACACCTGCTCTTAACGGCATGCGTATGCATCTTAAATTACGACACTTTGGTGTTGGCTCCCGTAT
Rt - 12F ATACGGGAGCCAACACCAGTGTCTTATGAATGTAGATGAGCTCAGATCGGAATTAGAGCAGGTGTGACGGAT
Rt - 12R ATCCGTCACACCTGCTCTAATTCCGATCTGAGCTCATCTACATTCATAAGACACTGGTGTTGGCTCCCGTAT
Rt-13F ATACGGGAGCCAACACCACACATCACATACCTTCAAGAGCGATGACGGCCCTTTATAGGCAGAGCAGGTGTGACGGAT
Rt-13R ATCCGTCACACCTGCTCTGCCTATAAAGGGCCGTCATCGCTCTTGAAGGTATGTGATGTGTGGTGTTGGCTCCCGTAT
Rt - 18F ATACGGGAGCCAACACCAATGTGGTGGATAGCAAACCCCCGACGATTGAGGATTAGAGCAGGTGTGACGGAT
Rt - 18R ATCCGTCACACCTGCTCTAATCCTCAATCGTC**GGGGG**TTTGCTATCCACCACATTGGTGTTGGCTCCCGTAT
Rt - 19F ATACGGGAGCCAACACCAGTTGAAGCTAGTACTGCGGAAGCATAGTCCATAAGTAGAGCAGGTGTGACGGAT
Rt - 19R ATCCGTCACACCTGCTCTACTTATGGACTATGCTTCCGCAGTACTAGCTTCAACTGGTGTTGGCTCCCGTAT
Rt - 20F ATACGGGAGCCAACACCAGCGAAATGAAGGTATGTTTTTGAATAATAATGTGGCAGAGCAGGTGTGACGGAT
Rt - 20R ATCCGTCACACCTGCTCTGCCACATTATTATTCAAAAACATACCTTCATTTCGCTGGTGTTGGCTCCCGTAT
Rt - 21F ATACGGGAGCCAACACCAAAATAGATCAAAACCGCATGCTGGAGCAGTTTTAGCAAGAGCAGGTGTGACGGAT
Rt - 21R ATCCGTCACACCTGCTCTTGCTAAAACTGCTCCAGCATGCGGTTTTGATCTATTTTGGTGTTGGCTCCCGTAT
Rt - 22F ATACGGGAGCCAACACCAATAATTGCTCGTTGATACTTATATAAAGTACAGGCAAGAGCAGGTGTGACGGAT
Rt - 22R ATCCGTCACACCTGCTCTTGCCTGTACTTTATATAAGTATCAACGAGCAATTATTGGTGTTGGCTCCCGTAT
Rt – 23F ATACGGGAGCCAACACCATCCAATGAGGCCATGGACCGGTAAACTCGGACGCGCAGAGCAGGTGTGACGGAT
Rt – 23R ATCCGTCACACCTGCTCTGCGCGTCCGAGTTTACCGGTCCATGGCCTCATTGGATGGTGTTGGCTCCCGTAT
Rt - 25F ATACGGGAGCCAACACCAAGACGATAAGAATAATATCGAAAATATATGTTTTCAGAGCAGGTGTGACGGAT
Rt - 25R ATCCGTCACACCTGCTCTGAAAACATATATTTTCGATATTATTCTTATCGTCTTGGTGTTGGCTCCCGTAT

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tube and tubes were gently mixed again for 10 min at RT. MBs were again collected on the magnetic rack for 1 min. MBs were washed 3 times for 2 min per wash in 1 ml of PBS and resuspended by gentle pipetting 3 times with magnetic collection for 1 min between each wash. The supernatant was removed and the MBs with aptamer-captured rickettsial cells were resupended in 500 µl of 0.25 µg/ml of streptavidin-horse radish peroxidase (Sav-HRP) in PBS per sample for 10 min at RT with gentle mixing. MBs were again collected using the magnetic rack for 1 min per sample and washed 3 times in PBS as before. Amplex® UltraRed (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 1:1,000 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 within the first 30 sec of development unless otherwise noted in the figure legends using the green (rhodamine) channel of a Quantifluor™ handheld fluorometer (Promega Corp.) set to its highest sensitivity.

