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Development and evaluation of aptamer magnetic capture assay in conjunction with real-time PCR for detection of *Campylobacter jejuni*

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ABSTRACT

A prototype method for the concentration and detection of *Campylobacter jejuni* was developed using a previously reported biotinylated DNA aptamer in conjunction with qPCR. The so-called aptamer-based magnetic capture-qPCR (AMC-qPCR) assay was compared to a similar immunomagnetic separation (IMS)-qPCR assay. In small volume experiments (300 μl) applied to serially diluted *C. jejuni* suspended in buffer containing a mixed culture of other common food borne pathogens, the lower detection limit of the AMC-qPCR method was 1.1 $\log_{10}$/300 μl *C. jejuni* cells, one $\log_{10}$ better (lower) than that of IMS-qPCR (2.1 $\log_{10}$ CFU/300 μl). AMC-qPCR capture efficiency was 10–13% at assay detection limit. In 10 ml scale-up experiments, the lower detection limit of AMC-qPCR was 2.0 $\log_{10}$ CFU/10 ml with corresponding capture efficiency of 4–7%. Nucleic acid aptamers are promising alternatives to antibodies for magnetic bead-based capture followed by qPCR detection.

1. Introduction

Campylobacter jejuni is a leading cause of food borne illness (Scallan, Hoekstra, Widdowson, Hall, & Griffin, 2011). Based on 2012 FoodNet figures, the annual incidence of *C. jejuni* infection in the United States is 14.3 per 100,000 population (CDC, 2013). In the European Union, it is estimated that there are approximately nine million cases of human campylobacteriosis per year (EFSA, 2011). Besides classic bacterial gastroenteritis, *C. jejuni* infection may also result in autoimmune neurological disorders such as Guillain-Barré syndrome and Miller Fisher syndrome; and less frequently in meningitis, pneumonia, and miscarriage (Yang, Jiang, Huang, Zhu, & Yin, 2003). Ingestion of as few as 500 *C. jejuni* cells has been reported to cause disease in humans (Robinson, 1981). The main sources of food borne transmission of *C. jejuni* are poultry and raw milk products, as well as untreated water (Altekruse, Stern, Fields, & Swerdlow, 1999).

Rapid and accurate detection of food borne pathogens is an important food safety tool. While culture-based methods are considered the gold standard, specific temperature and environmental requirements for the growth and recovery of *Campylobacter* spp. make these assays particularly cumbersome. Many studies have advocated that detection of food borne pathogens could be made more rapid if we were able to bypass, or even reduce, the time put into cultural enrichment. This can theoretically be accomplished by separating and concentrating the target organism(s) from the sample matrix prior to detection (Dwivedi & Jaykus, 2011). So-called pre-analytical sample processing could also provide reduction of sample size, making the use of molecular-based detection methods more feasible when applied to the larger sample sizes necessary for real-world applications.

Currently, immunomagnetic separation (IMS) is the most commonly used method for capturing and concentrating pathogens from complex sample matrices (Dwivedi & Jaykus, 2011). Nucleic acid aptamers, which are single stranded oligonucleotides that naturally fold into three-dimensional structures demonstrating target-specific binding affinity, have been proposed as alternatives to antibodies. Produced using an iterative process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Tuerk & Gold, 1990), aptamers provide potential advantages over antibodies in that they are inexpensive, stable, and can be synthetically manufactured and chemically manipulated with relative ease. In recent years, there has been an increasing interest in the application of aptamers for detection of microbial agents, including food borne pathogens (Suh, Brehm-Stecher, & Jaykus, 2013). However, comparative evaluation of the performance of aptamer versus antibody-based capture-detection assays for food

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borne pathogens has not yet been reported. The purpose of this study was to develop a prototype method to detect *C. jejuni* using a magnetic bead-bound DNA aptamer for target capture (aptamer magnetic capture or AMC) combined with quantitative real-time PCR (qPCR) for detection. Further, AMC-qPCR was compared in parallel to an immunomagnetic separation (IMS)-qPCR assay with respect to both detection limit and capture efficiency.

2. Materials & methods

2.1. Bacterial strains, culture conditions and preparation of cells

C. jejuni (A9a), a naturally occurring strain isolated from a poultry processing plant, was used for all experiments in this study (Malik-Kale et al., 2007). Cells were grown in *Brucella* broth (Becton–Dickinson and Co., Sparks, MD) for 48 h at 42 °C under microaerophilic conditions achieved using the GasPak™ EZ Campy Container System (Becton–Dickinson). The broth culture was centrifuged, washed and diluted in 1X phosphate buffered saline (PBS, pH 7.0) and cell concentrations were determined by plating serial dilutions on Campy Cefex agar (Hardy Diagnostics, Santa Maria, CA, USA). *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076), *Bacillus licheniformis* (ATCC 9789), *Shigella sonnei* (ATCC 25931), *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* Scott A and *Escherichia coli* O157:H7 (ATCC 43895) were used as background bacteria to assure the specificity of the aptamer. All non-*Campylobacter* strains were grown in Brain Heart Infusion (BHI) broth (Becton–Dickinson) by overnight incubation at 37 °C. The cultured cells were washed, centrifuged, and diluted in PBS and cell concentrations were determined by plating on agar-solidified BHI plates.

