Article

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ABSTRACT:

Vibrio Anguillarum is a bacterial pathogen that causes serious damages to aquatic fish, and its rapid detection and prevention is critical. DNAzymes are DNA-based catalysts with excellent stability. In this study, in vitro selection of DNAzymes was performed using the crude extracellular matrix (CEM) of *V. Anguillarum* as the target. Difference from previous selections targeting bacterial CEM, this work used an unmodified DNA library allowing easier adoption of the technology. After 7 rounds of selection, a DNAzyme named VAE-2 with high activity and specificity was obtained. It showed the highest activity towards *V. Anguillarum* among eight types of tested bacterial strains. Polyvalent metal ions are needed for its activity. Protease treatment of the CEM and filtration studies indicated that the target is a protein with a molecular weight between 50 k and 100 k Da. A fluorescent biosensor was designed for *V. anguillarum* with detection limit down to 4000 cfu/mL, and detection was demonstrated for real fish tissue and feeding water samples. Being the first work of DNAzyme-based sensing of aquatic bacteria, this study indicates that unmodified DNA can be used for targeting bacterial CEM, and it provides a new framework for developing other RNA-cleaving DNAzymes for rapid detection of pathogenic bacteria and water pollution.

KEYWORDS: *Vibrio Anguillarum;* DNAzymes; biosensors; aptamers

INTRODUCTION

Selective detection of bacteria is critical for water and food safety, environmental research, and microbiology. Many methods have been developed, such as fluorescent antibodies,[1] cell culturing,[2] ELISA,[3] and PCR.[4] However, most of them are time-consuming, expensive, and not suitable for onsite applications. For example, cell culturing often takes days to complete, while antibody-based assays need multiple washing steps and are expensive. PCR needs DNA extraction, multiple reagents and a thermocycler, and it may also pick up DNA from dead cells.

To address this analytical challenge, efforts have been made to isolate aptamer for bacterial cells, but non-specific binding is often a concern.[5-8] Currently, few bacterial binding aptamers have been widely used.[6, 9-12] DNAzymes are DNA oligonucleotides with catalytic activity. The first DNAzyme was reported in 1994 for RNA cleavage.[13] Since then, many different DNAzymes have been reported.[14, 15] RNA-cleaving DNAzymes have been most widely used for biosensor development,[16-18] and pharmaceutical research[19] due to their excellent activity.[20-22] DNAzymes have so far been most successful in metal sensing,[23-25] such as Pb^{2+},[13] Ca^{2+},[26, 27] Na^{+},[28, 29] Cu^{2+},[22] UO_2^{2+},[30] Ag^{+},[31, 32] and lanthanides.[33]

Since DNAzymes rely on catalytic reactions, they may be less susceptible to nonspecific binding. Instead of targeting cell surface molecules, Li and coworkers used crude extracellular mixtures (CEM) as the target for selecting DNAzymes. Fluorogenic DNAzymes were obtained to detect *Escherichia coli*,[34] and *Clostridium difficile*.[35, 36] A paper-based sensor was developed for *Klebsiella pneumoniae*.[37] These reports demonstrated some unique advantages of using DNAzymes for bacterial detection.

In Li's selection method, the selection libraries were always labeled with a fluorophore and a quencher right next to the RNA cleavage junction. While it allows immediate and sensitive signaling, such modifications are expensive and the selection procedures are complex. At the same time, the labels become part of the DNAzymes and they may directly involve in catalysis, making it difficult for the DNAzymes to be used for other types of signaling mechanisms. We are interested to see if an unmodified library can work for this purpose.

So far, most DNAzymes target terrestrial microorganisms that infect humans.[34, 38-41] *V.*

anguillarum is bacterial pathogen that causes serious damages to aquatic fish,[42] such as trouts, eels and turtles, often leading to a large economic loss.[43] Diseases caused by aquaculture animals can spread worldwide, leading to a huge economic loss. Therefore, quick analysis of such infections is important. In this work, we performed a conventional DNAzyme selection using the CEM from *V. anguillarum* and obtained a highly selective DNAzyme. This low cost yet effective DNAzyme may facilitate its adoption for further biosensor development and application.

MATERIALS AND METHODS

Materials. *Vibrio anguillarum*, *Vibrio vulnificus, Edwardsiella tarda, Vibrio parahemolyticus* and *Aeromonas salmonicida* were from China Center of Industrial Culture Collection (CICC). *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were provided by Marine Resources Development Institute of Jiangsu (Lianyungang). The in vitro selection and sensing related DNA sequences (Table1) and streptavidin-coated magnetic particles were from Sangon Biotech (Shanghai, China). Tris (hydroxymethyl) aminomethane, Tween-20 and all metal salts were from Sinopharm (China) at the highest purity available. N-(2-hydroxyethyl) piperazine-N'-(2- ethanesulfonic acid) (HEPES), 2-(N-morpholino)-ethanesulfonic acid (MES). T4-DNA ligase, deoxynucleotide (dNTP) mix, Taq DNA polymerase with 10×PCR buffer, 6×gel loading dye, and low molecular weight DNA ladder were from New England Biolabs. Gel-red (10,000×) was from BBI CO., LTD (Shanghai, China). Tryptone and yeast extract powder were from Oxoid. Agarose was from Biowest. All solutions were prepared using ultrapure water with an electric resistance >18.20 MΩ·cm.

