Journal of Biotechnology 167 (2013) 454–461

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Nucleic acid aptamers for capture and detection of *Listeria* spp

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ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 15 July 2013
Accepted 22 July 2013
Available online 11 August 2013

Keywords:
Listeria monocytogenes
Listeria spp
DNA aptamer
Aptamer magnetic capture
Immunomagnetic separation
Real-time PCR

ABSTRACT

The purpose of this study was to identify biotinylated single-stranded (ss) DNA aptamers with binding specificity to *Listeria* and use these for capture and subsequent qPCR detection of the organism. For aptamer selection, SELEX (systematic evolution of ligands by exponential enrichment) was applied to a biotin-labeled ssDNA combinatorial library. After multiple rounds of selection and counter-selection, aptamers separated, sequenced, and characterized by flow cytometry showed binding affinities to *L. monocytogenes* of 18–23%. Although selected for using *L. monocytogenes*, these aptamers showed similar binding affinity for other members of the *Listeria* genus and low binding affinity for non-*Listeria* species. One aptamer, Lbi-17, was chosen for development of a prototype capture and detection assay. When Lbi-17 was conjugated to magnetic beads and used in a combined aptamer magnetic capture (AMC)-qPCR assay, the pathogen could be detected at concentrations <60 CFU/500 μl buffer in the presence of a heterogeneous cocktail of non-*Listeria* bacterial cells, with a capture efficiency of 26–77%. Parallel experiments using immunomagnetic separation (IMS)-qPCR produced the same detection limit but lower capture efficiency (16–21%). Increasing assay volume to 10 and 50 ml resulted in reduced capture efficiency and higher limits of detection, at 2.7 and 4.8 $\log_{10}$ CFU *L. monocytogenes* per sample, respectively, for the AMC-qPCR assay. Biotinylated ssDNA aptamers are promising ligands for food-borne pathogen concentration prior to detection using molecular methods.

1. Introduction

Food-borne disease is an important public health problem worldwide. In the United States (U.S.) alone, it is estimated that there are 9.4 million episodes of food-borne illness annually, resulting in over 55,000 hospitalizations and 1300 deaths (Scallan et al., 2011). Although rare, *Listeria monocytogenes* infection (called human listeriosis) is of considerable concern by virtue of disease severity, and several large outbreaks of listeriosis have been documented over the last 30 years (McLauchlin et al., 2004). In response, the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) has issued new regulations that include testing mandates for various food products (Gottlieb et al., 2006). Depending on the sample type, this can include testing for the pathogen, *L. monocytogenes*, and/or testing for the entire *Listeria* genus, which is frequently used as an indicator for the potential presence of *L. monocytogenes* (Vazquez-Boland et al., 2001). *Listeria* and *L. monocytogenes* testing comprises a high proportion of the food-borne pathogen testing market, the other two important pathogens being *Salmonella* and *E. coli* O157:H7 (BCC Research, 2012). Because of the significance of *L. monocytogenes*, U.S. regulatory agencies have established a "zero tolerance" policy for the organism in ready-to-eat (RTE) foods (Chen et al., 2003).

Historically, standard methods for detecting *L. monocytogenes and Listeria* spp. in food and environmental samples have relied upon cultural enrichment and selective/differential plating followed by biochemical identification (Hudson et al., 2001; Skjerve et al., 1990; Uyttendaele et al., 2000). Over the last two decades, the cumbersome selective plating steps have been replaced by enzyme-linked immunosorbent assay (ELISA), DNA/RNA hybridization, or quantitative polymerase chain reaction (qPCR) (Farber and Peterkin, 1991). This has shortened the time to detection for negative samples from 5 days to 2–3 days, but presumptively positive samples must still be confirmed by cultural methods, taking upwards of a week. It is generally recognized that cultural enrichment remains the rate-limiting step for pathogen detection in foods.

It has been suggested that food-borne pathogen detection could be made more rapid if the target pathogens were separated, concentrated, and purified from the sample matrix before detection, so-called pre-analytical sample processing (Stevens and Jaykus, 2004). The immobilization of antibodies to magnetic beads, which forms the basis for immunomagnetic separation (IMS), has been widely used for the separation of target microorganisms from complex sample matrices (Dwivedi and Jaykus, 2011). Although

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http://dx.doi.org/10.1016/j.jbiotec.2013.07.027

Table 1
DNA sequences of the aptamer library and PCR primers and probes used in this study.

Designation	Oligonucleotide sequence
DNA aptamer library	5′- AGTATACGTATTACCTGCAGC - N_{40} - CGATATCTCGGAGATCTTGC -3′
Forward constant region primer	5′- AGTATACGTATTACCTGCAGC -3′
Biotinylated reverse constant region primer	5′-/5Biosg/- GCAAGATCTCCGAGATATCG -3′
L. monocytogenes hlyQ forward primer	5′-CATGGCACCACCAGCATCT-3′
L. monocytogenes hlyQ reverse primer	5′-ATCCGCGTGTTTCTTTTCGA-3′
hlyQ TaqMan probe	5′-/56-FAM/-CGCCTGCAAGTCCTAAGACGCCA/BHQ_1/-3′

antibodies are the most commonly used ligands for this pathogen concentration, they are difficult and expensive to produce, have relatively short shelf-lives, and have varying degrees of target specificity and avidity, all of which can impact capture efficiency and subsequent analytical detection sensitivity.

