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Communication

Simultaneous Detection of Foodborne Pathogens Using a Real-Time PCR Triplex High-Resolution Melt Assay

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Abstract: Foodborne pathogens pose risks to populations all over the world. Pathogens can be used as bioterrorism agents, causing an outbreak that affects many individuals through the consumption of a commonly affected food or beverage. A PCR assay can be used to identify pathogens through their unique melting points using a high-resolution melt assay. Assays can be used to detect the bacteria individually or from a mixture using species-specific primers. An assay was developed to detect and identify three pathogens that routinely cause multistate foodborne outbreaks, as documented by the U.S. Centers for Disease Control and Prevention, *Campylobacter jejuni* (*C. jejuni*), *Escherichia coli* (*E. coli*), and *Salmonella enterica* (*S. enterica*), in single bacterium assays and a multiplex. The primers were targeted to specific and unique gene sequences of each pathogen, including *cadF*, *yedN*, and *hilA*, respectively. Each pathogen was identified by its unique melting temperature in single assays: 78.10 ± 0.58 °C for *C. jejuni*, 81.96 ± 0.42 °C for *E. coli*, and 87.55 ± 0.37 °C for *S. enterica*. The multiplex successfully detected and identified all three of the pathogens with the distinctly separated melt peaks. The PCR high-resolution melt assay also proved to be specific, reproducible, fast, and sensitive in experiments.

Keywords: foodborne pathogens; bioterrorism agent; *Campylobacter jejuni*; *Escherichia coli*; *Salmonella enterica*; real-time PCR melt assay

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1. Introduction

Bacterial and viral pathogens can contaminate food and beverage sources unintentionally, or the pathogens can be intentionally introduced [1]. Foodborne pathogens are biological agents that can cause illness if ingested. It is estimated that one of every four to six Americans suffers a significant foodborne pathogen illness each year [2,3]. A foodborne pathogen outbreak is defined by two or more cases of the same foodborne illness resulting from ingestion of a common food or beverage. An outbreak involves a sudden rise in the number of cases of an infection or disease occurring at a community or geographical area beyond what is considered baseline for that area [4]. Bacterial or viral illnesses pose risks to the population by causing mild, severe, or life-threatening symptoms. Mutations in the original pathogens can lead to bigger threats. Mass food production produces huge risk factors for the population [2]. Over 250 pathogens have been discovered, which includes bacteria, viruses, parasites, and worms [3]. Most foodborne illness outbreaks caused by pathogens in the United States over the past fifteen years have been caused by *Salmonella* spp. and *Escherichia coli* (*E. coli*), but *Listeria monocytogenes (L. monocytogenes)*, *Vibrio parahaemolyticus (V. parahaemolyticus)*, and *Campylobacter* spp. are also common culprits [5]. Bacteria have contaminated meat, nuts, peanut butter, salads, flour, cake mix, donuts, and dairy products and have been employed as bioterror agents in reported cases [5–17].

The U.S. Centers for Disease Control and Prevention (CDC) reported that *Campylobacter* spp. caused the most incidences of foodborne illness in 2016 followed by *Salmonella* spp. at number two and *E. coli* at number four on the list [18]. *Salmonella* spp.,

E. coli, and *Campylobacter* spp. are three pathogens, among others, that have similar symptoms when ingested. These symptoms include diarrhea, which could be bloody, abdominal pain and cramps, fever, and, sometimes, vomiting [19–21]. *Salmonella* spp. often contaminates peanut butter, meats, seafood, salad, poultry, dairy products, fresh fruits, and vegetables [5]. *E. coli* often contaminates raw or undercooked ground meats, cake mix, flour, salads, cheeses, nut butter, and sprouts [5]. *Campylobacter* spp. is prevalent in raw or undercooked poultry, products that touched cattle, as well as seafood and produce [22]. *Campylobacter* spp. and *Salmonella* spp. infections have been increasing in both developed and developing countries [22]. The CDC recognizes the importance of rapid culture-independent diagnostic tests (CIDTs) [18].