Preparation of CEM. The *V. anguillarum* strain in lyophilized powder was rejuvenated in 20 mL LB culture (Luria Bertani Broth, 1% tryptone, 0.5% yeast extract powder, 1% NaCl, pH 7.5), and incubated at 25 °C with shaking at 180 rpm until the OD_{600} (optical density at 600 nm) approximated to 1. Then, a certain amount of bacteria were plated onto LB agar plates for detection and glycerol stocks (all steps of inoculation on a clean bench). The remaining bacterial cultures were packed into several 1.5 mL EP tubes and centrifuged at 11,000 g for 5 min at room temperature. The supernatant were transferred to multiple new 1.5 mL sterilized EP tubes and stored at -20 °C as the CEM of *V. anguillarum* (CEM-VA). The

CEM of other bacteria were prepared in a similar way except temperature and time.

Table 1. DNA sequences related to in vitro selection*.

DNA Names	Sequences and modifications (5′-3′)
Lib-N_{40}-pool	pGGAAGATGGCGAAACATCTT-N40-TAGTGACGGTAAG CTT
Primer1(P1)	AAGCTTACCGTCACTA
Primer2(P2)	**Biotin**-GTCACGAGTCACTAT**rA**GGAAGATGGCGAAACA
FAM-Substrate	**FAM**-GTCACGAGTCACTAT**rA**GGAAGATGGCGAAA
183601_F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATCTNTCACTATAGGAAGATGG CGAAACA
183601_R355	CAAGCAGAAGACGGCATACGAGATCAACGTGGGTGAC TGGAGTTCCTTGGCACCCGAGAATTCCAGTGCCAAGCT TACCGTCACTA

* The Lib-N40-pool DNA was phosphorylated on the 5′-end (denoted by the p); N_{40} denotes for the 40 random nucleotides. The cleavage site ribo-adenine is denoted by rA, FAM = carboxyfluorescein.

Preparation of the initial library. Lib-N_{40}-pool was amplified by PCR with P1 and P2 primers for preparation of the initial library. The PCR mixture (50 μL) included Lib-N_{40}-pool (< 100 ng), 0.2 μM (final concentration) each of P1 and P2, 200 μM dNTPs, 1× thermopol reation buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8), and 1.25 U/μL Taq DNA polymerase. The following thermocycling protocol was used: 94 °C for 5 min, 14 cycles of 94 °C for 30 s, 57.5 °C for 30 s, and 72 °C for 1 min; final extension at 72 °C for 2 min. The PCR product was analyzed by 2% agarose gel electrophoresis (stained with Gel-red). Finally, all PCR products were further concentrated via ethanol precipitation.

Positive selection. For the first round, 50 μL streptavidin-coated beads (50 mg beads/mL, diameter =0.5 μm) were washed three times with buffer A (10 mM Tris-HCl，1 mM EDTA，

1 M NaCl， 0.01%~0.1% Tween-20, pH 7.5). Then, 500 μL of biotinylated DNA library (from above PCR) was incubated with buffer A containing magnetic beads (5 mg/mL) for 30 min at 37 °C. After magnetic separation and washed twice with buffer A, 0.2 M NaOH was added to remove the unbiotinylated strand. After rinsing with Milli-Q water to ensure the pH was close to neutral, 150 μL CEM-VA and equal volume buffer B (100 mM HEPES, 300 mM NaCl, 30 mM $MgCl_2$, 0.02% Tween-20, pH 7.5) were added and the sample was incubated at 37 °C on a DNA rotary mixer for 1 h. After that, the concentration of the cleavage fragment (concentration = C) was measured on a Nanodrop instrument. The immobilized biotinylated DNA strands (concentration = C_0) were all released from magnetic beads (incubated for 2 min at 95 °C in 10 mM EDTA, pH 8.2 with 95% formamide will typically dissociate > 96% of immobilized biotinylated DNA[44]). The fraction of cleavage was calculated to be (C/C_0). Each sample was measured three times. The DNA concentration was measured using a Nanodrop instrument with 1.5 μL volume. A typical UV-vis spectrum for this quantification of cleaved DNA is shown in Figure S1. The cleavage fragment was subjected to ethanol precipitation, PCR amplification, and backed to the full length for the next round of selection (Figure 1A).

