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Saúde

Flavivirus NS1 protein: selection of a capture DNA aptamer using CE-SELEX

Proteína NS1 de Flavivírus: Seleção de um aptâmero de DNA de captura por CE-SELEX

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Abstract: The clinically important viruses in the genus *Flavivirus* include Zika virus (ZIKV), dengue virus (DENV), and yellow fever virus (YFV). Accurately diagnosing flaviviral infection is challenging, especially in regions where multiple flaviviruses co-circulate. Flavivirus nonstructural protein 1 (NS1) is a glycoprotein of approximately 48 kDa whose presence in patient sera is an early marker of infection. The aim of this study was to select a capture aptamer for flavivirus NS1 proteins capable of binding with high affinity to the NS1 in all medically important flaviviruses, with the goal of applying it in aptamer-based diagnostic assays. Aptamer selection was performed using a variation of the SELEX technique, termed CESELEX, which uses capillary electrophoresis (CE) in the separation step. ssDNA sequences obtained after the third CESELEX cycle were sequenced. Quantitative real-time polymerase chain reaction (qPCR) was used to quantify DNA in affinity and specificity assays. An aptamer (Flav8) with high affinity (Kd = 3.58 nM ± 0.22) was obtained that could bind to the NS1 proteins of YFV, ZIKV, and DENV (serotypes 1, 2, 3, and 4) equally. The Flav8 aptamer, owing to its high affinity and ability to recognize the NS1 of all clinically important flaviviruses, is suitable as a capture aptamer for use in Enzyme-Linked Oligonucleotide Assay (ELONA) sandwiches or similar assays.
Keywords: Aptamer, NS1 protein, flavivirus, diagnosis, CE-SELEX.

Resumo: Os vírus clinicamente importantes do gênero Flavivirus incluem o vírus Zika (ZIKV), o vírus da dengue (DENV) e o vírus da febre amarela (YFV). Diagnosticar com precisão a infecção por flavivírus é um desafio, especialmente em regiões onde ocorre a superposição da circulação de diferentes espécies. A proteína não estrutural 1 dos flavivírus (NS1) é uma glicoproteína de aproximadamente 48 kDa cuja presença no soro dos pacientes é um marcador precoce de infecção. O objetivo deste estudo foi selecionar um aptâmero de captura capaz de se ligar com alta afinidade à NS1 em todos os flavivírus clinicamente importantes, com o objetivo de aplicá-lo em ensaios diagnósticos baseados em aptâmeros. A seleção do aptâmero foi realizada utilizando uma variação da técnica SELEX, denominada CE-SELEX, que utiliza eletroforese capilar (CE) na etapa de separação. As sequências de ssDNA obtidas após o terceiro ciclo de CE-SELEX foram sequenciadas. A reação em cadeia da polimerase quantitativa em tempo real (qPCR) foi usada para quantificar o DNA em ensaios de afinidade e especificidade. Foi obtido um aptâmero (Flav8) com alta afinidade (Kd = 3,58 nM ± 0,22) capaz de se ligar às proteínas NS1 de YFV, ZIKV e DENV (sorotipos 1, 2, 3 e 4). O aptâmero Flav8, devido à sua alta afinidade e capacidade de reconhecer a NS1 de todos os flavivírus clinicamente importantes, é adequado para o uso como um aptâmero de captura de ensaios sanduíches em técnicas como ELONA (Enzyme-Linked Oligonucleotide Assay) ou similares.
Palavras-chave: Aptâmero, proteína NS1, flavivírus, diagnóstico, CE-SELEX.

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INTRODUCTION

Mosquito-transmitted viruses pose significant public health problems in several subtropical and tropical countries. Fifty-three virus species that are transmitted by mosquitoes belong to the genus *Flavivirus*. Among them, clinically important viruses in this genus include Zika virus (ZIKV), dengue virus (DENV), and yellow fever virus (Hitakarun et al., 2020).

In Brazil, the diagnosis of flaviviral infection is challenging because more than one flavivirus cocirculates and vaccination against YFV is extensive. During the initial stages and mild and medium phases of symptoms, DENV, ZIKV, and YFV infections exhibit very similar clinical presentations, including headaches, rash, fever, and fatigue, making it challenging to distinguish between them. Moreover, traditional serological diagnostic approaches also show relatively high antigenic cross-reactivity. Molecular techniques have been successfully applied for diagnosing flaviviral infections, offering advantages such as speed, sensitivity, and virus-specific identification. However, these techniques require professional expertise and expensive laboratory equipment and reagents, which are often not available in endemic regions (Moreli & Da Costa, 2013). Therefore, simpler, cheaper, and more accurate diagnostic methods are required.