Results

Figure 1 (left) illustrates the results of an aptamer – based "Western" blot of protein extracts from several species of *Rickettsia* (*R. bellii*, *R. conorii*, *R. parkeri*, *R. rickettsii*, and *R. typhi*) as well as *R. typhi* whole cell lysate at approximately equal weights per lane. The blot revealed that the Rt-18R aptamer strongly reacted with a target molecule (presumably a protein, although other surface molecules such as polysaccharides are possible (Silverman et al., 1978) having an estimated weight of ~ 84 kD, but only in the boiled whole cell sample (far right lane). Figure 1 (right side) also illustrates the lowest energy secondary structure of the Rt-18R aptamer, which is unremarkable except for its run of 5 guanines, because segments of 4-7 guanines are common among the highest affinity candidate aptamers (bolded and underlined in Table 1) as ranked by ELASA (Table 2).

A checkerboard matrix of the top 3 ranked aptamers was conducted as shown in Figure 2 (top) which revealed a typically > 4.0 signal to noise ratio (SNR) for sandwich combination number 5 (Figure 2 bottom graphic). Sandwich combination 5 represented the pairing of the Rt-18R with itself in both the capture and reporter roles. While other sandwich combinations yielded quite good SNRs, Rt-18 paired with itself proved to be consistently the best sandwich assay combination across numerous experiments (data not shown).

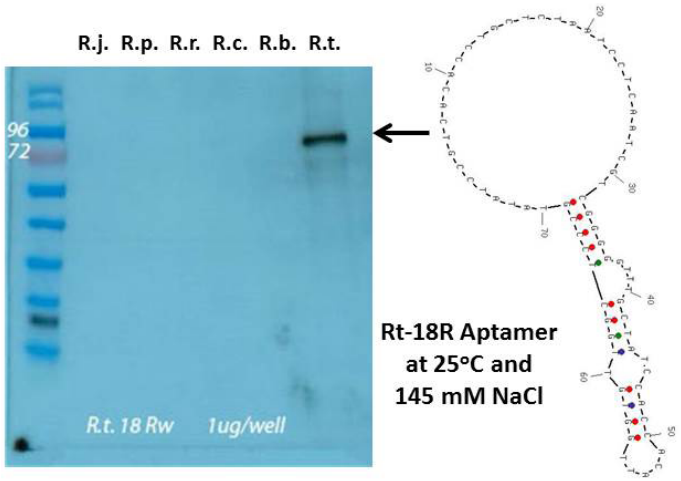

Figure 1: *Left panel – aptamer Western blot using the Rt-18R aptamer against protein extracts and R. typhi whole cell lysate all at ~ 1 µg/well. From left to right; molecular weight markers, protein extracts from R. typhi, R. parkeri, R. rickettsii, R. conorii, R. bellii, and an R. typhi whole cell lysate. Right panel – Secondary stem-loop structure of the Rt-18R aptamer determined by energy minimization using UNAFold software with 25°C, 137 mM NaCl, and DNA parameters*

The titration data presented in Figure 3, while not entirely consistent between the two trials, illustrate the same increasing trend in fluorescence as a function of *R. typhi* cell concentration and suggest a sensitive limit of detection (LOD) between 100 and 1,000 cells per ml. The prototype sandwich assay also appears to have a good dynamic range in which it responds to 6 logs of cell concentration from 10^2 to 10^8 cells per ml.