Deep sequencing. The PCR product from the final round was purified by 2% agarose gel (stained with Gel-red) and extracted from the gel using a kit (Biosci Biotech, China). Then the forward (183601_F) and reverse primers (183601_R355, see Table 1) were added for PCR. The PCR product was purified with 2% agarose gel, extracted, and eluted in 50 μL of Milli-Q water (DNA concentration was 208.9 ng/μL by UV-vis). The sequencing was performed by Sangon Biotech (Shanghai, China).

Activity assays. The activity assay of each candidate DNA sequence was performed by a dPAGE assay (denatured polyacrylamide gel electrophoresis) reported previously.[45] Briefly, a DNAzyme and the FAM-labeled substrate were annealed at 90 °C to form a DNAzyme complex (final concentration 5 μM). Then, 23 μL CEM were mixed with 2 μL of the 5 μM DNAzyme complex and incubated in 25 μL Buffer B for 1 h. The DNA was then precipitated by adding 3 M NaOAc (pH 5.5) and ethanol precipitation, and the pellet was dried in a speedvac at room temperature. The dried DNA was then dissolved with 20 μL Milli-Q water

and 10 μL gel loading buffer (2.5% Ficoll^{R}-400, 11 mM EDTA, 3.3 mM Tris-HCl, pH 8.0, 0.017% SDS, and 0.015% bromophenol blue). After running in 10% dPAGE at 120 V for 80 min, the cleavage percentage was calculated by Bio-Rad GelDoc^{TM} EZ imaging system, which can analyzed the percentage of each DNA band according to its intensity in the whole lane (the cleavage DNA plus un-cleavage equal to 100%).

Fluorescence-based CEM-VA sensing. Kinetics studies were performed in 96-well plates. The sensing complex was formed by annealing the FAM-labeled substrate (10 μM) and the quencher-labeled enzyme (15 μM) in the selection buffer (Table S1). Then, 4 μL of the above annealed DNAzyme was diluted with 86 μL buffer B for each well, and 10 μL of CEM-VA was added. The fluorescence intensity (F) were monitored by a microplate reader (Infinite M1000 Pro, Tecan, Switzerland). The fluorescence of the same concentration of DNAzyme complex (without quencher) was set as blank (marked F_0). Each sample was run in triplicate, and the activity was expressed as F/F_0 (excitation at 488 nm and emission collected at 520 nm). The kinetics of signaling was followed on the microplate reader at room temperature (~22 °C) with 30 s intervals.

Live fish experiments. The fish samples were infected by 0.2 mL *V. anguillarum* solution through dorsal muscle or intraperitoneal injection. Regular feeding and observation of changes in the body surface were made for the experimental group. Two types of samples were collected as the bacterial sources: one from fish bodies (using a sterile medical cotton ball to scrape the infection site or body surface) and another from the feeding water. All the collected bacterial sources were cultured in the Luria Bertani Broth at 25°C with shaking at 180 rpm for 24 h. Each sample was tested in triplicate, and all bacterial cultures were centrifuged at 11,000 g for 5 min at room temperature. The supernatants were the CEM to mix with the DNAzyme complex and tested as described above. The testing stopped when all the experimental group fish died.

RESULTS AND DISCUSSION

In vitro selection. We cultured *V. anguillarum* and removed the cells by centrifugation.

The resulting supernatant were the crude extracellular matrix (Figure 1A) and we named it (CEM-VA). To isolate DNAzymes that can be activated by CEM-VA, a 94-nucleotide (nt) library containing a 40-nucleotide random region was used (N40, Figure 1B). Our initial library had ~10^{14} random DNA sequences. Each sequence contained a single RNA linkage (rA denoting for ribo-adenine) serving as the cleavage site, and a 5′ biotin for immobilization on a strapavidin column. The immobilized library was incubated with the CEM-VA, and the active sequences might be cleaved and then release the 76-nucleotide fragment into the solution. After separation with a magnet, the solution was PCR amplified to rebuild the full-length library for the next round of selection (Figure 1C).

