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The influence of food matrices on aptamer selection by SELEX (systematic evolution of ligands by exponential enrichment) targeting the norovirus P-Domain

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ABSTRACT

This study investigates the enrichment of aptamers targeting the norovirus protruding domain in the presence of foods often associated with norovirus outbreaks. The goal is to explore if and how the presence of food alters *in vitro* selection of aptamers and target binding of the enriched oligonucleotides. Our study demonstrates that the introduction of food to SELEX (systematic evolution of ligands by exponential enrichment) is either detrimental to enrichment of oligonucleotides with target-specific binding, or facilitates enrichment of non-target-specific oligonucleotides. Moreover, a relationship between target binding of enriched oligonucleotides in presence of food and their selection condition was not observed. Our findings also suggest that a pathogen specific aptamer with application in food does not need to be selected in presence of the particular food, but may require properties beyond high affinity and selectivity to be applied for pathogen extraction and detection in undiluted food matrices.

1. Introduction

Aptamers, single-stranded nucleic acid molecules that specifically bind a target molecule (Ellington & Szostak, 1990), are an important emerging tool for food analytical applications (Amaya-González, de-los-Santos-Álvarez, Miranda-Ordieres, & Lobo-Castañón, 2013). They are derived from an *in vitro* selection process termed SELEX: systematic evolution of ligands by exponential enrichment (Ellington and Szostak, 1990; Tuerk & Gold, 1990). SELEX is an iterative process in which aptamers are selected from a diverse library of synthetic single-stranded nucleic acid molecules. Since the first description of SELEX, the method has not been streamlined, and is constantly being evolved depending on the downstream application of the aptamer (Zhuo et al., 2017). In each SELEX round, oligonucleotides which bind the target are separated from non-binding molecules and are amplified by polymerase chain reaction (PCR). To create the starting pool for the next round of SELEX, single-stranded-nucleic acid molecules are generated from the previous round's amplification products. Stringency is increased with each SELEX round and selection conditions are adjusted to continuously apply more selective pressure. This facilitates the selection of aptamers with a high affinity for the target. *In vitro* selection is completed when target-specific enrichment of the single-stranded-nucleic acid library is confirmed and no further improvements to enrichment are possible. SELEX is highly adaptable, enabling the selection of aptamers that bind immunogenic and toxic substances, and allows the use of a variety of selection environments beyond physiological conditions to which antibodies are restricted. Hence, the greatest advantage of aptamers over antibodies, in respect to food analytic applications, lies in the fundamental choice of selection conditions based on the assay or extraction environment. For aptamer mediated extraction and detection of pathogens, toxins, or allergens from food matrices, this translates into selecting aptamers in a milieu that represents the potential implicated food sources.

Foodborne viruses, such as norovirus (NoV), can contaminate a variety of food sources; the presumably low number of virions, in combination with complexity of food matrices, present a challenge for extraction and detection. NoV is the leading cause of acute gastroenteritis worldwide, with NoV GII.4 being the predominant strain in illness outbreaks between 2009 and 2013 (Havelaar, 2015; Vega et al., 2014). NoV is a non-enveloped virus and its capsid consists of 180 copies of the major capsid protein (VP1) (Jiang, Wang, Graham, & Estes, 1992). VP1 is defined by two regions: the contiguous shell region (S) and the protruding region (P-Domain) (Prasad et al., 1999). The P-Domain's protruding characteristic makes it an ideal target for NoV

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extraction. Moreover, the P-Domain is easily produced in *E. coli* (Koho et al., 2012) which makes it an attractive target molecule for SELEX. NoV is easily transmitted by person-to-person contact or through the consumption of virtually any contaminated food. Foodborne NoV illness is widely attributed to fresh and frozen berries (Mäde, Trübner, Neubert, Höhne, & Johne, 2013; Sarvikivi et al., 2012), leafy greens (Müller et al., 2016), and molluscan shellfish, especially oysters (Cho et al., 2016; Le Guyader, Bon, et al., 2006). The NoV capsid specifically binds to oyster diverticulum and, depending on the NoV genotype, and strain, to the oysters gills and mantle (Le Guyader, Loisy, et al., 2006; Maalouf et al., 2011). This could pose a challenge to selection of aptamer for NoV with intended application in oysters. Consequently, a counter selection that includes oyster tissue could help to identify aptamers that bind to a different site on the NoV capsid than the oyster tissue or are able to out-compete the NoV-oyster binding. The introduction of oyster, or any food matrix, should also increase stringency during SELEX, as it presents a challenge for oligonucleotides to bind their target.

Aptamers with specific affinity to NoV can be used in food related outbreak investigations and for extraction and detection of viral particles that need to be intact for downstream application. Although the use of aptamers in food analysis has been suggested, and aptamers have been selected for a variety of foodborne pathogens, including NoV, (Beier et al., 2014; Escudero-Abarca, Suh, Moore, Dwivedi, & Jaykus, 2014; Giamberardino et al., 2013; Moore, Escudero-Abarca, Suh, & Jaykus, 2015) the incorporation of food matrix into SELEX has yet to be evaluated. In the current study, we took the novel approach of investigating the influence of four food matrices that are highly associated with NoV outbreaks (frozen strawberries, lettuce, whole oyster, and oyster digestive diverticula) on the enrichment of aptamers targeting the NoV GII.4 P-Domain. Our hypothesis is that *in vitro* selection in presence of food matrix will lead to the enrichment of aptamers that can be used in the food in which they are selected. Additionally, we expect that successful aptamer target binding will be dependent on the presence of the food matrix used during selection, as each food matrix will have an effect on aptamer folding and its ability to interact with its target.