Nucleic acid aptamers are single-stranded DNA or RNA oligonucleotides that bind target molecules with high affinity and specificity. Aptamers are selected using an *in vitro* selection process termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX). SELEX is performed using selection cycles comprising target incubation with a random synthetic oligonucleotide library, partitioning, and amplification by polymerase chain reaction (PCR) (Tuerk & Gold, 1990). Usually, 6–15 rounds are reported to enable the selection of the best binders. Their low immunogenicity, batch-to-batch reproducibility, capacity for chemical modification, small size, and low production cost have enabled aptamers to replace antibodies in many applications. Consequently, aptamer-based approaches are being extensively investigated for diagnosing several viral diseases (Argondizzo et al., 2020; Chakraborty et al., 2022; Lou et al., 2022; Wandtke et al., 2022).

Flaviviruses are enveloped viruses with a positive-sense RNA genome that encodes seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) and three structural proteins (C, M, and E). Flavivirus nonstructural protein 1 (NS1) is a glycoprotein (with a molecular weight of approximately 48 kDa) found in the inner and outer membranes of infected cells and is secreted into the extracellular space. The presence of NS1 in serum is an early marker of infection in patients with DENV, ZIKV, and YFV (Lee & Zeng, 2017; Mok et al., 2023; Thevendran et al., 2023).

The objective of this study was to select a capture aptamer for flavivirus NS1 proteins capable of binding with high affinity to the NS1 protein of all medically important flaviviruses, with the goal of applying it in aptamer-based diagnostic assays. The capture aptamer functions by attaching the NS1 protein present in a biological sample to a substrate. Following this, another aptamer or antibody capable of distinguishing between the NS1 of different flaviviruses (DENV, ZIKV, and YFV) can be added for differential diagnosis.

Aptamer selection was performed using CESELEX, a variation of the SELEX technique that utilizes capillary electrophoresis (CE) to separate oligonucleotides that bind to the target during the separation step (Mendonsa & Bowser, 2004).

MATERIAL AND METHODS

NS1 Protein

Recombinant yellow fever virus NS1 protein expressed in mammalian HEK293 cells (Native Antigen Company, UK) was used.

Oligonucleotide Library Design

A random oligonucleotide library was purchased from IDT DNA Technologies (USA). The 80-mer library sequence (Berezovski et al., 2006) is as follows: *5´*-CTTCTGCCCGCCTCCTTCC-(39N)-GGAGACGAGATAGGCGGACACT-*3´*, where the central 39N represents the random part of the sequence.

CE-SELEX

Three CE-SELEX selection cycles were performed. Initially, 800 µM of ssDNA was heated at 95 °C for 5 min, snap-cooled on ice for 15 min, and left at room temperature for 10 min. Subsequently, it was incubated with 80 pmol of YFV NS1 protein in 60 mM of borate buffer (Sigma-Aldrich, USA) for 30 min at 25 °C under shaking. A sample of the incubation solution was subjected to CE separation using a neutral capillary in a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., USA). The mixture was separated under reverse polarity (26 kV) in borate buffer at 25 °C and monitored at 214 nm.

The NS1-aptamer complexes were eluted at different positions in relation to the oligonucleotide library and collected in a vial containing 8 µL of separation buffer. After collecting material from five runs, the bound sequences were amplified using PCR. The PCR amplification was performed using 0.2 µM forward primer (*5´*-CTTCTGCCCGCCTCCTTCC-*3´*), 0.2 µM reverse primer (*5´*-AGTGTCCGCCTATCTCGTCTCC-*3´*), 1 U Taq DNA polymerase (Invitrogen, USA), 50 mM $MgSO_4$ in 1× PCR buffer (Invitrogen), and a reaction master mix containing 200 µM dNTPs. The PCR thermocycling parameters were as follows: 94 °C for 2 min for denaturation, followed by 17 cycles of denaturation at 94 °C for 15 s, annealing at 72 °C for 30 s (-1 °C per cycle), and an extension at 68 °C for 30 s. Subsequently, 14 cycles were completed, involving denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and an extension at 68 °C for 30 s. Following the last cycle, a final extension step was performed at 68 °C for 2 min.

The single-stranded DNA of interest was obtained from the conventional PCR products using asymmetric PCR. This was achieved using a Master Mix reaction kit containing 0.2 µM forward primer, 0.02 µM reverse primer, 200 µM dNTPs, 1 U Taq DNA polymerase (Invitrogen), and 50 mM $MgSO_4$ in 1× High Fidelity PCR buffer (Invitrogen). The PCR thermocycling conditions are described above. Conventional and asymmetric PCR were performed using a Veriti Thermal Cycler (Applied Biosystems, USA). PCR amplification was verified by electrophoresis on 2% agarose gels (in Tris-borate-EDTA buffer) stained with ethidium bromide and imaged using a UV transilluminator BioDoc-It® Imaging Systems (Analytik Jena US LLC,USA).

Aptamer Sequencing

The ssDNA sequences obtained after the third cycle of the CE-SELEX procedure were sequenced on an Illumina MiSeq platform (Illumina Inc., USA). Sequencing and bioinformatic analyses were performed using GenXPro GmbH (Germany). Post-sequencing analyses were performed using the Galaxy server available at https://usegalaxy.org/ (Thiel, 2016).

