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Generation and characterization of nucleic acid aptamers targeting the capsid P domain of a human norovirus GII.4 strain

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ABSTRACT

Human noroviruses (NoV) are the leading cause of acute viral gastroenteritis worldwide. Significant antigenic diversity of NoV strains has limited the availability of broadly reactive ligands for design of detection assays. The purpose of this work was to produce and characterize single stranded (ss)DNA aptamers with binding specificity to human NoV using an easily produced NoV target—the P domain protein. Aptamer selection was done using SELEX (Systematic Evolution of Ligands by EXponential enrichment) directed against an *Escherichia coli*-expressed and purified epidemic NoV GII.4 strain P domain. Two of six unique aptamers (designated M1 and M6-2) were chosen for characterization. Inclusivity testing using an enzyme-linked aptamer sorbent assay (ELASA) against a panel of 14 virus-like particles (VLPs) showed these aptamers had broad reactivity and exhibited strong binding to GI.7, GII.2, two GII.4 strains, and GII.7 VLPs. Aptamer M6-2 exhibited at least low to moderate binding to all VLPs tested. Aptamers significantly ($p < 0.05$) bound virus in partially purified GII.4 New Orleans outbreak stool specimens as demonstrated by ELASA and aptamer magnetic capture (AMC) followed by RT-qPCR. This is the first demonstration of human NoV P domain protein as a functional target for the selection of nucleic acid aptamers that specifically bind and broadly recognize diverse human NoV strains.

1. Introduction[2]

Human noroviruses (NoV) are the most common cause of acute viral gastroenteritis worldwide (Glass et al., 2009) and the leading cause of foodborne illness in the United States (Scallan et al., 2011). Despite their public health significance, the availability of routine detection methods for these viruses is limited, in part due to the absence of an in vitro cultivation method. While molecular amplification (specifically reverse transcriptase quantitative PCR or RT-qPCR) is usually used for NoV detection and genome quantification by the public health sector, it is not commonly used in clinical diagnostics. Because of sample complexity (fecal matrix) and the need to remove PCR inhibitors, ligand-based detection methods are more appealing for clinical diagnostics.

Unfortunately, human NoV are genetically and antigenically diverse, complicating the identification of broadly reactive ligands (e.g., antibodies) that can be used for virus capture and/or detection. The lack of broad reactivity by antibodies to human NoV strains has been well documented (Burton-MacLeod et al., 2004; Shiota et al., 2007), and for this reason, enzyme immunoassays display poor sensitivity (Costantini et al., 2010; Kele et al., 2011). Other candidate NoV ligands have been explored, such as putative NoV infection co-factors known as histo-blood group antigens (HBGAs) (Cannon and Vinjé, 2008; Harrington et al., 2004) and porcine gastric mucin, which contains some HBGAs (Pan et al., 2012; Tian et al., 2008); peptides (Rogers et al., 2013); and single chain antibodies (Huang et al., 2014). While some of these react with multiple human NoV strains or VLPs, no completely inclusive human NoV ligand has been reported.

For both pathogen capture and purification, nucleic acid aptamers are a promising alternative ligand. Aptamers are short (20–80 mer) single-stranded DNA or RNA sequences that interact (bind) with their target through their three-dimensional structures. They offer advantages over antibody-based affinity molecules in their ease of production, purification, modification, physical stability, and lower cost (Brody and Gold, 2000; Murphy, 2003; Tombelli et al., 2007). Nucleic acid aptamers are selected in vitro based on affinity for a target molecule, protein, virus, or cell using a

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[2] NoV; norovirus(es). ELASA; enzyme-linked aptamer-sorbent assay. AMC; aptamer magnetic capture. RT-qPCR; reverse transcriptase quantitative polymerase chain reaction.

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molecular-based iterative enrichment method called SELEX (systematic evolution of ligands by exponential enrichment).

In the absence of a robust in vitro cultivation method, the only source of whole viruses for ligand selection is stool samples from infected individuals. As infectious virus in stool is a difficult sample to obtain and work with, virus-like particles (VLPs) are frequently used instead for many types of studies, from disinfection to immune response characterization (Cheetham et al., 2007; Lou et al., 2012; Nilsson et al., 2009; Souza et al., 2007; Vongpunsawad et al., 2013). VLPs demonstrate similar binding behavior to HBGAs as human NoV particles (Huang et al., 2003; White et al., 1996); however, their production and purification can be costly, time consuming, and variable (Koho et al., 2012). An alternative is to focus selection on a portion of the human NoV major capsid protein or VP1. Unlike VLPs for which the entire capsid [all 180 copies of the major capsid protein (VP1)] assembles as nucleic-acid free "ghosts," "P domain proteins" consist of proteins containing the outermost domain of the NoV VP1 capsid protein. Like VLPs and human NoVs, these proteins retain their antigenicity, can still bind to histo-blood group antigens and have been used for structural, binding, and vaccination studies (Cao et al., 2007; Koho et al., 2012; Tan et al., 2011). P domain proteins can easily be produced in a bacterial system (Tan and Jiang, 2005) and expressed and purified at low cost and with high yield, making them an attractive target for ligand selection. In this study, we describe the production of single stranded (ss)DNA aptamers with binding affinity to a representative human NoV strain by SELEX using a P domain protein. Once isolated and characterized, promising aptamer candidates were further tested for their degree of reactivity with a broad panel of human NoV VLPs. They were then used to develop prototype methods to capture and/or detect GII.4 human NoV in outbreak-associated fecal specimens.