Specificity was investigated using a variety of *Rickettsia* species from the typhus and spotted fever groups and *Orientia* from the scrub typhus group as well as unrelated *E. coli* and L929 host cells at various con centrations. The cross-reactivity results of three separate trials are presented in Figure 4 along with positive (Sav-HRP plus AUR only) and negative (PBS blank) controls. While the homogeneous Rt-18R sandwich assay responded even more strongly to *R. parkeri* and *Orientia* in some cases, it appears from the data that

Table 2: *ELASA Rankings of R. typhi aptamer candidates*

Candidate Aptamer	Trial 1	Trial 2	Average
Rt - 5/16F	1.582	1.477	1.530
Rt - 6R	1.556	1.448	1.502
Rt - 3R	1.451	1.546	1.499
Rt - 18R	1.587	1.312	1.450
Rt - 6F	1.479	1.399	1.439
Rt - 21R (73)	1.496	1.320	1.408
Rt - 4F	1.444	1.327	1.386
Rt - 22R	1.376	1.295	1.336
Rt - 25F (71)	1.400	1.221	1.311
Rt - 20F	1.343	1.249	1.296
Rt - 3F	1.417	1.086	1.252
Rt - 22F	1.287	1.216	1.252
Rt - 7bR	1.340	1.161	1.238
Rt - 8R	1.315	1.146	1.231
Rt - 7aF	1.405	1.004	1.205
Rt - 12R	1.256	1.144	1.200
Rt -1/9/17/24F	1.251	1.144	1.198
Rt - 11R	1.244	1.143	1.194
Rt - 23F	1.289	1.089	1.189
Rt - 4R	1.056	1.266	1.161
Rt - 2F (58)	1.229	1.087	1.158
Rt - 18F	1.301	1.004	1.153
Rt - 13F (78)	1.365	0.912	1.139
Rt - 23R	1.284	0.960	1.122
Rt - 10F	1.154	1.084	1.119
Rt - 13R (78)	1.157	1.043	1.100
Rt - 8F	1.156	1.001	1.079
Rt - 5/16R	1.205	0.910	1.058
Rt - 11F	0.956	1.101	1.029
Rt - 12F	1.044	0.903	0.974
Rt - 20R	1.041	0.890	0.966
Rt - 7aR	1.100	0.766	0.933
Rt - 19F	0.989	0.874	0.932
Rt - 19R	0.903	0.804	0.854
Rt -1/9/17/24R	0.882	0.686	0.784
Rt - 10R	0.832	0.707	0.770
Rt - 7bF	0.769	0.663	0.716
Rt - 25R (71)	0.818	0.563	0.691
Rt - 21F (73)	0.721	0.623	0.672
Rt - 2R (58)	0.690	0.513	0.602
Blanks (No rickettsia, Only Streptavidin-HRP + ABTS)	0.174	0.129	0.152

Notes: *Slashes indicate that the aptamer sequence emerged in more than one clone and is more frequent in the final aptamer pool than candidates with no slashes in their designations. Numbers in parentheses indicate an unusual aptamer length which differs from the expected 72 bases. F; forward and R; reverse primed DNA sequences.*

