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Complex target SELEX-based identification of DNA aptamers against *Bungarus caeruleus* venom for the detection of envenomation using a paper-based device

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ABSTRACT

Complex target SELEX always have been an intriguing approach to the scientific community, as it offers the potential discovery of novel biomarkers. We herein successfully performed SELEX on *Bungarus caeruleus* venom to develop a panel of highly affine aptamers that specifically recognizes the *B. caeruleus* (common krait) venom and was able to discriminate the *B. caeruleus* venom from Cobra, Russell's, and Saw-scaled viper's venom. The aptamers generated against the crude venom also lead to the identification of the specific component of the venom, which is β-Bungarotoxin, a toxin uniquely present in the *B. caeruleus* venom. The best performing aptamer candidates were used as a molecular recognition element in a paper-based device and were able to detect as low as 2 ng krait venom in human serum background. The developed aptamer-based paper device can be used for potential point-of-care venom detection applications due to its simplicity and affordability.

1. Introduction

The development of aptamers against complex targets or disease conditions where specific biomarkers are not known has always been an intriguing challenge for the scientific community (Shamah et al., 2008). Complex targets, like cells, with a myriad of possible targets expressed on their membranes (Takahashi, 2018), or heterogeneous mixture of proteins like secretome of cancer cells, infectious pathogens (Bonin-Debs et al., 2004; Ranganathan and Garg, 2009) and/or snake venoms (Tan et al., 2018; Warrell, 2012) provide an exciting possibility of developing aptamers against an unknown component of the heterogeneous mixture. This can also result in the aptamer-based discovery of novel biomarkers, which can then be used for diagnostic purposes (Berezovski et al., 2008).

In recent years, complex target SELEX has proven its utility to identify disease-specific biomarkers from the secretome of cancer cells. Using this approach, an aptamer that could discriminate between the secretome of pancreatic cancer and non-cancerous cells was identified. By utilizing biochemical purification methods and mass-spectrometric

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analysis aptamer target was identified as cyclophilin B, a biomarker uniquely present in the secretome of pancreatic cancer cells (Ray et al., 2012). Later, the same biomarker was identified in the serum of pancreatic cancer patients. Besides, using a complex target SELEX strategy, aptamers against protozoan and bacterial pathogens have also been designed leading to the discovery of unique pathogen-specific biomarkers (Shamah et al., 2008; Ulrich and Wrenger, 2009).

Snake venom is a rather more complex mixture as it consists of several different proteins, peptides, amines, carbohydrates, and various other substances (Warrell, 2010). The development of binders (eg. antibodies) against a specific venom component requires the same in its purified form. However, the purification of the aforementioned individual components from crude venom is very challenging. Further, cloning, expression and purification of various proteins present in venom also pose challenges as gene cloning procedure require mRNA from venom glands for that one need to sacrifice the snake which is not permissible as per wildlife act in several countries.

A large body of literature suggests that antibodies developed in response to inactivated crude venom of particular species are highly cross-reactive due to the high molecular weight of proteins and common epitopes present in the venom of other species (Fernandes et al., 2000; Ledsgaard et al., 2018; Stábeli et al., 2005). On the other hand, lethal toxins may be species-specific but they are poorly immunogenic owing to their low molecular weight (Knudsen and Laustsen, 2018; Laustsen et al., 2017). Considering the complexity of snake venom, complex target SELEX offers an exciting opportunity to screen species-specific aptamers from a large aptamer library that can target species-specific uniquely present small molecular weight toxins. Such aptamers can be a valuable tool for assessing the true burden of snake envenomation and conducting forensic investigations (Brunda et al., 2006; Theakston and Laing, 2014). Snake envenomation is a major public health concern around the world, particularly in tropical countries where they are a major contributor to mortality and morbidity (Murray et al., 2015; Suraweera et al., 2020). In large parts of India, the majority of mortality is attributable to the “Big Four” venomous species, which comprise the Indian or spectacled cobra (*Naja naja*), common krait *(Bungarus caeruleus*), saw-scaled viper (*Echis carinatus*) and Russell’s viper (*Daboia russelii*) (Choudhury et al., 2017; David A Warrell, 1999; Puzari and Mukherjee, 2020) with an estimated annual average death count of 58, 000 (Suraweera et al., 2020). Thus, for effective snakebite management, rapid and accurate identification of the envenoming species is the key to reduce the severity and fatality associated with the envenoming (Mohapatra et al., 2011). The current diagnosis regimen primarily involves clinical examination that mainly relies upon symptoms (Ariaratnam et al., 2009; Sano-Martins et al., 1994; Warrell, 2012) and further confirmation involves antibody-based detection in some cases (Warrell, 2010). However, using an antibody for molecular recognition has many limitations associated with it; for instance, the development of antibodies depends on biological systems (mainly animals), provides batch-to-batch variation, high cost of development, and requirement of refrigeration for storage to name a few (Chatterjee et al., 2020; Kaur et al., 2018). Many of these limitations posed by the antibodies can be readily ameliorated by aptamers, the chemical surrogates of antibodies.

Aptamers are single-stranded nucleic acid molecules that form a variety of secondary structures functionally mimicking the epitope binding sites of antibodies. These polynucleotides are chemically synthesized so are relatively very cheap and lacking any batch-to-batch variation. Once developed against a target, they can be rapidly mass-produced in a short time and do not require any stringent storage conditions (Dhiman et al., 2017; Kaur et al., 2019; Taneja et al., 2020).

In the recent past, several assays and diagnostic tests based on different techniques and principles were evolved which includes, bioassays, immunodiffusion, immunoelectrophoresis, immunofluorescence, haemagglutination, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and optical immunoassay (Gopalakrishnakone et al., 2015). However, there exists a major gap in the non-availability of a low-cost point-of-care (POC) device that can diagnose snake venom using body fluid is the major hurdle in the effective treatment of snakebite through the administration of specific antivenom (Michael, 2013). Microfluidic, paper-based and lateral flow devices are the possible solution for POC application for the rapid and affordable detection of snake-venom (Slagboom et al., 2018; Zancolli et al., 2017). There are few reports on microfluidic way of profiling snake-venom. However, none for the rapid detection of snake bite. There are few reports on the development of lateral flow-based immunochromatographic assay (Pawade et al., 2016) and dot ELISA-based (Shaikh et al., 2017) specific snake venom detection. Both the methods employed antibodies of rat and rabbit origin and are time-consuming. Although, a preliminary data on the paper-based sensor was reported by our group but this sensor had utilized antibodies as a molecular recognition element that evinced batch-to-batch variation thus limits its scale-up and POC application (Michael, 2013). This limitation can easily be overcome by aptamers.

Using a complex target SELEX strategy, we herein, report the development of a panel of highly affine aptamers against the crude venom of *B. caeruleus* (Common Indian Krait). To the best of our knowledge, this is the first report of successful SELEX on crude snake venom. The SELEX was performed against the crude venom of *B. caeruleus*, which is a heterogeneous mixture of proteins, peptides, enzymes, amines, carbohydrates, and various other substances. The developed aptamers have shown highly selective binding for the venom of *B. caeruleus* and were able to discriminate it from the venom of other snakes as well as red scorpion venom. The best performing aptamer candidate was able to detect *B. caeruleus* venom in both buffer as well as human serum background with a limit of detection of as low as 2 ng. The molecular target was also identified using biochemical and mass-spectrometric analysis. Further, to demonstrate its possible POC application we have also adapted aptamers onto a paper-based device.

2. Materials and methods

2.1. Reagents and chemicals

All routine reagents were procured from Sigma Aldrich, USA. Oligonucleotides used in the study were procured from Integrated DNA Technologies (IDT, USA). Ninty-six well plates (MaxiSorp™) were procured from Thermo Fischer Scientific, U.S.A. 3,3′,5,5′-tetramethylbenzidine (TMB,BD OptEIA™) was procured from BD Biosciences, USA.