Aptamer similarity analysis and secondary structure prediction

The phylogenetic tree of the selected aptamers was constructed using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993). The initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with the highest log likelihood value. All analyses were performed using MEGA 11 software under default parameters (Tamura et al., 2021), available at https://www.megasoftware.net/. The predicted secondary structure and Gibbs free energy of the selected aptamers were determined using the Mfold web server, available at http://unafold.rna.albany.edu (Zuker, 2003); under room temperature (25 °C) and 60 mM Na^+ conditions.

Equilibrium Dissociation Constant (Kd) Determination

The YFV NS1 protein (40 pmol) was incubated with different aptamer concentrations in 60 mM borate buffer at 25 °C for 30 min under shaking. The sequences bound to the target were separated from the unbound sequences through filtration using Amicon 0.5 mL Filters for DNA Purification and Concentration – 50K (Merck, Germany), according to the manufacturer's instructions (Ramos et al., 2007).

The amount of bound sequences in the samples was quantified by qPCR using Go-Taq® qPCR Master Mix (Promega, USA), according to the manufacturer's instructions. The amplification parameters were as follows: 95 °C for 2 min for

denaturation, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing at 60 °C for 1 min, and a final step at 95 °C for 15 s, followed by the melting stage. The reactions were performed using a qTOWER3 Real-time Thermal Cycler (Analytik Jena, Germany). A standard curve was constructed for each aptamer ranging from 0.05 ng to 0.00005 ng, with a dilution factor of 1:50. The samples were processed in triplicates.

The aptamer mass (ng) in each elution sample was calculated. The results were used to plot saturation curves. Data were analyzed by nonlinear regression analysis (one-site-specific binding with Hill slope) using GraphPad Prism 5 software (GraphPad Software, USA).

Specificity Assay

50 nM of the aptamer in the selection buffer was incubated with 30 pmol of each NS1 protein (DENV serotypes 1, 2, 3, 4, YFV, or ZIKV) in 60 mM borate buffer for 30 min, under shaking, at 25 °C. Bound and unbound sequences were separated as previously described by filtration using Amicon 0.5 mL filters. The number of bound sequences in the samples was quantified using qPCR, as previously described**.**

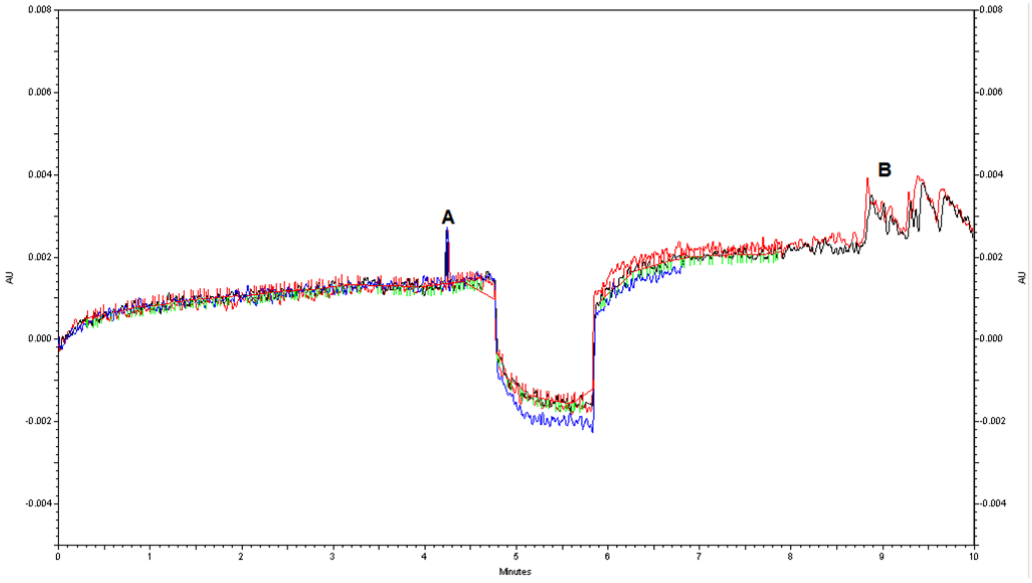
RESULTS

CE-SELEX was used for aptamer selection in the present study. An illustrative CE electropherogram is shown in **Fig. 1**. The elution conditions used allowed the separation of peaks corresponding to oligonucleotides that did not bind to the target (as indicated by peak B) and NS1-aptamer complexes (as indicated by peak A), allowing them to be collected individually. Four runs were performed to accumulate oligonucleotides for the next PCR amplification step, and these runs were overlaid and presented in Figure 1. After the third selection cycle, the obtained ssDNA pools were sequenced.

Figure 2 shows a phylogenetic tree of the 27 most prevalent sequences obtained during the sequencing step. Seven sequences were chosen for further characterization based on the number of reads obtained in the sequencing, the lowest ΔG value at 25 °C, and their distance in the phylogenetic tree.

