5′-Nuclease PCR Assays for Foodborne Pathogen Detection using the Agilent-Stratagene Mx3000P Q-PCR System

Application: Food Safety

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Abstract

Nucleic acid-based assays are increasingly being applied as a rapid alternative to conventional culture techniques to test for pathogenic microbial contaminants in food. This study details the use of real-time quantitative PCR as a tool to detect four major foodborne bacteria that cause human disease. Primers and probes were used to develop assays that specifically target the *invA*, *rfbE*, *hlyA*, and *Cj0414* genes from *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Campylobacter jejuni* respectively. The detection limit for these assays was between 1-10 fg genomic DNA per PCR reaction, quantification was linear over 6-7 log units. Results can be obtained with these assays in 2.5 h following enrichment. When used in tandem with an appropriate sample preparation technique, this method may be suitable for food safety applications.

Introduction

Foodborne disease is estimated to cause 76 million illnesses and 5,000 deaths annually in the United States [1]. Of the known pathogens that cause foodborne illness, *Salmonella*, *Escherichia coli* O157:H7, *Listeria*, and *Campylobacter* are among the short list of micro-organisms that exert the largest disease burden.

Bacteriological culturing has traditionally been used to detect microbial pathogens in food, and this technique is still in widespread use today. However, the time-consuming and low-throughput nature of culture-based detection methods warrant their replacement by more rapid methods based on biomolecular analysis, like the polymerase chain reaction (PCR). PCR, predicated on DNA synthesis and amplification, is an established, core technique in molecular biology, microbiology, diagnostics, genetics and environmental science applications.

A derivative of PCR, real-time quantitative PCR (Q-PCR) kinetically monitors the reaction in ‘real time’. Q-PCR confers significant advantages over conventional PCR, including increased reaction speed, sensitivity and specificity; it also eliminates the need to open the reaction tubes for post-PCR analysis, thus preventing cross-contamination. Q-PCR is a fluorogenic probe-based 5′-nuclease assay that provides a higher level of specificity than conventional PCR, thus assuring the correct DNA target is amplified, and it is the preferred method to

measure starting amounts of DNA in a sample.

In this study, we describe the development and composition of kinetic 5′-nuclease PCR assays designed to individually detect four major food-borne pathogens. The assays were designed by researchers at the USDA Western Regional Research Center in Albany, CA and are being tested as part of an on-going collaboration to develop rapid detection approaches to food-borne pathogens. The first generation assays are intended for use in research laboratories, but these may also find utility as tools to aid in the USDA's response to outbreaks of foodborne illnesses. The assays were conducted using an existing Agilent-Stratagene Q-PCR platform (Brilliant Q-PCR Master Mix and the Mx3000P thermal cycler system), and assay performance was evaluated using the Agilent-Stratagene MxPro software.

Materials and Methods

DNA, primers, and probes.

Genomic DNA from four major food-borne pathogens was purchased from ATCC (Table 1). Ten-fold serial dilutions of genomic DNA were prepared in 1X TE (10mM Tris, 1 mM EDTA, pH 8.0). A primer and TaqMan®* probe set was designed for *Salmonella enterica* using Primer Express v2.0 (Applied Biosystems, Inc.) to target the chromosomal invasion gene invA, which mediates *Salmonella* entry into epithelial cells [2]. PCR primers were constructed from the regions including positions 1317 to 1344 (forward) and 1384 to 1404 (reverse). The predicted 88 bp amplicon contains sequence complimentary to the detection probe at positions 1346 to 1382 (Table 2). The primerprobe sets used for the specific detection of *Escherichia coli* O157:H7, *Listeria,* and *Campylobacter* were extracted from previously published research [3,4,5]. The TaqMan® probe reporter dye for all assays was 6-carboxyfluorescein (FAM), and the quencher was a minor groove binding non-fluorescent quencher (MGBNFQ) (Applied Biosystems, Inc.).

Species	Serotype	Strain	ATCC number
Salmonella enterica	Typhimurium	LT2	700720D-5
Escherichia coli	O157:H7	RIMD 0509952	BAA-460D-5
Listeria monocytogenes		Li 2	19115D
Campylobacter jejuni		RM3193	BAA-1234D-5

Table 1. Bacterial isolates used as a source of genomic DNA

5′-nuclease PCR assay.

The amplification reaction mixtures (25 µl) contained template DNA (1000 pg-0.001 pg in 1 µl), 1X Brilliant Multiplex Q-PCR Master Mix (Stratagene), 30 nM ROX reference dye, 0.3 µM each primer, and 0.1 µM TaqMan® probe (0.05 µM for the *C. jejuni* assay). All reactions were performed in triplicate and run on the Mx3000P thermal cycler system (Stratagene). The amplification profile for all assays was 1 cycle at 95°C for 10 min., followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. Fluorescent signals were read using 516 nm (FAM) and 610 nm (ROX) filters at the end of each annealing/extension step. Data analysis was carried out with MxPro software (Stratagene).