2.2. Snake venom collection and procurement

Snake venom was obtained from wild specimens collected under permit numbers 5141/WL/4R-6/2017, A.33,011/5/2011-CWLW/305, and W.L./Research Study/WLM/2341 issued by West Bengal, Mizoram and Himachal Pradesh Forest Departments, India and their specific identity were confirmed by professional herpetologists. The specimens were handled according to relevant guidelines or regulations and were released after milking. For the current work, ethical permission was obtained from Institutional Ethics Committee, Bangor University, U.K. and Translational Health Science and Technology Institute, India. In addition to this, venom from the ‘Big Four’ species was also procured from KV Institute, Uttar Pradesh, India and the Irula Snake Catchers Industrial Co-operative Society (ISCICS), Vadanemmeli village, Kancheepuram District, Tamil Nadu, India. Biosafety permissions to handle snake venom at the institute were also obtained from the Institutional Biosafety Committee, THSTI, Faridabad-121,001, Haryana, India.

2.3. Aptamer development through SELEX

The G-quadruplex biased and completely random libraries as described in recent work from our group (Kalra et al., 2018) were used to

screen aptamers against venom of *B. caeruleus*. These libraries have identical 18 nucleotide long primer binding sites for DNA amplification. For PCR amplification DRF (Forward- 5′ GTC TTG ACT AGT TAC GCC 3′) and DRR (Reverse - 5′ GAG GCG CCA ACT GAA TGA-3′) primers were used to prime the template and nascent strands, respectively. For single-stranded (ss)DNA generation, PCR was performed using 5′ FAM-labeled DRF (Fluorescent in nature) and 3′ rA-modified DRR (5′ GAG GCG CCA ACT GAA TGrA-3′) primers. The process of ssDNA generation was same as described recently (Chatterjee et al., 2020; Dhiman et al., 2018; Kalra et al., 2018). To develop aptamers against the crude venom of *B. caeruleus*, a nitrocellulose membrane (NCM)-based subtractive Systematic Evolution of Ligands through EXponential enrichment (SELEX) approach was adopted.

Experimentally, a mixture of DNA libraries containing 1500 pmol of each library in selection buffer (SB, 10 mM Tris pH 7.5 supplemented with 10 mM $MgCl_2$, 50 mM KCl and 25 mM NaCl) was heated at 92 °C, followed by snap chilling on ice, and then brought to room temperature (RT). Such prepared libraries were then incubated for 1 hour at RT with a cocktail of venom from *N. naja, D. russelii and E. carinatus* dissolved in Nuclease Free Water (NFW) immobilized on NCM, as a counter-selection step. The unbound sequences were then incubated with NCM-immobilized *B. caeruleus* venom for 1 hour at RT. After incubation, the membrane was washed with washing buffer (SB supplemented with 0.5 % Tween-20) to remove the unbound and loosely bound sequences. Venom-bound sequences were then eluted by heating the membrane in NFW at 92 °C for 10 min, and the resultant solution was used for PCR amplification of the eluted sequences. Amplification of aptamers followed by generation of ssDNA population was performed as described recently by our group (Chatterjee et al., 2020; Dhiman et al., 2018; Taneja et al., 2020).

The selection pressure was gradually increased with every successive round by increasing the amount of the counter-selection venoms and simultaneously reducing the amount of target venom. The number of washes and the strength of Tween-20 (T-20) in the washing buffer were also increased up to 1.5 % as selection progresses. Additionally, salmon sperm DNA and dextran sulfate were added to reduce the non-specific interactions during SELEX. In addition, counter selection was also given with human serum, and the volume of human serum was gradually increased from round 1–8. After eight rounds of SELEX, the archived aptamer pools from rounds 2, 4, 6 and 8 were evaluated using an Aptamer Linked Immobilized Sorbent Assay (ALISA) to monitor the progress of aptamer selection. The aptamer pool of the round displaying the highest binding towards the target venom was sent for Next Generation Sequencing (NGS) at Eurofins Genomics service, India.

2.4. Aptamer Linked Immobilized Sorbent Assay (ALISA)

ALISA was used to evaluate the binding of the developed aptamer candidates towards *B. caeruleus* venom. Experimentally, 500 ng snake venom (krait or other species) was coated onto a 96-well plate using standard 100 mM carbonate-bicarbonate buffer, pH 9.6 at 37 °C for 1.5 hour. After this, the coating solution was discarded in 1% bleach and marginal sites were blocked with 5 % skimmed milk supplemented with 0.25 % T-20 in SB.

After blocking, wells were washed with SB once and then 100 pmol of 5' biotin-labeled aptamer were added to each well for 1 hour at RT. Following this, the plates were washed with SB and SB supplemented with 1 % T-20 (v/v). Next, biotinylated aptamer bound to venom was probed with 1:3000 (v/v) streptavidin-horseradish peroxidase (Sigma Aldrich U.S.A.) and was further incubated for 1 hour at RT. Finally, 100 μL of TMB substrate was added to each well and incubated for 3–5 min at RT. The reaction was quenched using 5 % H_2SO_4 and optical density (OD) was measured at 450 nm. ΔOD_{450} obtained, by subtracting OD_{450} of appropriate negative controls (aptamer control and antigen control) was plotted.

2.5. Assessment of cross-reactivity of aptamers

To assess the cross-reactivity of SELEX-derived aptamers, 10 aptamer candidates were screened by ALISA for their ability to bind with the venom of the 'Big Four' species. Based on the outcome of this experiment, two aptamer candidates were selected and their binding was assessed for eight different snake venoms including 'Big Four', namely venom obtained from *N. naja, N. kaouthia, N. oxiana, D. russelii, B. caeruleus, B. fasciatus, B. niger, and E. carinatus.* In addition to snake venoms, we have also assessed cross-reactivity of aptamers for red scorpion (*Hottentotta tamulus, H. tamulus*) venom as well. These species were chosen based on their distribution and importance in terms of snake and scorpion bite in the Indian subcontinent. The ALISA was performed as described in the previous section.

2.6. Circular Dichroism (CD)

The CD experiments were performed on J-815 Spectropolarimeter (JASCO, Tokyo Japan) to determine the secondary structure of two selected aptamer candidates. A quartz cuvette with 0.2 cm path length was used to record the spectra of samples containing 20 μM of each aptamer in binding buffer, using an average of three scans.

2.7. Truncation of aptamers

Based on the NUPACK (http://www.nupack.org) predicted secondary structure truncated variants of two selected aptamers were designed and their binding was assessed and compared with their respective parents' counterparts in an ALISA.

2.8. Determination of apparent dissociation constant (K_d)

The dissociation constant (K_d) of the selected aptamers (B6, B8 and combination of both) was gauged with ALISA for *B. caeruleus* venom. The ALISA was performed as described in the earlier section. Briefly, a fixed venom amount was subjected to various aptamer concentrations ranging from 2 to 500 nM. The absorbance at 450 nm was plotted as a function of aptamer concentration and K_d was measured using the following equation in Graph-pad Prism version 7:

$$Y = Bmax\frac{X}{Kd + X}$$

Here, Y represents the aptamer binding; X is aptamer concentration and Bmax is maximum binding.

2.9. Limit of detection of selected aptamers

The limit of detection (LOD) of venom of *B. caeruleus* for selected aptamers (B6, B8 and combination of both) was determined by ALISA. For this ALISA was performed with different amounts of *B. caeruleus* venom ranging from 2 to 1000 ng/well. Rest protocol was followed as described under section 2.4.

2.10. Evaluation of aptamer candidates to detect geographically distinct krait venom

Venom variability among geographically distinct populations of same species might pose a challenge in the species-specific diagnosis of venom (Casewell et al., 2020; Chippaux et al., 1991). To address this challenge, we have also assessed the aptamer binding against krait venom obtained from three geographically distinct populations (Central, East and Southern India) using ALISA as described in the aforementioned section. To determine the effect of venom collection time along with geography we have performed ALISA with 5 samples of *B. caeruleus* venom collected at three different sites between years 2017–2021 (Detailed information given in result section and Figure S3

in supplementary material).