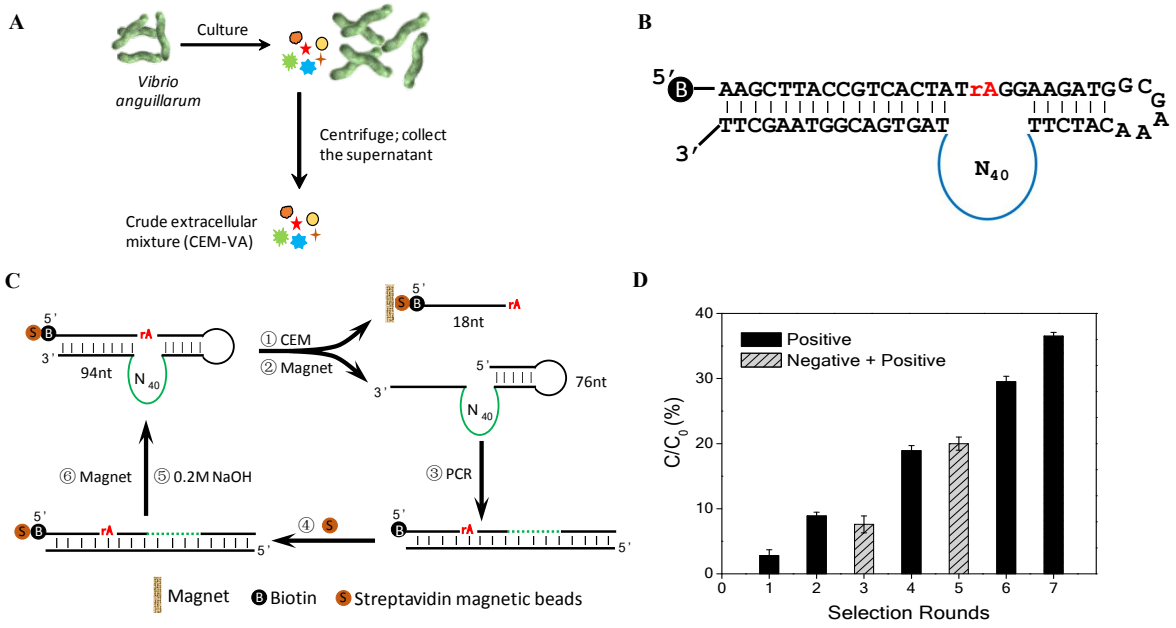

Figure 1. (A) Schematic illustration of preparing the CEM-VA. **(B)** The initial library for our in vitro selection. The cleavage site is at the rAG junction and a biotin was labeled on the 5′ end. **(C)** Selection for CEM-VA-specific DNAzymes. The initial library was immobilized and the active sequences were cleaved in the presence of CEM-VA. After PCR, the cleavage fragments were recovered to the original full-length library to seed the next round selection. **(D)** The progress of our selection measured by a UV-vis spectrophotometer (C = concentration of the cleaved library in the supernatant; C_0 = concentration of all of the DNA immobilized on the magnetic beads of each round).

A total of 7 rounds of selection were carried out. For round 3 and 5, a negative selection

step was also introduced by first incubating the library with CEM-VV (e.g. the CEM from *Vibrio vulnificus*, a potential interfering bacterium), where the cleaved sequences were discarded and the uncleaved ones were then incubated with the CEM-VA for the normal selection. The cleavage yield of each round gradually increased (Figure 1D), reaching nearly 40% at round 7. We stopped at this point, and the PCR products of round 7 were sequenced.

Sequence analysis. We obtained 46,421 sequences, and the first two most abundant ones accounted for 36.3% and 20.2% of them (Figure 2A). Therefore, the library was highly converged. We aligned the first 10 most abundant sequences with the help of Cluster Omega (all above 1% and totaled ~80%, Figure 2A), and some conserved nucleotides can be observed, suggesting that the selection might be successful.

Their cleavage activities were measured by a gel-based assay in the presence of CEM-VA. Only the first and second candidate DNAzymes showed cleavage bands (Figure 2B), and we named them VAE-1 and VAE-2, respectively. We then predicted their secondary structures with the IDT Oligonanlyzer 3.1 software and both them had a stem loop structure (Figure 2C, 2D). The sequences in the loop in red are conserved in both enzymes. Since VAE-2 DNAzyme had better activity, most of our subsequent studies were performed with it.