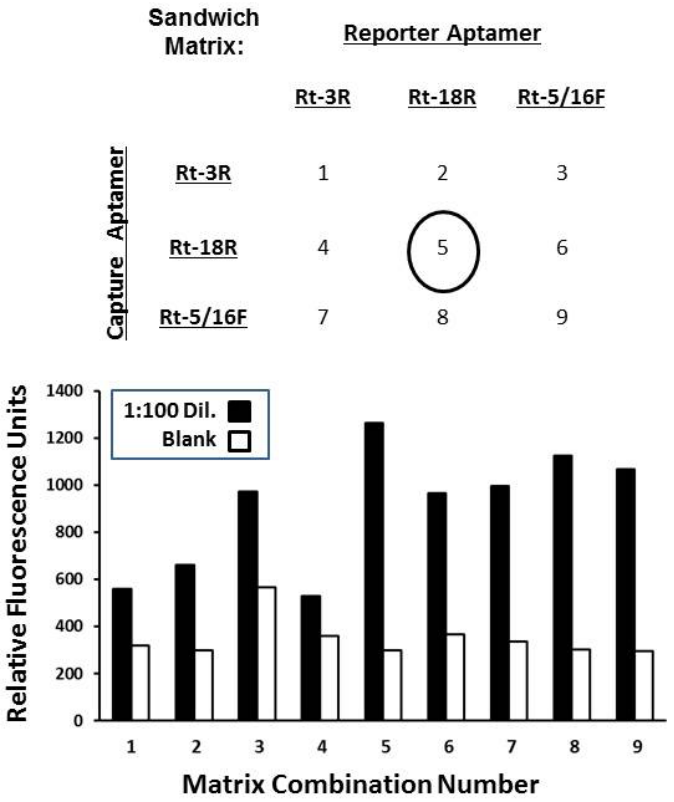

Figure 2: *Top panel – Experimental checkerboard matrix matching the top 3 R. typhi whole cell aptamers from ELASA screening in Table 2. Sandwich assay capture and reporter aptamer pairs were assigned combination numbers 1-9 as shown. Bottom panel – results of a typical screening experiment in which the fluorescent enzyme-linked aptamer-magnetic bead sandwich assay was assessed for each combination shown in the above panel with zero (blank, white bars) and a 1:100 dilution of stock R. typhi cells (~ 1,000 cells per ml, black bars).*

this prototype assay is fairly specific for *Rickettsia* and *Orientia* as a group (same family), because there was low or insignificant fluorescence noted with the L929 host cells and *E. coli*. The positive controls consisting of only Sav-HRP plus AUR yielded strong fluorescence in all cases. Variations in the actual fluorescence levels between trials are likely due to differences in actual timing of the readings.

Data shown in Figure 5 were acquired after a longer (5 min) AUR development period, but illustrate that the sandwich assay also works when conducted in ≤ 100% human serum. The 1:1,000 dilution of *R. typhi* whole cells (~ 1,000 cells per ml) was consistently detectable in 25%-100% serum and the addition of 100% serum actually appeared to aid in bringing the assay back to its baseline level in 0% serum (PBS only). Similarly, detection was shown to be quite possible in a dog tick homogenate for 1:100 (~10^4 cells/ml) and 1:1,000

(~10^3 cells/ml) dilutions of *R. typhi* whole cell stock as demonstrated by the data in Figure 6.

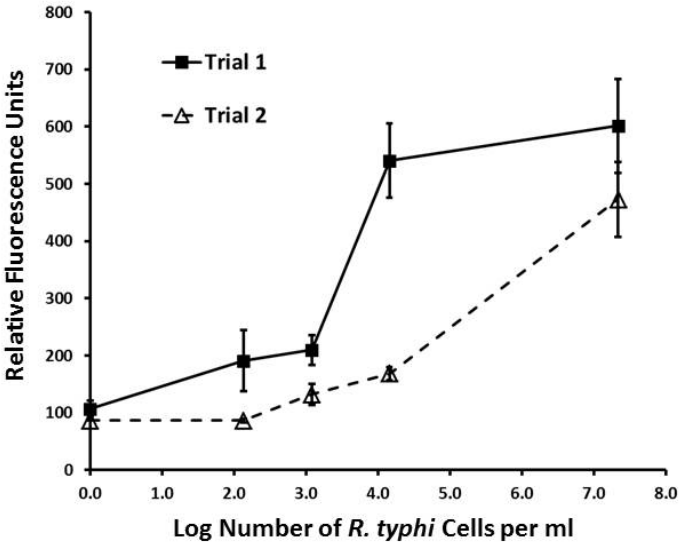

Figure 3: *Titration results for 2 separate trials for the lyophilized homogeneous Rt-18R fluorescent aptamer-magnetic bead sandwich assay (combination 5 in Figure. 2) versus the concentration of R. typhi cells shown. Means and standard deviations of 3 readings are shown for each data point.*

Discussion

DNA and RNA aptamers as a class of binding reagents still show great promise for diagnostic applications (Jayasena, 1999). The present work further illustrates this point because a sensitive prototype assay (LOD between 100 and 1,000 cells/ml) was demonstrated for *R. typhi*. Admittedly, despite the rounds of negative selection (absorption) with *Rickettsia* from the spotted fever group, we were not able to produce an entirely *R. typhi* specific assay. However, the assay does appear to respond solely to members of the Rickettsiaceae and not to an unrelated bacterial species (*E. coli*) and the host cells (L929) in which *R. typhi* cells were cultured.