2.2. Preparation of ligand-bound magnetic beads

The 81 mer biotinylated aptamer 229, previously identified and characterized using whole-cell SELEX (Jaykus, Dwivedi, & Smiley, 2011), was used in studies because of its high binding affinity to *C. jejuni*. The highly purified biotinylated oligonucleotide was procured from Integrated DNA Technologies (IDT, Coralville, IA, USA) and diluted using diethylpyrocarbonate (DEPC)-treated water. To prepare aptamer-bound magnetic beads, a solution of biotinylated aptamer was first denatured at 90 °C for 5 min and flash cooled for 10 min on ice. Conjugation to streptavidin-coated magnetic particles (Promega, Madison, WI) was done as per manufacturer instructions using a two-fold excess concentration of aptamer 229 (0.1 nmol aptamer per 50 μg magnetic beads) as optimized in earlier experiments (data not shown).

For comparison purposes, anti-*Campylobacter* biotinylated polyclonal antibody (Thermo Scientific, Rockford, IL) was conjugated to streptavidin-coated magnetic beads (Promega) using a concentration of 7 μg antibody per 50 μg beads. To minimize non-specific binding, the aptamer and antibody-conjugated magnetic particles were blocked with 2% bovine serum albumin suspended in 1X PBS containing 0.05% Tween 20 (PBST) for 2 h at room temperature with gentle rotation. After the blocking, the ligand (aptamer or antibody)-conjugated magnetic beads were washed with 1X PBS using magnetic pull-down, and stored at 4 °C until used for the assay.

2.3. Aptamer magnetic capture (AMC) and immunomagnetic separation (IMS) of C. jejuni

The AMC capture steps were done using two different sample volumes (300 μl and 10 ml) and ligand-coated bead volumes optimized in preliminary studies (data not shown). For the small sample volume (300 μl) studies, a 48 h culture of *C. jejuni* A9a was 10-fold serially diluted in PBST to yield concentrations ranging from 10^2 to 10^7 CFU/ml. Nine hundred microlitres of select solutions (containing 10^2, 10^3, 10^5 or 10^7 CFU *C. jejuni*/ml) were mixed with a suspension (100 μl) of four other common food borne pathogens (i.e. *S. enterica* serovar Enteritidis, *B. licheniformis*, *S. sonnei*, and *E. coli* O157:H7) which were held at a concentration of 10^4 CFU/ml. A 100 μl aliquot of this cocktail was mixed with 150 μl of PBST and 50 μl (or 50 μg) of either aptamer-conjugated magnetic beads or antibody-conjugated magnetic beads followed by incubation for 45 min at room temperature with gentle rotation. After incubation, the *C. jejuni* bound beads were pulled down using a magnetic separation stand (Promega) and washed thrice to remove unbound cells using 500 μl of 1X PBST. The scale-up studies were performed in a similar manner but using 300 μl (or 300 μg) of aptamer–bead complex as applied to 10 ml buffer system (PBST) containing 10^2, 10^3, 10^4, 10^5 and 10^6 *C. jejuni* CFU and 10^3 CFU mixed microflora. After capture for 45 min, the *C. jejuni* bound magnetic beads were washed and subjected to DNA extraction and qPCR.

2.4. DNA extraction and quantitative real-time PCR

DNA was extracted from recovered beads using the MasterPure™ DNA Purification kit (Epicentre, Madison, WI) in accordance with manufacturer instructions. Detection of *C. jejuni* was done using a Taqman™ quantitative real-time PCR (qPCR) protocol targeting a 126 bp region of the *gly*A gene. Primers (Forward 5′- TAA TGT TCA GCC TAA TTC AGG TTC TC-3′; Reverse 5′- GAA GAA CTT ACT TTT GCA CCA TGA GT -3′) and TaqMan™ probe (5′-/FAM/AAT CAA AGC CGC ATA AAC ACC TTG ATT AGC/TAMRA/-3′) used for DNA amplification were those reported by Jensen, Andersen, Dalsgaard, Baggesen, and Nielsen (2005) and procured from IDT. The qPCR was carried out in the SmartCycler® PCR system (Cepheid, Sunnyvale, CA, USA). A 25 μl PCR reaction containing 1X PCR buffer, 5 mM $MgCl_2$ (Invitrogen/Life Technologies, Carlsbad, CA, USA), 0.4 mM dNTP mix (Applied Biosystems, Foster City, CA, USA), 300 nM forward primer, 300 nM reverse primer, 200 nM Taqman probe, 1.75 U Platinum® Taq DNA Polymerase (Invitrogen) and 2.5 μl of *C. jejuni* DNA was used. The two-step thermocycling protocol was as follows: initial denaturation at 95 °C for 120 s, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s.

Capture efficiency was estimated according to the method previously reported by Joshi et al. (2009). Briefly, a standard curve was constructed using 10-fold serial dilutions of an overnight culture of *C. jejuni* cells that were enumerated culturally and also extracted for DNA isolation and subjected to qPCR. The data were plotted as $\log_{10}$ CFU (*X* axis) vs. Ct value (*Y* axis). The approximate CFU recovered after ligand-mediated magnetic pull-down was estimated from the standard curve based on resulting Ct values obtained from qPCR after capture. The percent capture efficiency (% CE) was calculated as the ratio of the estimated CFU (after capture and detection by qPCR) to the total input CFU per sample, multiplied by 100.

2.5. Data analysis

Statistical comparisons between capture efficiency at each inoculum level were done by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test using statistical analysis software (SAS ver. 9.2, Cary, NC) ($^*p < 0.01$).

3. Results

3.1. Quantitative real-time PCR standard curve for C. jejuni

The qPCR standard curve demonstrated log linear detection in the range of 1–8 $\log_{10}$ CFU *C. jejuni* per reaction with an R^2 value of