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Development of DNA Aptamers that Detect *Clonorchis Sinensis* Adult Fluke and Egg Proteins by Enzyme-Linked Microplate Assay

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Abstract

Development of DNA aptamers through ten rounds of selection and PCR amplification (SELEX) against recombinant adult Cs44 and Egg proteins of *Clonorchis sinensis* liver flukes is described along with the top three aptamer DNA sequences for each target derived from next generation sequencing. These 6 top candidate aptamer DNA sequences were previously shown to stain *C. sinensis* adult flukes and eggs by confocal fluorescence microscopy and lateral flow assays (Pharmaceuticals 15:693, 2022). Herein these aptamers are shown to detect less than 125 ng of each target *C. sinensis* protein via ELISA-like aptamer-based enzymatic microplate colorimetric assay with no cross-reactivity against bovine serum albumin or two other *Cyclospora* parasite recombinant proteins at 125 ng.

Introduction

Liver fluke infections are relatively common in Asia due to human consumption of raw or undercooked fish. In particular, up to 20 million people in China, N. and S. Korea and Vietnam may be infected at present with *Clonorchis sinensis* [1-3] which can cause chronic inflammation of human bile ducts leading to cholangiocarcinoma [4]. *Opisthorchis viverrini* is another liver fluke species of similar concern for human health that is primarily found in fish in Thailand and other parts of Asia. But, here we focus on *C. sinensis* adult parasites and eggs and their surface (Cs44 and Egg) proteins for initial Enzyme-Linked Immunosorbent Assay (ELISA)-like test development. While there are several commercial ELISA kits and descriptions of antibody-based ELISAs for *C. sinensis* antigens in the academic literature, this is the first report of a DNA aptamer-based ELISA or an "ELASA" (Enzyme-Linked Aptamer Sorbent Assay) alternative to antibody-based ELISAs.

Materials and Methods

Recombinant Proteins

Recombinant Cs44 and egg protein were obtained from Bioclone Inc. (San Diego, CA. USA). Table 1 below gives the amino acid sequences of these recombinant proteins.

Protein Attachment to Magnetic Beads (MBs) and Systematic Evolution of Ligands by EXponential enrichment (SELEX) Aptamer Development

Ten µg of target protein was added to 30 µl of stock tosyl-activated M280 (2.8 µm diameter) MBs from Dynal Corporation (~ 2 X 10^9 MBs per ml stock) in 1 ml of sterile phosphate buffered saline (PBS; pH 7.2). MBs were incubated for 2 hours at 35°C with periodic mixing in an incubator. Magnetic separation was achieved with a Dynal MPC-S magnetic rack. Supernates were carefully siphoned to remove excess target protein with-

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Table 1: Amino acid sequences of recombinant protein targets and controls from Bioclone, Inc.

***Clonorchis sinensis* Cs44 Antigen (a.a.18-274); Cat. # RPL-0146; Swiss-Protein #Q9UB18**
MKFLKLVIIG ALFLNVLCLD GGAQPPKSGA QRPFSHWIAG WFLVPLEVKA SDHF
***Clonorchis sinensis* Egg protein (a.a.17-253); Cat. #RPL-0147; Swiss-Protein #Q8I8I3**
MKPICLLLVG LVSISLTSGY KRGYNFGLED GRVATGRFYR GGYGDATGGE VSGYDYDLEG DLSASGSSAH AGRFGKQRHE EDDGFYTQGG SFYVSGKARR DDGYGITAGL KAKGNFYGTG TEGEGSQYEH VTTFRRGGGH DTKGKKKHYN EYDSYGQAKK YGDKKVANNF DLRGILKAKG KFDGYGKSDV SSEFEKYGKL GYSGSSKGYG GRDVYGKLKG KSEYDAYGKL KGYGSQNDYS
***Cyclospora cayetanensis* TA4 Antigen-like protein; NCBI Reference Sequence: XP_026191783.1**
MRFFGDLKKR RGNGNSDSMK GEGNEAYLYS TGAAGHAGHL AGMSFCGSLG QGSHKYLYCS PNRLQKMAPF SLLSLASASL LLAHGAFAED TSVGTEVDCT TAMNALRKKA GLEAFTIHTS VDAYHLPVGT HLAGDKVTTD KKEEVKELCT KILGNTADTS KRVDGDKVNL VAVQEGLSAD CSAAVDYWRG AFPTFTGKPQ KFTGNQYDGK QVSFLGLLNP KSGASVNCAY YNCAKESGEG SFNGLICRTQ MNVNDGELFT DEQWDKIVQA FDSGAAALPT MMAIGAAFVG LFVY
***Cyclospora cayetanensis* Oocyst Wall Protein-2 or WP2; GenBank: OEH78485**
MVDGLCITPA APVLECPNGY INICKAKDRA ESPCCAKGHT AEKIARCREG MQSQDGYCTS IVAHEAVTEC PAGYALINHG LQCIKQERGQ AAAACVAPDV LSAEGDSCLR TMQQGYEYIC PDEYQCIAYA HTKKKYSPVC SACAKTTEMQ PLCGCPEGQI EVQGYCFEED VYGVCQRHQG MPRKQAPSKR QPVKPTKKGE EAPEPSCSPV GRVSCSCEES YTLHCTSNIC TCINREIIPV VPICRGELDE SGKCMAQVKT PLLYTCAEGF TCDVVNKKGR CHCVRVAIAE PSARCAAGEP HKGKCMEVVR EQKIVECPQG YSETCCDNHC SCTKTHLATR EVKCASGAVS IQGECVYVSQ PSPGCEVVSL AIDLVALSA

