P-T211

Design and Validation of a Lyophilized Multiplex Real-Time PCR Assay for *Campylobacter* Pathogen Detection and Speciation in Fast PCR conditions

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ABSTRACT

Background: *Campylobacter jejuni, C. coli*, and *C. lari* are among the most frequently reported food-borne pathogens. To facilitate the identification of human-pathogenic species, we designed and validated a sensitive and specific multiplex real-time FAST PCR assay to detect and identify *C. jejuni, C. coli*, and *C. lari* isolates. The assay included an internal positive control (IPC) and used a five-dye configuration in a lyophilized format. PCR methods provide rapid, sensitive and culture-independent approach that is important for detection of pathogens that are sensitive to different environmental and growth conditions. *C. jejuni, C. coli,* and *C. lari* were detected either individually or in mixtures at 5-100 CFU/mL. Each species was specifically detected in the presence or absence of other species. The *Campylobacter* multiplex assay showed 100% specificity for all targets analyzed and no detection of an exclusion panel that included nearest neighbors. Multiplex real-time PCR can simplify pathogen detection and quantification and reduce cost since 3 species can be analyzed in a single reaction. *Campy* bacteria detection in on-filter concentration and lysis experiments was successfully tested directly from chicken rinse. This method increased sensitivity of detection about 1000 fold. Fully automated sample preparation showed the detection at 10-100 CFU/per mL from Campylobacter inoculated chicken rinses. Campy detection was successfully validated for chicken meat, deli meat, eggs, raw whole milk and direct chicken rinses.

MATERIALS AND METHODS

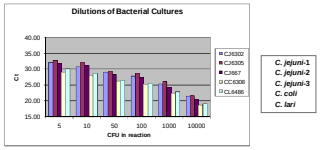
Campylobacter DNA sequence information was obtained from public and Applied Biosystems databases. The sequences were aligned to identify regions of consensus for selecting Real-time PCR and sequencing primers. Multiple *Campylobacter* isolates were sequenced to verify candidate target TaqMan® sites across multiple strains within each species to design specific primers and probes. Two forward and two reverse sequencing primers were selected and used for sequencing of target gene fragments. First, DNA was amplified using True Allele PCR Premix, (Applied Biosystems), cleaned with ExoSAP-IT® (USB), and 1 µL was used for DNA sequencing following the manufacturer's recommendation (BigDye® Terminator v1.1, Applied Biosystems). Sequencing reactions were analyzed by the the 3100 Genetic Analyzer (Applied Biosystems) and by using "ClustalW multiple sequence alignment". The assay used a five-dye configuration in a lyophilized format. *Campylobacter* Multiplex: TaqMan® Real-Time PCR assays were designed for each *Campylobacter* species and lyophilized in a single reaction tube. A 30 µl volume reaction was set up by reconstitution of the lyophilized mix which contained, optimized for lyophilization, 2X environmental master mix version 2 (EMM v.2) and TaqMan® assay mix (target primers and probes, as well as Internal positive control (IPC) primers, probe and a template), with up to 30 uL of target DNA solution. The samples were amplified and detected on the Applied Biosystems 7500Fast Real-Time PCR System using FAST cycling conditions (95ºC for 2 min, followed by 40 cycles at 95ºC for 3 sec and 60ºC for 30 sec).

RESULTS

Figure 1. *Campylobacter* multiplex detects, differentiates, and quantifies *C. coli, C. jejuni*, and *C. lari* in a single reaction tube.

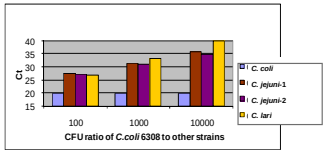
•FAM	*C.coli*	target gene: *gyrB*
•VIC	*C.Jejuni*	target gene: *rnpB*
•LIZ	*C.lari*	target gene: *gyrB*
•NED	IPC (Internal Positive Control)	
•ROX	Reference Dye	

Figure 2. *Campylobacter* multiplex detects bacteria individually or in mixtures at 5-100 CFU/mL



Designed for 7500FAST Real-Time PCR Instrument

C. jejuni, C. coli, and *C. lari* were detected either individually or in mixtures at 5-100 CFU/mL. Each species was specifically detected in the presence or absence of other species. The *Campylobacter* multiplex assay showed close to 100% efficiency for all targets analyzed.



