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Performance of a Genus Specific *Campylobacter* spp. Real Time PCR Assay In Testing Broiler Chicken Carcass Rinses with Accompanying Cultural Confirmation

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Abstract

Following exhaustive genus/species inclusivity (n=55) and exclusivity (n=67) testing, a real-time polymerase chain reaction (PCR) assay specific for the *Campylobacter* genus 16S ribosomal RNA (rRNA) gene was used to test rinse samples from broiler chicken carcasses sampled directly from a poultry processing plant. Broiler carcass rinses (n=30) were tested for *Campylobacter* spp. both by real-time PCR (1 h per 10 enriched samples) and by standard 72 hour cultural methodology. Samples obtained over the period of two shifts from before (pre) and after (post) the submersion chiller were all culture positive for *Campylobacter*. After an 18 hour enrichment, all samples were positive by real-time PCR. The limit-of-detection (LOD) of the PCR assay was ~ 800 cfu/ml rinsate of target organism. Real-time PCR analysis of the enrichments indicated viable *Campylobacter* spp. were present in greater quantities in pre-chiller carcasses (mean Cycle Threshold, C_T = 35.74 for non-enriched and 17.00 for enriched) than in post-chiller rinse samples (mean C_T=36.37 for non-enriched and 25.00 for enriched). Non-enriched samples resulted in a signal below the threshold of detection, thus highlighting the need for enrichment prior to PCR amplification. These results are congruent with plate count data. Assay response curves generated with pre-chiller and post-chiller rinsates spiked with known amounts of *Campylobacter jejuni* were not significantly different, indicating assay robustness across a variety of carcass samples. Attempts to concentrate cells by centrifugation were of limited value. Collectively the data highlight the need for continued efforts in non-cultural enrichment of food samples immediately prior to real-time PCR analysis in order to exceed the threshold of detection. Real-time PCR is an efficacious means to accurately detect the presence of *Campylobacter* spp. in overnight enrichment cultures of chicken carcass rinse samples.

Introduction

Campylobacter spp. are a major causative agent of human food-borne gastroenteritis and subsequent adverse human health sequelae (1, 2). A significant source and reservoir of this pathogen, both through ingestion and in-home transmission, is raw and undercooked poultry (4,7,8, 9, 10, 13, 14,16, 20). Reliable and timely *Campylobacter* spp. analysis is an important first step in reducing disease due to this bacterium as well as its transmission through the food supply.

Current cultural methodologies, while effective for most types of poultry carcass samples, are notoriously time-consuming (up to five days), tedious, often subjective and in some instances, unreliable. With the advent of real-time PCR, testing for this pathogen as well as other food-borne bacterial disease agents, is undergoing changes that are presenting new alternatives to strictly culture- based methods. A variety of PCR assays for *Campylobacter jejuni, C. coli* and *C. lari* have been reported (6, 11, 13, 15). The R.A.P.I.D. System is a highly versatile real-time PCR platform which includes the means to further evaluate the amplicon by melting temperature analysis after the detection assay is finished (19). A similar approach has been used to speciate *Campyloabcter* (12) as well as to detect gyrA mutations in antibiotic resistant *C. coli* isolates (5). Ultimately, using rapid detection platforms such as real-time PCR will augment the testing process by providing a fast and reliable screen of a sample that can subsequently be cultured in order to obtain actual bacterial isolates for further study.

In this work we report progress on the development and validation of a real-time PCR assay designed to detect members of the *Campylobacter* genus while excluding closely- related phyla such as *Arcobacter*, and *Helicobacter*. This approach utilizes freeze dried reagents in a user-friendly format amenable to a variety of lab settings and user expertise levels.

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*Mention of a trade name, proprietary product or specific equipment is necessary to report factually on available data: however, the USDA neither guarantees nor warrants the standard of the product and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Materials and Methods

Carcass Rinse Samples. Processed broiler carcasses were sampled from a large FSIS-inspected processing plant. Chicken carcasses were sampled before (pre) and after (post) the chlorinated chiller baths. Carcasses were collected and individually rinsed in 300mL of Butterfield's phosphate diluent using an automated carcass shaker. The rinsate was transferred to a filtered stomacher bag to remove large particles of fat, skin, and tissue pieces. Thirty (30) ml of each filtrate was stored in sterile conical tubes and held on ice during the culturing and DNA extraction steps.

Selective Enumeration. Individual carcass samples were direct plated on Campy-CEFEX agar (250 ?l per plate x 4 plates and an additional 100 ?l per plate on two plates for a total plated volume of 1.2 ml). Plates were placed in zip lock plastic bags and gassed (3x flush) with *Campylobacter* gas-mixture (85% N_2, 10% CO_2, and 5% O_2) and incubated 72 hours at 42°C under microaerobic growth conditions (85% N_2, 10% CO_2, and 5% O_2). Presumptive *Campylobacter* spp. colonies were counted and selected isolates picked for further confirmation as *Campylobacter* based on cellular morphology and serology.