2.11. Fabrication of paper-based devices

All paper microfluidic devices used in the current work were fabricated on Whatman® qualitative filter paper (Grade 4, thickness 0.205 mm, Cytiva, India). All the paper-based devices used in this work were designed using the CorelDraw X8 software (Corel Corporation, Ottawa, Canada). The design was printed using a wax printer (ColorQube 8570, Xerox India Ltd., India). The printed devices were kept on the hot plate at 120 °C for 2 min to melt the wax so that it can percolate down to the other side of the paper and forms the hydrophobic barrier. The printed wax devices were then cut using a computer-controlled laser cutting and engraving machine (Model CMA 6040, GD Han's Yueming Laser Group Co., Ltd, China) or with simple scissors for further use. All the paper-based devices used in this work are used without any further modifications.

2.12. Development of aptamer and paper-based devices

To detect the *B. caeruleus* venom on a paper-based device, initially, a known amount of snake venom was coated on the surface of the hydrophilic reaction zone, the white circled area (CA) of a paper-strip using the pipette. Uncoated CA was served as a venom control (VC). After coating, the strip was dried at RT for 5 min. Thereafter, the CA was blocked with 5 % skimmed milk supplemented with 0.25 % T-20 in SB for 30 min. CA was then washed one time with SB followed by the addition of 100 pmol of biotinylated B6 and B8 aptamers in reaction volume of 10 μL followed by an incubation of 30 min at RT. Thereafter, the CA was washed twice with SB supplemented with 1 % T-20 followed by the addition of streptavidin-HRP (1:2000; 10 μL/CA) and incubated for 30 min at RT. Next to this, paper-based device was washed as described above. Finally, the TMB substrate (10 μL/CA) was used as the substrate for HRP, which gives blue color. The blue CA indicating the presence of *B. caeruleus* venom while no such color was observed in absence of snake venom. Finally, the image of the paper strip was captured by using an android smart phone camera (Samsung A series).

2.13. Limit of detection

To determine the low-end detection limit of *B. caeruleus* venom on paper-based device, a range (1000-2 ng) of venom was spiked in the coating solution. The rest of the steps such as blocking, washing, incubation time, amount of biotinylated B6/B8 aptamers, and streptavidin-HRP were followed as mentioned previously. Further, to study the effect of sample matrix on krait specific aptamer venom interaction, human serum sample obtained from a healthy individual was diluted (1:100 in coating solution), and then it was spiked with a range of (1000-2 ng) of *B. caeruleus* venom. Following this, the appearance of blue color was used to determine the lowest possible amount of venom that can be detected in the serum.

2.14. Sample preparation for LC-MS/MS

Ten micrograms of crude venom were resolved by SDS-PAGE. The gel was stained with Coomassie brilliant blue and destained with water. The specific band of ~55 kDa was cut from the gel. Forty mM ammonium bicarbonate (ABC) in 40 % acetonitrile (ACN) was used to destain the excised bands. Reduction and alkylation were done by 5 mM dithiothreitol (DTT) at 60 °C for 45 min and 10 mM iodoacetamide (IAA) on the gel bands respectively. The gel pieces were dehydrated using 100 % ACN and dried further for 10 min at room temperature (RT). The in-gel digestion was carried out as explained previously (Goel et al., 2013). Trypsin (Gold mass-spectrometry trypsin; Promega, Madison, WI) was added in ice-cold tubes and kept at 37 °C for 10–12 hour. Peptides were removed from the gel pieces by adding 50 % ACN with 0.1 % formic acid (FA) in the tubes. Finally, the same step was carried with 100 % ACN and 0.1 % FA. The peptides were lyophilized and kept at −80 °C until LC-MS/MS analysis.

2.15. LC-MS/MS analysis

Digested samples were reconstituted in 0.1 % FA and analyzed by reverse-phase high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry using an Ekspert-nanoLC 415 system (Eksigent; Dublin, CA) which is directly connected to a Sciex 5600 Triple-TOF (SCIEX; Concord, Canada) mass spectrometer.

Mobile phase A consisted of 2 % acetonitrile/98 % of 0.1 % formic acid (v/v) in water, and 98 % acetonitrile/2 % of 0.1 % formic acid (v/v) in water was used as mobile phase B during reverse-phase high-pressure liquid chromatography. The analytical column (75um x 15 cm) from Eksigent used for the peptides separation and retention time drift was maintained by keeping the temperature constant at 35 °C. The acquired raw files were saved in.wiff format. Autocalibration of MS and MS-MS were done with 25 fmol b-gal, and 20 μm SilicaTip electrospray PicoTip emitter (New Objective Cat. No. FS360-20-10-N-5-C7-CT) was used to inject the peptides into the mass spectrometer.

A high-resolution TOF-MS scan over a mass range 350–1250 m/z was used for Data-Dependent Acquisition (DDA) and intensity greater than 150 cps and charge state between +2 and +5, was used to select the parent ion. Once the parent ion and isotopes were fragmented by MS/MS, they were excluded for 12 s from further MS/MS fragmentation. Rolling collision energy was used to trigger collision-induced dissociation. Accumulation time of 250 ms and 70 ms ions was used for MS and MS/MS respectively.

2.16. Database search

MS/MS spectra were searched in Protein Pilot software v. 5.0.1 (SCIEX). The following settings were used for Paragon search: Sample type: Identification; Dithiothreitol, Iodoacetamide were used for reduction and alkylation respectively, Digestion: Trypsin; TripleTOF 5600 as instrument type: Species: *B. caeruleus*; Thorough ID for Search effort. Carbamidomethylation was used as a fixed modification. A confidence score of >0.05 was used for peptide identification for consideration for further analysis. *B caeruleus* sequences were fetched from the UniProt website (www.uniprot.org) and contamination list was added to this. False discovery rate analysis was also performed. A peptide and product ion tolerance of 0.05 Da was used in searches. The output file from this search contains protein identification, UniProt accession number, cleaved and modified peptide sequences, relative intensity, precursor and fragment ion charge and unused Protscore. Wiff files were also processed with MaxQuant using the protein identification parameter (Cox and Mann, 2008). All other parameters were same as used in the Protein Pilot software.

2.17. Competitive Aptamer Linked Immobilized Sorbent Assay (ALISA)

To substantiate the mass spectrometry data and to map the possible binding target, a competitive ALISA was performed. In this assay, 5′ biotin-labeled aptamers (B6 and B8) were challenged with a range (50–6400 pmol) of previously reported β-bungarotoxin specific unlabelled aptamer (βB-1; 5′-GTTTTCCCCTTGTCGCTTTTGGTTCGTTCT GCCTCTATCT-3′) in an ALISA (Ye et al., 2014). The reverse competitive ALISA was also performed with a range (50–6400 pmol) of unlabelled B6 and B8 aptamers challenged with 5′ biotin-labeled βB-1 aptamer. The binding of aptamers was quantified in terms of % binding considering unchallenged aptamer OD_{450} value as 100 % binding.

3. Results

3.1. Generation of aptamers

As described in Fig. 1a, a NCM based subtractive SELEX approach was used to develop aptamers against the *B. caeruleus* venom. A total of 8 rounds of SELEX were performed. After eight iterative rounds of selection, the binding of the SELEX representative population from various rounds (2, 4, 6 and 8) was assessed for their binding with *B. caeruleus* venom. It is evident from Fig. 1b that the aptamer population from round 6 (R6) displayed the highest binding, having approximately five times higher binding than the initial library mix. Thus, the pool of R6 was further amplified and was subjected to NGS. Based on the NGS data, ten unique aptamer sequences (Table S1) with the highest copy numbers were selected for further study.