We hypothesize that the assay specificity can probably be improved by development of new longer aptamers with a randomized region greater than 36 bases to enable multiple binding sites for various epitopes. The rationale for this hypothesis is that antibodies have 3 hypervariable binding regions in their complementarity determining regions (CDRs) which may lead to greater selectivity because the probability of binding a longer multivalent aptamer to each epitope is multiplicative or more restrictive and specific in nature for each epitope that is bound (Bruno, 2013) versus

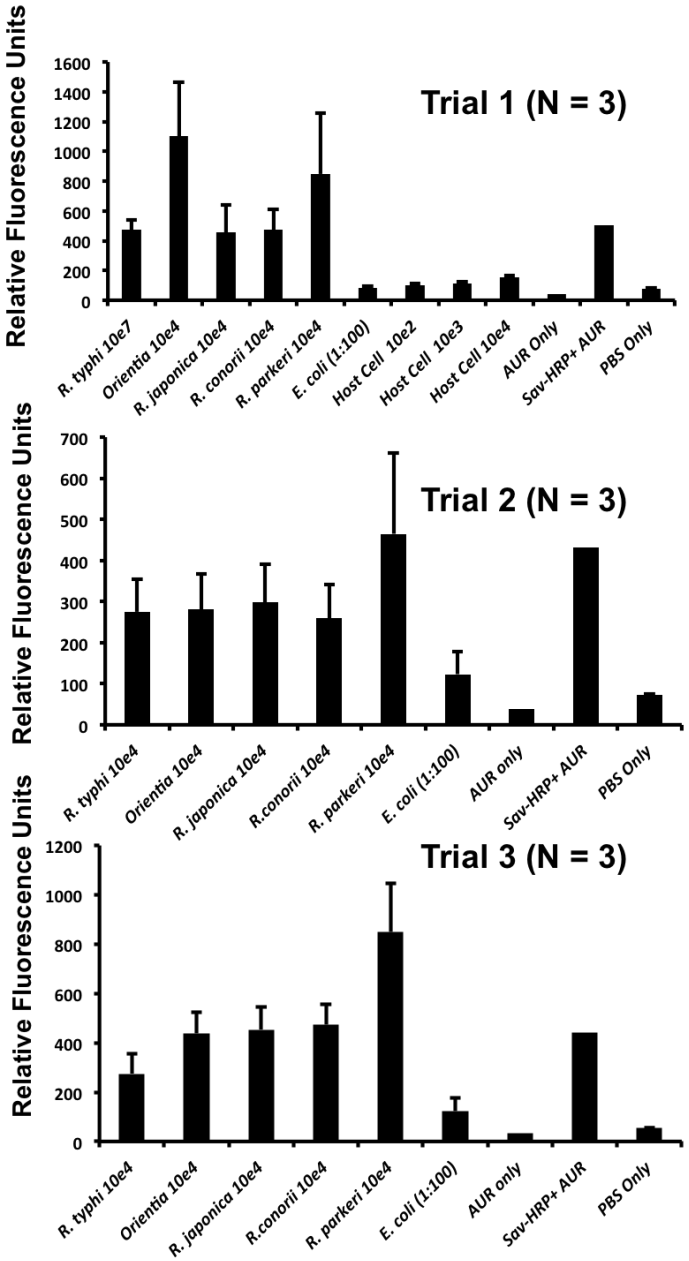

Figure 4: *Cross-reactivity assessment of the lyophilized homogeneous Rt-18R assay versus various species of Rickettsia and Orientia, E. coli and L929 host cells at the cell concentrations (per ml) shown and with various positive and negative controls. Means and standard deviations of 3 independent readings are shown for each of the 3 trials.*

binding to just one epitope with a shorter aptamer. Shorter 60-72 base aptamers have demonstrated superb specificity for small molecules (Bruno et al., 2009b; Jenison et al., 1994) and peptides (Bruno et al., 2011). However, larger and more complex proteins with epitopes that span several species or even genera appear to present a problem for shorter aptamers in terms of specificity unless a unique epitope can be found on the target protein. New multivalent aptamers are already proving to possess superior affinities

and specificity based on reports from the literature (Hasegawa et al., 2008; Mallikaratchy et al. 2011; McNamara et al., 2008).