Presence/ Absence Testing, Enrichment Culture. 10ml carcass rinse aliquots were inoculated into 10mL of Bolton's non-selective *Campylobacter* enrichment broth in a T-25 tissue culture flask and incubated 18 hours at 42°C under microaerobic conditions. Following incubation, enrichment aliquots were plated on Campy-CEFEX and presumptive colonies picked and confirmed.

Results

BACTERIA DETECTED (n=55)

Campylobacter jejuni (ATCC 29428)	*Campylobacter spp. field isolates C1-C50*
Campylobacter jejuni (Chicken source, n=24)	*Campylobacter spp. isolated from poultry crate*
Campylobacter jejuni (Environmental, n=5)	*Campylobacter spp. isolated from turkey*
Campylobacter jejuni (Other sources, n=17)	*Campylobacter spp. isolated from insect*
Campylobacter jejuni (ATCC 33250)	*Campylobacter coli isolated from porcine*
Campylobacter coli (ATCC 49941)	*Arcobacter butzleri ATCC 49616**
Campylobacter lari (ATCC 43675)	* Genbank match for *C. jejuni*, mismatch for *A. butzleri*
Campylobacter upsaliensis (ATCC 43954)	

BACTERIA NOT DETECTED (n=67)

Aeromonas hydrophila	*Helicobacter species from bird (ATCC 51800)*
Acinetobacter baumannii	*Klebsiella pneumonia*
Arcanobacterium pyogenes	*Lactobacillus acidophilus*
Arcobacter butzleri (USDA field isolate 5365)	*Lactobacillus casei*
Arcobacter butzleri (USDA field isolate 5511)	*Listeria grayi (ATCC 700545)*
Arcobacter butzleri (USDA field isolate 4097)	*Listeria innocua (ATCC 51742)*
Arcobacter cryaerophilus (ATCC 43158)	*Listeria ivanovii (ATCC 49954)*
Bacillus anthracis, sterne	*Listeria monocytogenes (isolate J1-225)*
Bacillus cereus	*Listeria monocytogenes (isolate C1-109)*
Bacillus globigii	*Listeria seeligeri (ATCC 51334)*
Bifidobacteria longum	*Listeria welshimeri (ATCC 43551)*
Brevundimonas diminuta	*Micrococcus luteus*
Carnobacterium piscicola	*Morganella morganii*
Citrobacter freundii	*Ochrobactrum species*
Clostridium perfringens	*Pantoea agglomerans*
Enterobacter cloacae	*Pseudomonas fluorescens*
Enterococcus faecium	*Rhodococcus equi*
Escherichia coli Reference Collection 1	*Salmonella Reference Collection, SarC 1*
Escherichia coli Reference Collection 27	*Salmonella Reference Collection, SarC 2*
Escherichia coli Reference Collection 50	*Serratia liquefaciens*
Escherichia coli Reference Collection 51	*Serratia marcescens*
Escherichia coli Reference Collection 60	*Shewanella putrefaciens*
Flavobacterium odoratum	*Shigella flexneri*
Flexispira rappini (ATCC 43968)	*Shigella sonnei*
Hafnia alvei	*Sphingomonas paucimobilis*
Helicobacter acinonychis (ATCC 51101)	*Staphylococcus aureus*
Helicobacter bilis (ATCC 51632)	*Vibrio cholerae*
Helicobacter pametensis (ATCC 51478)	*Vibrio parahaemolyticus*
Helicobacter pylori (ATCC 43504)	*Yersinia entercolitica*
Helicobacter pylori (CDC isolate 5/001)	*Helicobacter pylori (CDC isolate 5/005)*
Helicobacter pylori (CDC isolate 5/002)	*Helicobacter pylori (CDC isolate 5/006)*
Helicobacter pylori (CDC isolate 5/003)	*Helicobacter pylori (CDC isolate 5/007*

Table 1. **Inclusivity and exclusivity data for the R.A.P.I.D. real-time PCR *Campylobacter* assay.**

				R.A.P.I.D. *Campylobacter* assay Ct result	
Sample code	Presump Campy Count cfu/ml	Presump Campy Count log10 cfu/ml	Cultural Enrichment	No Enrichment	24 h Enrichment
1 pre chiller	310	2.49	Pos	36.50	15.5
2 pre chiller	385	2.59	Pos	35.68	16.93
3 pre chiller	185	2.27	Pos	35.71	17.66
4 pre chiller	110	2.04	Pos	36.57	18.8
5 pre chiller	585	2.77	Pos	35.55	16.84
1 post chiller	15	1.18	Pos	36.56	24.97
2 post chiller	28	1.45	Pos	36.83	24.65
3 post chiller	14	1.15	Pos	36.95	22.59
4 post chiller	1	0.00	Pos	37.11	23.33
5 post chiler	13	1.11	Pos	37.28	29.13

