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Aptamer Selection against a *Trichomonas vaginalis* Adhesion Protein for Diagnostic Applications

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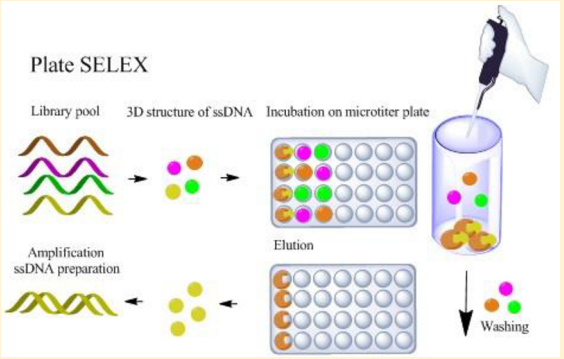
Supporting Information

ABSTRACT: Trichomoniasis, caused by *Trichomonas vaginalis*, is the leading nonviral sexually transmitted infection worldwide. We report the selection of a DNA aptamer against a *T. vaginalis* adhesion protein, AP65, using a microtiter plate-based in vitro combinatorial chemistry process termed systematic evolution of ligands by exponential enrichment. The enriched library pool was sequenced by next-generation sequencing, and several aptamer candidates with high affinity and specificity were identified. The aptamer with the highest affinity and specificity had a K_D in the low nanomolar range, as confirmed by three different techniques: surface plasmon resonance, enzyme-linked aptamer assay, and biolayer interferometry. The selected aptamer was demonstrated to have a high specificity to the AP65 protein and to *T. vaginalis* cells with no cross-reactivity to other enteric and urogenital microorganisms. Current work is focused on the development of inexpensive and easy-to-use aptamer-based diagnostic assays for the reliable and rapid detection of *T. vaginalis* in vaginal swabs.



KEYWORDS: *aptamer, Trichomonas vaginalis, sexually transmitted disease, adhesion protein 65*

Trichomonas vaginalis infection or trichomoniasis is the leading nonviral sexually transmitted infection (STI) worldwide.[1] The World Health Organization estimated a global incidence of 248.5 million cases in 2005 and 276.4 million cases in 2008 in adults between 15 and 49 years of age.[2] The clinical manifestations of the disease in women include purulent vaginal discharge, pruritus, dysuria, dyspareunia, and "strawberry cervix".[3] In men, they are usually asymptomatic but may present with urethritis.[4,5] The complications of *T. vaginalis* infection include pelvic inflammatory disease, cervical cancer, infertility, and adverse pregnancy outcomes in women and epididymitis, prostatitis, and balanitis in men. The infection is also known to increase the risk for human immunodeficiency virus (HIV) transmission.[3,6] Hence, a correct, rapid, and cost-effective diagnosis is critical so that appropriate treatment may be prescribed, preventing further dissemination of the disease. However, current detection methods do not meet these requirements, as the infection is difficult to diagnose clinically, since the associated symptoms may also be present in other urogenital infections and sexually transmitted diseases.[3,5] Current diagnostic methods available for *T. vaginalis* detection include direct visualization of the trophozoite by wet mount microscopy, culture methods, immunoassay techniques, and nucleic acid amplification tests (NAAT). On the one hand, the most widely used method, the wet mount microscopy, is rapid and inexpensive but suffers from a low sensitivity of only 38−82%.[3,7,8] The culture method, on the other hand, is considered as the gold standard but requires at least 2−7 d of incubation.[5,9] While NAAT methods, such as polymerase chain reaction (PCR) amplification, provide highly reliable results,[10−20] they require expensive equipment, reagents, infrastructure, and trained personnel.

Various immunoassay techniques have been developed for the detection of *T. vaginalis*, employing the use of antibodies as recognition probes to detect specific antigen markers. In *T. vaginalis* infection in humans, the adhesion protein 65 (AP65) is the primary antigen detected.[21]

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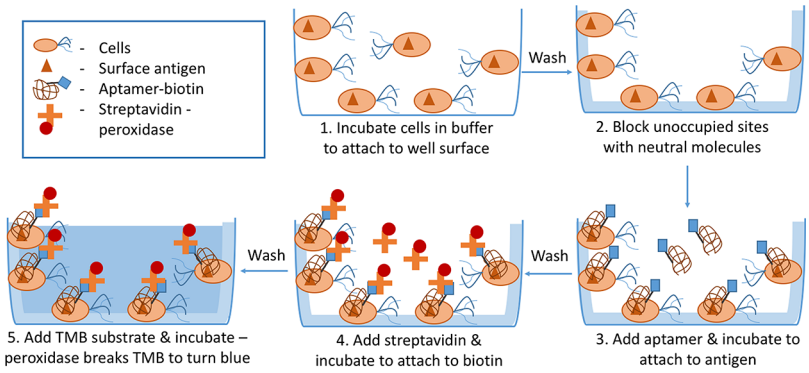

Figure 1. AP65 aptamer-ELAA method used in this study showing step-by-step immobilization of molecules on microtiter plate surface for the detection of *T. vaginalis* cells.

In antigen-based immunoassays, cell surface proteins are potentially good candidates as target molecules for detection of pathogen cells. The AP65 of *T. vaginalis* is a prominent adhesin that is located on the parasite's cell surface and is secreted to the extracellular environment. It plays a role in the adhesion of the parasite to host cell[22] and to iron-rich heme and hemoglobin.[23,24] AP65 is believed to be a unique protein of *T. vaginalis*, since previous experiments have shown no cross-hybridization and immuno-crossreactivity of AP65 to other trichomonads found primarily in animals, for example, *Trichomonas suis, Pentatrichomonas hominis,* and *Tritrichomonas fetus*.[25] One of the most successful immunoassay-based tests developed is the OSOM Trichomonas test marketed by Sekisuki Diagnostics,[26−31] but there are no reports of rapid tests that can be deployed at the point of need. Since the first reports of aptamers in the early 1990s, there has been an increasing interest in their use as an alternative to antibodies. Aptamers are oligonucleotides, either single- or double-stranded DNAs or RNAs that can bind to a variety of molecules with high affinity and specificity,[32] exhibiting several properties that make them interesting as an alternative to antibodies as tools for analytical applications. Aptamers, being inherently nucleic acid in nature, are far more flexible, stable, and cost-effective as compared to antibodies. Aptamers are selected using an in vitro process, that is, selection can be performed in nonphysiological conditions, thus avoiding the need to sacrifice animals. Furthermore, while the aptamer selection process can be expensive, once the aptamer has been selected, its production is several orders of magnitude less expensive than that of its antibody counterpart. Aptamers can be easily modified and immobilized and can be exploited in a plethora of analytical applications, including molecular aptamer beacons and the combination of aptamers and nucleic acid amplification for ultrasensitive detection,[33] which are not feasible with antibodies.