Figure 1

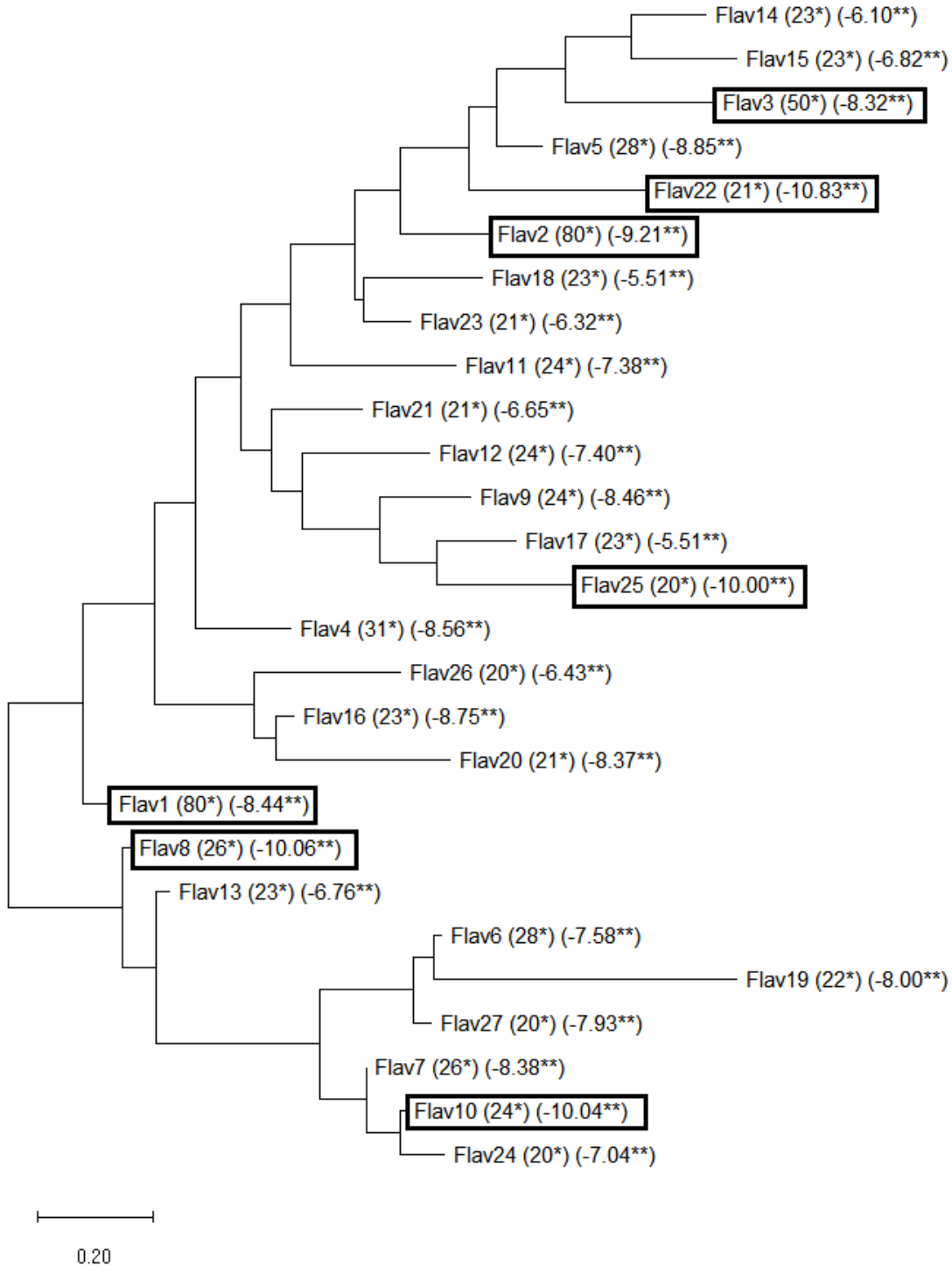
Illustrative electropherogram of CE-SELEX separation



Note: Peak A corresponds to the NS1-aptamer complexes, while peak B represents oligonucleotides that did not bind to the target. Four runs, displayed using distinctly colored lines, were performed to accumulate sufficient DNA for PCR amplification. The runs are overlaid on the graph. The *x* axis on the graph represents minutes, while the *y* axis represents absorbance units (A.U.)