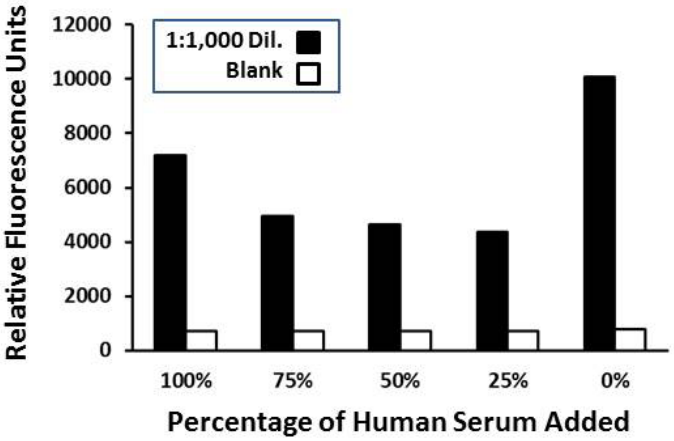

Figure 5: *Results of the fresh reagent Rt-18R sandwich assay conducted in serum at the percentages shown with a 1:1,000 dilution of R. typhi cells (~ 100 cells per ml, black bars) versus zero added cells (blanks, white bars). AUR development was extended to ~ 2 mins which resulted in higher relative fluorescence values.*

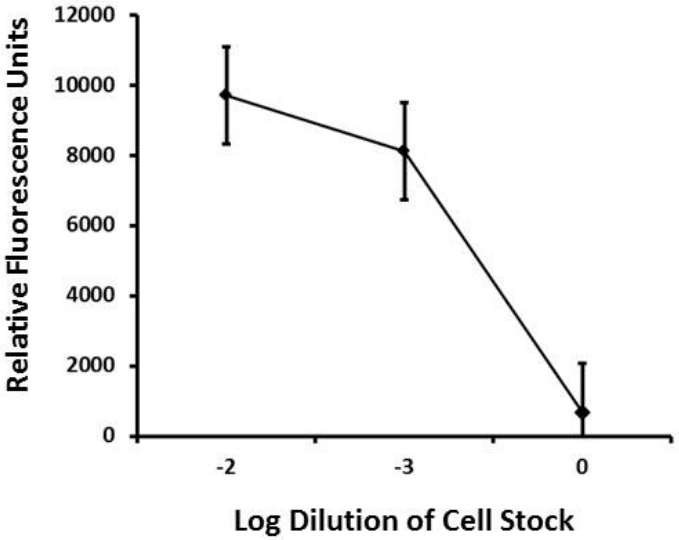

Figure 6: *Results for use of the Rt-18R sandwich assay conducted in dog tick homogenate (5 medium-sized ticks ground into 10 ml of PBS) and then spiked with a 10^{-2} (~ 1,000 cells/ml) or 10^{-3} (~ 100 cells/ml) dilutions of R. typhi whole cells versus a zero blank. Data points represent the means and standard deviations of 3 readings.*

Specificity among *Rickettsia* immunoassays is fairly good (Kovacova and Kazar, 2000; La Scola and Raoult, 1997), but *R. typhi* has been shown to possess only 24 unique proteins of 776 proteins examined from genomic studies of *R. typhi* and its closest relatives (McLeod et al., 2004). Of course, the very nature of the Weil-Felix test also illustrates that *Rickettsia* cross-react with proteins from *Proteus* species (Amano et al., 1995) and if *Rickettsia* are truly related to ancestral mitochondria, then any anti-rickettsial cell binding reagent (aptamer or antibody) may somewhat cross-react with eukaryotic host cells carrying mitochondria. However, we did not observe strong cross-reactivity with L929 cells using our Rt-18R-based assay (Figure 4).