Species	Oligo‡	Sequence (5′ - 3′)	Target	Amplicon size (bp)	Ref.
S. enterica	F	CGT GTC CTT TGG TAT TAA TCC AAC AAT C	*invA*	88	-
	R	CCG GAG TTT CTC CCC CTC TTC			
	probe	ATC AGC AAG GTA GCA GTC AGT ATT TCT GGG TAA CGC A			
E. coli O157:H7	F	TTT CAC ACT TAT TGG ATG GTC TCA	*rfbE*	85	3
	R	TGA GTT TAT CTG CAA GGT GAT TCC			
	probe	TTC TAA CTA GGA CCG CAG AGG AAA GAG AGG AAT TA			
L. monocytogenes	F	TGC AAG TCC TAA GAC GCC A	*hylA*	113	4
	R	CAC TGC ATC TCC GTG GTA TAC TAA			
	probe	CGA TTT CAT CCG CGT GTT TCT TTT CG			
C. jejuni	F	CTG AAT TTG ATA CCT TAA GTG CAG C	*Cj0414*	86	5
	R	AGG CAC GCC TAA ACC TAT AGC T			
	probe	TCT CCT TGC TCA TCT TTA GGA TAA ATT CTT TCA CA			

‡ The 5′-reporter dye for all probes is FAM; the 3′-quencher dye for all probes is MGBNFQ

Table 2. Primers and TaqMan® probes used in real-time quantitative PCR

Results and Discussion

Individual primer-probe sets were used in 5'-nuclease Q-PCR reactions containing purified genomic DNA to successfully amplify and detect the target gene from each of the four foodborne pathogens (Figure 1). A fluorescent signal was observed for each reaction that contained DNA, whereas the no template control (NTC) samples all gave negative results.

To evaluate the sensitivity of the primer-probe combinations, DNA from each species was serially diluted 10-fold in buffer and each dilution was subjected to PCR. Seven dilutions for Q-PCR amplification were created for each species, utilizing a dynamic range that spanned seven log units between 1000 and 0.001 pg DNA/reaction. Only the *Campylobacter* assay was sensitive enough to detect a signal in the most dilute sample; this assay has a detection limit of 1 fg/rxn. The *Salmonella*, *Escherichia coli* O157:H7, and Listeria assays all resulted in a detection limit of 10 fg/rxn. The most sensitive Q-PCR assays that use genomic DNA to detect single copy genes have detection limits between 1-10 fg/rxn.

The quantitative aspects of each assay were evaluated by correlating log input DNA with threshold cycle signals (Ct) for each dilution series (Figure 2). Linear regression analysis was performed on each resultant standard curve in order to obtain a regression square coefficient, all of which fell within 0.996 and 1.000. Thus, the regression line for each assay approximates real data points with little error. The slopes of the curves were used to calculate the Q-PCR reaction efficiencies, which were found to be between 93.5-101.3%, depending on the assay. These results suggest that these assays could be used to accurately measure the load of contamination by these four infectious agents in unknown samples by interpolation of