2. Materials and methods

2.1. Viruses, virus-free fecal specimens, and virus-like particles

A GII.4 outbreak-derived human clinical (fecal) sample [sequence-confirmed to be the "2006b" cluster of GII.4 epidemic strains (Tsai et al., 2014; Yang et al., 2010)] was obtained courtesy of S.R. Greene (North Carolina Department of Health and Human Services, Raleigh, NC) and suspended 20% in phosphate-buffered saline (PBS). Human NoV-negative stool samples derived pre-exposure from individuals participating in a human challenge study were kindly provided by C.L. Moe (Emory University, Atlanta, GA). In some instances, stool suspensions were used without further processing. In other cases, the suspensions were partially purified by chloroform extraction (Shin and Sobsey, 2008). All suspensions were stored at −80 °C until use in experiments. Virus-like particles (VLPs), which consisted of purified virus capsid without the viral genome, were provided courtesy of R. Atmar (Baylor College of Medicine, Houston, TX). The following VLPs were available for this study: GI.1, GI.4, GI.6, GI.7, GI.8, GII.1, GII.2, GII.3, GII.4 (2 strains), GII.6, GII.7, GII.12, and GII.17.

2.2. Preparation of P-domain protein

The clinical outbreak stool specimen used for creation of the P domain was confirmed to belong to the 2006b GII.4 cluster by RT-PCR amplification and sequencing (data not shown). Primers specific to the P domain region (nt 5744–6704), which included flanking *Bam*HI and *Not*I restriction enzyme sites, were designed using the GII.4 2006b sequence [accession number: JN400603; (Tsai et al., 2014)] based on the locations of previously reported primers without a hinge (Tan and Jiang, 2005; and Table 1). These were used to produce cDNA using the RETROscript kit (Ambion/Applied Biosystems) and amplified in PCR with the designed primers [GII.4 P domain forward/reverse, Table 1] and the Platinum *Taq* system (Invitrogen). The products were cleaned with the QIAquick PCR purification kit (Qiagen) and restriction digested with *Bam*HI and *Not*I (New England BioLabs, Ipswtich, MA). This was ligated into a similarly digested pGEX-4T-1 plasmid (GE Healthcare, Piscataway, NJ) containing an *N*-terminal glutathione-*S*-transferase (GST) tag with a 2:1 insert: vector ratio. The vector was then electroporated into electrocompetent *E. coli* BL21(DE3) cells [E. cloni EXPRESS; Lucigen, Middleton, WI]. Successful transformants were screened by colony PCR and confirmed by sequencing (Genewiz, Inc.).

P domain–GST fusion protein and GST-only cultures were grown overnight in 2X yeast extract tryptone ampicillin (YTA) broth incubated at 37 °C. Thereafter, the bacteria were pelleted, reconstituted in 2X YTA, and used to seed a larger 2X YTA culture that was grown at 37 °C to an OD_{600} of 0.6–0.9. The cultures were then induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left overnight at 25 °C with gentle shaking. Cells were purified by centrifugation and lysed with 106 μm acid-washed beads (Sigma) and a mini bead beater (Biospec Products Inc., Bartlesville, OK). For further purification, the lysate was incubated 1:1 (v/v) in 50% glutathione sepharose 4B agarose bead solution (GE Healthcare, Little Chalfont, United Kingdom) for 30–45 min at 22 °C, followed by centrifugation and washing of the bead-protein complexes. Elution from the fusion protein was done using 50 mM Tris-HCl/10 mM reduced glutathione buffer (pH 8.0) mixed 1:1 with the bead volume and incubated for 15–20 min at 22 °C followed by centrifugation. Presence of the P domain protein in the lysate and eluate was confirmed by Western blotting on nitrocellulose membranes using anti-GST primary antibody (Thermo Fisher Scientific, Waltham, MA) and anti-GII.4 primary antibody (ab80024, Abcam, Cambridge, England).

2.3. Aptamer selection (SELEX) and characterization

2.3.1. Preparation of DNA library

An 81-base combinatorial DNA library having a 40 nt variable region was obtained from Integrated DNA Technologies (IDT, Coralville, IA). The library was prepared for SELEX by producing an 81 bp double-stranded (ds)DNA molecule that was unlabeled at the 5′ end and labeled at 3′ end with biotin by PCR using a forward constant region primer and a biotinylated reverse constant region primer (Table 1), as described previously by Dwivedi et al. (2010). For separating the biotinylated DNA strand from its complementary strand, the labeled dsDNA was coupled with Streptavidin MagneSphere® Paramagnetic particles (Promega) and captured by magnet (MPC-M magnetic particle concentrator, Dynal A.S. Oslo, Norway). The captured dsDNA was denatured by treatment with 0.15 M sodium hydroxide and after three washes with Tris-EDTA (TE), the immobilized biotinylated strand was released by incubating beads in 28% ammonium hydroxide at 85 °C for 10 min. Removal of residual ammonium hydroxide was achieved using Vivaspin 500 filters (10,000 molecular weight cut-off, Sartorius Stedim Biotech, Cedex, France) with two washes of nuclease-free water. The purified ssDNA was stored in −80 °C until use.

2.3.2. Selection of aptamers using GII.4 human NoV P domain protein

SELEX and counter-SELEX were performed using the P domain–GST fusion protein and the GST tag with NoV-negative human stool and bead matrix as targets, respectively. Briefly, 300–500 pmol of the library was pre-heated at 90 °C for 10 min and cooled on ice for 10 min. For counter-SELEX, the library was exposed to a 125 microliter (μl) bed volume of the GST beads for 1 h at 22 °C with end-over-end mixing. The mixture was cen-

Table 1
Oligonucleotides used in the selection and characterization of aptamers with binding affinity to human NoV.