2. Experimental section

2.1. PCR reagents and oligonucleotides

All PCR reagents and cloning kits were purchased from Life Technologies (Carlsbad CA, USA), if not otherwise noted. All primers and the sequence library were purchased from Integrated DNA Technologies (Coralville IA, USA). The DNA library consisted of 40 randomized nucleotides flanked by two constant primer binding regions with standard desalting and handmixing of the random bases (1 μmole scale).

2.2. Amplification and cloning of the P-Domain gene into expression vector

A clinical stool sample positive for NoV genogroup II was partially purified by chloroform extraction, and viral RNA was extracted using the QIAamp Viral RNA Minikit from Qiagen (Valencia CA, USA), following the manufacturer's recommendations. The VP1 gene was amplified from genomic RNA by reverse transcriptase (RT)-PCR in a nested two-step reaction using two different sets of primers (Supplementary material 1). The amplified VP1 gene was cloned into a pCR™4-TOPO vector using the TOPO® TA Cloning Kit for Sequencing. The P-Domain gene was then amplified from linearized recombinant plasmid using the Platinum Pfx ® polymerase to generate a blunt end PCR product (Cycling conditions: 94 °C for 300 s, 35 cycles of: 94 °C for 30 s, 60.0 °C for 30 s, 68 °C for 90 s, final extension: 72 °C 420 s) with previously described P-Domain primers (Koho et al., 2012). The PCR product was cloned into the expression vector pET100/D-TOPO® of the Champion™ pET *Directional TOPO®* Expression Kit by Fisher Scientific (Suwanee GA, USA) and subsequently sequenced and characterized as GII.4 New Orleans 2009. (For detailed methodology, refer to Supplementary material 1).

2.3. Protein production, purification, and characterization

The P-domain was produced in *E. coli* strain BL 21 Star™ as previously described (Koho et al., 2012) using the Champion™ pET *Directional TOPO®* Expression Kit by Fisher Scientific (Suwanee GA, USA). The control protein of the Champion™ pET *Directional TOPO®* Expression Kit was produced per manufacturer's instruction and the recombinant protein purified and isolated as described for the P-Domain. (For detailed methodology for Section 2.3, please refer to Supplementary material 1).

2.4. Immobilization of protein to paramagnetic particles

The purified dialyzed P-Domain and control protein (for counter selections) was immobilized on Dynabeads® His-Tag Isolation & Pulldown (Fisher Scientific, Suwanee GA, USA), using 500–750 μl protein solution (average concentration 68 μg/ml) and 25–50 μl bead suspension (1–2 mg beads) as described by the manufacturer with small variations. Briefly, for each SELEX round, beads were freshly prepared and incubated with protein solution for 10 min, washed twice with wash buffer (as directed by the manufacturer, but without Tween ®-20), and twice with SELEX binding buffer (BB); 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 10 mM Tris Base, 100 mM NaCl, pH 7.4. Washed beads were suspended in 50 μl BB. The protein concentration was measured before and after immobilization using the Qubit® Protein Assay Kit (Fisher Scientific, Suwanee GA, USA).

2.5. Preparation of food matrix

To prepare the whole oyster (*Crassostrea virginica*) matrix, 10 locally harvested oysters (Fowl River, AL, USA) were homogenized. For diverticular (div) matrices, the diverticula of about 40 oysters were dissected and homogenized. Frozen strawberries were purchased from a local supermarket and 40 g homogenized by blending. Using the food homogenates, 10% suspensions (4 g in 40 ml) were prepared in BB. The pH of the suspensions was adjusted to 7.4, as needed. To prepare the lettuce matrix, romaine lettuce was torn in uniform pieces by hand (diameter: 0.5″), and 50 g of lettuce was rinsed with 50 ml of BB, with orbital shaking at 50 rpm for 15 min in a Whirl-Pak ® sampling bag (6″ × 9″). The rinse solution was transferred to a new container and used as lettuce matrix. The pH of the lettuce matrix was tested, but did not require adjustment. The absence of NoV in food matrix preparations was confirmed by RT-qPCR as previously described (DePaola et al., 2010). Matrix preparations were aliquoted and frozen until further use.

2.6. SELEX

The SELEX was started with 2.5 nmol oligonucleotide library divided in five microcentrifuge tubes each containing 500 pmol oligonucleotide library (Fig. 1). Prior to each round the library was heated in 500 μl BB for 5 min at 95 °C, and immediately cooled on ice for 15 min. Bovine Serum Albumin (BSA) stock solution (10 mg/L BSA in BB) was added to a final concentration of 0.01%. The P-Domain coated Dynabeads® suspension was then added to the library. The P-Domain amount for the first round was 390 pmol per 500 pmol library, brought up to a final volume of 700 μl BB. The amounts of target protein were gradually reduced to 55 pmol, with increasing number of SELEX rounds (for detailed information, refer to Supplementary material 2). After 30 min incubation at room temperature under tilt rotation on setting 4.5 using the Bellco Roller Drum by Bellco Glass Inc. (Vineland, New Jersey, USA), the beads were collected using a magnet rack and washed with