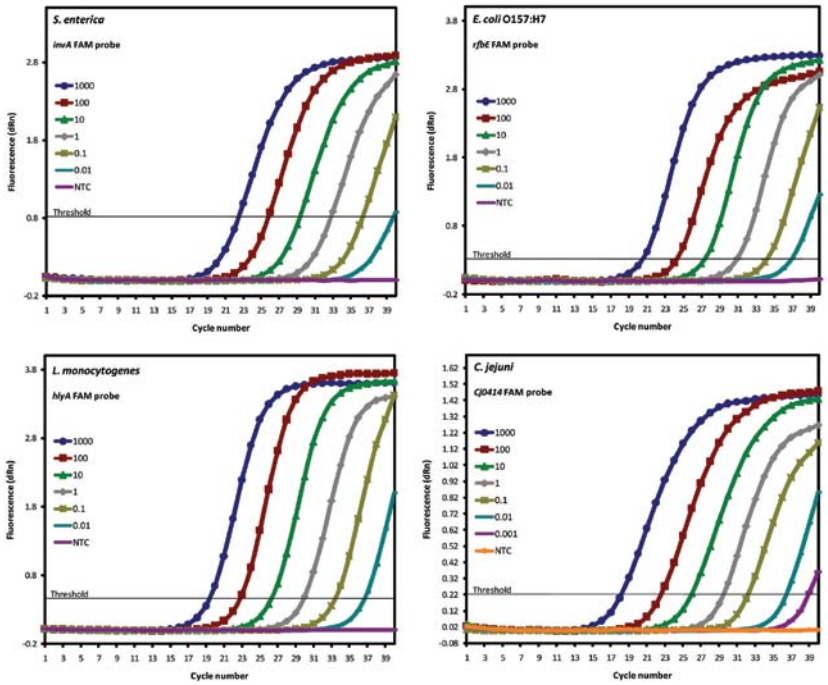

Figure 1. Amplification sensitivity for the 5'-nuclease PCR foodborne pathogen detection assays. A range of known concentrations of genomic DNA from *S. enterica*, *E. coli* O157:H7, *L. monocytogenes* (all at 1000 pg – 0.01 pg) and *C. jejuni* (1000 pg – 0.001 pg) were used in singleplex 5' nuclease PCR assays containing the primer-probe set for the target gene. Each trace represents the average of triplicate PCR reactions. The cycle number is plotted as a function of the baseline subtracted fluorescent reading normalized to the reference dye (dRn). NTC, no template control.

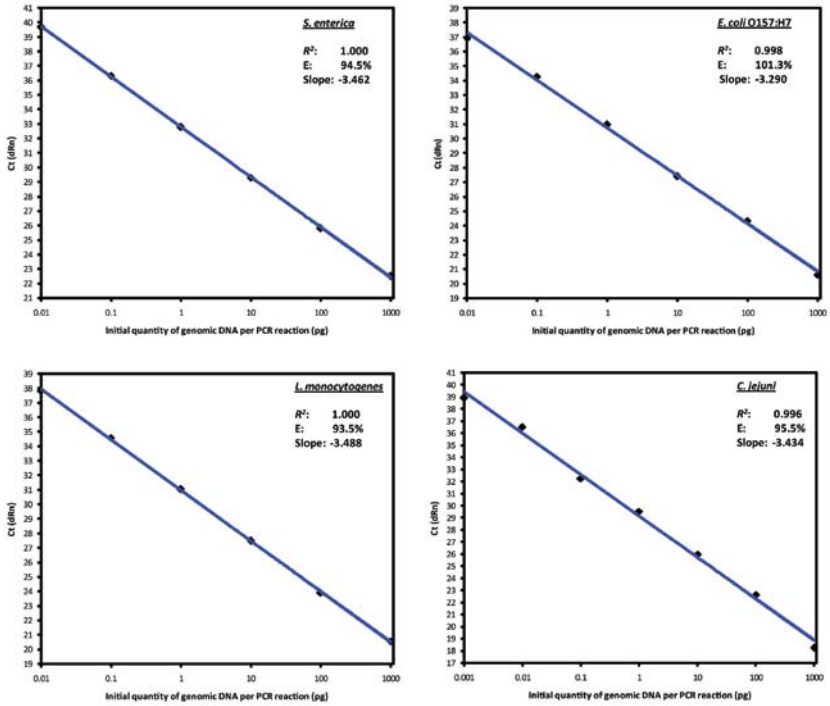

Figure 2. Threshold cycle analysis of serial 10-fold dilutions of foodborne pathogen DNA. A range of known concentrations of genomic DNA from *S. enterica*, *E. coli* O157:H7, *L. monocytogenes* (all at 1000 pg – 0.01 pg) and C. jejuni (1000 pg – 0.001 pg) were used in singleplex 5' nuclease PCR assays containing the primer-probe set for the target gene. The starting quantity of DNA from each serial dilution is plotted as a function of threshold cycle (Ct) values to obtain a standard curve. Each trace represents the average of triplicate PCR reactions. The straight line is calculated by linear regression.

similar standard curves. These results also demonstrate the advantage of Q-PCR over conventional and real-time PCR in obtaining a model with high predictive power that can be used to detect and quantify pathogen load. Food samples require an enrichment step that incubates microbes, if present, before DNA extraction and analysis. Hence, absolute quantification that precisely measures the extent of contamination in the initial raw sample is normally not possible for any detection method, but Q-PCR analysis does allow absolute quantification of enriched pathogens in unknown samples and thus gives a relative measure of contamination in the original samples.

Conclusions

This study describes the development of sensitive and specific detection assays for four common foodborne pathogens. A key component of the assays is combining gene amplification, detection and data analysis steps by using the Agilent-Stratagene Mx3000P Q-PCR platform. Relative to traditional microbiological culturing methods for food testing, our molecular detection approach offers enormous potential for reducing food analysis time from days to hours. Next generation multiplexed detection assays will incorporate the integrated four color technology of the Agilent-Stratagene Mx3000P Q-PCR system to provide simultaneous detection of multiple pathogens in one reaction tube.

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