Aptamers were first introduced in 1990 and were selected in an in vitro combinatorial chemistry process called systematic evolution of ligands by exponential enrichment or SELEX.[34−36] SELEX starts with an initial library pool of oligonucleotides with a random region flanked at the two ends with constant sequences where primers will be attached. Each round of SELEX for the selection of DNA aptamers involves five steps: incubation of the library pool of single-stranded DNA with the target molecule, separation of the nonbinding oligonucleotides, elution, amplification of bound oligonucleotides, and single-strand formation to constitute the library pool of the next round.[37] Following the completion of the selection process, the enriched DNA pool is sequenced, and individual aptamer candidates are investigated for their binding abilities to the target. Aptamers can be then truncated to eliminate nonessential nucleotides and potentially increase the binding functionality, specificity, and affinity, but they may also result in the removal of nucleotides important for folding into the desired three-dimensional (3D) conformation for binding to the target.

In this work, we describe the selection of aptamers against adhesion protein AP65 of *T. vaginalis* using microtiter plate SELEX (p-SELEX). At the end of SELEX, the enriched DNA pool was sequenced using next-generation sequencing (NGS), and various aptamer candidates were identified, analyzed, and characterized using surface plasmon resonance (SPR). The binding properties of the aptamer with the highest affinity and specificity were evaluated using SPR, biolayer interferometry (BLI), and enzyme-linked aptamer assay (ELAA). SPR and BLI are two well-established detection platforms for monitoring biomolecular interactions in real time, while ELAA provides an inexpensive, easy, and rapid way to determine the binding properties of aptamers. Truncation studies were performed to identify high binding affinity domains after the upstream aptamer selection process was performed. In addition, a comparison of binding affinities of the selected AP65_A1 aptamer and a commercially available polyclonal AP65 antibody was also performed. Finally, the ability of AP65_A1 aptamer to distinguish between control proteins as well as between other enteric and urogenital microorganisms was tested, confirming the robustness and functionality of the selected AP65_A1 aptamer.

RESULTS AND DISCUSSION

Aptamer Selection. Microtiter plates have been extensively used in reporter-linked aptamer assays similar to enzyme-linked immunosorbent assay (ELISA) techniques, but there are only a few reports of their use in immobilization and partitioning steps in SELEX.[41,42] Although different immobilization techniques may arise to different aptamers binding at various sites of the target protein, as compared to the conventional magnetic beads-based SELEX methods, p-SELEX could provide the following advantages:

(1) The immobilization step is easily done with fresh sample in each round in p-SELEX unlike in magnetic beads-based SELEX, where immobilization of target is usually prepared in large batch for the entire SELEX process and

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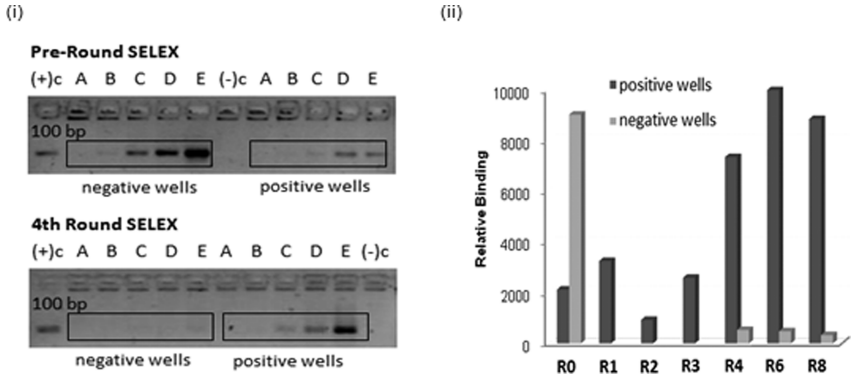

Figure 2. Evolution of the AP65 aptamer during plate SELEX. Gel electrophoresis results from the pilot PCR studies of the preround (R0) and 4th round (R4) of selection (i). Well numbers A−E correspond to the amplifications of eluted aptamers from the negative and positive selection steps, respectively, with increasing PCR cycles from 7, 9, 11, 13, and 15. Wells (+) c and (−) c correspond to the positive and negative PCR amplification controls, respectively, at 15 PCR cycles. The intensity of bands obtained by gel electrophoresis from different rounds are represented as relative binding (ii).

Table 1. Full-Length Aptamer Sequences (A1−A6)

aptamers	aptamer sequence (5′-3′)	*N*	MW (g mol^{-1})	G-score
A1	AGC TCC AGA AGA TAA ATT ACA GGT GAG GGC GGG CGG GTG GTT GTA ATA TGA TCG AAT GGT ATA TGT GTG TTT GCA ACT AGG ATA CTA TGA CCC CG	95	29 690	21
A2	AGC TCC AGA AGA TAA ATT ACA GGG GCC GGG GTG GCT CAG GCA AGG GGT TGA CCT GTC GTA GGG ATT GTT TTA ACA ACT AGG ATA CTA TGA CCCC	94	29 276	20
A3	AGC TCC AGA AGA TAA ATT ACA GGT GGG TGG GTG GGC GGT GGA ATT TAG CGG CGG AGC TCT GTG TGT GTT AGG GCA ACT AGG ATA CTA TGA CCCC	94	29 420	21
A4	AGC TCC AGA AGA TAA ATT ACA GGG GAT CAG TAA GGT TGA GAC GGC CTG AAT CTA TCG TGG AGA CCA CGC GAC GCA ACT AGG ATA CTA TGA CCCC	94	29 176	13
A5	AGC TCC AGA AGA TAA ATT ACA GGG AGA GTA AAC TTT GCA AAC ACA ACA ATA CCA TTC CGG AAC GTT CTT AAC ACA ACT AGG ATA CTA TGA CCCC	94	28 975	
A6	AGC TCC AGA AGA TAA ATT ACA GGG GCG GGG GGG CGG GGG AGG CGG AAG GCC TGC TAA AGT CGT TGT GAG CGA ACC AAC TAG GAT ACT ATG ACC CC	95	29 725	42.20

stored at 4 °C, which may result in target degradation or denaturation.