Figure 2

Phylogenetic tree of selected aptamers

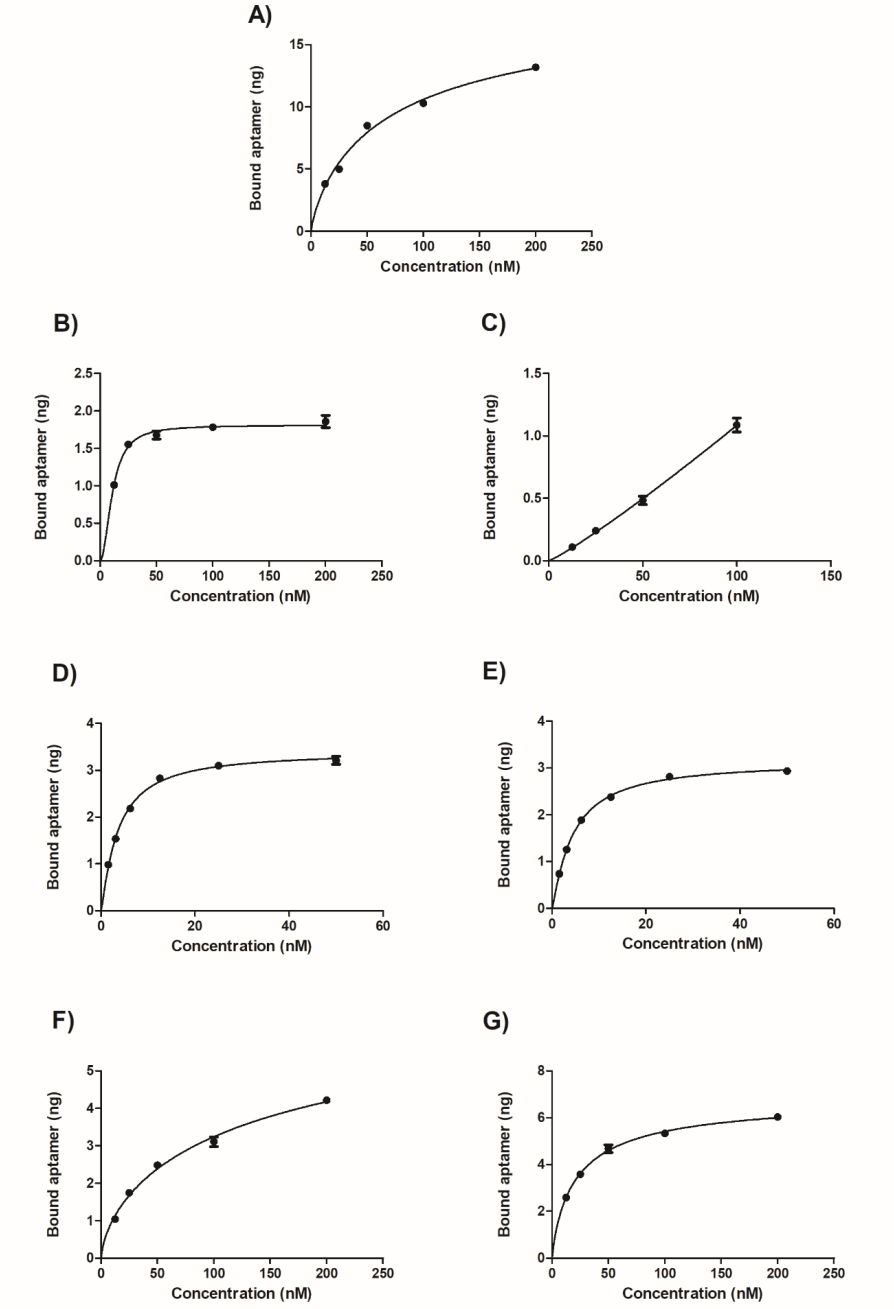


Note: The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 27 nucleotide sequences. A total of 81 positions were used in the final dataset. The chosen sequences are highlighted in boxes. Evolutionary analyses were conducted using MEGA 11 software. (*Number of reads, **Gibbs free energy of the secondary structure).

The affinity of the aptamer for the target is represented by the equilibrium dissociation constant (Kd). The dissociation constants of the seven selected aptamers were calculated using saturation binding assays. Specific binding curves representing the quantity (ng) of aptamers bound to the NS1 protein relative to the corresponding aptamer concentration are shown in Figure 3. For Flav3 (C), it was not possible to obtain a saturation curve, indicating low or no affinity for the target. Flav8 (D) and Flav10 (E) aptamers exhibited the best affinity, with Kd values of 3.58 ± 0.22 nM and 4.42 ± 0.19 nM, respectively. The remaining Kd values in ascending order were: 11.01 ± 0.69 nM (Flav2, B), 22.42 ± 2.78 nM (Flav25, G), 76.99 ± 31.1 nM (Flav1, A), and 176.3 ± 25.9 nM (Flav22).

Figure 3

Dissociation constant (Kd) determination of NS1 aptamers

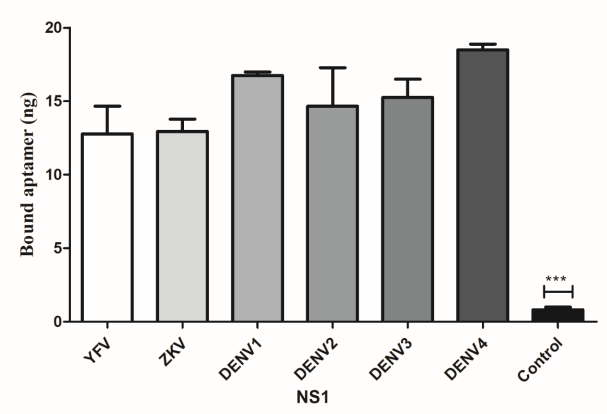


Note: The saturation curve was obtained by plotting the aptamer concentration (nM) versus the total ssDNA (ng) bound to NS1 protein. Protein-bound aptamer was quantified using a quantitative real-time polymerase chain reaction (qPCR). Non-linear regression analysis (one-site-specific binding with hill slope (GraphPad Prism 5) was used to determine the Kd. **A)** Flav1 (76.99 ± 31.1 nM); **B)** Flav2 (11.01 ± 0.69 nM); **C)** Flav3 (undetermined); **D)** Flav8 (3.58 ± 0.22 nM); **E)** Flav10 (4.42 ± 0.19 nM); **F)** Flav22 (176.3 ± 25.9 nM); and **G)** Flav25 (22.42 ± 2.78 nM).