Table 2: DNA oligonucleotide primers and SELEX template sequences. mino acid sequences of recombinant protein targets and controls from Bioclone, Inc.

Oligonucleotide	Sequence	Notes
Primer 1 (Forward or F)	5′-ATACGGGAGCCAACACCA-3′	0.6 µM working conc.
Primer 2 (Reverse or R)	5′-ATCCGTCACACCTGCTCT-3′	0.6 µM working conc.
SELEX Template	5′-ATACGGGAGCCAACACCA-**(N)**$_{36}$-TGGTGTTGGCTCCCGTAT-3′	$\mathbf{N}_{36}$ = 36 randomized dNTPs (equal 25% A,C,G, & T)

Note: All oligonucleotides were obtained from Integrated DNA Technologies, Inc. (IDT; Coralville, IA).

out disrupting the concentrated MBs. Protein-coated MBs were blocked with 1 ml of 10% ethanolamine in sterile PBS for 1 hour at 35°C with periodic mixing. Thereafter, protein-coated MBs were resuspened and washed 3 times in 1.5 ml of sterile PBS for 10 minutes per wash with continual mixing. The final purified protein-coated MBs were collected by magnetic separation and stored in a refrigerator until being used.

(Table 2) below gives the 72 base SELEX DNA template with 36 randomized mid-region bases designated as "N" (N = 25% A, 25% C, 25% G and 25% T) and primer DNA sequences that were used for aptamer development.

The SELEX DNA template (72 base randomized library shown in Table 2 above) was dissolved in enough sterile nuclease-free PBS to produce a 100 nanomole/ml concentration. Primers were dissolved in sterile nuclease-free water at 0.6 uM each. The SELEX template solution was added to a sterile microfuge tube and heated at 95°C for 5 minutes to generate a completely Single-Stranded (ss) DNA library for target binding. The hot library template solution was added to 100 µl of Cs44 or Egg protein-coated MBs (~ 2 x 10^7 MBs). The DNA library-target protein-MB suspension was gently mixed at Room Temperature (RT) for 1 hour.

Target-MBs with any bound DNA were collected by magnetic separation and washed in sterile PBS 3 times as before. Following the third wash, the DNA-target-MB suspension was heated at 95°C for 5 minutes in 100 µl of sterile nuclease-free deionized water to release bound DNA and the hot supernate containing aptamer DNA was collected. The supernate was allowed to cool to RT and 20 µl of supernate was used to prepare several 50 µl tubes for Polymerase Chain Reaction (PCR) amplification using 1 minute at 95°C, 1 min at 60°C, 1 minute at 72°C for 30 cycles followed by 72°C for 10 minutes and refrigeration. Five µl of PCR amplicons from each of the PCR amplicon tubes was added to 1 uL of 6X gel loading buffer for agarose gel electrophoresis to verify the presence of the correct length (72 bp) aptamer PCR product in a 2% agarose submarine gel with 3 µl of 10 mg/ml ethidium bromide (EtBr) added to 45 mL of molten gel prior to casting the gel and running it at 100V in cold 1X TAE (Tris-Acetate-EDTA) buffer with 5 µl of an appropriate DNA ladder standard. The gels were then digitally photographed on a UV transilluminator with an orange Wratten filter.