(2) Immobilization of target protein in each round of the p-SELEX is easily confirmed by a standard direct or indirect ELISA test using antibodies.

(3) The partitioning step is more efficient in p-SELEX with complete removal of solution containing the unbound oligonucleotides.

(4) The magnetic bead-based SELEX contains more matrix components, requiring implementation of a rigorous negative selection step.

While we used magnetic bead-based SELEX on many occasions, we decided to pursue microtiter plate SELEX for the selection of an aptamer/aptamers against AP65. To prevent the evolution of the library toward the matrix, a negative selection step was performed already from the first selection round. However, implementing a negative SELEX so soon in the process can increase the risk of eliminating high-binding aptamer candidates.[43] Therefore, after the first round, three rounds of only positive selection were performed, to increase the possibility of selecting DNA specific to the target (Figure 1). A negative selection was again incorporated in the fourth selection round and in each round from there on.

The evolution of the selection process of p-SELEX was monitored with the direct plate PCR assay and gel electrophoresis (Figure 2), and evolution was already clearly evident from the fourth round, with a clear increase in the intensity of the band from DNA eluted in each of the positive selection steps, whereas negligible interaction was detected with control naked plate well, indicating enrichment of the pool with these sequences exhibiting binding to AP65.

Identification of Aptamer Candidates. At the end of the selection process the enriched pool of oligonucleotides was sequenced using NGS. Short reads obtained from NGS were removed using data filtering by length (80−100 bp) to obtain sequences of the correct size (94 bp). One aptamer sequence, AP65_A1, was significantly dominant and represented ∼9% of the p-SELEX pool, and ∼10% of remaining sequences also corresponded to AP65_A1 but with different point mutations. The top six over-represented individual sequences (AP65_A1−A6) were selected for further analysis. Sequences of full-length aptamer candidates are shown in Table 1 and reveal a high percentage of guanine content. The QGRS mapper, used to predict putative G-quadruplex forming guanines,[44] calculated a G-score of more than 20 for AP65A1-AP65A3 and AP65A6. G-score is the statistical parameter for predicting the probability of finding a G-quadruplex motif; the higher the G-score, the higher the probability is. G-Quadruplex formation is frequently found in aptamers and offers advantages such as higher stability and resistance over unstructured sequences.[45]

Screening of AP65 Aptamer Candidates. The screening of binding affinities of AP65 aptamer candidates was studied by SPR. AP65 protein and control proteins (bovine serum albumin (BSA), streptavidin) were immobilized on separate channels of a CM5 chip, and AP65 aptamer candidates were injected and flowed over the surface. A one-to-one Langmuir model was used to analyze the binding constants and

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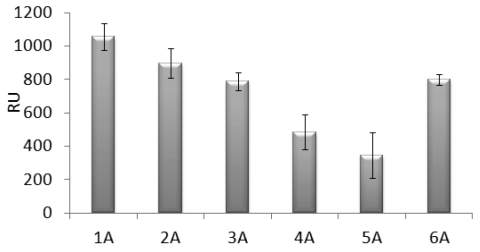
(i)



(ii)

Aptamer candidates	K_D (nM)	Chi^2
AP65_A1	**56**	3,21
AP65_A2	**153**	6,89
AP65_A3	**283**	1,18
AP65_A4	**444**	2,69
AP65_A5	**1205**	4,89
AP65_A6	**179**	0,886

Figure 3. Evaluation of the full-length AP65 aptamer candidates (A1−A6). Screening of AP65 aptamer candidates by SPR (i). Affinity dissociation constants K_D of the full-length aptamer candidates calculated by SPR (ii).