A specificity assay **(Fig. 4)** was performed for the aptamer with the highest affinity (Flav8), which revealed that Flav8 could bind equally to the NS1 proteins of DENV (serotypes 1, 2, 3, and 4), ZIKV, and YFV.

Figure 4

Specificity assay



Note: The binding of the Flav8 aptamer to flavivirus NS1 proteins was assessed using qPCR. The graph shows the amount of ssDNA (ng) recovered after incubating 50 nM of Flav8 aptamer with 30 pmol of each protein. Statistical analyses were performed using GraphPad Prism 5 software (**p < 0.01). The control represents the Flav8 aptamer without NS1 protein incubation.

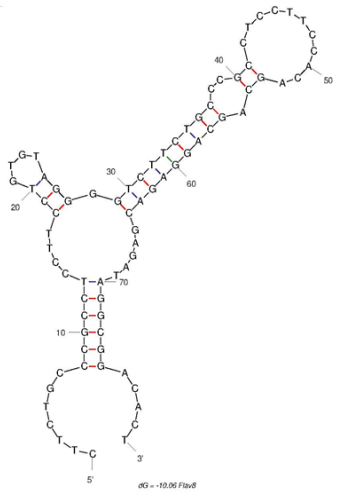
These results demonstrate that Flav8 is suitable as a capture aptamer for use in enzyme-linked oligonucleotide assay (ELONA) sandwiches or similar assays, owing to its high affinity and ability to recognize the NS1 protein of all clinically important flaviviruses. The Flav8 sequence and its predicted secondary structure are shown in Figure 5.

Figure 5

The Flav8 sequence and its predicted secondary structure

A) **5'CTTCTGCCCGCCTCCTTCC**TGTGTAGGGGTCTTCTGCCCGCCTC
CTTCCACAGCAGCA**GGAGACGAGATAGGCGGACACT3'**

B)



Note: **(A)** The Flav8 aptamer sequence with the conserved regions shown in bold. **(B)** The secondary structure of the Flav8 aptamer predicted using the Mfold server (ΔG = -10.06 kcal/mol).

DISCUSSION

Nucleic acid aptamers are usually developed *in vitro* using a molecular evolution process termed SELEX, which is based on iterative selection-amplification steps (Tuerk & Gold, 1990). This methodology involves exposing a chemically synthesized library of random sequences (10^{14}–10^{15} sequences) to the target molecule, enabling the interaction of all target-binding oligonucleotides. The next step involves separating sequences that bind to the target from the unbound sequences for subsequent PCR amplification. Following this, the PCR products are exposed to the target again. The selection, separation, and amplification steps comprise one SELEX round. After the last round, the PCR products are cloned and sequenced to identify the binding sequences (Wang et al., 2019).

CE-SELEX is a powerful SELEX variant in which capillary electrophoresis (CE) is used to separate the oligonucleotides that bind to a target from free oligonucleotides (Mendonsa & Bowser, 2004) based on the difference in mobility between these molecules under specific conditions. CE separations are based on the presence of an electrically induced flow, called electroosmotic flow (EOF). The capillary is filled with a buffer solution and its ends are immersed in the same buffer. An electric field is applied at the ends, generating current inside the capillary with consequent movement of the analytes towards the electrodes. It is essential to standardize CE conditions so that free and bound oligonucleotides elute in separate positions in the electropherogram, allowing their collection for subsequent PCR amplification. To this end, variables such as type of capillary, buffer, pH and polarity (standard or reverse) can be explored. In the present study, the best results were obtained using a neutral capillary associated with reverse polarity in borate buffer.

The CE technique has advantages such as speed, versatility, low cost per analysis, high separation power and minimum consumption of samples and solvents. However, the technique is not suitable for use with non-polar and volatile compounds. During the CE process, there is no steric impairment for the aptamers to bind to the target because the molecules remain in solution throughout the separation (Gao et al., 2020). Moreover, the protein targets retain their native conformations. The main advantage of CE separation is that high-affinity aptamers can be obtained in only three to five selection rounds (Mendonsa & Bowser, 2004). In this study, high-affinity aptamers were obtained using only three CE-SELEX rounds, thus demonstrating their efficiency.

The diagnosis of flaviviral diseases can be difficult due to several factors: presence of clinically asymptomatic or oligosymptomatic disease; clinical similarity with other diseases; phylogenetic cross-reactions that can occur between serological tests (especially in endemic regions) and difficulty in accessing reference laboratories that can perform differential diagnosis. Specificity is one of the main problems related to the diagnosis of flavivirus infection.