The top ten aptamers were evaluated using ALISA for their ability to bind with the venom of *B. caeruleus*. The binding aptitude was experimentally recorded in terms of ΔOD_{450} (OD_{450} of the test set-OD_{450} of antigen control). Higher ΔOD_{450} reflects the higher binding of DNA aptamers for the venom of *B. caeruleus.* All the selected aptamers displayed impressive binding towards the *B. caeruleus* venom (Fig. 1c).

3.2. Assessment of selectivity of aptamers

The selectivity of the developed aptamers was evaluated with ALISA. For this, the aptamers were checked with the venom of Common krait (*Bungarus caeruleus*), Indian cobra (*Naja naja*), Russell's viper (*Daboia russelii*), and Saw-scaled viper (*Echis carinatus*). Except for B1, B2, B3 and B10, most of the developed aptamers displayed excellent selectivity for *B. caeruleus* venom (Fig. 2a–j). The B2 and B3 aptamers displayed the highest level of cross-reactivity against *N. naja* and *D. russelii* venom while B1 and B10 evinced high cross-reactivity with *D. russelii* venom but marginal cross-reactivity was observed for *N. naja* venom as well. Owing to the high cross-reactivity these four aptamers (B1, B2, B3, and B10) were not considered for further studies.

Based on this data, two aptamer candidates (B6 and B8) were then assessed for their ability to bind a range of snake venom along with red scorpion venom. For this study, the aptamers were subjected to the venom of *B. caeruleus, N. naja, N. kaouthia, N. oxiana, D. russelii, B. fasciatus, B. niger, E. carinatus* and *Hottentotta tamulus* (*H. tamulus*, Red scorpion). Both the tested aptamers (B6 and B8) displayed the highest binding and selectivity towards the *B. caeruleus* venom (Fig. 3a–b). However, marginal cross-reactivity was observed with the venom of *B. fasciatus* which is another krait species.

3.3. Secondary structure of the aptamers

The secondary structures of the B6 and B8 aptamers were first predicted with a NUPACK web server (http://www.nupack.org/). The NUPACK predicted structure of B6 and B8 displayed stem-loop-like structures. B6 aptamer evinced two stem-loop-like motifs while a single stem-loop was observed in the case of B8 aptamer (Fig. 4a and b) with minimum free energy (MFE) of −3.89 and −0.76 kcal/mol for B6 and B8 aptamers respectively. These *in-silico* predictions were further validated with CD studies. The CD studies revealed the presence of negative peaks at ~257 nm and ~256 nm, and positive peaks at ~302 nm and ~292 nm for B6 and B8 respectively (Fig. 4c and d). These peaks confirm the presence of stem-loop structure (Kypr et al., 2009; Sharma et al., 2017).

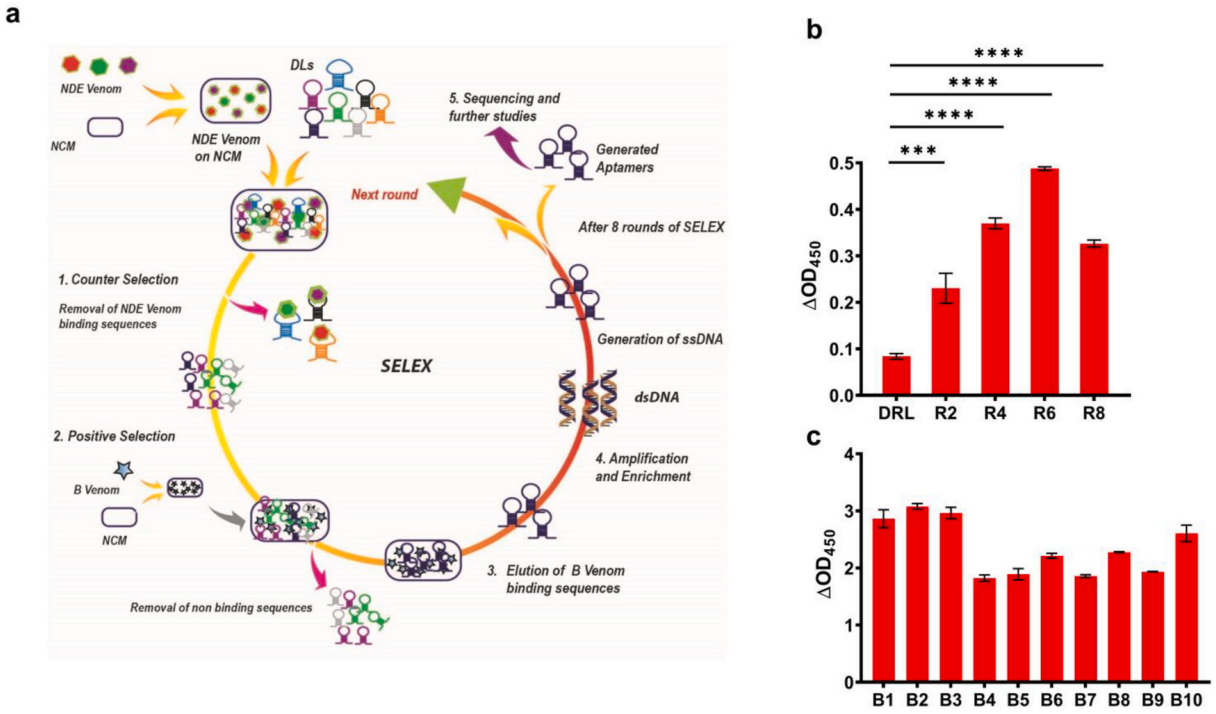

Fig. 1. (a) A schematic representation of the SELEX strategy. (1) Counter-selection: DNA libraries (DLs; G-quadruplex biased and unbiased) were incubated with the nitrocellulose membrane preimmobilized with *Naja naja* (N), *Daboia russelii* (D), and *Echis carinatus* (E) venoms (NDE) to remove any sequences with the binding tendency to these, (2) Positive selection: Unbound sequences are then incubated with *Bungarus caeruleus* venom (B) to select sequences with an affinity towards it; (3) Elution of binders; (4) Amplification: PCR products were made single-stranded and used for the next round of SELEX. The whole process of counter selection and the selection is repeated eight times. (5) Sequencing: Finally, the aptamer pool from each round with the highest affinity was subjected to NGS to identify aptamers sequences. **(b)** The binding propensity of the mixture of DNA libraries in comparison to various round sequence pools. **(c)** The relative binding aptitude of the developed aptamers with the *B. caeruleus* venom. All the developed aptamers depicted good binding ability with the *B. caeruleus* venom. Here higher ΔOD_{450} reflects the higher binding propensity of the aptamers. Bars represent mean ± SD. To compare the binding one-way ANOVA with multiple comparison was applied. **** represent statistical significance at p value (*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$, ****$p < 0.0001$).