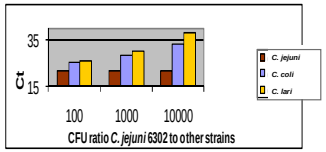


Figure 3. Sample preparation methods tested

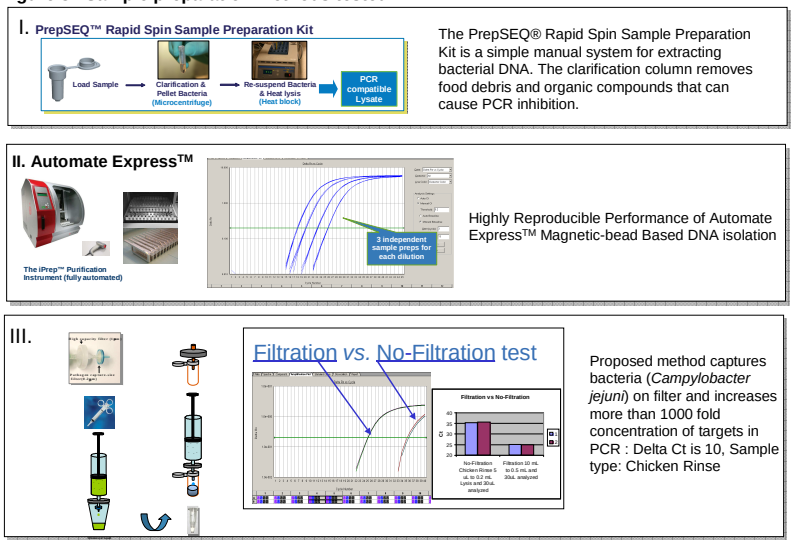
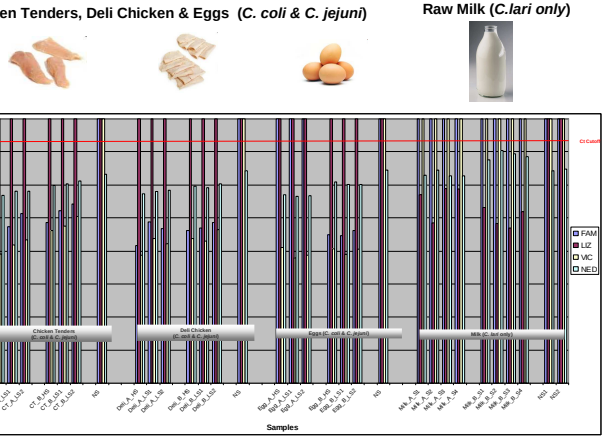


Figure 4. *Campylobacter* multiplex detects bacteria in food samples (24 hour enrichment) with high sensitivity

Spike Levels per 25 g

Organism	Plate 1 (cfu/100uL)	Plate 2 (cfu/100uL)	Plate 3 (cfu/100uL)	Average cfu/100uL
C.jejuni	6	3	5	4.8 LS 48 HS
C. coli	8	3	4	4.8 LS 48 HS
C. lari	1	2	2	2 -3

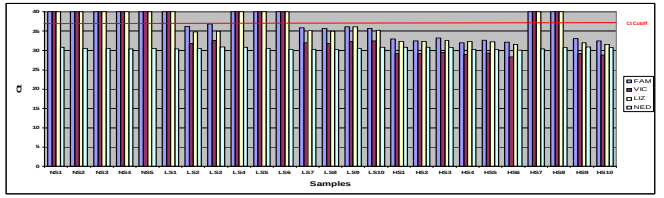
Chicken Tenders, Deli Chicken & Eggs (*C. coli & C. jejuni*) Raw Milk (*C.lari only*)



NS – no spike, (A) – Standard PrepSEQ™ RapidSpin protocol; (B) – PrepSEQ™ RapidSpin protocol with Bead Beating

Figure 5. *Campylobacter* multiplex detects bacteria in direct chicken rinses (no enrichment) samples with high sensitivity

Organism	Matrix	Low-Level Inoculum CFU/whole chicken			High-Level Inoculum CFU/whole chicken		
Campylobacter coli *Campylobacter lari* *Campylobacter jejuni*	Direct Chicken Rinse	125	133	140	865	799	833



NS – no spike, (L) – Low-Level Inoculum; (H) – High-Level Inoculum

TRADEMARKS/LICENSING

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