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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-