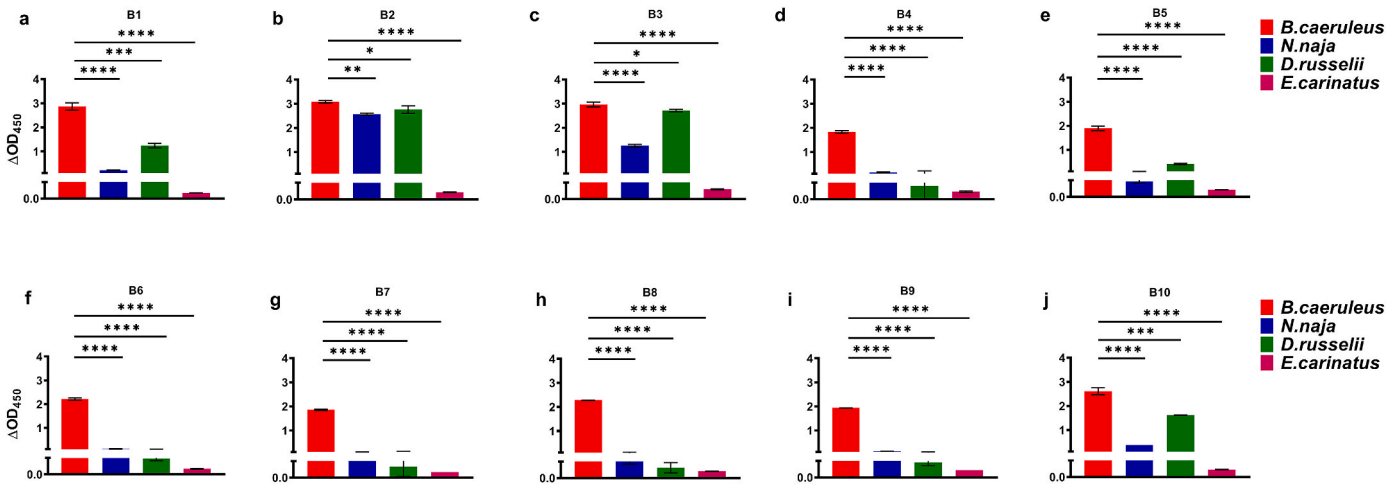

Fig. 2. (a–j) Relative binding of 10 SELEX derived aptamers against the venom of 'Big Four'. All the developed aptamers largely displayed selectivity against *B. caeruleus* venom. To compare the binding one-way ANOVA with multiple comparison was applied. **** represent statistical significance at p value (*p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.0001) Bars represent mean ± SD.

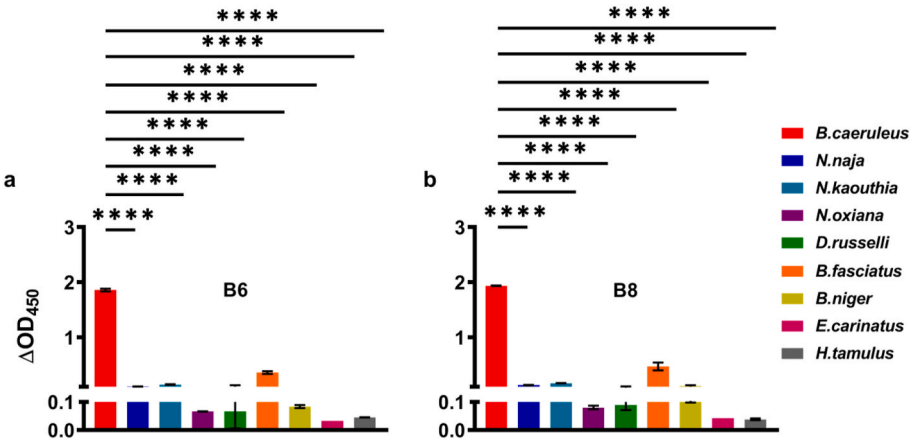

Fig. 3. (a–b) The relative binding (ΔOD_{450}) of 2 best-performing aptamers with the venom of *N. naja, N. kaouthia, N. oxiana, D. russelii, B. caeruleus, B. fasciatus, B. niger, E. carinatus* and Indian Red Scorpion (*H. tamulus*). Both the aptamers, B6 and B8 displayed the highest level of binding and selectivity. To compare the binding one-way ANOVA with multiple comparison was applied. **** represent statistical significance at p value (*p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.0001) Bars represent mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Truncation of aptamers

It is evident from the literature that all nucleotides present in a particular aptamer do not take part in its interaction with the cognate target and only a specific portion do. However, the flanking region may provide the overall stability to the structured portion of the aptamer thus truncation of such sequences may diminish the aptamer binding (Dhiman et al., 2018; Hasegawa et al., 2016). To elucidate the structure-activity relationship, two NUPACK web derived secondary structure-guided truncated variants of B6 (13-mer B6-T1 and 10-mer B6-T2) and B8 aptamer (10-mer, B8-T1) were designed and synthesized with 5' biotin label (Table S2). When comparing the binding of these truncated variants with their respective parent aptamer, considering parent aptamer binding as 100 %, it is evident (Fig. 4e and f) that truncated variants of B6 (13-mer B6-T1 and 10-mer B6-T2) evinced ~90 % reduction in binding while 14-mer B8-T1, a truncated variant of B8, lost around 75 % binding. This data suggests the contribution of the truncated portion of aptamers in their binding to the venom of *B. caeruleus*. Therefore, for subsequent experiments, the parent aptamers were used.

3.5. Determination of apparent dissociation constant (K_d) of aptamer candidates

The affinity of the developed B6 and B8 aptamers were measured in terms of the dissociation constant (K_d) of the aptamers. K_d was determined by exposing B6 and B8 aptamers at concentrations ranging from 2 to 500 nM to a constant amount of *B. caeruleus* venom (Fig. 5a). The study reveals the respective K_d value of B6 and B8 as 148.6 nM and 19.87 nM. The K_d values suggest that the aptamer B8 is ~7.4-fold more affine than B6 aptamer. By combining B6 and B8 aptamers in equimolar concentrations ranging from 2 to 500 nM, the affinity of the two aptamers was assessed in combination. It is evident from Figure S1a that the affinity of B6 and B8 combination is ~2 fold better than the B6 aptamer alone. This data also suggests that the B8 aptamer, which has a higher affinity than the B6 aptamer alone and the B6 and B8 combination, is a substantial contributor to the improved affinity.

3.6. Limit of detection (LOD) of B6 and B8 aptamers

The selected B6 and B8 aptamers were evaluated for their ability to detect the lowest possible amount of *B. caeruleus* venom in an ALISA. Aptamer response was plotted as a function of venom amount. It is evident from Fig. 5b, that the aptamer response increased from 2 to 64 ng venom. However, this response achieves the saturation plateau at 125 ng, and beyond that, not much increase in aptamer response was observed even after increasing the amount of venom. This results show that these aptamers can detect as low as 2 ng venom (equivalent to 20 pg/μL) of *B. caeruleus*. However, B8 evinced a better signal intensity in comparison to B6 aptamer. In addition to this, we have also determined