In the clinical setting, early diagnosis of flavivirus infection would be more effective for controlling the epidemic and for timely treatment. Reverse transcription PCR (RT-PCR) has become the gold standard for molecular detection of viruses due to its high selectivity and relatively high sensitivity. However, elaborate laboratories and highly qualified specialists are required, available only in large medical centers and central laboratory facilities. RT-PCR has

also some inherent disadvantages, including false-negative results from new strains or false-positive results due to sample contamination. Finally, molecular diagnosis is only possible in the acute phase of the disease (Moreli & Da Costa, 2013). The development of portable reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) devices are promising efforts for molecular diagnostics in the field, but applications of these assays remain limited. For these reasons, serological assays continue to be important alternatives for detection, particularly when focusing on field-ready assays (Varghese et al., 2023). However, serological tests are often unable to specifically identify viruses of the same genus, particularly in the case of immunoglobulin G (IgG), not allowing a specific diagnosis.

Antibodies are regularly used in current diagnostic strategies for NS1 detection (Hermann et al., 2014). Compared to antibodies, aptamers have lower production costs, reversible denaturing properties, and are stable at room temperature for long-term storage. In addition, aptamers typically do not exhibit batch-to-batch variation. The drawbacks associated with antibodies have motivated efforts to shift towards NS1 protein-aptamer-based diagnostics. The advantages of aptamers over antibodies allow them to serve as molecular recognition elements in various diagnostic platforms (Argondizzo et al., 2020; Chakraborty et al., 2022; Lou et al., 2022; Wandtke et al., 2022).

ELONA is a bioassay that employs oligonucleotides, such as aptamers, instead of antibodies to quantify a target using conventional plate readers (Drolet et al., 1996). The ELONA sandwich is a variation of this technique that uses a pair of aptamers, one for capture and the other for detection. Essentially, the capture aptamer retains and concentrates the protein target on the plate, and the detection aptamer detects and identifies the immobilized protein (Lee & Zeng, 2017; Mok et al., 2023). Although a similar diagnosis can be performed using the sandwich ELISA (Enzyme-Linked Immunosorbent Assay), once specific antibodies have been obtained, the use of aptamers is more promising for the reasons discussed above.

In a previous study using CE-SELEX, our research group selected the ZIKV60 detection aptamer (Almeida et al., 2022). ZIKV60 exhibited high binding affinity to the ZIKV NS1 protein (Kd = 2.28 ± 0.28 nM) and could distinguish between ZIKV NS1 and NS1 from DENV (serotypes 1, 2, 3, and 4) and YFV. To achieve this specificity, counter-selection steps (negative selection) were included for NS1 proteins from other flaviviruses. The aim of the present study was to obtain a capture aptamer for use in combination with ZIKV60 in an ELONA sandwich assay for ZIKV diagnosis. The Flav8 aptamer that was successfully obtained met the affinity and specificity requirements for this application. Currently, we are in the process of selecting detection aptamers for YFV NS1 and DENV NS1 (serotypes 1, 2, 3, and 4) for use with Flav8 and ZIKV60 in a single diagnostic test kit capable of distinguishing between all the aforementioned diseases.

CONCLUSION

The present study presents an important step towards the development of a practical diagnostic test for the differential diagnosis of clinically relevant flaviviruses, which is vital for the adequate management of these infectious diseases.

REFERENCES

Almeida, N. B. F., De Sousa, T. A. S. L., Santos, V. C. F. D., Lacerda, C. M. S., Silva, T. G., Grenfell, R. F. Q., Plentz, F., & De Andrade, A. S. R. (2022, September 2). DNA aptamer selection and construction of an aptasensor based on graphene FETs for Zika virus NS1 protein detection. *Beilstein J. Nanotechnol.*, *13*, 873-881. https://doi.org/10.3762/bjnano.13.78

Argondizzo, A. P. C., Silva, D., & Missailidis, S. (2020, December 26). Application of Aptamer-Based Assays to the Diagnosis of Arboviruses Important for Public Health in Brazil. *Int. J. Mol. Sci., 22*(1), 159. https://doi.org/10.3390/ijms22010159

Berezovski, M. V., Musheev, M. U., Drabovich, A. P., Jitkova, J., & Krylov, S. N. (2006, August 1). Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides. *Nature Protocols, 1*, 1359-1369. https://doi.org/10.1038/nprot.2006.200

Chakraborty, B., Das, S., Gupta, A., Xiong, Y., T-v, V., Kizer, M. E., Duan, J., Chandrasekaran, A. R., & Wang, X. (2022, February 28). Aptamers for Viral Detection and Inhibition. *ACS Infectious Diseases*, *8*(4), 667-692. https://doi.org/10.1021/acsinfecdis.1c00546

Drolet, D., Moon-McDermott, L., & Romig, T. S. (1996, August 1). An enzyme-linked oligonucleotide assay. *Nature Biotechnology, 14*, 1021-1025. https://doi.org/10.1038/nbt0896-1021