Name	Sequence 5–3′′
DNA aptamer library	AGTATACGTATTACCTGCAGC-N40-CGATATCTCGGAGATCTTGC
Biotin–reverse constant	Biotin-GCAAGATCTCCGAGATATCG
Forward constant	AGTATACGTATTACCTGCAGC
Reverse constant	GCAAGATCTCCGAGATATCG
GII.4 P domain forward[a]	GCACGGATCCTCAAGAACTAAACCATTTACTGTC
GII.4 P domain reverse	GGACGCGGCCGCTTATAAAGCACGTCTACGCCC
JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG
G2SKR	CCRCCNGCATRHCCRTTRTACAT
COG2R	CCRCCNGCATRHCCRTTRTACAT
Ring 2P probe	56-FAM TGGGAGGGCGATCGCAATCT-3BHQ 1
T7GII.4F	TAATACGACTCAACTATAGCAAGAGTCAATGTTTAGGTGGATGAG
GII.4R2	GTTGGGAAATTCGGTGGGACTG

[a] Underlined sequences are restriction enzyme recognition sites.

trifuged at 500 × *g* for 5 min and the supernatant reserved. DNA was purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation [10% (v/v) 3 M sodium acetate, 200% (v/v) 100% ethanol, and 50 μg/ml Ambion GlycoBlue (Life Technologies, Grand Island, NY)] with reconstitution of the pellet in 25 μl DEPC-treated water. The DNA concentration was adjusted to 20–40 ng/μl and amplified by PCR using 2 μl of the template and primers described in Table 1. The reactions of 50 μl contained 1X Go Taq® Buffer (Promega), 500 nM of Conserved Forward Primer, 500 nM biotinylated conserved reverse primer, 0.2 mM Promega PCR Nucleotide mix (Promega), 0.5 μg single-stranded DNA binding protein (Promega), and 2 U Go Taq® DNA polymerase (Promega). A Bio-Rad T100 Thermal Cycler was used for the PCR (Bio-Rad Laboratories, Hercules, CA) with an initial 95 °C step for 2 min followed by 30 cycles of 95 °C for 30 s, 50–65 °C for 30 s (see below), and 72 °C for 15 s; and a final extension at 72 °C for 5 min. After every round of SELEX and counter-SELEX, an initial annealing gradient (from 50 to 65 °C) using the cycling conditions above was used to determine the optimal annealing temperature prior to the larger regeneration of the remaining pool. This temperature optimization was required to reduce concatamers and primer dimers. The amplified pool was then made into biotin-labeled ssDNA as described above.

The initial counter-SELEX was followed by seven rounds of positive selection which were performed in the same manner as the counter-SELEX described above except that the P domain-GST fusion protein lysate was used instead of the GST lysate; unbound sequences were removed by washing; and the protein-aptamer complexes were eluted from the beads using a glutathione elution buffer [50 mM Tris–HCl/10 mM reduced glutathione buffer (pH 8.0)] followed by phenol–chloroform extraction and ethanol precipitation. Prior to sequencing, another counter-SELEX round was performed using GST lysate and human NoV-negative human stool. The amplified pool was then resolved on a 2% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen). The purified pool was cloned via electroporation using the TOPO® TA Cloning Kit (Invitrogen). Colonies were selected, grown, plasmid-extracted, and screened by PCR. Selected colony plasmids were then sequenced (Genewiz, Inc.).

2.3.3. Analysis of aptamer sequences, structural folding, and stability

Usable aptamer sequences obtained were grouped into identical/similar sequences, and the proportion of each sequence in the pool determined. Structural folding analysis and ΔG prediction of the candidate aptamer sequences was performed using the DNA Mfold online server (http://mfold.rna.albany.edu/) using 0.5 mM magnesium, 1 mM sodium, and 23 °C as input parameters (Zuker, 2003). Candidate sequences from the pool were selected on the basis of how many times they repeated in the pool, low ΔG value (stability), and uniqueness and formation of loops in the secondary structure. Motif analysis was done for aptamers M1, M5, and M6-2 using the MEME Suite 4.10.0 online server (http://meme.nbcr.net/meme/tools/meme) with the criteria of a minimum motif length of 6 bases having no more than two base mismatches (Bailey and Elkan, 1994; Bailey et al., 2009).