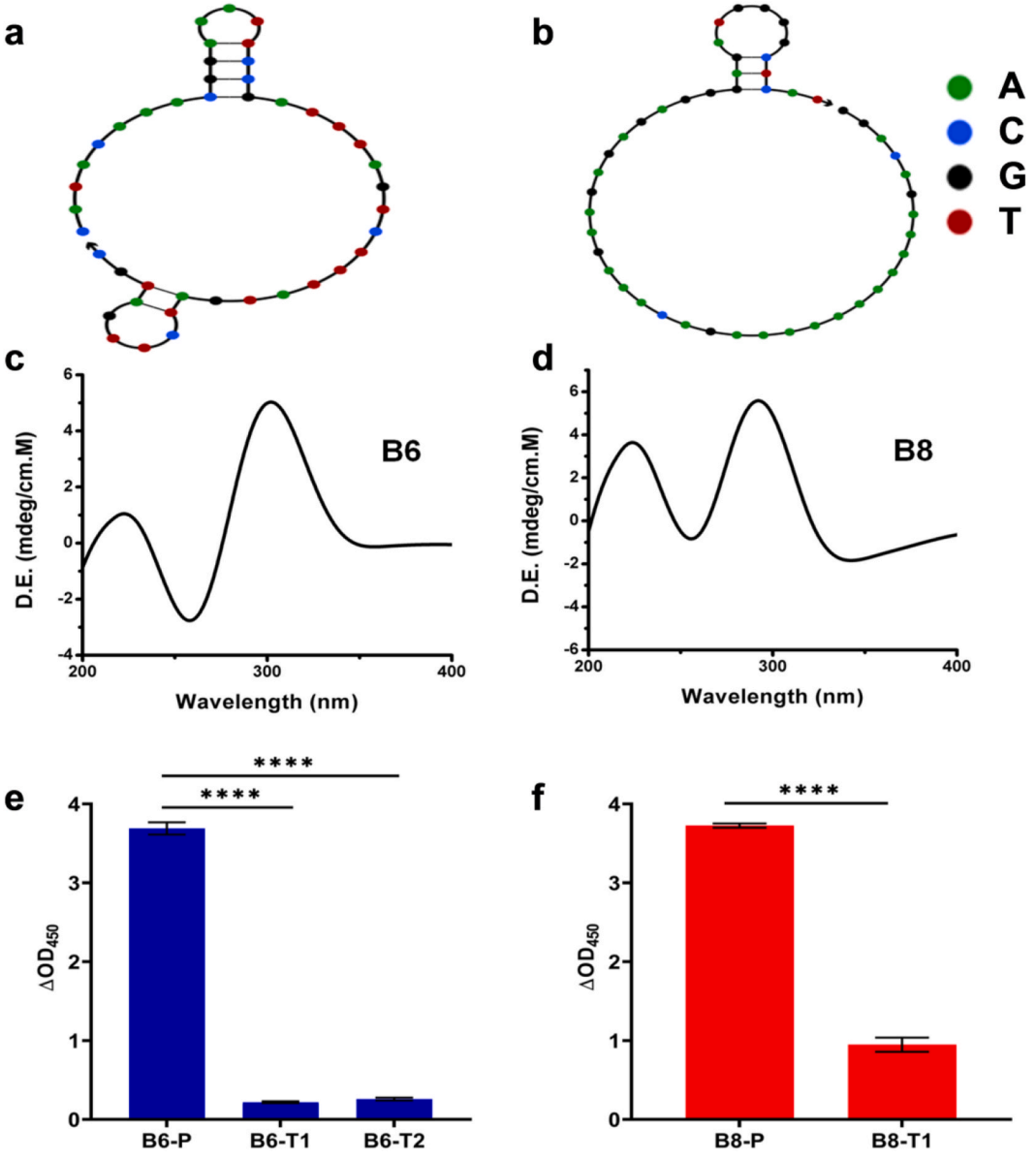

Fig. 4. The secondary structure of different aptamers. The secondary structure is predicted by NUPACK for aptamer **a)** B6 and **b)** B8. Both the aptamers have a stem and loop structure. The CD spectrum of the **c)** B6 and **d)** B8 aptamer, **(e–f)** Comparison of binding of truncated aptamers with their respective parent aptamers. To compare the binding one-way ANOVA with multiple comparison was applied. **** represent statistical significance at p value (*p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.0001) Bars represent mean ± SD.

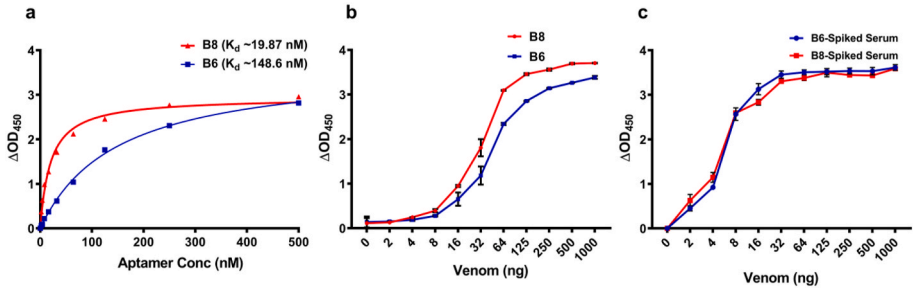

Fig. 5. a) The Apparent dissociation constant curve derived through non-linear regression representing binding affinity (K_d) of B6 and B8 aptamers for *B. caeruleus* venom **b)** The response of the aptamers when subjected to the various venom amount. Both the aptamers were able to detect up to 20 pg/μL of *B. caeruleus* venom. Limit of detection of **(c)** B6 and B8 aptamers in human serum spiked with *B. caeruleus* venom. Both the aptamers were able to detect up to 20 pg/μL of *B. caeruleus* venom.

the LOD of B6 and B8 in combination. Figure S1b indicates that the OD value of B6 and B8 combination was marginally increased only at higher venom amount (125–1000 ng) in comparison to B6 alone. Overall, no significant improvement was observed in terms of LOD at lower venom amount (2–64 ng) when combination of B6 and B8 aptamers were used in comparison to B6 and B8 alone.

3.7. Evaluation of aptamer performance in serum background

As the current study also aims to develop aptamers for diagnostics and epidemiological applications for krait envenomation, the ability of aptamers to detect *B. caeruleus* venom in human serum background was also assessed. Both B6 and B8 displayed impressive binding with the *B. caeruleus* venom in human serum. The aptamers showed minimal interference by the serum components, marking their potential to be used in clinical settings. The aptamers charted a LOD of ~2 ng (equivalent to ~20 pg/μL) for both B6 and B8 (Fig. 5c).

3.8. Evaluation of the effect of venom variability in terms of geographical variation on the aptamers binding

The aptamers were also assessed for their ability to detect the *B. caeruleus* venom from different geographical locations to evaluate the effect of geographical variations (Casewell et al., 2020; Kalita and Mukherjee, 2019; Senji Laxme et al., 2021). The aptamers displayed differential binding with the venom from different geographical locations (three different regions of India; Central, Eastern and Southern). The binding pattern of both the aptamers was also varied for each geographical variation of venom. The aptamer B8 displayed a better binding propensity with each geographic variety of the venom than B6, which is in agreement with the earlier results in this study. Interestingly when a mixture of both the aptamers was gauged for their binding aptitude towards the venom obtained from various geographical regions, its binding response was marginally improved from the B6 alone suggesting the contribution of B8 aptamer in improvement of binding to venom (Figure S2).

To evaluate the effect of venom collection time and geography we have assessed the aptamer binding against 5 different venom samples of *B. caeruleus*. These samples were collected between years 2017–2021 at Hooghly, West Bengal, India, K.V. Institute Uttar Pradesh, India and Irula Snake Catchers Industrial Co-operative Society (ISCICS), Vadanemmeli village, Kancheepuram District, Tamil Nadu, India. ALISA data clearly demonstrate that developed aptamers were able to bind to all 5 samples and as expected, B8 was found to be superior than B6 (Figure S3). The binding of B6 and B8 combination was superior to B6 and comparable with B8 aptamer. This data indicates that higher binding of B6 and B8 combination than B6 alone is due to the contribution of B8 aptamer in improving the affinity. This data also indicates that aptamers binding, particularly B8 was minimally affected by the time of venom collection and geographical variation.

3.9. ALISA-based detection of snake venom on a paper-based device

Next, we assessed the selectivity and sensitivity of B6 and B8 aptamers in an indirect format assay comparable to ALISA but in a simple (instrument less), fast (>2hr), cost-effectivewith a visual readout on a paper-based device with high suitability for point-of-care testing (POCT). Fig. 6a shows two sets of experiments with B6 and B8 aptamers. The first two sub-set in both the images with blue color indicates the B6 and B8 aptamer interact specifically with venom. The other two sets represent venom control (VC) with no-color i.e. negative control, indicating that both the aptamer do not show any non-specific binding with paper surface or with blocking and washing buffers. Next, we assessed the selectivity of both the aptamers in a similar fashion by immobilizing equal quantity of venoms from different snakes such as *N. naja, N. kaouthia, N. oxiana, D. russelii, B. fasciatus, B. niger, E. carinatus*, and the scorpion venom namely *H. tamulus* (the Indian Red scorpion). No blue color was observed in any of the snake and scorpion venom except *B. caeruleus* indicating high selectivity of B6 and B8 aptamers respectively (Fig. 6b). Further to establish the sensitivity of the present assay different quantity of *B. caeruleus* venom was immobilized ranging from 1000 ng to 2 ng ("0" served as VC). It was observed that the intensity of blue color was highest at 1000 ng and visible up to 2 ng for both B6 and B8 aptamers (Fig. 6c). These results demonstrate the high capability of B6 and B8 aptamers for *B. caeruleus* venom detection via assay performed with paper-based device. The present aptasensor offer an instrument less assay for venom detection and one can easily see the output just by observing the change in color of the test zone. Further, being a simple and handy device it has a high potential to be used as a POC assay for snake venom detection.