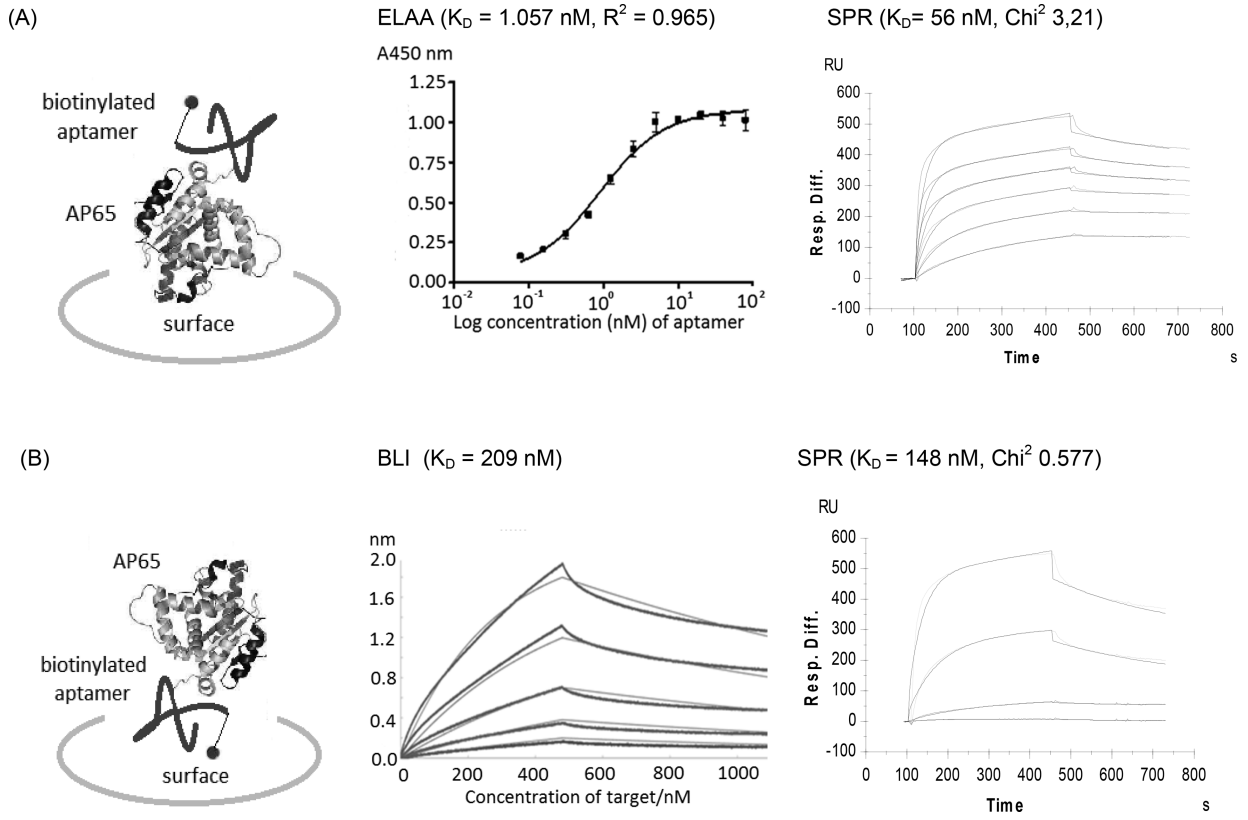


Figure 4. Dissociation constants of AP65_A1/AP65 obtained by different methods with relevant statistics. (A) Absorbance reading of biotinylated AP65_A1 aptamer binding to AP65 by ELAA and SPR sensogram showing the binding of AP65_A1 to immobilized AP65 represented in RU. (B) BLI and SPR sensogram showing the binding of AP65 to immobilized biotinylated AP65_A1 represented in nanometers and RU, respectively.

determine K_D using a range of AP65 concentrations (370 to 10 000 nM). Figure 3 shows the evaluation of the full-length aptamer candidates with the K_D values calculated for each candidate ranging from low nanomolar to low micromolar dissociation constants. Lower K_D values were obtained for sequences with higher G-score motif indicating some role of the G-quadruplex motif in the binding to the target. The aptamer AP65_A1 was observed to be the aptamer with highest affinity, with a K_D of 56 nM (Figure 4A), and a good fit for the model was obtained as demonstrated by the Chi^2 value of 3.21. No interaction with control proteins was observed (data not shown), demonstrating the specificity of AP65_A1 aptamer candidate.

Binding Affinity Studies of AP65_A1 Aptamer. The binding affinity of the selected AP65_A1 aptamer was further studied using ELAA. This method is based on an indirect ELISA immunoassay-type format, with AP65_A1 5′-biotinylated used as the biorecognition element and Streptavidin-horseradish peroxidase (SA-HRP) used as the reporter probe. The wells of a microtiter plate were saturated with AP65, and a range of concentrations of the biotinylated aptamer was used. A K_D value of 1.057 nM was obtained using the sigmoidal dose-response curve model of the GraphPad Prism software, further confirming a low nanomolar range K_D value of the aptamer with AP65 immobilized on the surface. No interactions with control proteins were observed, again corroborating the results obtained using SPR (Figure 4A).

While the K_D values obtained using SPR and ELAA differed by an order of magnitude, this is easily explained by the fact that different immobilization strategies were employed, with the AP65 protein being immobilized via cross-linking chemistry in SPR, while absorption was used in ELAA. Taking this into consideration, the results obtained are consistent, and similar phenomena have been reported previously.[46]

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Table 2. Truncated Aptamer Species of Aptamer A1

aptamers	aptamer sequence (5′-3′)	*N*	MW (g mol^{-1})	G-score
A1	AGC TCC AGA AGA TAA ATT ACA GGT GAG GGC GGG CGG GTG GTT GTA ATA TGA TCG AAT GGT ATA TGT GTG TTT GCA ACT AGG ATA CTA TGA CCC CG	95	29 690	21
A1_WP	TGA GGG CGG GCG GGT GGT TGT AAT ATG ATC GAA TGG TAT ATG TGT GTT TG	50	15 788	21
A1_WF	TGA GGG CGG GCG GGT GGT TGT AAT ATG ATC GAA TGG TAT ATG TGT GTT TGC AAC TAG GAT ACT ATG ACC CCG G	73	22 867	21
A1_WR	AGC TCC AGA AGG TAA ATT ACA GGT GAG GGC GGG CGG GTG GTT GTA ATA TGA TCG AAT GGT ATA TGT GTG TTT G	73	22 955	21
A1_13	GGC GGG CGG GTGG	13	4191	20
A1_14	GGG CGG GCG GGT GG	14	4520	21
A1_15	GGT GAG GGC GGG CGG	15	4834	20
A1_16	GGT GAG GGC GGG CGGG	16	5163	20
A1_19	GGT GAG GGC GGG CGG GTGG	19	6126	17
A1_22	GGT AAA TTA CAG GTG AGG GCGG	22	6984	
A1_23	GGT AAA TTA CAG GTG AGG GCG GG	23	7313	
A1_26	GGT AAA TTA CAG GTG AGG GCG GGC GG	26	8260	

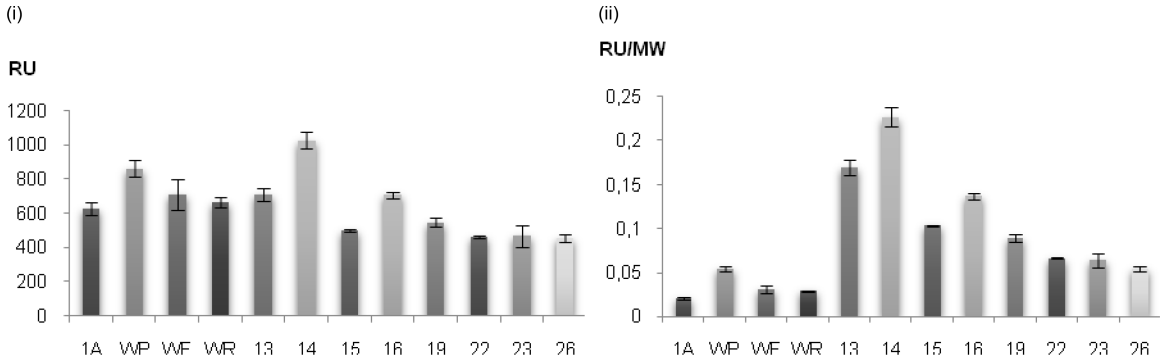

Figure 5. SPR experiments showing the interaction between truncated aptamer sequences of AP65_A1 aptamer to AP65 protein immobilized on the surface of the CM5 Biacore chip. Full-length aptamer (1A), 1A without primer regions (WP), 1A without forward (WF), 1A without reverse (WR), and short versions of 1A aptamer (13−26 mers). The binding of sequences is represented in RU (i) and normalized by the molecular weight of each sequence (ii).