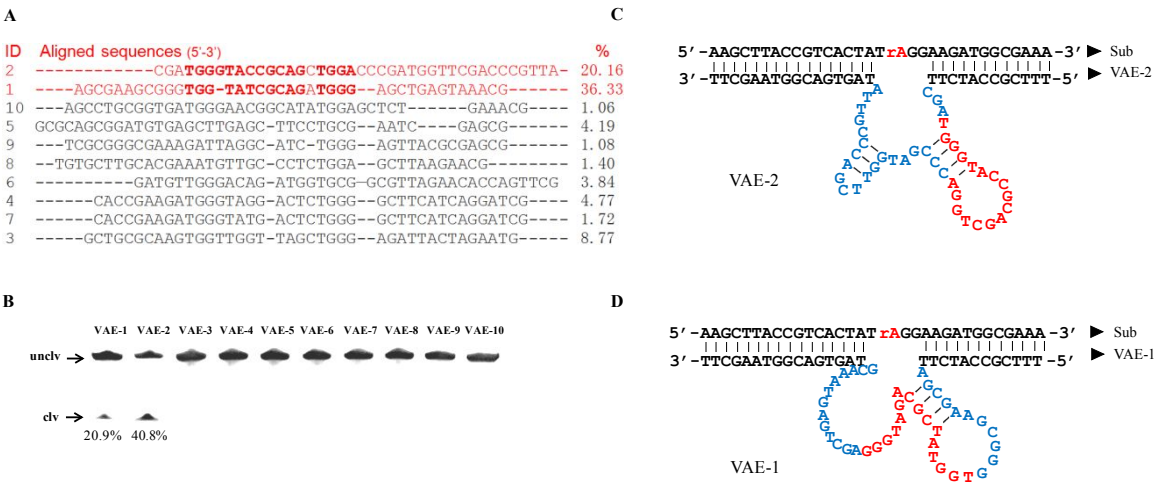

Figure 2. (A) The most abundant 10 DNA sequences from deep sequencing. The bases in boldface are for the highly aligned VAE-1 and 2 sequences. **(B)** A gel micrograph showing the activity of each DNAzyme in buffer B for 1 h. The predicted secondary structures of **(C)** VAE-2 and **(D)** VAE-1 DNAzymes.

Highly specific detection. We then constructed a molecular beacon like sensor by labeling a quencher on the 3′ end of the VAE-2 enzyme strand and a FAM fluorophore on the 5′ end of substrate (Figure 3A). The fluorescence was quenched when they were annealed to form a complex. In the presence of CEM-VA, the substrate was cleaved and release of the fluorophore-bearing fragment can lead to fluorescence enhancement.[46]

When the sensor was incubated with the blank culture medium without CEM-VA (the lane marked LB), no cleavage was observed (Figure 3B). Therefore, the above cleavage was not caused by the medium. We then incubated the sensor in the CEM of seven different bacteria. Among these, only *V. vulnificus* showed a moderate cleavage (~30% of that by CEM-VA). The same conclusion was also obtained from the biosensor-based assay (Figure 3C). *V. vulnificus* is also a marine bacterium and they may share some common CEM. Overall, the VAE-2 DNAzyme has a high specificity to *V. anguillarum* and can be useful for its detection.

Highly sensitive detection. To evaluate the sensitivity of the VAE-2 based biosensor towards *V. anguillarum*, we made a 10-fold serial dilution of the initial CEM-VA (original concentration 4×10^8 cfu/mL) to yield bacterial suspensions of different concentrations. Then, all of them were prepared as described in experimental section. The quantification followed the literature method)[45]. The cleavage yield of VAE-2 increased with increase of the CEM-VA concentration (Figure 3D). The same test was also performed using the biosensor and the fluorescence intensity of each sample was monitored for 1 h. A concentration-dependent response was also obtained (Figure 3E). The fluorescence intensity increased with increasing *V. anguillarum* concentration from 1×10^3 to 1×10^8 cfu/mL, and the detection limit was calculated to be 4,000 cfu/mL.