The 72 bp DNA aptamer bands were excised from the gel using a fresh, autoclaved razor blade and eluted overnight at 4°C in a sterile microfuge tube. A Qiagen gel purification kit was used to purify the 72 bp PCR products which were eluted off of the column into 50 µl of elution buffer. To begin the second round and all subsequent rounds (up to round 10) of MB-SELEX, the 4 PCR tubes were pooled and heated at 95°C in sterile deionized nuclease-free water for 5 minutes to release bound DNA (aptamers) from the target protein-coated MBs.

Next Generation Sequencing (NGS)

Following round 10 of MB-SELEX, the Cs44 and Egg protein aptamers were pooled and sent to Base Pair Biotechnologies Inc. (Pearland, TX, USA) for Illumina NGS. Table 3 summarizes the top 3 aptamer DNA sequences for Cs44 and Egg protein. Complete sequencing results are given in the supplemental file.

Aptamer Synthesis and ELASA

Each of the 6 DNA sequences shown in Table 3 was synthesized at Integrated DNA Technologies (IDT) in Coralville, IA, USA) with a 5′-biotin and 6 carbon linker to enable ELISA-like (ELASA) microplate experiments. The ELASA technique has been de-

scribed in detail by the authors in other publications [5,6]. But briefly, the target proteins (recombinant Cs44 and Egg protein) were immobilized in 0.1M sodium bicarbonate buffer (pH 8.5) at the concentrations indicated in the figures or figure legends overnight at 4˚C. The following day, the wells were evacuated and washed three times in 200 µl of PBS, followed by blocking with 200 µl of 2% ethanolamine in PBS for 1 hour, 3 washes in 200 µl of PBS, and addition of 100 µl of 1 mg/ml of each 5'-biotinylated candidate aptamer as shown in the figures for 1 hour with gentle mixing. The wells were decanted and washed 3 more times in 200 µl of PBS plus 0.1% Tween 20 followed by addition of 100 µl of 1 mg/ml streptavidin-horseradish peroxidase conjugate (Thermo Fisher Inc.), followed by 3 more washes in 200 µl of PBS-Tween 20 and addition of 100 µl of KPL ABTS® 1-Component Microwell Peroxidase Substrate from SeraCare/LGC Clinical Diagnostics (Gaithersburg, MD, USA). The green reaction was stopped after 10 minutes by addition of 100 µl of stop solution from SeraCare per well and absorbance was compared visually after digital photography.

Results

Figure 1 depicts an EtBr-stained 2% agarose electrophoresis gel result that validates the expected presence of approximately 72 base pair aptamer amplicons between the 50 and 100 bp ladder standard bands following round 10 of MB-SELEX for both the Cs44 and Egg protein aptamer pools. Table 3 summarizes the top 3 most frequent consensus sequences for the Cs44 and Egg protein designated Cs44 F1-3 and Egg F1, 2 and 4 as shown in the table.

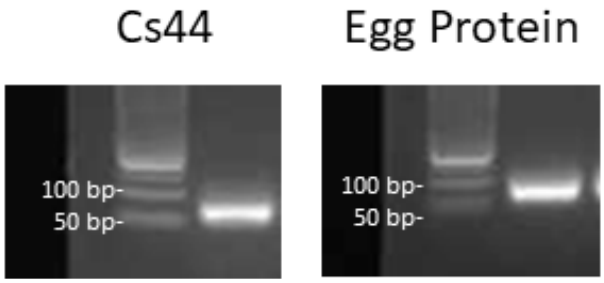

Figure 1: Validation of the round 10 SELEX aptamer amplicon expected sizes (72 bp) in EtBr-stained 2% agarose electrophoresis gels.

Table 2: Most frequent consensus aptamer DNA sequences from Illumina NGS.