The opposite format with aptamer being immobilized on the surface and target used as ligand was also studied using both SPR and BLI (Figure 4B). In SPR biotinylated AP65_A1 aptamer was immobilized on a streptavidin-coated sensor chip, and different concentrations of AP65 were flowed over the surface. The K_D of the AP65 protein was estimated using a one-to-one Langmuir model via the analysis of the binding of AP65 (37−1000 nM). The resulting K_D was 148 nM, and a good fit for the model was obtained with Chi^2 value of 0.577. These results were corroborated by BLI experiment, where K_D of 209 nM was obtained highlighting the robustness of AP65_A1 aptamer, also when used in an immobilized format. It is quite normal to see differences in the K_D of aptamers free in solution and immobilized, as the immobilization process may in fact, to some extent, impede the folding of the aptamer into its optimum 3D structure for target binding. This is often overcome using spacers to extend the aptamer from the surface and thus facilitate its 3D formation for optimum target binding, and this will be pursued when developing assays for detection of *T. vaginalis*.

Truncation Studies of AP65_A1 Aptamer. The AP65_A1 candidate was further used for truncation studies to eliminate the nonessential nucleotides, potentially improving the K_D and improving specificity. The first strategy explored was the removal of the constant regions at either the 3′ end or 5′ end, or at both extremes. A second truncation strategy investigated was based on the use of GQRS Mapper, for predicting G-quadruplexes in nucleotide sequences. In total, 11 shortened sequences were selected and analyzed for their ability to bind to AP65 (Table 2). AP65 target was immobilized on a CM5 chip, and sequences corresponding to truncated versions of AP65_A1 were passed over the chip surface. As can be clearly seen in Figure 5, removal of the constant regions at one or both extremes has no effect on the binding properties of the AP65_A1 aptamer. The highest binding was observed for a 14-mer with the highest G-score 21, while lower binding affinities were observed for truncated sequences with lower G-scores. A similar G-score was obtained for other well-known short aptamers that bind with high affinity, such as the thrombin binding aptamer (TBA)[47] and the β-conglutin binding aptamer I (β-CBAI).[48]

AP65 Aptamer Comparison with Antibody. To demonstrate the binding affinity of the full-length AP65_1A aptamer, the K_D of a commercially available polyclonal antibody to AP65 was determined for comparison. The method used was a standard ELISA, and the test was performed simultaneously and under the same conditions as the ELAA used to determine the K_D value of the aptamer to AP65. The result, as shown in Figure S3, shows that the aptamer has better binding affinity to AP65 than the tested polyclonal antibody, with the K_D of the antibody to AP65 being 12.41 nM as compared to 1.057 nM for the aptamer. The use of the polyclonal antibody as compared to the selected aptamer was then compared using indirect assay formats,

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where a range of concentrations of AP65 (0.02−18 nM) were immobilized on the wells of a microtiter plate. In the case of the aptamer, and ELAA was used, where biotinylated aptamer, followed by an excess of SA-HRP was added to each well, while for the antibody an ELISA was used, where the PAb anti-AP65 was added to each well, followed by an excess of antirabbit IgG-HRP. Very similar limits of detection (LODs) of 3.2×10^{-11} and 4.5×10^{-11} M were obtained for the aptamer and antibody, respectively, while better sensitivity was achieved with the ELAA (Figure S4A).

The same ELAA format was applied to the detection of *T. vaginalis* cells. A range of cell concentrations, starting with 33 500 and serially diluting 1:2, were immobilized on the wells of a microtiter plate, and an LOD of 8.3×10^3 cells/mL was obtained (Figure S4B). In comparison to the LOD of 100 trichomonads/mL reported using an indirect ELISA in 1986,[49] the preliminary AP65 ELAA developed in this study has a lower sensitivity. However, the commercially available OSOM kit based on an antibody sandwich assay has an LOD of ∼2500 organisms per milliliter, and generally the minimal concentration of 1×10^4 organisms per milliliter of vaginal fluid appears to be necessary for identification of the protozoan by wet mount.[50] Additionally, it must be emphasized that the assay reported here is a preliminary one, simply demonstrating the ability of the aptamer to be used for the detection of *T. vaginalis* cells; various assay formats will be pursued in an effort to reach ultralow limits of detection, using the selected aptamer.[51]

Cross-Reactivity to Enteric and Urogenital Microorganisms. The ELAA test was conducted on culture isolates of *T. vaginalis* and other enteric and urogenital tract microorganisms, including *Bacillus subtilis, Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Neisseria gonorrhoeae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica*, and *Staphylococcus aureus*. As biotin and biotin-like compounds are present in microorganisms, the microorganisms studied were also incubated with SA-HRP alone,[52] and the response obtained was subtracted from that obtained with AP65 + SA-HRP, to eliminate this background signal. In the future development of assays for the detection of AP65, aptamer directly linked to HRP will be used, avoiding this background interaction between streptavidin and biotin present in cells. As can be seen in Figure 6, a high specificity of the AP65 aptamer to *T. vaginalis* with low cross-reactivity to *N. gonorrhoeaee* at concentrations higher than 1×10^8 organisms per milliliter and minimal cross-reactivity to the other microorganisms, as observed, highlighting its potential use in cost-effective assays for the rapid detection of *T. vaginalis*. The detection of *T. vaginalis* by antibodies in the OSOM kit showed cross-reactivity to *S. aureus* at 1×10^8 organisms per mL, indicating that at very high concentrations this organism can interfere with the test results. Ongoing work, focusing on different sandwich formats with dual aptamers as well as mixed assays combining aptamers and antibody, should increase the affinity and achieve even better specificity due to the binding of capture and detecting molecules to different epitopes on AP65, and thus on *T. vaginalis* cells.