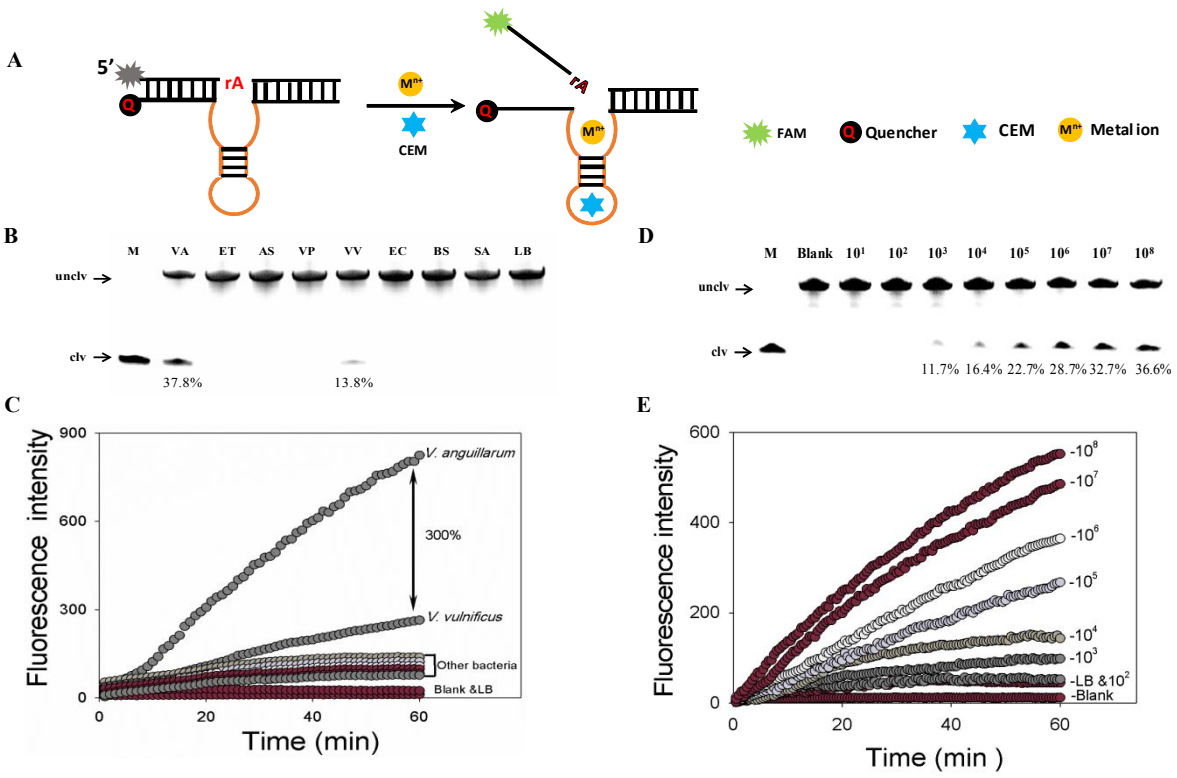

Figure 3. (A) A molecular beacon design for DNAzyme sensor. **(B)** A cleaving gel image for VAE-2 specificity detection within 1 h. LB: Blank culture medium, ET: *Edwardsiella tarda*, VV: *Vibrio vulnificus*, VP: *Vibrio parahemolyticus*, AS: *Aeromonas salmonicida*, EC: *Escherichia coli*, BS: *Bacillus subtilis*, SA: *Staphylococcus aureus*, VA: *Vibrio anguillarum*. **(C)** A biosensor-based assays responded to VAE-2 in blank culture medium and seven bacteria. **(D)** The gel image and **(E)** biosensor assay of VAE-2 in the CEM-VA from different concentrations of *V. anguillarum*.

A protein-dependent DNAzyme. Since the CEM is a complex mixture, it is difficult to know the exact molecule responsible for promoting the reaction. Li and coworkers performed extensive analysis and found that a transcription factor (a protein) to be responsible for one of their DNAzymes.[35] For most other DNAzymes selected using the CEM of cells, the target molecules have yet to be identified.

We first tested whether our target is a protein or not. We treated CEM-VA with trypsin to cleave proteins before mixing it with the VAE-2 DNAzyme. The results were measured by both gel-based and biosensor-based assays, but neither showed cleavage (Figure 4A). Therefore, the target is likely to be a protein. We then attempted to estimate the molecular

weight of this protein. CEM-VA was filtered through centrifugal columns with different molecular weight cut-offs from 10 k to 100 k Daltons. Only the filtrates of the 100 k induced cleavage (Figure 4B). There, the molecular weight of the target protein is between 50 k and 100 k.

A metal-dependent DNAzyme. Since most DNAzymes require metal ions for activity, we also studied the effect of metal ions.[47, 48] We first added EDTA to chelate polyvalent metal ions and this has inhibited the cleavage (Figure 4C, the red bar). The EDTA added sample still contained Na^+, and possibly other free monovalent metal ions. Therefore, it is likely that polyvalent metal ions are required for its cleavage. We then added extra monovalent metal ions (Li^+, Na^+, K^+, Ag^+, Tl^+), where Ag^+ and Tl^+ inhibited the reaction. We also added extra divalent metals (Ca^{2+}, Cu^{2+}, Ba^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Pb^{2+}, Zn^{2+}). Only the samples with Ba^{2+}, Ca^{2+}, Mn^{2+} showed cleavage, while the rest metals all inhibited the reaction. It is likely that soft transition metal ions can interact with the target protein and inhibit the DNAzyme activity.

We also studied the effect of pH (Figure 4D), and the cleavage activity gradually increased with higher pH. This is consistent with the general mechanism of RNA cleavage. According to this result, we used pH 8.0 for most of this study. To some extent, it is quite interesting that we were able to obtain a new DNAzyme with such unmodified library. In most such selections, the 8-17 motif is often obtained.[49] This study demonstrated the possibility of obtaining new DNAzymes with a complex selection medium.