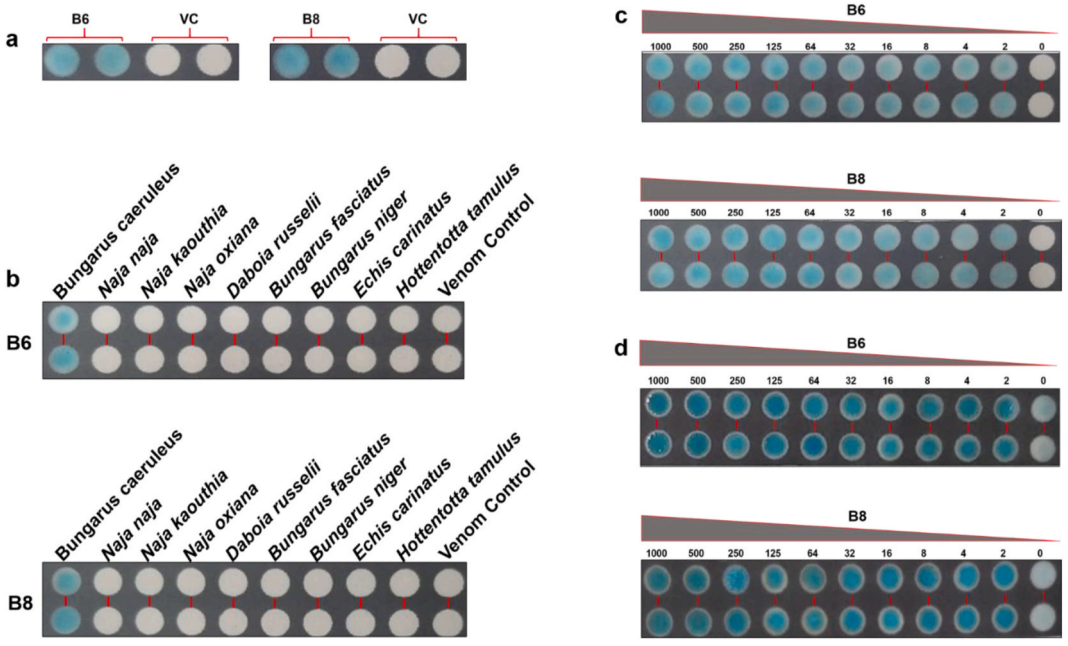

Fig. 6. Paper-based device for detection of snake venom **(a)** The reaction zones with B6 and B8 showing TMB color in the images, while no color appears in venom control (VC) with no venom samples, **(b)** the selectivity test on paper-based devices indicates that both B6 and B8 do not have any cross-reactivity with other venom samples. Analytical sensitivity of paper-based device for venom detection **(c)** in crude venom and **(d)** in Human serum background. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.10. Determining limit of venom detection in human serum on paper-based device

The sensitivity of venom detection in human serum background was established using a paper-based device. A range of *B. caeruleus* venom (1000-2 ng) spiked into human serum background (obtained from a healthy individual) was evaluated to assess the effect of the clinical sample matrix on sensor sensitivity. The result indicated that aptamers B6 and B8 were able to detect as low as 2 ng of venom in serum (Fig. 6d) and also show a significant difference when compared to the serum sample without antigen designated as "0" in Fig. 6d. Notably, the aptasensor exhibited a similar low-end detection limit of 2 ng venom in both the cases *i.e.,* in coating solution as well as in human serum background. Overall, these results exhibited the high selectivity and sensitivity of B6 and B8 aptamers against *B. caeruleus* venom with paper-based devices. However, in relative terms B8 evinced better color intensity on paper in comparison to its B6 counterpart.

3.11. LC-MS/MS analysis

The component of the venom binding with the B6 and B8 aptamers was first identified with Western blot, developed by interacting the best performing aptamer candidates (B6 and B8) with the resolved protein components on the NCM (Figure S4). Results suggest that the developed aptamers bind to a venom component with a molecular mass of around ~55 kd (Figure S4). Since, B8 was emerged as a best performing aptamer candidate having higher binding and affinity in comparison to B6 thus it was subjected to detailed MS analysis. To investigate the venom component binding with the B8 aptamer, the aforementioned protein was excised from the gel and were subjected to LC-MS/MS analysis. LC-MS/MS analysis identified Basic phospholipase A2 β-bungarotoxin A2 chain by using ProteinPilot and MaxQuant search engines. Both software identified the peptides TAALCFGDSEYIGAHK, TIICYGAAGTCGR and TWGHYADYGCYCGAGGSGTPVDALDR, which correspond to β-bungarotoxin A2 (Figure S5). This data suggests the target of the aptamer is β-bungarotoxin, however, B8 also binds with a different unique epitope as reported above.

3.12. Competitive Aptamer Linked Immobilized Sorbent Assay

To substantiate the MS findings we have performed competitive ALISA where previously reported aptamer, βB-1 (developed against β bungarotoxin of *B. multicinctus)* was challenged with B6 and B8 aptamers (aptamers developed in the current study) and vice versa. It is important to note that β-bungarotoxin of *B. multicinctus* and *B. caeruleus* is highly similar (~99 % similarity, Figure S6). As expected, binding of βB-1 aptamer was reduced when it was challenged with B6 and B8 aptamers in a competitive ALISA (Figure S7). Interestingly when order of the aptamers was reversed i.e. when B6 and B8 aptamers were challenged with βB-1 aptamer no significant reduction in B8 aptamer binding was observed. However, on the other hand, binding of B6 aptamer was reduced. This data suggests that B8 aptamer has better affinity than the previously reported βB-1 aptamer (Ye et al., 2014).

Further, these observations are strongly supported by the K_d data of B6 and B8 aptamers (Figure S7). As K_d value of B8 aptamer ($K_d \sim 19.87$ nM) was superior than the previously reported βB-1 aptamer ($K_d \sim 65.9$ nM) of *B. multicinctus* thus, it was able to displace the βB-1 aptamer in competitive ALISA format while βB-1 was unable to do so due to its comparatively lower affinity than B8 aptamer. On the other hand, as affinity of B6 is slightly lower (~2 fold lower than βB-1 aptamer) thus it was able to displace βB-1 only at the higher concentrations while B6 was displaced by βB-1 even at the lower concentrations owing to the comparatively higher affinity of βB-1. This data clearly indicates that cognate target of B6 and B8 aptamers is β-bungarotoxin.

4. Discussion

We herein report the success of the employed NCM-assisted SELEX for a complex target i.e., crude venom of *B. caeruleus*. The feat of the SELEX can be simply gauged by the observed high binding propensity of the developed aptamers with *B. caeruleus* venom. The pool of various SELEX rounds showed an incremental binding until R6. It then showed a loss of binding propensity during the 8th round of SELEX, which can be attributed to the loss of binders, reduction in the aptamer pool complexity, or a combination of both, during the SELEX (Wang et al., 2019). Based on NGS data, the ten aptamer candidates showing the highest multiplicity (thus enrichment during SELEX) were assessed for their binding against *B. caeruleus* venom. These ten SELEX derived aptamer candidates were having varied length. We have used PCR master mix containing Taq DNA polymerase, having a high error rate (2.2×10^{-5} per nt per cycle) to promote diversity of the aptamer library during amplification cycle. Some SELEX derived aptamers are slightly larger than the original length of the library (central random region of library). This is possibly because of the extendase activity of DNA polymerase (hu, 1993). Slightly smaller length aptamer possibly generated because of the mutation introducing nature of polymerase used or mispriming event or polymerase pausing that could happen during PCR (Kalra et al., 2018; Li et al., 1990; Ranu, 1994; Westberg et al., 1999).