Alternative ligands for pathogen capture are nucleic acid aptamers, which are short (20–80 mer) single-stranded (ss)DNA or RNA molecules that specifically interact (bind) to their target through their 3-dimensional structure; intermolecular hydrogen bonding also contributes to their binding affinity and specificity (Jayasena, 1999). These molecules offer advantages over traditional antibody-based affinity molecules in their ease of production and stability, largely due to the chemical properties of nucleic acids versus amino acids. Numerous advantages of aptamers over antibodies are extensively reviewed by others (Bunka and Stockley, 2006; Khati, 2010). The objective of this study was to identify and characterize ssDNA aptamers with binding affinity to *Listeria monocytogenes*. A candidate aptamer was then used for development of a prototype capture method as proof-of-concept that aptamers can be used in pre-analytical sample processing prior to the application of qPCR for detection.

2. Materials and methods

2.1. Bacterial strains, culture conditions and preparation of cells

The aptamer selection procedure, whole cell SELEX (systematic evolution of ligands by exponential enrichment) was performed targeting *Listeria monocytogenes* ATCC19115. Other *Listeria* spp. used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and included *L. innocua* ATCC33091, *L. ivanovii* ATCC19119 and *L. grayi* ATCC25401. Naturally-occurring *L. welshimeri* and *L. seeligeri* strains isolated from foods (courtesy of Dr. Sophia Kathariou, NCSU) were used in inclusivity studies. All *Listeria* strains were grown in Trypticase Soy Broth (TSB, Becton-Dickinson and Co., Sparks, MD) with overnight incubation at 37 °C. Non-*Listeria* strains, including *E. coli* O157:H7 ATCC43895, *Bacillus cereus* ATCC49063, *Salmonella enterica* subsp. enterica serovar Enteritidis ATCC13076, *Staphylococcus aureus* ATCC23235, *Pseudomonas aeruginosa* ATCC23993, *Shigella flexneri* ATCC12022, *Brochothrix thermosphacta* ATCC11509 and *Lactococcus lactis* MG1363, were used for counter SELEX and in exclusivity studies. Brain Heart Infusion (BHI) broth (Becton–Dickinson) with overnight incubation at 37 °C was used to cultivate all non-*Listeria* spp. strains except *B. thermosphacta*, which was grown at 26 °C.

2.2. DNA aptamer selection process

2.2.1. Preparation of DNA library for initial SELEX screening

For the first round of SELEX, an 81-mer combinatorial DNA library consisting of a 40 nucleotide random region was obtained from Integrated DNA Technologies (IDT, Coralville, IA). A typical combinatorial library of 1 μmol scale such as the one used here is limited to 10^{14}–10^{15} individual sequences (Jayasena, 1999). The DNA library was 5′ end-labeled by PCR using a biotinylated reverse constant region primer. Briefly, a 50 μl reaction master mix containing 5 μl of aptamer library (10 μM), 1× GoTaq® buffer (Promega Corp., Madison, WI), 0.2 mM GeneAmp® dNTP mix (Applied Biosystems, Foster City, CA), 5 U Go Taq® DNA Polymerase (Promega), 500 nM unlabeled forward constant region primer, and 500 nM biotin labeled reverse constant region primer was amplified using a three-step thermal protocol of initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min using a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/Bio-Rad Laboratories, Hercules, CA). The DNA library and all primers used in this study are described in Table 1. The double-stranded PCR product was coupled to Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega) as per manufacturer recommendations. The forward ssDNA moieties were separated from the biotinylated ssDNA bound to the magnetic particles by alkaline denaturation in 0.15 M NaOH, and the magnetic beads bound to biotinylated ssDNA were collected using a Dynal MPC®-M magnetic particle concentrator (Dynal A.S, Oslo, Norway). Two additional alkaline denaturations were then performed in 0.15 M NaOH to completely remove the forward strand from the 5′ biotinylated complimentary strand. The purified 5′ biotinylated strands coupled with magnetic particles were washed thrice in 1× Tris-EDTA (10 mM Tris/Tris–HCl, 1 mM EDTA) and transferred into a clean microcentrifuge tube prior to a final alkaline denaturation, the purpose of which was to separate the biotinylated strand from the magnetic particles. The final alkaline denaturation was done in 28% ammonium hydroxide (Sigma–Aldrich, St. Louis, MO) for 10 min at 90 °C. The supernatant obtained after magnetic pull-down was mixed with twice the amount of water, then the 5′ biotinylated strand was collected by ethanol precipitation with reconstitution of the DNA pellet in 50 μl of nuclease-free water. The density of ssDNA library was determined by NanoPhotometer Pearl (Implen GmbH, Munich, Germany) and adjusted to 500 pmoles for the first round of selection.

2.2.2. In vitro selection process (SELEX)

The whole cell SELEX aptamer selection protocol used in this study was adapted from the work of Dwivedi et al. (2010) with modifications. Six rounds of positive-SELEX targeting *L. monocytogenes* were performed, while two rounds of negative (counter)-SELEX were also done. In brief, approximately 500 pmoles (corresponding to $1.8 \sim 3.0 \times 10^{14}$ ss DNA copies) of the biotinylated ssDNA library was denatured by heating at 90 °C for 5 min and renatured by flash cooling on ice for 10 min to allow intra-strand base pairing. The ssDNA aptamer library was incubated with overnight cultured *L. monocytogenes* cells at concentration of $\sim 10^{8-9}$ cells at room temperature (RT) for 1 h with gentle rotation. Prior to the incubation, *L. monocytogenes* cells were washed 3 times with 1× phosphate buffered saline solution (PBS) and resuspended in binding buffer (1× PBST, 0.05% Tween20 in 1× PBS). The ssDNA-bound cells were recovered by centrifugation at 7000 × *g* and washed to remove unbound and non-specifically bound ssDNA moieties. The ssDNA (aptamers) bound to the cells were released by heat (90 °C) prior to enrichment by PCR, and the double-stranded PCR product was separated and converted to ssDNA for another round of SELEX, as described above.