Table 2. **Cultural and R.A.P.I.D. real-time PCR *Campylobacter* assay analysis of non-enriched and enriched poultry carcass rinse samples obtained from pre and post chiller stage of processing.**

Materials and Methods

DNA Extraction. Genomic DNA was extracted from test samples for real-time PCR analysis. Volumes of 200 ?L of direct whole carcass rinse sample (filtered in a filtered stomacher bag) or enrichment were extracted using the IT-1-2-3 DNA Purification Kit? (Idaho Technology, Inc., Salt lake City, UT). DNA was eluted with 100 ?L of elution buffer.

Real-time PCR. Real-time PCR analysis was performed using Idaho Technology's Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.?) System . Briefly, 40 ?l of each extracted DNA sample was added to one vial of freeze-dried IT BioReagent? designed specifically for amplification of sequences within the 16S rRNA gene of the *Campylobacter* spp. Each vial of IT BioReagent is sufficient for two 20?l PCR reactions complete with specific primers, hybridization probes, stabilization buffer, $MgCl_2$ buffer, dNTPs, KlenTaq1? DNA polymerase, and TaqStart? antibody. The freeze-dried reagent was completely rehydrated with the sample then transferred to two Roche LightCycler? capillary tubes and pulse-centrifuged. The filled capillary tubes were loaded into the R.A.P.I.D. instrument for amplification and detection. Details of the R.A.P.I.D. System are available on the Idaho Technology website at http://www.idahotech.com/ (19).

Establishing the Limits-of-Detection. Pre- and post-chiller carcass rinses with low presumptive *Campylobacter* counts were selected as background media and spiked with known quantities of *Campylobacter jejuni* in order to determine the limits-of-detection of the real-time PCR assay. To control for background *Campylobacter* contribution from the rinses, BPD was also used as a background medium. This experiment was repeated three times, each time using different low-count carcass rinses as the background media. Carcass rinses were spiked with 100-fold dilutions of *C. jejuni* culture followed by direct plating to Campy-CEFEX and simultaneous analysis by real-time PCR.

Results

				R.A.P.I.D. *Campylobacter* assay Ct result		
Sample code	Presump Campy Count cfu/ml	Presump Campy Count log10 cfu/ml	Cultural Enrichment	No Concentratoin	15x Concentrtation	100 x Concentration
Pre-Chiller	347	2.54	Pos	36.75	33.09	--
Post-Chiller	75	1.88	Pos	36.62	34.74	--
Pre-Chiller	7329	3.87	Pos	33.40	--	30.348
Post-Chiller	34	1.53	Pos	33.24	--	32.896

Table 3. **R.A.P.I.D. real-time PCR *Campylobacter* assay results of centrifugal concentration of non-enriched poultry carcass rinse samples.**

	Presump. Campylobacter Count log_{10} cfu/ml			
	Sample			
Hour	pre 11	pre 12	post 11	post12
0	2.27	2.89	1.78	1.18
1	2.51	2.87	2.11	0.70
2	2.65	3.19	2.18	*nd*
3	*nd*	*nd*	*nd*	*nd*
4	3.05	3.54	2.18	1.70
5	*nd*	*nd*	*nd*	*nd*
7	*nd*	*nd*	*nd*	*nd*
8	*nd*	*nd*	*nd*	*nd*
20	9.32	10.16	9.38	8.94

Table 4. **Presumptive *Campylobacter* plate counts to accompany real-time PCR data from Figure 3.**

Results

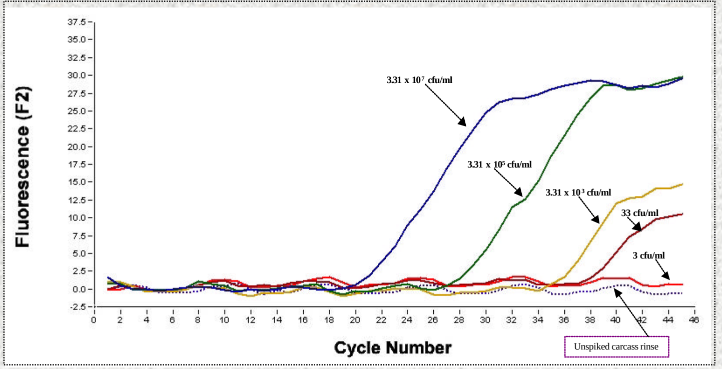

Figure 1. **Typical assay for *Campylobacter* inoculated into a pre-chiller carcass rinsate with serial dilutions of a pure culture of *C. jejuni* determined with the R.A.P.I.D. real-time PCR *Campylobacter* assay.**