We were somewhat surprised by the ~84 kD band that emerged in our aptamer Western blots (e.g., Figure 1), because the 84kD band only appeared in the boiled whole cell sample, but not the extracted protein samples for *R. typhi*. In addition, to the best of our knowledge, no ~ 84 kD *R. typhi*-discriminatory surface protein had been described previously (Kovacova and Kazar, 2000; La Scola and Raoult, 1997; Raoult and Dasch, 1995; Uchiyama et al., 1995; Chao et al., 2008). Figure 1 suggested that the assay for *R. typhi* using the Rt-18R aptamer might be specific for *R. typhi*, but subsequent assay results (Figure 4) revealed that Rt-18R was not entirely specific for *R. typhi*. It is of interest to note that immunoassays have emerged which utilize a 17 kD outer membrane protein (OMP) for discrimination of rickettsial groups (i.e., spotted fever versus typhus and scrub typhus (Kovacova and Kazar, 2000; La Scola and Raoult, 1997). However, an~ 80 kD protein from *R. typhi* has been reported by Amano, et al., 1995 in immunoblots using patient antisera. Furthermore, Kowalczewska, et al. (2012) alluded to an OMP of up to 768 amino acids in *R. prowazekii* and we identified a 768 amino acid (MW ~ 84.5 kD) found in *R. typhi* ATCC-VR144/Wilmington strain from the PubMed Protein database (GenBank: AAU03634.1) which may be the OMP detected in Figure 1.

It is also noteworthy that the *R. typhi* assay was a homogeneous sandwich assay involving the use of only one type of aptamer (Rt-18R) instead of two different aptamers. It thus stands to reason that the ~ 80 kD protein associated with *R. typhi* and detected by Rt-18R was sufficiently abundant on the cell surface to enable multiple bindings to the Rt-18R aptamer acting in both the capture and reporter roles simultaneously. The homogeneous assay involving Rt-18R was selected based on its marginally greater signal to noise performance data shown in Figure 2 (combination 5). However, greater specificity may be developed from some of the other top heterogeneous aptamer combinations shown in Figure 2. Further exploration of

the specificity of these other heterogeneous aptamer sandwich combinations is the topic of future research.

The prototype assay appeared to function well in both diluted and undiluted human serum with undiluted serum actually appearing to aid the assay's performance somewhat (Figure 5). In fact, there was an odd increasing trend in fluorescence as a function of serum concentration from 25% to 100% added serum during the capture phase. So, serum may have some stabilizing effect on the assay, but it is difficult to reconcile this observation with what appears to be superior performance in pure buffer (0% serum in Figure 5). This observation will form the basis for future investigations in serum, if specificity can be improved by lengthier aptamers. Still, we were able to demonstrate *R. typhi* detection in the 100 to 1,000 cells per ml range in buffer, serum, and tick homogenates (Figures 3, 4, 5 and 6).

Conclusions

The concept of an ultrasensitive fluorescent enzyme-linked assay has existed for decades (Yolken and Stopa, 1979). In the present report, we describe the use of a newer AUR-based aptamer-magnetic bead-based sandwich assay for sensitive (100-1,000 cells/ml) detection of *R. typhi* and related *Rickettsia* and *Orientia* species in buffer, serum and tick homogenates. The present work illustrates an improvement in terms of reproducibility in biological fluids and matrices by the use of a fluorescent enzymatic reporter system versus the previous QD-based aptamer-magnetic bead sandwich assays. However, assay specificity can, and probably will be, improved by development of longer (> 72 base) multivalent aptamers with several binding sites in their anticipated structures. Indeed, more recent data generated by our laboratories (not presented), suggests that longer 100-200 base aptamers appear to have the ability to discriminate *Rickettsia* species from the Typhus group versus the SFG. Overall, the present work illustrates the potential for ultimate development of ultrasensitive, rapid (< 1 h), and highly portable or even handheld nucleic acid aptamer-based assays and diagnostic systems for detection of infectious disease agents at the point of care and in the field.

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Author Contributions

JGB conceived of and directed the project and wrote the draft manuscript. TP and AE developed the *Rickettsia* aptamers, screened aptamers by ELASA, and conducted some of the aptamer sandwich assays. JCS performed aptamer Western blots. CCC, ZZ, and WMC conducted assay verification including limit of detection and cross-reactivity studies at the NMRC in MD and reviewed the manuscript.

Conflicts of Interest

The authors declare that no conflicts of interest exist.

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