Polymerase chain reaction (PCR) tests are examples of rapid CIDTs [3,18]. PCR tests have been developed to detect gene sequences unique to several pathogens, including *Campylobacter* spp., *Salmonella* spp., and *E. coli* strains and have been reviewed previously [3,9–11,13,16,17,23]. In situ, post-PCR, melt analysis performed to gradually increase the temperature to melt double-stranded DNA has been shown to differentiate sequence variations, allowing the differentiation of species and strains [8–11,13,16,17,23]. The melt temperature reflects the percentage of GC content as well as the amplicon length [9]. The melt temperature of the PCR amplicon can be detected using a covalently labelled fluorescent dye [8] or a saturating intercalating dye such as EvaGreen or LC Green Plus [9,11,17]. PCR high-resolution melt (HRM) analysis is a fast and effective method and utilizes real-time PCR instrumentation that is found in most clinical diagnostic and forensic laboratories. It is a versatile tool that can be modified for application to many different pathogens, including viruses [24] and bacteria [23].

The aim of this research was to develop a multiplex PCR HRM assay to simultaneously detect and identify the *Campylobacter jejuni (C. jejuni*), *E. coli,* and *Salmonella enterica* (*S. enterica*) pathogens that very often cause foodborne illness. Bacterial DNA from each of the species was tested separately and together in a triplex multiplex, and specificity, sensitivity, and reproducibility were also tested.

2. Materials and Methods

2.1. Bacterial Strains

Extracted DNA from *C. jejuni*, subspecies jejuni, *E. coli*, str. MG1655, and *S. enterica*, subspecies enterica, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Additional pathogens for specificity testing were also obtained from ATCC. These samples included *Bacillus cereus*, str. NRRL B-568, *Bacillus subtilis*, str. 168, *Bacillus thurigiensis* strain USDA H522, *Clostridium difficile*, str. 90556-M6S, *L. monocytogenes*, str. EDGe, *Shigella flexneri* type 2, str. 24570, and *V. parahaemolyticus*, str. EB101. Tested bacterial strains are listed alphabetically in Table 1. The lyophilized DNA stocks were reconstituted in nuclease-free water and diluted to 1 ng/µL.

Table 1. Tested bacterial strains used in this study.

Bacterial Strain	Source
Bacillus cereus, str. NRRL B-568	ATCC (10876D-5)
Bacillus subtilis, str. 168	ATCC (23857D-5)
Bacillus thurigiensis, str. USDA H522	ATCC (35646D-5)
Campylobacter jejuni, subsp. jejuni	ATCC (33560D-5)
Clostridium difficile, str. 90556-M6S	ATCC (9689D-5)
Escherichia coli, str. MG1655	ATCC (700926D-5)
Listeria monocytogenes, str. EGDe	ATCC (BAA-679D-5)
Salmonella enterica, subsp. enterica	ATCC (700720)
Shigella flexneri type 2, str. 24570	ATCC (29903D-5)
Vibrio parahaemolyticus, str. EB101	ATCC (17802D-5)

2.2. PCR Primers

Published PCR primers for *C. jejuni*, *E. coli*, and *S. enterica* were used in the triplex assay [9,11]. The primer sequences and gene locations are listed in Table 2. The primers were tested using the NCBI Basic and Local Alignment Search Tool (BLAST) using the default settings to evaluate their specificity *in silico*. The primers were synthesized by and purchased from IDT (Coralville, IA, USA), reconstituted in nuclease-free water, quantified using a NanoDrop 2000 (ThermoFisher, Frederick, MD, USA), and diluted to 5 µM stocks for the PCR assays.

Table 2. PCR primers and genes for PCR assays in this study.