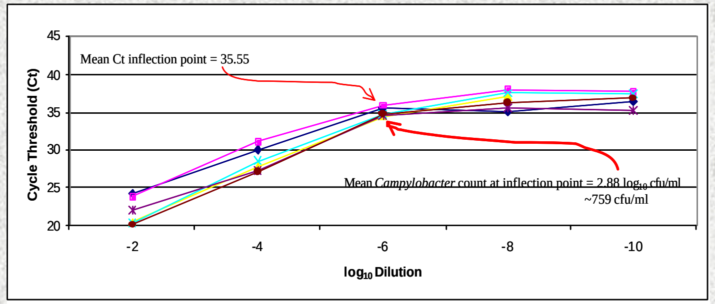

Figure 2. **Dilution series plots of *Campylobacter jejuni* inoculated into six different carcass rinsates (pre and post chiller) and detected using R.A.P.I.D. real-time PCR for detection.**

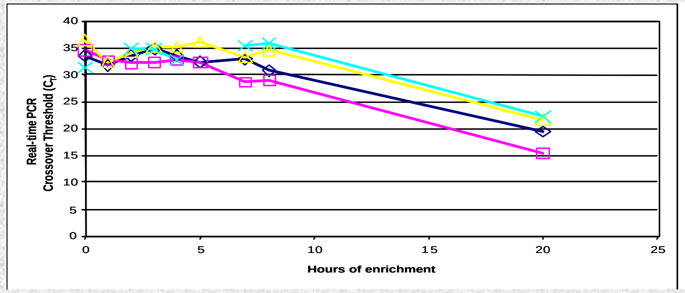

Figure 3. **Time course analysis of poultry carcass rinse enrichment cultures for *Campylobacter* in pre and post chiller carcass rinses using the R.A.P.I.D. real-time PCR *Campylobacter* assay.**

Results and Discussion

The primers and hybridization probes used in this assay were specific for the genus *Campylobacter* (*Table 1*) and able to detect as few as 33 cfu/ml of spiked rinsate (*Figure 1*). C_T's greater than 35 were observed for all carcass rinses prior to enrichment regardless of the *Campylobacter* viable count (*Table 2*). The background, detected as late-amplifying real-time PCR products, was also evident in a spiking experiment (*Figure 2*) with six rinses (three each of pre and post chiller) and likely due to low levels of non-viable *Campylobacter* in the rinsate. In this study, all rinses were positive for *Campylobacter* as determined by enrichment and cultural confirmation. A 16-24 hour enrichment resulted in C_T's less than 30 and within a more reliable real-time PCR reactivity range (*Table 2*). The real-time PCR C_T's after enrichment (*Table 2*) were consistent with a higher level of *Campylobacter* in pre-chiller samples (C_T mean = 17.1) and a lower level in post-chiller (C_T mean = 24.9). Time course experiments with samples taken during the enrichment period (*Figure 3*) indicated that while C_T's numerically decreased and viable *Campylobacter* counts increased (*Table 4*), the 16-24 hour incubation was still required for reliable determinations.

Concentrating the samples prior to DNA extraction (*Table 3*) by centrifugation in microfuge tubes did result in lower C_T's congruent with the presumptive *Campylobacter* counts, however, these experiments were preliminary and inconsistent. It is likely that the efficiency of the DNA extraction procedure is inconsistently affected by varying levels of extraneous material such as fat, tissue, protein, chlorine etc.

An advantage of this and other PCR-based food pathogen detection assays is the allowance of non-selective enrichments prior to analysis (3, 15) . For samples that are a low target density or sub-lethally injured, the non-selective growth period offers optimal conditions which will likely result in more reliable presence/absence testing. A non-cultural microbial enrichment of food samples could lead to detection within a single work day. Important to the control of *Campylobacter* would be a means to monitor actual levels on carcass rinses rapidly to assess incoming loads as well as processing efficiency. Combining real time PCR, with a quantitatively reliable concentration and extraction format would greatly advance the solution to this analytical challenge.

Conclusions

- **Real-time PCR detection of *Campylobacter* using freeze-dried I.T. BioReagents is an effective tool to verify the presence of *Campylobacter* in enriched poultry carcass rinses.**
- **This method is rapid. Following an overnight non-selective enrichment, it is possible to test ten samples within one hour.**
- **Combining non-selective enrichment with a real-time PCR assay offers is a means to determine the presence of *Campylobacter* in samples that might otherwise require significantly greater grow out times under selective culture.**

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