Aptamer	DNA Sequence (5' → 3')
Cs44 F1	ATA CGG GAG CCA ACA CCA AAC AGC GAA AAA ATG ACA AGC ACT GCA GTT AAA TAG TCT GGT GTT GGC TCC CGT AT
Cs44 F2	ATA CGG GAG CCA ACA CCA AGT TTA ACA GGG CCA TTT CCT AAG CGG ATT TCC CCC TGG TGT TGG CTC CCG TAT
Cs44 F3	ATA CGG GAG CCA ACA CCA CCA GAT AAC GAA GTG TAA GAA AGC TGA CAT CCT AGG ATG GTG TTG GCT CCC GTA T
Egg F1	ATA CGG GAG CCA ACA CCA CAC AGC AGA CAT AAG GAT TGT AAA TCA CAC GGA TGG TGG TGT TGG CTC CCG TAT
Egg F2	ATA CGG GAG CCA ACA CCA CAC GAT TAC TGT GAC TAG GAC AGC TAT AAC ATG TTG TGG TGT TGG CTC CCG TAT
Egg F4	ATA CGG GAG CCA ACA CCA CTA TAG GGT GTA GCT GAT CCG CTC CCT TCT CCC AGG TGG TGT TGG CTC CCG TAT

Figure 2 illustrates a limit of detection (LOD) ELASA titration (serial two-fold dilutions of the immobilized proteins) compared to blank wells without the recombinant protein targets. The lowest level of recombinant Cs44 or Egg protein analyzed capped at 125 ng, but it is clear that the LOD is lower than that because the green color could be lighter and yet still visible over the nearly clear zero target blanks for all 6 aptamers analyzed.

Figure 3 illustrates very low or no cross-reactivity of the top aptamers for each of these targets versus 125 ng each of immobilized Bovine Serum Albumin (BSA) as well as 2 other recombinant proteins (TA4 Antigen-like and Wall Protein (WP)-2) from the oocysts of the parasite *Cyclospora cayetanensis* [7] based on comparison with the much darker green colors in Figure 2 against the cognate targets also at 125 ng.

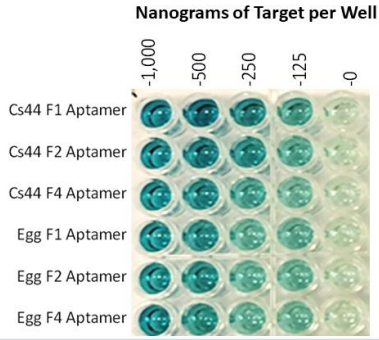

Figure 2: Limit of detection titration of the Cs44 and Egg recombinant proteins from 1,000 to 125 and zero ng per well with each of the respective 6 aptamer candidates.

Discussion

The top anti-*C. sinensis* Cs44 adult and egg protein DNA aptamer sequences listed in Table 3 were part of the SELEX round 10 aptamer pools already demonstrated to stain the respective surfaces of adult *C. sinensis* flukes and eggs by Jilin Univ. in China using confocal fluorescence microscopy with *Fasciola hepatica* sheep fluke negative controls in a previous publication [8]. So, here we have expanded upon those results by adding the identity of the top candidate aptamer DNA sequences, NGS data and demonstrating the top aptamer utility in ELISA-like (ELASA) microplate assays in which at least 125 ng of recombinant Cs44 and Egg proteins were reliably detected with essentially no cross-reactivity with BSA or two different *Cyclospora* recombinant protein targets.

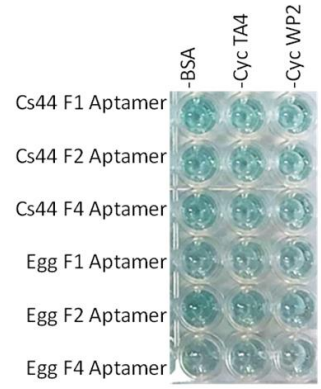

Figure 3: Cross-reactivity results of the 6 aptamer candidates versus Bovine Serum Albumin (BSA) and 2 Cyclospora (Cyc) recombinant antigens from Table 1.

Conclusions

While several commercial and academic ELISA kits for *C. sinensis* exist, the DNA aptamers described herein provide a viable alternative to antibody-based ELISAs for use in an ELASA approach. The ELASA has a LOD of less than 125 ng of *C. sinensis* Cs44 and Egg protein and no visible cross-reactivity with BSA or the 2 *Cyclospora* proteins tested. In addition, revelation of the aptamer DNA sequences and raw NGS data in the supplemental files will enable the broader scientific community to conduct more experiments in this important diagnostic area to hopefully improve liver fluke detection in environmental and perhaps human patient samples.

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