Specie	Gene Target	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
Escherichia coli	*yedN*	TCCTGGATTGAGGTGCTTTATC	CTACGGAGACCTGGGTAATTCC
Campylobacter jejuni	*cadF*	TGCTATTAAAGGTATTGATGTAGGTGA	CAGCATTTGAAAAATCCTCAT
Salmonella enterica	*hilA*	CAGGGCTATCGGTTTAATCGTCC	GCAGACTCTCGGATTGAACCTG

2.3. PCR Reaction Conditions and HRM Analysis

In each single PCR reaction, the components included 1 µL of the forward and reverse primers for each specie, 8 µL of 2.5X LightScanner master mix (BioFire Defense, Murray, UT, USA), 9 µL of molecular biology grade nuclease-free water, and 1 µL of 1 ng of the target DNA for a total of 20 µL. Each assay was tested at least eight times and a no-template control with no input DNA was tested in each experiment. Assay sensitivity was tested in quadruplicate with 1 µL of input DNA serially diluted from 1 ng/µL to 0.5 ng/µL, 0.1 ng/µL, 0.05 ng/µL, 0.01 ng/µL, 0.005 ng/µL, 0.001 ng/µL, and 0.0005 ng/µL.

In each triplex PCR reaction, the components included 1 µL of the forward and reverse primers for each specie, 8 µL of 2.5X LightScanner master mix, 3 µL of molecular biology grade nuclease-free water, and 1 µL of each target DNA for a total of 20 µL. The triplex assay was tested five times and a no-template control with no input DNA was also tested.

The PCR was conducted using a Rotor-Gene Q real-time PCR instrument (Qiagen, Hilden, Germany), detecting Green. The PCR cycling conditions were as follows: initial hold at 95 °C for 10 min, 40 cycles of 90 °C for 15 s denaturation, 60 °C for 15 s of primer annealing, and 72 °C for 15 s primer extension, a second hold at 72 °C for 5 min and a third hold at 45 °C for 1 min prior to the melt. The melt analysis was performed using the settings of 55 to 95 °C, increasing by 0.3 °C in 3 s intervals.

3. Results

3.1. Results with Single-Specie PCR Melt Assays

Each of the primers was specific for its target specie in BLAST. Single-specie melt PCR assays were evaluated for *C. jejuni*, *E. coli*, and *S. enterica* (using the DNA obtained from ATCC described in Table 1) using the reaction conditions described in the Materials and Methods at least eight times. The primers that were used targeted the specific genes *cadF*, *yedN*, and *hilA,* for *C. jejuni*, *E. coli*, and *S. enterica*, respectively (Table 2). Each specie produced an amplicon with a unique melt temperature (Figure 1). Each was tested eight to ten times over a period of weeks in this work and was found to be reproducible. *C. jejuni* produced a melt at 78.10 ± 0.58 °C (n = 8) with the primers developed for it. *E. coli* led to an amplicon that melted at 81.96 ± 0.42 °C (n = 10) with its primer set. *S. enterica* led to an amplicon that melted at 87.55 ± 0.37 °C (n = 10) with its primer set. The no-template controls did not amplify.

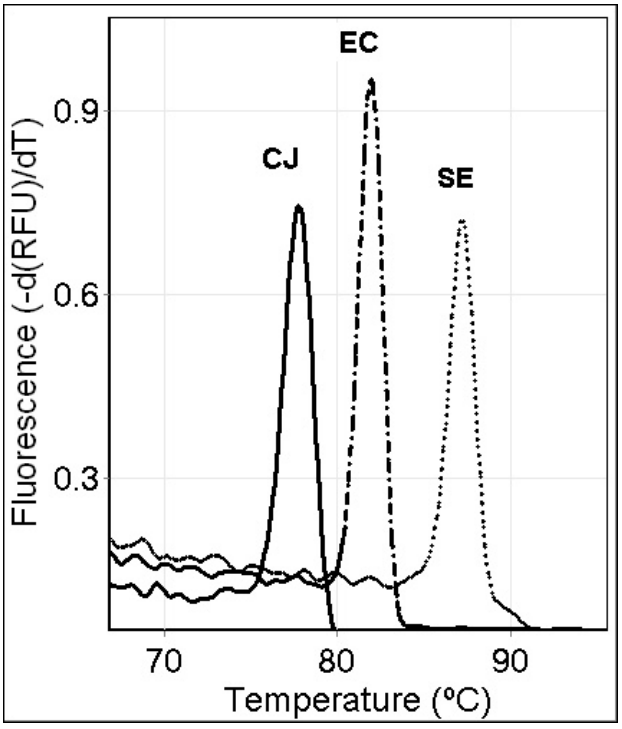


Figure 1. Representative single PCR high-resolution melt assay results for detecting and identifying *C. jejuni* (CJ), *E. coli* (EC), and *S. enterica* (SE), individually.