Gao, T., Luo, Y., Li, W., Cao, Y., & Pei, R. (2020, January 1). Progress in the isolation of aptamers to light-up the dyes and the applications. *Analyst, 3*(145), 701-718. https://doi.org/10.1039/c9an01825e

Hermann, L., Thaisomboonsuk, B., Poolpanichupatam, Y., Jarman, R. G., Kalayanarooj, S., Nisalak, A., Yoon, I. K., & Fernandez, S. (2014, October 2). Evaluation of a Dengue NS1 Antigen Detection Assay Sensitivity and Specificity for the Diagnosis of Acute Dengue Virus Infection. *PLoS Negl Trop Dis 8*(10): e3193. https://doi.org/10.1371/journal.pntd.0003193

Hitakarun, A., Khongwichit, S., Wikan, N., Roytrakul, S., Yoksan, S., Rajakam, S., Davidson, A. D., & Smith, D. R. (2020, January 30). Evaluation of the antiviral activity of orlistat (tetrahydrolipstatin) against dengue virus, Japanese encephalitis virus, Zika virus and chikungunya virus. *Scientific Reports, 10*(1499). https://doi.org/10.1038/s41598-020-58468-8

Lee, K. H., & Zeng, H. (2017, November 21). Aptamer-Based ELISA Assay for Highly Specific and Sensitive Detection of Zika NS1 Protein. *Analytical Chemistry, 89*(23). https://doi.org/10.1021/acs.analchem.7b02862

Lou, B., Liu, Y., Shi, M., Chen, J., Li, K., Tan, Y., Chen, L., Wu, Y., Wang, T., Liu, X., Jiang, T., Peng, D., & Liu, Z. (2022, December 1). Aptamer-based biosensors for virus protein detection. *TrAC Trends in Analytical Chemistry, 157*. https://doi.org/10.1016/j.trac.2022.116738

Mendonsa, S. D., & Bowser, M. T. (2004, August 12). In Vitro Selection of High-Affinity DNA Ligands for Human IgE Using Capillary Electrophoresis. *Analytical Chemistry, 76*(18). https://doi.org/10.1021/ac049857v

Mok, J., Kim, E., Kang, M., Jeon, J., & Ban, C. (2023, February 1). *Development of an optical sandwich ELONA using a pair of DNA aptamers for yellow fever virus NS1*. Talanta. https://doi.org/10.1016/j.talanta.2022.123979

Moreli, M. L., & Da Costa, V. G. (2013, January 1). A systematic review of molecular diagnostic methods for the detection of arboviruses in clinical specimens in Brazil and the importance of a differential diagnosis. *Virology Discovery*, *1*(1). https://doi.org/10.7243/2052-6202-1-1

Ramos, E., Piñeiro, D., Soto, M., Abánades, D. R., Martín, M. E., Salinas, M., & González, V. (2007, May 1). A DNA aptamer population specifically detects Leishmania infantum H2A antigen. *Laboratory Investigation*.

Tamura, K., & Nei, M. (1993, May 1). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10*(3), 512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tamura, K., Stecher, G., & Kumar, S. (2021, April 23). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution, 38*(7), 3022-3027. https://doi.org/10.1093/molbev/msab120

Thevendran, R., Rogini, S., Leighton, G. J. T., Mutombwera, A., Sarah, S., Tang, T., & Citartan, M. (2023, May 15). The Diagnostic Potential of RNA Aptamers against the NS1 Protein of Dengue Virus Serotype 2. *Biology 2023, 12*(5), 722. https://doi.org/10.3390/biology12050722

Thiel, W. H. (2016, January 1). Galaxy Workflows for Web-based Bioinformatics Analysis of Aptamer High-throughput Sequencing Data. *Molecular Therapy – Nucleic Acids, 5*, e345. https://doi.org/10.1038/mtna.2016.54

Tuerk, C., & Gold, L. (1990, August 3). Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase. *Science, 249*(4968), 505-510. https://doi.org/10.1126/science.2200121

Varghese, J., De Silva, I., & Millar, D. S. (2023). Latest Advances in Arbovirus Diagnostics. *Microorganisms, 11*(5), 1159. https://doi.org/10.3390/microorganisms11051159

Wandtke, T., Wędrowska, E., Szczur, M., Przybylski, G., Libura, M., & Kopiński, P. (2022, January 26). Aptamers—Diagnostic and Therapeutic Solution in SARS-CoV-2. *Int. J. Mol. Sci. 2022, 23*(3), 1412. https://doi.org/10.3390/ijms23031412

Wang, T., Chen, C., Larcher, L. M., Barrero, R. A., & Veedu, R. N. (2019, January 1). Three decades of nucleic acid aptamer technologies: Lessons learned, progress and opportunities on aptamer development. *Biotechnology Advances, 37*(1), 28-50. https://doi.org/10.1016/j.biotechadv.2018.11.001

Zuker, M. (2003, July 1). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research, 31*(13), 3406-3415. https://doi.org/10.1093/nar/gkg595