2.3.4. Binding analysis using enzyme-linked aptamer sorbent assay (ELASA)

Binding affinity assays were done using the candidate aptamers (M1 and M6-2) and a panel of virus-like particles (VLPs) corresponding to genogroup I [GI.1 (Norwalk virus), GI.2, GI.4, GI.6, GI.7 and GI.8] and genogroup II [GII.1, GII.2 (SMV), GII.3, GII.4 (Houston and Grimsby), GII.6, GII.7, GII.12 and GII.17] human NoV, and also for chloroform extracted 20% stool suspensions derived from a patient confirmed to have GII.4 New Orleans infection. This was done using a previously reported ELISA-like method (Escudero-Abarca et al., 2014; Moe et al., 2004; Rogers et al., 2013) that we refer to as enzyme-linked aptamer-sorbent assay (ELASA). Briefly, VLP suspensions (1.3–4.3 mg/ml) were adjusted to a concentration of 3 μg/ml in PBS; in the case of whole virus, 10-fold serial dilutions of chloroform extracted 20% GII.4 New Orleans stool solutions were made. One hundred μl aliquots of VLP or diluted stool were placed on flat-bottom polystyrene 96 well plates (Costar 3591, Fisher, Pittsburg, PA) and incubated overnight at 4 °C. After removal of the fluid, the wells were blocked with 200 μl of 5% skim milk in PBS-Tween 20 (0.05%) (PBST) with a 10 nM mix of unrelated DNA oligonucleotides [*Listeria monocytogenes* primers hlyQF/R and L23SQF/R (Rodríguez-Lázaro et al., 2004)] for 2 h at 22 °C with gentle shaking. Blocking solution was discarded and three washes of 200 μl PBST per well were performed. Next, 100 μl of biotinylated aptamer (1 μM) was added to each well, and the plate was incubated for 1 h at 22 °C with gentle shaking. After removal of the liquid, the plates were washed 4 times with PBST. One hundred μl of ELISA-grade streptavidin-horseradish peroxidase (1 mg/ml, 1:5000, Invitrogen, Carlsbad, CA) was added per well with incubation for 15 min at 22 °C with shaking. After removing the unbound enzyme and rewashing with PBST, 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) microwell peroxidase substrate system [solution A:B (1:1), KPL, Gaithersburg, MD] was added for color development, and absorbance at 450 nm was recorded using a microplate reader (Tecan Infinite M200pro, Tecan Group Ltd., Männedorf, Switzerland).

All ELASAs were replicated on three separate occasions with at least three wells per replicate. Results were expressed as the ratio between the absorbance values for test samples divided by those for the negative control (no VLP). As per convention (Ebel et al., 2002; Escudero-Abarca et al., 2014; Hirneisen and Kniel, 2012), a VLP/No VLP ratio of less than 2.0 was considered low to no binding (−); 2.0–5.0 was considered low binding (±); 5.0–10.0 was considered medium binding (+); and >10.0 was considered strong binding (++).

Table 2
Aptamer sequences obtained after SELEX against NoV GII.4 P domain.

Name	ΔG	Variable region sequence[a]	Occurrence in pool
M1[b]	**−7.11**	**TGTTTATGGGGATAAACGTATCTAATTCGTGTACTAATCA**	**3/11**
M9-2	−4.12	TGTTAAGGGGAATTAATAATGATAATCCGTCTACTAATCA	2/11
M12-2	−3.95	TGTTAGGGGGAATTAATAATGGATAATCCGTCTACTAATCA	1/11
M13-2	−8.13	TGGGGGGTGGTGCGGTGTGTGGCAGGGGAGCATAGCCGGGGGCCCCCT	1/11
M6-2[b]	**−8.33**	**TGGGAAGAGGTCCGGTAAATGCAGGGTCAGCCCGGAGAG**	**1/11**
M5[b]	−6.43	TGGGGGGTGGTGCGGTGTGTGACAAGGGAGCATAGCCGGGGGCCCCCT	3/11

[a] Bolded sequences were two candidates chosen for further characterization.
[b] Sequences chosen for motif analysis using MEME.

Means and standard deviations for ratios associated with replicate experiments were calculated using Microsoft Excel. Additionally, for plates containing positive and negative chloroform-extracted stool, statistical comparison was performed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison using GraphPad Prism version 5.0d (San Diego, CA).

2.4. Aptamer magnetic capture (AMC)-RT-qPCR for detection

2.4.1. Aptamer magnetic capture (AMC)

As proof-of-concept, biotinylated aptamers were used to concentrate human NoV from stool samples. Thirty μg of Dynabeads® MyOne Streptavidin C1 magnetic beads (Invitrogen-Dynal AS, Oslo, Norway) were diluted in 1 ml PBS + 0.05% PBST, mixed, and recaptured using the Dynal MPC-M magnetic particle concentrator (Invitrogen-Dynal). The beads were resuspended in 1 ml of 5% skim milk and blocked overnight at 4 °C with rotation. The beads were then twice washed with 500 μl PBST, resuspended in 50 μl PBST, and stored at 4 °C until use. These beads will hereafter be referred to as "blocked beads."

Aptamer capture of human NoV from stool was performed based on the protocol of Cannon and Vinjé (2008) with substitution of aptamers for purified histo-blood group antigens (HBGAs). Ten-fold serial dilutions of a previously aliquoted 20% GII.4 stool suspension were prepared in PBS and 100 μl of each dilution was placed into a dedicated tube containing 900 μl PBST and 15 μl of biotinylated aptamer (100 μM, ~5.9 ng total). The contents were mixed by end-over-end rotation for 1 h at 22 °C. Fifty μl of the blocked beads were then added, and the tubes incubated for another hour with flipping at 22 °C. Beads were magnetically recovered and washed once with 500 μl PBST followed by one wash with 500 μl PBS. Beads were resuspended in 100 μl PBS and stored at −80 °C until RNA extraction. Negative controls consisted of tubes containing 450 μl PBST, 450 μl Superblock T20 (Thermo Fisher Scientific, Waltham, MA), 100 μl of diluted sample, and 50 μl of blocked beads. RNA extraction was done using the NucliSENS® easyMAG system (bioMerieux SA, Marcy l'Etoile, France) according to the manufacturer's instructions with a 40 μl final elution volume. The eluted RNA was immediately stored at −80 °C until use in RT-qPCR (below).