3.2. Sensitivity of the Single-Specie PCR Melt Assays

Each primer set was tested with a serial dilution the target DNA from 1 ng/µL to 0.0005 ng/µL at least four times. Representative results are shown in Figure 2. The *S. enterica* assay detected the 0.001 ng input DNA in repeated trials. The *E. coli* and *C. jejuni* assays detected the 0.0005 ng input under the reaction conditions, used two and three times, respectively.

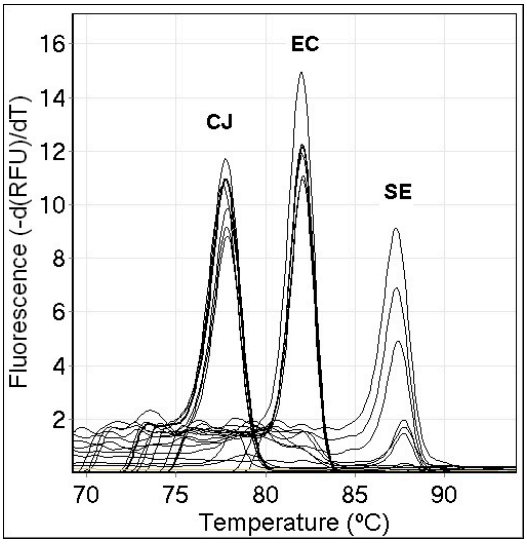
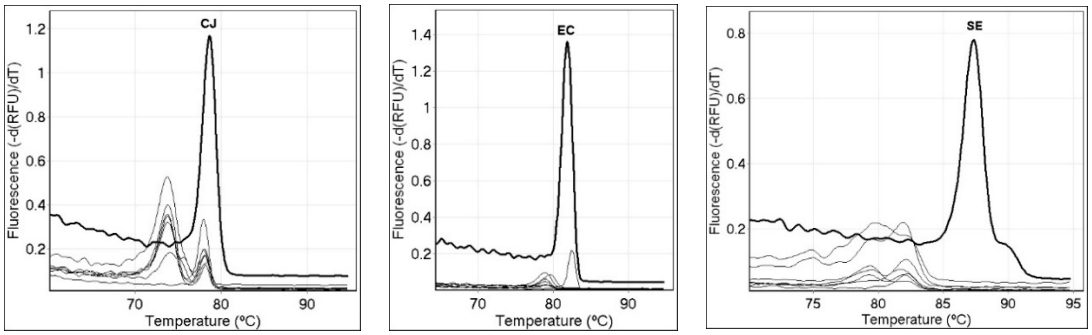


Figure 2. Representative single PCR high-resolution melt assay sensitivity results. The highest input of DNA typically leads to a higher melt peak, although this can be inconsistent for some concentrations due to stochastic amplification.

3.3. Specificity of the Single-Specie PCR Melt Assays

Specificity tests were conducted for each unique primer set using the ten DNA standards obtained from ATCC. The non-target bacterial samples did not lead to amplification

of the target amplicon although some non-specific and other amplification was observed with different and lower fluorescence minor melt peaks (Figure 3).



(**a**) *C. jejuni* (**b**) *E. coli* (**c**) *S. enterica*

Figure 3. Representative specificity results for the assays detecting and identifying (**a**) *C. jejuni* (CJ), (**b**) *E. coli* (EC), and (**c**) *S. enterica* (SE).