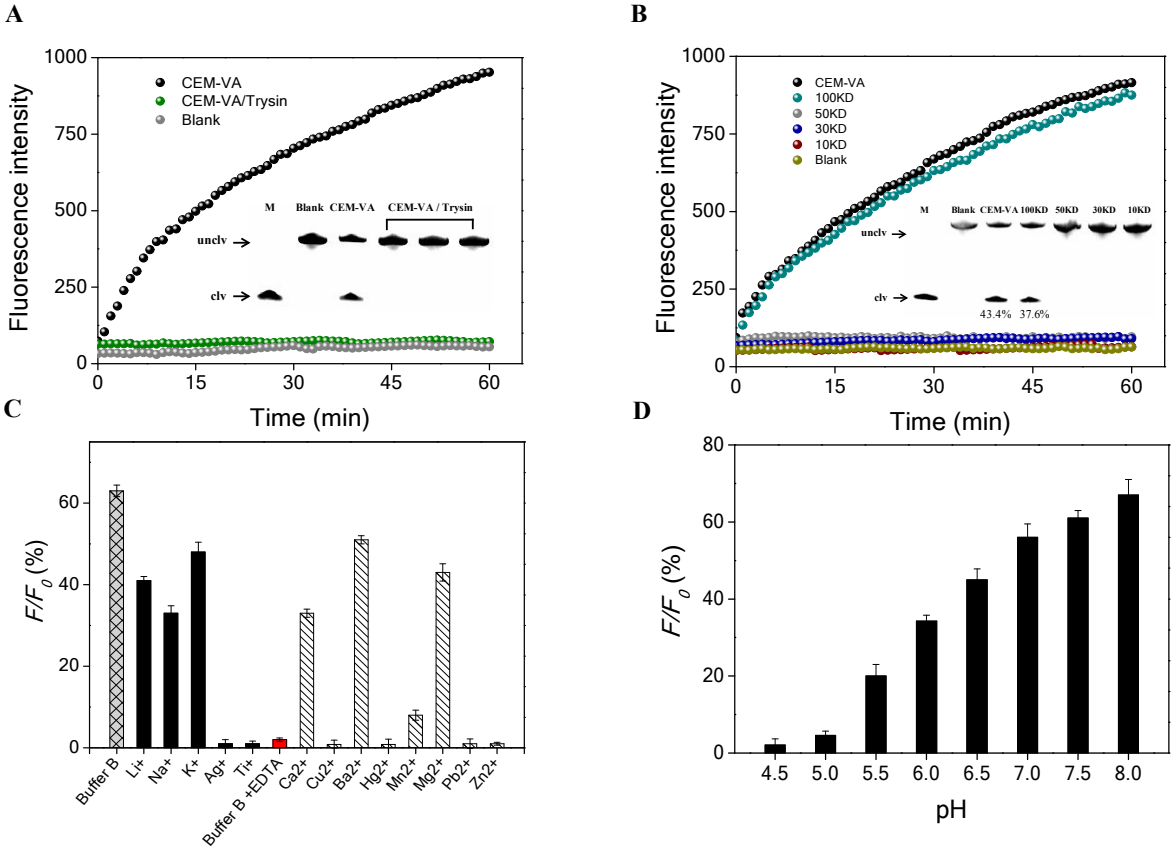

Figure 4. (A) Trypsin treated CEM-VA showed no signal to the VAE-2 based biosensor. Inset: a gel micrograph showing the activity. **(B)** Assessment of the molecular weight of the target protein. Inset: gel images responded to each part and all the cleavage reactions was 1 h in buffer B. **(C)** Effect of various monovalent and divalent metal ions on the cleavage activity. Adding 300 mM EDTA fully inhibited the cleavage (all DNAzyme complexes were formed in Buffer B). **(D)** The cleavage at different pH.

Test of real samples. *V. anguillarum* is also called *Listonella anguillarum*, a pathogen of the most serious vibrio disease in aquaculture.[50, 51] Due to the high morbidity and mortality rates, many efforts have been made to detect this pathogen. In this study, we developed a new strategy for fast detection of *V. anguillarum*. To confirm its application, we fed 9 crucians as the experimental group, while another 8 were set as controls, all of which were fed in different fishponds. Figure 5A and 5B showed the representing photographs of the experimental and control groups after the feeding days, and bleeding spots appeared on the body of the experimental group (Figure S2), consistent with those reported in the literature.[50,

[52, 53] We then chose the surface of fish bodies and the water for feeding as the bacterial sources. After culturing for 24 h, their CEM samples were tested for DNAzyme cleavage.

For both the CEM from the fish bodies (Figure 5C) and the feeding water (Figure 5D), we observed growing sensor signals from the sample group but the controls stayed at the background level. Since a lot of bacteria existed on the infection sites of the fish body, they also exist in the feeding water. Test of feeding water is sometimes preferred due to its non-invasive sampling and convenience. We have also followed the kinetics of cleavage and the signal growth with the feeding time was also observed (Figure S3). This experiment demonstrated that the VAE-2 DNAzyme can achieve the real sample testing and has the potential to be used as a simple biosensor for onsite *V. anguillarum* detection in resource-limited farms.

