

Real-Time PCR Detection of *Campylobacter* spp. from Chicken Carcasses Using the R.A.P.I.D.[®] System



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Summary

This study describes a rapid method for detecting *Campylobacter* spp. present on chicken carcasses. This method is a combination of an overnight non-selective enrichment of carcass rinsate and real-time PCR using the R.A.P.I.D. System. The total time to result after the 18-hour enrichment is 1 hour for 10 samples.

Abstract

R.A.P.I.D. real-time PCR analysis for *Campylobacter* spp. in enriched chicken carcass samples was congruent with the 72-hour presence/absence cultural method for all samples (n = 30). Whole carcass rinses were obtained from a commercial broiler processing plant using the F.S.I.S. method of shaking in 300 mL of Butterfield's phosphate diluent in an automated carcass shaker. Rinsate was pre-filtered through a filtered stomacher bag then analyzed by direct plating on selective agar and by non-selective enrichment. Real-time PCR amplification, detection, and analysis were done using the R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) System. DNA extractions of both the rinsate and the 18 hour enriched culture were performed using the IT-1-2-3 R.A.P.I.D. DNA Purification Kit[™] and subjected to real-time PCR analysis using freeze-dried hybridization probe BioReagents[™] targeting the *Campylobacter* spp. 16S ribosomal RNA gene. The total time to result after the 18-hour enrichment was 1 hour for 10 samples. Direct plating of the carcass rinsate indicated that pre-chiller carcasses contain approximately ten-fold more viable *Campylobacter* than post-chiller carcasses. Non-enriched samples fell below the threshold of detection of the PCR assay. However, real-time PCR analysis of the carcass rinsate enrichments indicated that viable *Campylobacter* spp. were present in greater quantities in pre-chiller carcasses than post-chiller carcasses as indicated by the PCR cycle at which the target is detected in enriched samples (cycle 17 pre v. cycle 25 post). Based on direct plating quantitation of rinses spiked with known concentrations of *Campylobacter jejuni*, the limits-of-detection of this assay were approximately 600 cfu per mL of carcass rinse (corresponding to approximately 25 cfu of gene copy equivalent per PCR reaction). Direct plating quantification of pre- and post-chiller carcass rinsate ranged from 40 (post) to 350 (pre) cfu of *Campylobacter* spp. per mL. This underscores the need for sample enrichment prior to PCR analysis in order to exceed the threshold of detection of the real-time PCR assay. In summary, the R.A.P.I.D. System was an effective tool for accurately detecting the presence of *Campylobacter* spp. in overnight enrichment cultures of chicken carcass rinse samples.

Background

Detection of the presence of *Campylobacter* on chicken carcasses is typically done using standard culturing and characterization methods. Briefly, this entails rinsing the carcasses followed by enrichment, isolation and biochemical analysis, which can take up to 5 days (1,2). Perishable food items such as chicken carcasses require a more rapid method of monitoring for the presence of pathogens like *Campylobacter*. An alternative to culturing methods is real-time polymerase chain reaction (PCR).

In order to develop a specific and sensitive PCR assay to detect *Campylobacter* spp., a region of homology among all members of the *Campylobacter* genus must be evaluated. The 16S ribosomal RNA gene is a good candidate region because the genetic sequence tends to be conserved within a species (inclusivity), while having considerable variation between different bacterial species (exclusivity), thus contributing to the specificity of the reaction. A real-time PCR assay targeting the 16S rRNA gene was developed and optimized on the R.A.P.I.D. System. This system incorporates the use of fluorescently-labeled hybridization probes to detect amplification of a DNA target in real time (3) (Figure 1). This assay was validated against a DNA panel of *Campylobacter* spp. as well as closely related and unrelated species (Table 1). The validated assay components were then freeze-dried into a single reaction vessel.

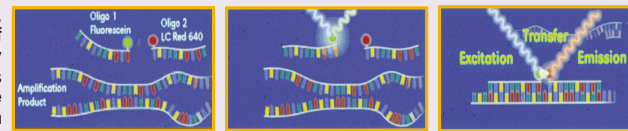


Figure 1. Detection of PCR amplification using hybridization probes.