3.4. Triplex PCR Assay

The triplex PCR multiplex assay was tested five times on different days and the results were reproducible. *C. jejuni* produced a melt peak at 77.31 ± 0.95 °C. *E. coli* produced a melt peak at 82.05 ± 0.79 °C, and *S. enterica* produced a melt peak at 87.70 ± 0.55 °C. A representative result is shown in Figure 4.

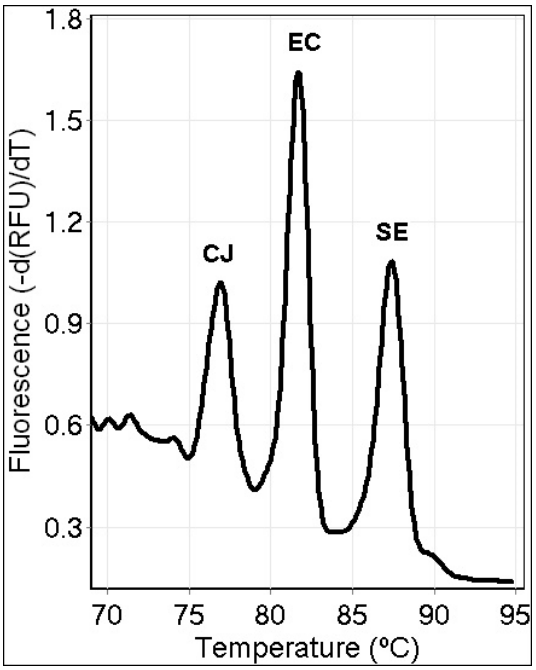


Figure 4. Representative triplex PCR high-resolution melt assay result for the assay detecting and identifying *C. jejuni* (CJ), *E. coli* (EC), and *S. enterica* (SE), simultaneously.

4. Discussion

The goal of this research was to create an HRM assay that could detect and differentiate between three foodborne pathogens that have similar symptoms, could possibly be used as bioterror threats, and cause a large proportion of foodborne illness annually. *Campylobacter* spp. detection and identification by traditional stool culture is slow and is prone to high false-negative results (28%), but PCR yielded more accurate results and better diagnosis [25]. HRM analysis is an excellent tool for species differentiation [26] and to

detect species simultaneously in a multiplex reaction by using their unique melting temperatures [9,17,23]. *Campylobacter* spp., *Salmonella* spp., and *E. coli* are common pathogens that can and have caused outbreaks in the human population [5], and *Salmonella* spp. has been used as a bioterrorism agent [6]. The species were differentiated by their melt temperatures using the LC Green Plus dye included in the LightScanner master mix. Each specie amplified well with the primer set designed to amplify its DNA and was specific using BLAST. The single-specie assays were sensitive and specific. The single-specie amplification reactions produced melt amplicons consistent with prior studies [9,11], although the reaction conditions were altered to produce the multiplex assay described in this study. In a previous study, the *C. jejuni* amplicon melt was detected at 76.6–76.72 °C [11] on an Illumina Eco qPCR Qiagen or Corbett Rotor-Gene® 3000, while the *E. coli* and *S. enterica* single assay melts were detected at 82.41 ± 0.43 °C and 86.91 °C, respectively, on the Qiagen Rotor-Gene Q [9] with the HRM setting. The multiplex assay was able to test and detect all three of the pathogens on the same instrument, as well as in the same mixture, rapidly, in under 2 h using the reported conditions. This assay tool is a fast and effective method for detecting foodborne pathogens to reduce time as well as labor costs in an emergency context [4]. The *C. jejuni* and *E. coli* assays have been used in food testing for the bacteria in chicken and apple cider, respectively [11,17].

5. Conclusions

The multiplex assay that was developed for *C. jejuni*, *S. enterica*, and *E. coli* was effective in the detection of all three bacterial species and allowed for the simultaneous identification of the three species using their melt temperatures. The multiplex provides a fast and effective way to distinguish between foodborne pathogens that produce similar symptoms when they are consumed. Since the symptoms of illness from the pathogens are so similar, the HRM multiplex enables efficient detection of one or more of the culprits at the same time. Future work will involve the testing of additional food products using the multiplex.

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