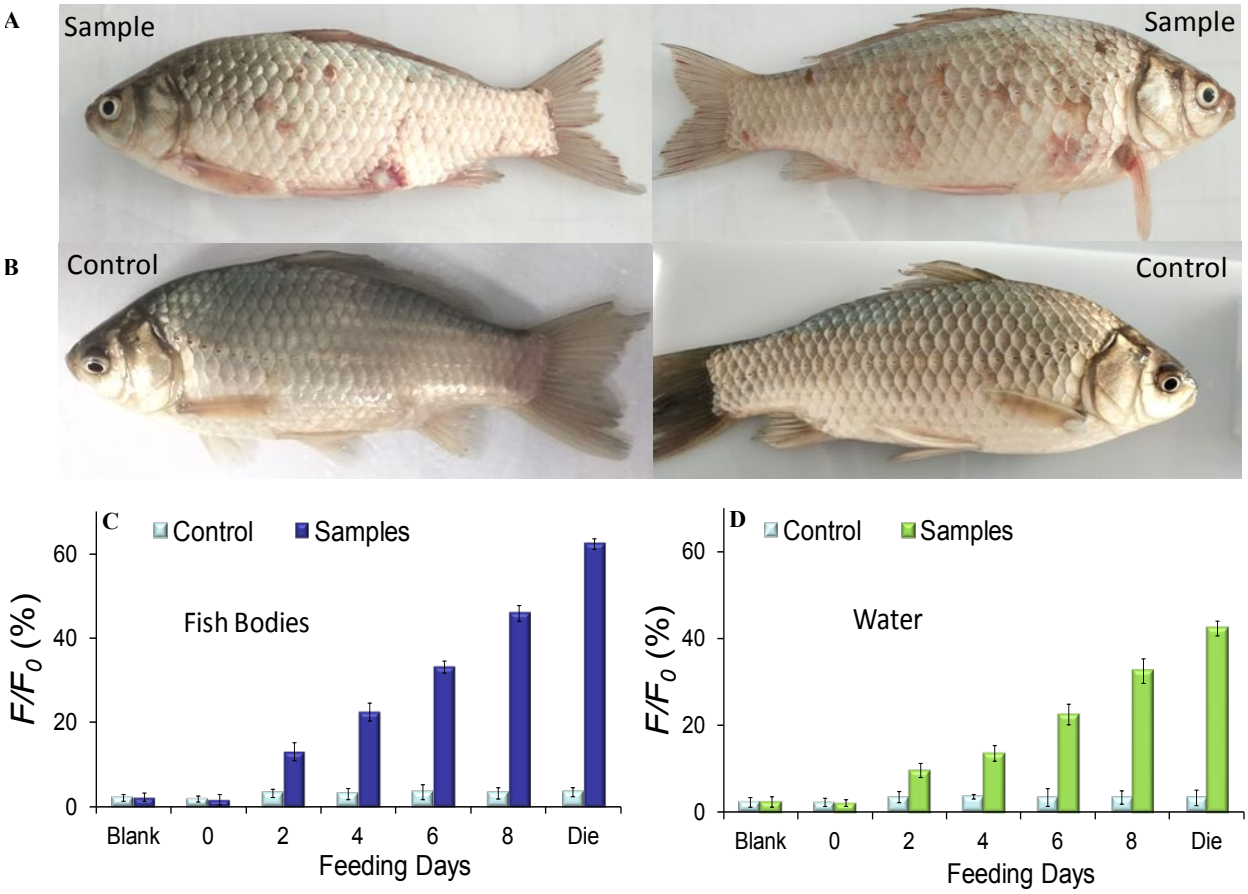

Figure 5. Photographs showing the bodies of (A) the *V. anguillarum* infected experimental group and (B) the control group after feeding for 11 days. (C) DNAzyme cleavage activity comparison of the control and infected samples as a function of feeding time for the CEM obtained from (C) the fish bodies and (D) the feeding water. After 11 days, the infected fish

died and the experiment stopped.

CONCLUSIONS

In summary, we reported an efficient RNA-cleaving DNAzyme for *V. anguillarum* detection obtained by in vitro selection. Different from previous work, this DNAzyme was selected from an unmodified library, and it showed high specificity and sensitivity to the CEM-VA. A FRET-based biosensor was design to detect *Vbrio anguillarum* with a detection limit of 4×10^3 cfu/mL. Infected fish can be detected from samples collected from its body or the feeding water. This work is also the first example of using marine bacteria as a target for DNAzyme selection, demonstrating the feasibility of developing other RNA-cleaving DNAzymes in this area. Our results will be useful for the further development of bioanalytical chemistry using DNAzymes for managing contamination and reducing human health risks.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.xxxxx.

DNA sequences, UV-vis spectra, changes in the surface tissue of a crucian infected by *V. anguillarum* and kinetics of the DNAzyme signaling for the infected fish and the control samples (PDF)

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interests regarding this paper.

ETHICAL APPROVAL

This article does not contain any studies with animals performed by any of the authors.

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