CONCLUSION

In the work reported here, we detail the selection and characterization of an aptamer against the AP65 adhesive protein, which can be used for the detection of the sexually transmitted parasite *T. vaginalis*. The selected aptamer was tested in three independent laboratories using three different methods, with each indicating a low nanomolar K_D. A preliminary assay for the detection of AP65, as well as of *T. vaginalis* cells, was developed and showed no cross-reactivity of the aptamer to control proteins and negligible cross-reactivity to enteric and urogenital tract microorganisms. This is the first report of an aptamer that can be used for the detection of *T. vaginalis* and fully fits with the requirements of cost, sensitivity, and specificity for its use in a cost-effective, rapid, and easy-to-use point of care device, and ongoing work is looking at the development of microtiter plate assays and lateral flow assays and the complete validation of these assays using vaginal swab samples and deploying these assays for implementation at the point of need.

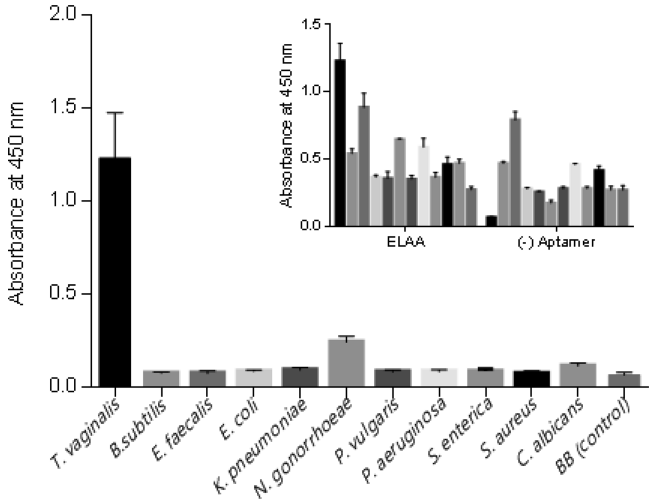

Figure 6. Cross-reactivity of AP65_A1 aptamer to the enteric and urogenital microorganisms with relative absorbance values obtained by ELAA. Relative absorbance ratio is the ratio of the absorbance value obtained by ELAA to the absorbance value obtained during the control ELAA without the presence of aptamer (-) multiplied by a factor. BB (binding buffer). The cell density of *T. Vaginalis* was determined to be 1×10^7 cells/mL, while *C. albicans* was 1×10^8 cells/mL, and the concentration of bacterial samples was $\sim 1 \times 10^8$ cells/mL.

METHODS

Reagents. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) purified recombinant *T. vaginalis* AP65 at 0.48 mg mL^{-1} in phosphate-buffered saline (PBS) and 2 M urea buffer solution and the affinity column purified polyclonal rabbit antibody to the recombinant AP65 at 0.80 mg mL^{-1} in PBS containing 50 mM glycine (pH 8.0) were purchased from Bioassay Plus, Inc. The high-performance liquid chromatography (HPLC) purified and lyophilized oligonucleotides (initial aptamer library, primers, and specific aptamer sequences) were purchased from BIOMERS. All microorganisms were obtained and prepared at the Pathogen-Host-Environment Interactions Research Laboratory at the Natural Sciences Research Institute of the University of the Philippines Diliman. The ELISA reagents including SA-HRP, HRP-linked secondary antibody (antirabbit IgG), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma. PBS buffer (10 mM phosphate, 138 mM NaCl and 2.7 mM KCl at pH 7.4), PBS-tween buffer (0.05% v/v tween, 10 mM phosphate, 138 mM NaCl and 2.7 mM KCl at pH 7.4) mix, ethanolamine−HCl (1 M, pH 8.5), 1-ethyl-3-(3-

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dimethylaminopropyl) carbodiimide (EDC), and, *N*-hydrosuccinimide (NHS) were also purchased from Sigma. All PCR reagents including the *Tfi* DNA polymerase were purchased from Invitrogen. The λ-exonuclease was purchased from Fisher Scientific. The Certified Low Range Ultra Agarose was purchased from Bio-Rad. The nucleic acid GelRed stain was purchased from Biotium. The ethanol, magnesium chloride ($MgCl_2$), sodium acetate (NaAc), sodium chloride (NaCl), sodium hydroxide (NaOH) solution, hydrochloric acid (HCl) solution, and sulfuric acid (H_2SO_4) were purchased from Scharlau Chemie S.A. All solutions were prepared with high-purity water obtained from Milli-Q RG system.

Aptamer Selection. Fifty microliters of 50 μg mL^{-1} AP65 target diluted in PBS was added to two wells of a NUNC Maxisorp microtiter plate and incubated at 37 °C for 30 min with gentle shaking. Negative control wells were also prepared by incubating 50 μL of PBS buffer. Following immobilization of the target, the solution in each well was removed, and 200 μL of PBS-tween buffer was added and incubated at 37 °C for a further 30 min with gentle shaking to block surfaces that were not coated with the target protein. After blocking, the wells were washed thrice with 200 μL of PBS-Tween buffer.

Immobilization of AP65 was confirmed qualitatively by a standard indirect ELISA method using the polyclonal antibody to the recombinant AP65 and an enzyme-linked secondary antibody (HRP-antirabbit IgG). Fifty microliters of 25 μg mL^{-1} of the AP65 antibody was added to the wells coated with AP65 as well as to the negative control well. The antibody was incubated at 37 °C for 30 min with gentle shaking to allow the antibody to bind with the target protein, followed by thorough washing, thrice with 200 μL of PBS-Tween. Fifty microliters of a 1/10 000 dilution of 1 mg mL^{-1} of the HRP-antirabbit IgG in PBS buffer was then added to the wells and incubated at 37 °C for 30 min, again with gentle shaking, followed by thorough washing, thrice with 200 μL of PBS-Tween. After it was washed, 50 μL of TMB was added to each well, producing a blue color, while the solution without the target protein remained transparent. The color development reaction was stopped by adding 50 μL of 1 M H_2SO_4.

The initial ssDNA library pool used was made of diverse 94-mer DNA sequences containing a random region of 50 nucleotides flanked by primer binding regions: 5′-AGCTCCAGAAGATAAATTACAGG-N(50)-CAACTAGG-ATACTATGACCCC-3′. In the first round, the initial library was diluted to 3 μM with binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$ at pH 6.4). Before the start of each round, the library pool was heated to 95 °C for 3 min and allowed to cool immediately to 20 °C to let the ssDNA sequences denature and fold into various 3D structures. After denaturation and folding, 100 μL of the library pool was added to the well that was coated with AP65 and left to incubate at 37 °C for 30 min with gentle shaking. Following removal of the unbound ssDNAs, the well was washed thrice with 200 μL of binding buffer. Thirty microliters of hot water (95 °C) was added to elute DNA sequences bound to the target protein, and this eluted DNA was stored at −20 °C.