All the developed aptamers displayed a high binding aptitude towards the *B. caeruleus* venom. In terms of selectivity, though, there was variation in terms of binding tendencies towards the venom of other tested species. The observed cross-recognition by certain aptamers can be attributed to similarity in the structure of epitopes recognized by them (Chadwick, 2008; Dasgupta, 2019). Conversely, the selectivity can be explained by the complete absence or sparse presence of the same or similar epitope in the venom of other species (Chadwick, 2008; Dasgupta, 2019). As the composition of the venom varies with snake species, the complete absence or sparsity of such epitopes seems normal (Choudhury et al., 2017; Gutiérrez et al., 2017; Warrell, 2010, 2012). Nevertheless, aptamer B6 and B8 displayed the highest performance among the initially selected ten aptamers when considered both binding proclivity and selectivity towards the *B. caeruleus* venom. These aptamers were also able to recognize *B. caeruleus* venom in a human serum background. This underlines the potential of the developed aptamer to be used as a tool to detect the presence of snake venom. Further development of aptamer-based diagnostics can obviate many limitations with the current antibody-based assays, notably, the difficulty in generating antibodies against low immunogenic but highly toxic components of the venom (Berm et al., 2018; Warrell, 2010). The current practice of developing antibodies in mice, chicken-egg yolk, or equines largely produces antibodies against large molecular weight antigens but fails to produce quality antibodies against small molecular weight neurotoxins (Pereira et al., 2019). However, aptamer development does not depend on the immunogenicity of the target, and as shown in the present study they can easily be generated against any target regardless of its molecular weight and structural complexity and may offer a reliable diagnostic tool (Chopra et al., 2014; Dhiman et al., 2017; Liu et al., 2018; Parashar, 2016; Sharma et al., 2017; Toh et al., 2014) The aptamers developed here are also important from the point of view that this is the first time a SELEX has been performed on crude snake venom, rather than purified components of the venom (Ye et al., 2014).

Discovery and screening of biomarkers with aptamers have been gaining attention in recent times (Berezovski et al., 2008; Gold et al., 2010; Jin et al., 2016; Kim et al., 2009; Ma et al., 2019; Ostroff et al., 2010; Ulrich and Wrenger, 2009), owing to their obvious advantages over antibodies (Gold et al., 2010; Kaur et al., 2018). One specific advantage is that once developed against a specific protein (potential biomarker), aptamers can be used in high throughput protein profiling, which then can be used to profile biomarkers or disease identification (Gold et al., 2010). We, herein also identified the target of the best

performing aptamer. The mass study revealed the target as basic phospholipase A2 β-bungarotoxin A2 chain, a biomarker common in krait venoms (Oh et al., 2017). To ascertain the finding of mass study, a competitive ALISA was performed using previously reported *B. multicinctus*' β-bungarotoxin binding aptamer (βB-1). Aptamer affinity data in combination with competitive ALISA further attest the β-bungarotoxin as a cognate target of best performing aptamer candidates generated in the current study.

Another interesting finding of this study is that the developed aptamers were specific to the venom of *B. caeruleus* and did not evince any significant cross-reactivity with the venom of the congeneric *B. niger* and *B. fasciatus,* despite β-bungarotoxin being an integral component of their venom (Silva et al., 2016). However, the composition of the β-bungarotoxin itself varies with the species (Khow et al., 2002; Setiyawan, 2013; Yanoshita et al., 2006) and with the geographical variation (Hia et al., 2020; Rusmili et al., 2019) within the same species. As revealed with mass study, the aptamer binds with the basic phospholipase A2 beta-bungarotoxin A2 chain, which might be missing from the venom of *B. niger* and *B. fasciatus,* or simply they have subtle changes in the amino acid sequences giving rise to different epitopes (Oh et al., 2017; Rusmili et al., 2014). Similarly, the differential binding of the B6 and B8 aptamer with the venom from varied geographical sources can also be explained, as geographical variations affect the venom composition via a variety of mechanisms including but not limited to seasonal variation, diet, habitat, age-dependent change, and sexual dimorphism (Chippaux et al., 1991a; Kalita et al., 2018). This might lead to the reduced amount of β-bungarotoxin present in the crude venom or minor variations in amino acid sequences leading to the generation of different epitopes or altered structure of the existing epitopes (Oh et al., 2017). Taken together, this study has generated a panel of aptamers that can provide a constant source of uniform-quality reagent for the detection of snake envenomation for clinical diagnostics and epidemiological purposes. One potential application where these aptamers can be utilized is the forensic investigation to ascertain the cause of envenomation or death. For example, the Government of Andhra Pradesh, India recently launched a scheme called "Apathbandhu" to provide monitory benefits to dependants of snakebite victims who die as a result of envenomation. The aptamers developed in this study can potentially be used as a tool in autopsy specimens to confirm the real cause of death and to prevent false claims (Brunda et al., 2006). In addition, compared to their conventional counterpart, ALISA experiments performed using the aptamers with paper-based devices shows promising results. As they can be accomplished with minimal use of sample and reagents, are less time consuming and have shown equal, if not better, sensitivity and selectivity, they will be of particular benefit in resource-constrained settings.

One limitation of this study is that due to the lack of availability of the venom of other krait species (*B. sindanus* and *B. walli* which are likely to be co-distributed with *B. caeruleus* in large parts of western, central and eastern regions of India) performance of B6 and B8 aptamers were not evaluated against these venoms. However, we are planning to evaluate the performance of our aptamers against the aforementioned venoms in near future as soon as we have access to these venoms.

5. Conclusions

In conclusion, we have successfully performed SELEX on a complex target, i.e., crude venom, and developed highly affine aptamers, stringently selective against its target, *B. caeruleus* venom. The developed aptamers were specific against *B. caeruleus* venom, and do not evince any cross-reactivity with the venom of other snake and scorpion species tested. The best performing aptamer candidate, B8, shows high binding in a serum background as well, demonstrating its potential to be used as a diagnostic tool in clinical, epidemiological studies, and forensic investigation. The success of this study and the demonstration of successful transfer of conventional ALISA method to a paper-based ALISA paves the way for similar studies to identify highly selective aptamers using complex targets, like crude venom. Further, an improve paper-based point-of-care (POC) device for the qualitative and quantitative detection of venom would allow for more affordable, rapid venom identification and better treatment modalities.

LC-MS/MS data availability

The raw data derived from this study is available at two public data repositories. The MS/MS data described in this study is freely available from Massive (ftp://massive.ucsd.edu/MSV000085338/) and Proteome Xchange database by using the following ID PXD019262.

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CRediT authorship contribution statement

Anjali Anand: Methodology, Data curation, Formal analysis, analysis and, Writing – original draft. **Bandhan Chatterjee:** Data curation, and, Writing – original draft. **Abhijeet Dhiman:** Data curation. **Renu Goel:** Data curation, and, Formal analysis, MS data analysis. **Eshan Khan:** Data curation. **Anita Malhotra:** Resources, Writing – review & editing, venom collection Writing - review and editing. **Vishal Santra:** Resources, Writing – review & editing, venom collection writing-review and editing. **Nitin Salvi:** Writing – review & editing, venom collection Writing-review and editing. **M.V. Khadilkar:** Writing – review & editing, venom collection writing-review and editing. **Ira Bhatnagar:** Data curation. **Amit Kumar:** Formal analysis, CD data analysis, Writing – review & editing, writing-review and editing. **Amit Asthana:** Writing – review & editing, review & editing, Supervision. **Tarun Kumar Sharma:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113523.

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