2.4.2. Quantification of virus recovery by RT-qPCR

RNA was amplified by one step RT-qPCR using the Superscript III Platinum One-Step kit (Invitrogen). Reactions of 25 μl were made containing 12.5 μl 2x Reaction Mix, 0.5 μl SuperScript III Reverse Transcriptase/Platinum *Taq* mix, 200 nM JJV2F primer, 200 nM COG2R primer, 200 nM Ring2P probe (Jothikumar et al., 2005), 5.5 μl nuclease-free water, and 5 μl template. Reverse transcription was done at 50 °C for 15 min followed by enzyme inactivation at 95 °C for 2 min. Amplification was done for 45 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 30 s. Quantification of genomic copies was based upon a standard curve using an in vitro transcribed GII.4 New Orleans amplicon covering a 460 nt range of the genome containing the JJV2F-COG2R primer target region. The amplicon was quantified using a Nano Photometer™ Pearl (Denville Scientific, Inc., South Plainfield, NJ), serially diluted, and used to construct a standard curve to estimate genomic copies. Amplifiable RT-qPCR units were estimated based on a standard curve of Ct values from serial 100 μl dilutions of the 20% GII.4 New Orleans stool isolate used for the AMC assay that had their genomic RNA extracted and amplified using RT-qPCR as described above in Section 2.4.1 and 2.4.2.

3. Results

3.1. Aptamer candidates

After seven rounds of SELEX and two rounds of counter-SELEX, aptamer pools were sequenced as in Section 2.3.2. Eleven sequences were identified, six of which were unique (Table 2). Candidates M1 and M6-2 were selected for further characterization based on the number of times the sequence occurred in the pool of sequences [3/11 and 1/11, respectively], low ΔG values [$\Delta G = -7.11$ and $\Delta G = -8.33$ for M1 and M6-2, respectively] (Table 3), and similarities in secondary structure (Fig. 1). MEME analysis of the aptamers showed multiple overlapping motifs and multiple motifs involved in loop regions or the formation of loop regions. More specifically, when comparing M1 and M6-2, three potential motifs of at least 6 bases were shared between the two aptamers. Motif 1 contained the sequence TAAA[C,T]G[T,C]A, where base mismatches are in brackets in respective order of aptamer M1 and M-2; for example, aptamer M1 contains motif 1 as TAAACGTA and M6-2 has TAAATGCA. Motif 1 was involved in the stem-loop regions for the major loop of both aptamers (Fig. 1). Motif 2 was shared between

Table 3
Binding affinity of selected aptamers (M1 and M6-2) to a broad panel of VLPs based on ELASA.

VLP	Aptamer	
	Mean VLP/No VLP ratio (ratio standard deviation)[a]	
	M1	M6-2
GI.1 Norwalk	3.40 (0.67) (±)[b]	4.28 (0.52) (±)
GI.4	3.17 (0.43) (±)	3.36 (0.24) (±)
GI.6	1.98 (0.38) (−)	2.75 (0.45) (±)
GI.7	7.32 (2.41) (+)	7.56 (2.45) (+)
GI.8	2.61 (0.26) (±)	4.55 (0.81) (±)
GII.1	3.59 (1.55) (±)	4.52 (0.44) (±)
GII.2 Snow Mountain	10.68 (0.70) (++)	12.00 (1.10) (++)
GII.3	2.94 (1.90) (±)	3.16 (0.72) (±)
GII.4 Grimsby	7.59 (0.46) (+)	11.54 (1.70) (++)
GII.4 Houston	10.41 (1.23) (++)	12.98 (1.76) (++)
GII.6	2.38 (0.78) (±)	4.40 (0.85) (+)
GII.7	7.47 (1.15) (+)	8.02 (1.99) (+)
GII.12	4.47 (0.54) (±)	5.55 (0.10) (+)
GII.17	3.94 (1.03) (±)	5.24 (0.75) (+)

[a] Values indicate the ratio between absorbance readings for test VLP sample versus negative control (VLP wells absorbance/No VLP wells absorbance) for each aptamer. Values obtained for the negative control were in the range of 0.1–0.4.
[b] Results less than 2.0 are considered negative per convention (−); 2.0–5.0 low binding (±); 5.0–10.0 medium binding (+); and >10.0 strong binding (++).