After three rounds of SELEX, a negative selection step was introduced prior to the positive selection step of incubating the library pool to the well coated with target protein. In the negative selection step, the library pool was incubated to the well without the target protein to eliminate nonspecific DNA. The DNA sequences that bound to the microtiter plate matrix in the negative selection step were also eluted using the same method as used in the positive selection step.

PCR Amplification. Amplification of the eluted sequences was performed in 100 μL of PCR solution (1 × *Tfi* PCR buffer, 0.35 mM $MgCl_2$, 0.2 mM dNTPs, 0.10 μM forward primer (5′-AGCTCCAGAAGATAAATTACAGG-3′), 0.10 μM reverse phosphorylated primer (5′-P-GGGGTC-ATAGTATCCTAGTTG-3′), 0.5 mg mL^{-1} BSA, 10 U *Tfi* polymerase) with 2 μL of template solution containing the eluted DNA. Amplification was performed in an iCycler thermocycler (Biorad) programmed with the following protocol: 2 min at 95 °C, followed by 6−20 repetitions of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C.

Prior to the main PCR amplification step, a pilot PCR was performed after each round with the DNA eluted in both the positive and negative selection steps. Aliquots of 20 μL were taken from 100 μL of PCR mixture at a certain number of cycle intervals, and the quantity and quality of the PCR products were evaluated using gel electrophoresis. Six microliters of the PCR products were run with 4 μL of loading buffer on a 2.4% w/v agarose gel and Tris/borate/EDTA (TBE) buffer at 100 mV for 20 min.

Generation of ssDNA. Following PCR amplification, ssDNA was generated by combination of asymmetric PCR (A-PCR) and exonuclease digestion of the phosphorylated reverse strand for use in subsequent rounds of SELEX as recommended previously.[38] Two tubes of 100 μL of A-PCR solution (1 × *Tfi* PCR buffer, 0.35 mM $MgCl_2$, 0.2 mM dNTPs, 0.30 μM forward primer, 0.5 mg mL^{-1} BSA, 10 U of *Tfi* polymerase), added to 10−15 μL of template solution containing the amplified PCR products, were used. The A-PCR was performed using the same thermocycling protocol as the PCR with the exception that the extension time was increased from 30 s to 12 min at 12 loop repetitions. Following A-PCR, 5X λ-exonuclease buffer was added to each tube to a final concentration of 1X. Ten units of λ-exonuclease was then added to each tube and incubated at 37 °C for 60 min before deactivation at 80 °C for 10 min using the thermocycler. Following enzyme digestion of the phosphorylated strands, the product quality and quantity were checked using gel electrophoresis. Ten microliters of the ssDNA generated was run with 4 μL of loading buffer and 2 μL of 150 mM NaOH on a 2.4% w/v agarose gel and TBE buffer at 100 mV for 20 min.

The ssDNA generated was collected from the two tubes via the sodium acetate−ethanol precipitation method to ensure that an adequate amount of DNA was available in the library pool for the next round.

Next Generation Sequencing. The DNA pool from the final round of selection was amplified and cleaned with the QIAEX II kit (Qiagen). The cleaned aptamer pool was used for Ion Torrent Next-Generation Sequencing, and the data were analyzed using Galaxy server[39] to identify aptamer candidates sequences.

Screening and Binding Affinity Studies. SPR was performed with a BIAcore 3000 (Biacore Inc.). Targets (AP65 and control proteins such as streptavidin and BSA) were immobilized on separate channels of a CM5 sensor chip activated with EDC/NHS (30 μL of a 1:1 mixture of EDC (400 mM) and NHS (100 mM)) followed by injection of 200 μg mL^{-1} target at a flow rate of 5 μL min^{-1}. After immobilization of the targets, unreacted NHS esters were deactivated via injection of an excess of ethanolamine

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hydrochloride (1 M). Unbound targets were then washed and removed from the surface using 2 M NaCl and 10 mM NaOH. The aptamer candidates were diluted to a final concentration of 2 μM (screening of aptamer candidates) or 1 μM (screening of shortened versions of aptamer AP65_A1) in binding buffer and injected during 6 min at a flow rate of 5 μL min^{-1} followed by 3 min of stabilization time and 10 min of dissociation time. For the calculation of K_D, a range of concentrations of aptamer candidates was prepared from a starting concentration of 1 μM by serial dilution (1:2) in binding buffer. The binding of DNA was analyzed with BIA evaluation software through corresponding changes in the refractive index of optical signals and expressed as resonance units (RU). The K_D was obtained using the one-to-one Langmuir binding model with subtraction of the readings from the control channel.

ELAA was another method used to determine the K_D of selected aptamer. The steps are the same as those in Figure 1, except that AP65 was immobilized on the well surface instead of *T. vaginalis* cells (Figure S1). Each well was saturated with AP65 by incubation of 50 μL of 10 μg mL^{-1} in PBS buffer, and both these wells and the uncoated wells were blocked with PBS-Tween. The wells were then thoroughly washed, and following washing, 50 μL of a range of concentrations of aptamer (biotinylated at the 5′ end), prepared in binding buffer, obtained by serial dilution (1 μM, 1/10 dilutions), were added to each of the separate wells. After this was washed three times with binding buffer, 50 μL of a 1/10 000 dilution of 0.50 mg mL^{-1} of streptavidin-HRP, prepared in binding buffer, was added to each well and incubated for 30 min at 25 °C. Following another thorough washing with the binding buffer, 50 μL of TMB was added to each well and allowed to react for 3 min for color development. The reaction was stopped by adding 50 μL of H_2SO_4. The absorbance was read using a SpectraMax 340PC (Molecular Devices) microtiter plate reader at 450 nm, and the data were analyzed using the sigmoidal log-dose−response model of the GraphPad Prism software. The LOD was calculated, defined as the corresponding analyte concentration at the signal value of the blank plus three standard deviations.[40]

SPR with biotinylated aptamer immobilized on the surface of streptavidin-coated chip was also used to analyze the aptamer sequences. Briefly, the surface of a CM5 chip was activated by addition of 30 μL of a 1:1 mixture of EDC (400 mM) and NHS (100 mM) at a flow rate of 5 μL min^{-1}. Streptavidin (200 μg mL^{-1}) was then added, followed by 30 μL of the blocking agent ethanolamine (1 M) and a final wash with 15 μL of 2 M NaCl and 10 mM NaOH at a flow rate of 5 μL min^{-1}. Finally, biotinylated aptamer (5 μM) was injected to the streptavidin-coated chip, and the binding of a range of concentrations of AP65 (serial dilution (1:2) starting with 1 μM) was analyzed as described above.