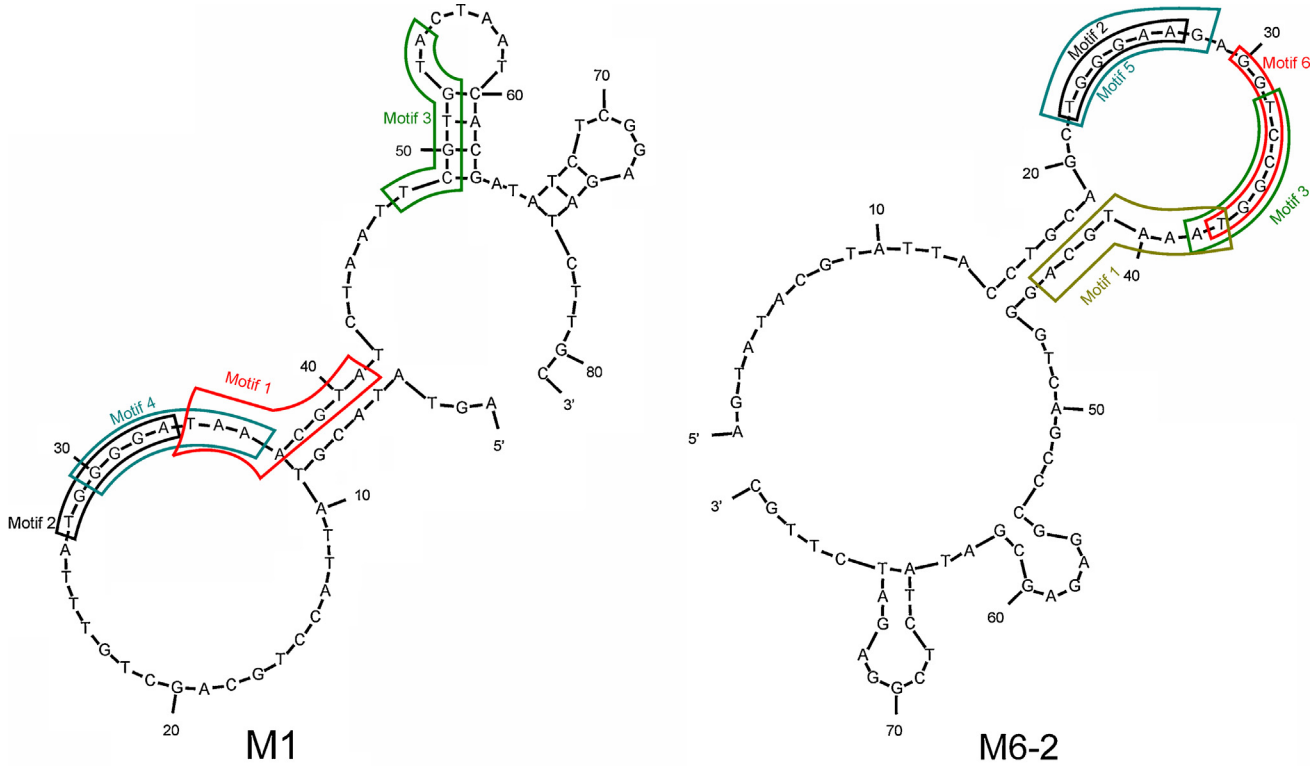

Fig. 1. Predicted secondary structures of the M1 and M6-2 ssDNA aptamers generated against the GII.4 P domain with common aptamer pool motifs circled. Secondary structures of two aptamers generated against the human NoV P domain were selected for further characterization and their secondary structures predicted with the Mfold server (Zuker, 2003). Additionally, common sequence motifs among all aptamer sequences obtained in the pool were identified with the MEME suite (Bailey and Elkan, 1994; Bailey et al., 2009). These commonly occurring motif sequences found in the aptamer pool are circled.

aptamers M1 and M6-2, and contained the sequence TGGG[G,A]A. Motif 3 (sequence TC[G,C][T,G]GTA) occurred in the major loops of both aptamers. Motifs 4-6 (not shown) on M1 or M6-2 were shared with aptamer M5. Some of these motifs overlap motifs shared between M1 and M6-2, and are also involved in stem-loops.

3.2. Aptamer binding inclusivity

Both aptamer candidates exhibited relatively stronger binding to VLPs representing GII human NoV genotypes over GI genotypes. Strong binding (++) for both aptamers was observed by ELASA for GII.2 and GII.4 VLPs. Ratios indicating higher binding under the "medium" binding category for both aptamers were observed for GI.7, GII.4 Grimsby (M1 only) and GII.7. Based on an absorbance ratio cutoff of 2.0, aptamer M6-2 exhibited broader recognition compared to M1, with some degree of binding to all of the VLPs tested. Positive signals were quite low for GI.6 and GII.3 VLPs. On the other hand, aptamer M1 did not appear to bind to GI.6, and had relatively low signals for GI.8, GII.3, and GII.6 VLPs. Overall, M6-2 had higher VLP/No VLP ratios compared to M1. As expected, assays using GII.4 VLPs provided some of the highest signal ratios. Interestingly, both aptamers also had GII.2 ratios about as high as the GII.4 (highest) VLPs.

3.3. Aptamers bind to partially purified human stool samples obtained from infected individuals

Both the M1 and M6-2 aptamers exhibited binding to serially diluted partially purified 20% stool specimens obtained from infected individuals (Fig. 2). Binding was statistically significant ($p < 0.05$) relative to human NoV negative stool when the samples were diluted 10^{-2} or 10^{-3}. These differences were not statistically significant for the 10^{-1} dilutions of stool, likely due to matrix-associated non-specific binding. When stool samples were diluted 10^{-4} or more, signal was lost, presumably because of dilution-associated depletion of virus, approaching the assay limit of detection.

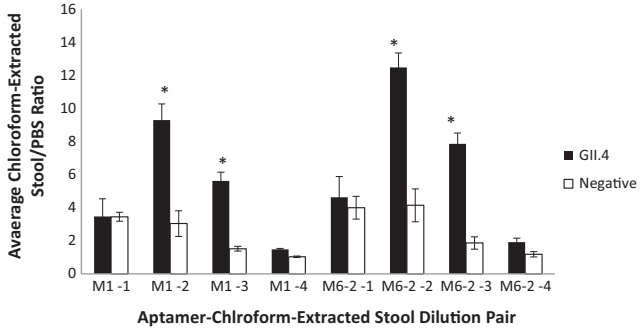

Fig. 2. Binding ratios of partially purified, serially diluted 20% GII.4 and human NoV-negative stool samples to selected aptamers by ELASA. Data are presented as ratio of absorbance for test samples (serially diluted stool) versus the PBS negative control wells. The asterisk indicates a statistically significant difference ($p < 0.05$) between GII.4 positive stool and stool confirmed negative for human NoV. Error bars represent one standard deviation above/below the mean. *X* axis is labeled by aptamer designation and dilution of stool.

3.4. Aptamer magnetic capture (AMC) coupled to RT-qPCR applied to outbreak stool specimens

Selected aptamers were used to concentrate HuNoV from diluted GII.4 New Orleans clinical stool isolates using magnetic nanoparticles. Concentrated viruses were then quantified by RT-qPCR. Both aptamers concentrated significantly ($p < 0.05$) more

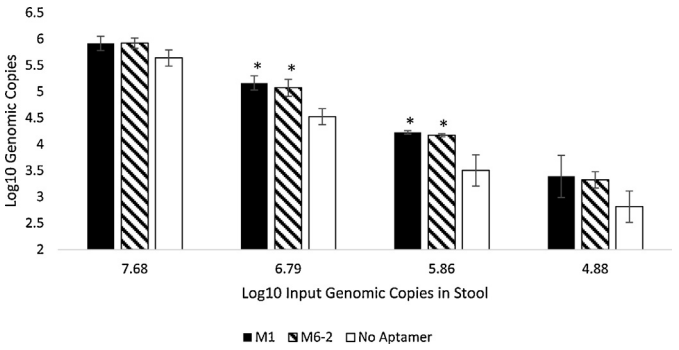

Fig. 3. Capture of GII.4 New Orleans in stool using aptamer magnetic capture (AMC). Biotinylated aptamers were incubated with serially diluted 20% suspensions of GII.4 New Orleans containing stool. Aptamer-virus conjugates were then captured with streptavidin-coated magnetic nanoparticles and quantified by RT-qPCR. AMC using blocked beads without aptamers served as negative control. Statistically significant differences between aptamer and control samples ($p < 0.05$) are designated by an asterisk.

virus than no aptamer controls at concentrations of 6.79 and 5.86 $\log_{10}$ human NoV genomic copies per ml of stool (Fig. 3).

4. Discussion

In this study, the P domain cloned and expressed from the genome of a 2007 GII.4 human NoV clinical stool isolate was used as the target in SELEX for production of ssDNA aptamers. A GII.4 strain was considered relevant because it belongs to the epidemic genotype that has been causing the largest numbers of cases over the last two decades (Bok et al., 2009; Noel et al., 1999). Furthermore, the P2 subdomain of the P domain is thought to be involved in host cell binding, is hypervariable, and likely responsible for antigenic drift of GII.4 strains (Lindesmith et al., 2008). Given these features, it is not surprising that the aptamers produced in this study did not strongly bind to all human NoV VLPs screened. However, they did exhibit binding to a majority of the VLPs tested, with generally better binding demonstrated for GII versus GI VLPs. A notable difference between aptamers M1 and M6-2 was observed for GI.8, GII.6, and GII.17 VLPs (Table 3). Different binding patterns for these two aptamers suggest that they may bind to different regions of the P domain. It could be hypothesized that aptamer M1 binds a less conserved region of the P domain because it was not as broadly reactive as M6-2, and overall showed lower signal intensity. As expected, both aptamers bound well to the GII.4 VLPs, as the aptamer target was a GII.4 2006b strain. Also as expected, both aptamers displayed stronger binding to the more recent and sequentially similar GII.4 Houston strain (2001) compared to the older and less sequentially similar GII.4 Grimsby (1996) strain (Glass et al., 2009; Shanker et al., 2011). Interestingly, M6-2 showed some degree of binding to all of the VLPs tested, suggesting that it likely binds a part of the P1 subdomain of NoV, where other fairly broadly reactive antibodies have been found to bind (Kitamoto et al., 2002; Parker et al., 2005; Shiota et al., 2007).

Multiple common sequence motifs within the variable region of aptamers M-1 and M6-2 were identified. Many of these motifs are involved in hairpin-loop or loop structures (Fig. 1) and may be implicated in aptamer binding to human NoV, as loop and stem-loop structures are often involved in binding (Kato et al., 2000; Kaur and Yung, 2012). Such motif analysis can inform future studies. For example, characterization of the nature of the aptamer binding domain(s) could be further investigated using nucleotide substitution. Further, identification of common motifs in aptamers M1 and M6-2, in addition to other aptamers might allow the production of truncated aptamers that could be combined into a chimeric aptamer (Kanwar et al., 2011) to create an even more effective broadly reactive ligand.

Recently, three studies have reported the development of DNA aptamers having binding affinity to NoV. Giamberardino et al. (2013) produced aptamers targeting the murine norovirus (MNV) surrogate using whole virus SELEX, finding that one also bound GII.3 NoV VLPs. This aptamer was used as a recognition element in a voltammetry-based biosensor. Beier et al. (2014) created DNA aptamers using an unspecified GII.4 strain's entire major capsid protein (VP1) by a different SELEX process than ours. However, the work focused primarily on innovations in bioinformatic analysis and protein-aptamer modeling rather than the functional binding characterization reported here. Interestingly, the aptamers produced by Giamberardino et al. (2013) and Beier et al. (2014) had ΔG values similar to M1 and M6-2, but the sequences and secondary structures differed from ours. In both papers, the aptamers produced were never applied for capture or detection of human NoV in outbreak-derived stool specimens, and aptamer binding to the intact capsid of only one genotype of human NoV was confirmed for any of the reported aptamers.