Biolayer interferometry (BLI) was performed by 2Bind GmBH (http://2bind.de/molecularinteractions/) and was performed with an Octet K2 BLI instrument (Pall Forte Bio) based on biotin−streptavidin interactions. Biotinylated aptamer (AP65_A1) was immobilized on high-sensitivity streptavidin-activated biosensors (SAX) at a concentration of 3 μg mL^{-1} in binding buffer. Following immobilization, the sensors were blocked with biocytin (10 μg mL^{-1}). A serial dilution of AP65 was prepared in assay buffer (PBS + 1,5 mM $MgCl_2$, pH 6.0, 0.05% Tween-20), ranging from 800 to 50 nM. The samples were analyzed at 30 °C. As a control reference, a sample without AP65 was used. The binding was analyzed through the signal shift in nanometers, and data were fitted globally to a 1:1 binding model.

ELAA for the detection of AP65 and *T. vaginalis* cells. The ELAA method was tested to analyze different concentrations of AP65 and *T. vaginalis* cells. AP65 (0.02−18 nM) was immobilized in individual wells of a microtiter plate, and 20 nM of biotinylated AP65 aptamer with 1/10 000 dilution of 0.50 mg mL^{-1} of streptavidin-HRP in binding buffer was added (Figure S1). Three-day old *T. vaginalis* culture cells were harvested by centrifugation at 10 000 rpm for 2 min and washed twice with PBS buffer. Fifty microliters of different concentrations of *T. vaginalis* cells ((65−335) × 10^4 cells/mL) were prepared by serial dilution (1:2). The *T. vaginalis* cell concentration was determined by direct counting using a hemocytometer. Detection was performed with biotinylated aptamer and streptavidin-HRP as described above (Figure 1).

Enzyme Linked Immunoassay. A standard ELISA method (Figure S2) was performed using the commercially available polyclonal antibody. Following the immobilization of AP65 by incubation of 50 μL of 10 μg mL^{-1} in PBS buffer, a range of concentrations of polyclonal antibody was added (serial dilution (1:10), starting with 1 μM). After the samples were incubated and washed, 50 μL of a 1/10 000 dilution of 1 mg mL^{-1} of antirabbit IgG HRP in PBS was added. The experiment was performed in parallel with and in the same manner as the ELAA to compare aptamer and antibody binding affinities.

Cross-Reactivity to Enteric and Urogenital Microorganisms. The indirect ELAA was tested on culture isolates of the *T. vaginalis* and other enteric and urogenital tract microorganisms including *Bacillus subtilis, Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Neisseria gonorrhea, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica*, and *Staphylococcus aureus*. The *T. vaginalis* isolate used in the study is a long-term culture of the Pathogen-Host-Environment Interactions Research Laboratory (PHEIRL) at the Natural Sciences Research Institute of the University of the Philippines Diliman. It was isolated from a female sex worker in 2013 and has been maintained in complete BI-S-33 medium. The identification of the isolate was by culture/wet-mount method and confirmed by 18S rDNA sequencing. The *C. albicans* and *N. gonorrhoeae* were clinical isolates donated by the late Dr. D. L. Valle, Jr. of the Makati Medical Center, Philippines. Similarly, the bacterial strains used in the study were maintained in the general bacterial culture medium tryptic soy broth.

Seventy-two hours old *T. vaginalis* culture and twenty-four hours old *C. albicans* and bacterial cultures were harvested by centrifugation at 10 000 rpm for 2 min and washed twice with PBS buffer. The concentration of bacterial samples used was standardized using 0.5% v/v McFarland buffer, and the concentrations of cells of *T. vaginalis* and *C. albicans* were determined using heamocytometer. Fifty microliters of microbial samples was used to coat individual wells of a microtiter plate. Fifty microliters of 20 nM of biotinylated aptamer was used. A negative aptamer control was also included to determine if there is significant nonspecific binding of the streptavidin-HRP to the microbial samples.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00065.

Indirect ELAA for the detection of AP65 immobilized on the surface of microtiter plate; indirect ELISA for the detection of AP65 immobilized on the surface of microtiter plate; absorbance readings of biotinylated AP65_A1 aptamer /polyclonal AP65 antibody binding to AP65 by ELAA/ELISA; absorbance readings of AP65 ELAA/ELISA at different amounts of AP65. Absorbance readings of aptamer interaction with *T. vaginalis* cells immobilized on individual wells of a microtiter plate, with negative control in the absence of *T. vaginalis* cells (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DNA, Deoxyribonucleic acid; SELEX, Systematic Evolution of Ligands by EXponential enrichment; K_D, Dissociation constant; SPR, Surface Plasmon Resonance; ELAA, Enzyme Linked Aptamer Assay; BLI, BioLayer Interferometry; STI, Sexually Transmitted Infection; HIV, Human Immunodeficiency Virus; NAAT, Nucleic Acid Amplification Test; PCR, Polymerase Chain Reaction; AP65, Trichomonas vaginalis Adhesion Protein 65; p-SELEX, Microtiter plate SELEX; GQRS, Quadruplex forming G Rich Sequences; CM5, Carbocymethylcellulose Biacore chip; ELISA, Enzyme Linked ImmunoSorbent Assay; LOD, Limit of Detection; SDS-PAGE, Sodium dodecyl sulfate poly(acrylamide) gel electrophoresis; PBS, Phosphate Buffered Saline; HPLC, High Performane Liquid Chromatography; SA-HRP, Streptavidin-horseradish peroxidase; HRP, Horseradish peroxidase; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, N-Hydrosuccinimide; ssDNA, single-stranded DNA; SAX, streptavidin-activated biosensors.

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