In a study similar to this one, Escudero-Abarca et al. (2014) created aptamers using partially purified infectious GII.2 Snow Mountain virus from stool (whole virus SELEX), as juxtaposed to the GII.4 P domain target in this work. The aptamers described in that study and those reported in this paper exhibited similar broad reactivity and high signal-to-noise ratios, despite the differences in target. The aptamers also had similar ΔG values and motifs that occurred in hairpin-loops. Likewise, similar binding signals were observed using partially-purified GII.4 stool isolate in the ELASA assay. Aptamers M1 and M6-2 exhibited lower capture efficiencies in AMC-RT-qPCR compared to aptamer 25 reported by Escudero-Abarca et al. (2014). This may be a function of differences in the counter-selection process, as Escudero-Abarca et al. (2014) performed more counter-SELEX rounds against a greater number of targets, which likely reduced nonspecific aptamer binding to magnetic particles and stool components. Nonetheless, the aptamers M1 and M6-2 displayed a reasonably good capture efficiency at a range of 4.88–6.79 $\log_{10}$ input genomic copies of virus. Because of the similar performance of the M1, M6-2, and the Escudero-Abarca et al. (2014) aptamers by ELASA and AMC-RT-qPCR, it is possible that they all bind to a conserved NoV region, but further analysis would be necessary to support this hypothesis. The limit of detection of the AMC-RT-qPCR assay was 4.88 $\log_{10}$ input genomic copies, which corresponded to about 2–3 $\log_{10}$ RT-qPCR amplifiable units in the input stool sample.

Unlike any of the previous reports of aptamers generated against NoV, this paper is the first report of aptamers developed with a biotin label during the selection process. Label modifications made after the development of aptamers have the potential to alter the three-dimensional structure resulting in reduced aptamer binding affinity (Jiang et al., 2004; Wang et al., 2005); thus selection using a functional biotin label allows for many downstream diagnostic and detection applications with less risk of losing aptamer functionality. For example, the colorimetric ELASA assay presented here could be further optimized and its sensitivity increased with a chemiluminescent or fluorescent assay; both of which are compatible for use with biotinylated ligands and the proper streptavidin/avidin conjugates (Lewkowich et al., 2001; Yu et al., 2011).

Not only were aptamers M1 and M6-2 able to bind to multiple human NoV VLPs, they also bound to stool samples previously confirmed as positive for human NoV as evaluated by both the ELASA and AMC assay. It was, however, necessary to purify and dilute the stool specimens in order to achieve reliable detection signals, suggesting that matrix-associated interference with ligand binding occurred when samples were too "dirty." This may be due to a degree of non-specific binding and/or association of

the aptamers with the extracted stool matrix. This phenomenon has been observed in similar types of assays done by other investigators for both aptamers (Escudero-Abarca et al., 2014) and other ligands (Burton-MacLeod et al., 2004; Huang et al., 2014; Li et al., 2012). Interestingly, the dilution of the chloroform-extracted stool to about 0.2% original stool content for GII.4 New Orleans used here is similar to the optimal 1% stool dilution reported by Huang et al. (2014) when detecting NoV GII.4 in ELISA using phages displaying single-chain antibodies. When it comes to AMC, non-specific binding to the paramagnetic beads is commonly observed, as has been reported by others for bacteria (Rijpens et al., 1999; Tomoyasu, 1998) and NoV (Escudero-Abarca et al., 2014; Gilpatrick et al., 2000). All told, regardless of the ligand or assay design, non-specific binding virtually always impacts analytical sensitivity and this remains a recalcitrant issue for development of rapid, reliable, and sensitive human NoV detection methods.

The human NoV capsid protein is under constant selective pressure, especially GII.4 strains, and strain emergence occurs every few years (Bull et al., 2010; Debbink et al., 2012). With respect to development of advanced detection and vaccination strategies that can cover emerging strains, it is important to have a readily available target for product development purposes. A functional human NoV P domain can be easily cloned, expressed and purified in *E. coli* with only capsid sequence information needed, thus resulting in the production of high concentrations of protein at low cost with relative ease. In short, the method described here can provide a cost-effective, rapid, and easily implemented means to create large quantities of ligands with high affinity to emerging human NoV strains. As rapid, microfluidic SELEX processes emerge, this may become an even simpler and faster means by which to select ligands with binding specificity to protein targets (Huang et al., 2010; Lou et al., 2009).

5. Conclusion

In summary, we isolated and characterized ssDNA aptamers with binding specificity to a broad range of human NoV VLPs and outbreak strains using an *E. coli*-expressed viral capsid protein, and demonstrated that they could be used as capture ligands in both ELISA-type and aptamer-mediated magnetic capture-RT-qPCR assays. The aptamers reported here are among the broadest reacting ligands to human NoV identified to date (Escudero-Abarca et al., 2014; Hardy et al., 1996; Huang et al., 2014; Kitamoto et al., 2002; Kou et al., 2014; Li et al., 2010; Shiota et al., 2007; Yoda et al., 2003, 2001). With further development, the aptamers may be useful in novel detection platforms such as biosensors (reviewed in Torres-Chavolla and Alocilja 2009). For example, the flexibility of chemical modification and stability of the presented aptamers makes them ideal candidates for use in combination with previously reported broadly reactive aptamers for detection of human NoV in complex samples using Luminex xMAP technology (Bergervoet et al., 2008; Porschewski et al., 2006). The presented aptamers may also have utility in antiviral or therapeutic applications (Jeon et al., 2004; Khati et al., 2003; Yoon et al., 2010). This is the first report to demonstrate that broadly reactive aptamers binding human NoV can be easily and cost-effectively produced using SELEX directed against P domain of these viruses.

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