Materials and Methods

Obtaining the Carcass Rinses. A total of thirty whole chicken carcasses were sampled before (pre) and after (post) the chlorinated chiller baths in the carcass preparation process. Each carcass was rinsed in 300 mL of Butterfield's phosphate diluent (BPD) in an automated carcass shaker, as per F.S.I.S. guidelines. The rinsate was pre-filtered through a Miracloth filtered stomacher bag in order to eliminate large organic matter that may interfere with the culturing and DNA extraction protocols. 30mL of each filtrate was kept in conical tubes on ice during the subsequent culturing and DNA extraction steps.

Microbial Analysis. Filtered carcass rinses were analyzed by direct plating on selective agar and by non-selective enrichment. For each carcass rinse, 250µL of rinsate x 4 and 100µL of rinsate x 2 were directly plated to Cefex media and incubated 72 hours at 42°C under microaerobic growth conditions (85% N₂, 10% CO₂, and 5% O₂). *Campylobacter* positive colonies were visually identified and enumerated.

In addition, 10mL of rinsate was added to 10mL of non-selective *Campylobacter* enrichment broth in a vented T-25 tissue culture flask and incubated 18 hours at 42°C under microaerobic conditions. Enrichments were analyzed by real-time PCR (see below).

DNA Extraction. Prior to PCR analysis, the bacteria present in the carcass rinse filtrate were extracted for their genomic DNA. 200µL of either direct filtrate or enrichment was extracted using the IT-1-2-3 R.A.P.I.D. DNA Purification Kit (Figure 2). The liquid sample was mechanically lysed with silica beads for five minutes using a Vortex Genie[®] 2 with Turbomix[™] adaptor. Binding buffer was added to the lysed sample and the entire volume was transferred to a spin filter and centrifuged until all of the liquid passed through. Wash buffer was added to the spin filter and centrifuged through. A subsequent centrifugation of the spin filter eliminated any residual wash buffer. DNA was eluted from the spin filter with 100µL of elution buffer.

Polymerase Chain Reaction. Real-time PCR analysis was performed using the R.A.P.I.D. System (Figure 3). 40µL of each extracted DNA sample was added to one vial of freeze-dried BioReagent (Figure 4) specific for amplification and detection of the 16S ribosomal RNA gene of *Campylobacter* spp. Each vial of BioReagent was enough for two 20µL PCR reactions and contained specific primers and hybridization probes, stabilization buffer, MgCl₂ buffer, dNTPs, KlenTaq[™], TaqStart[™] antibody, and enzyme diluent. Reagent completely rehydrated with the sample was transferred to two LightCycler[®] capillary tubes and pulse-centrifuged. The capillary tubes were loaded into the R.A.P.I.D. instrument for amplification and detection.

Establishing the Limits-of-Detection. To determine the limits-of-detection by both culture and PCR, pre- and post-chiller rinses with low cfu/mL were selected as background media for spiking in quantitated dilutions of *Campylobacter jejuni*. 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 rinses as the background media. Rinses were spiked with 100-fold dilutions of *C. jejuni* culture and directly plated to Cefex media as well as 200µL extracted and analyzed by PCR.



Figure 3. R.A.P.I.D. System



Figure 4. Freeze-dried BioReagent

Results

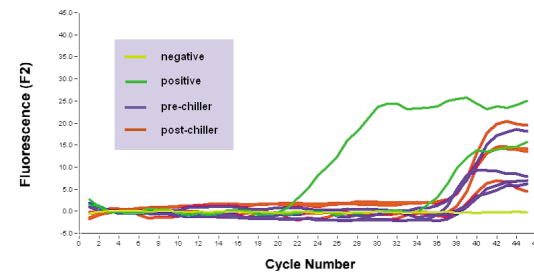


Figure 5. R.A.P.I.D. real-time PCR analysis of carcass rinses sampled pre- and post-chiller bath.

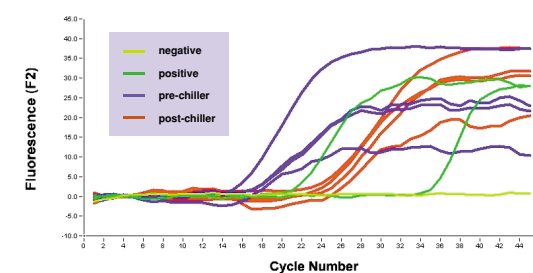


Figure 6. R.A.P.I.D. real-time PCR analysis of pre and post carcass rinse samples enriched for 18 hours.

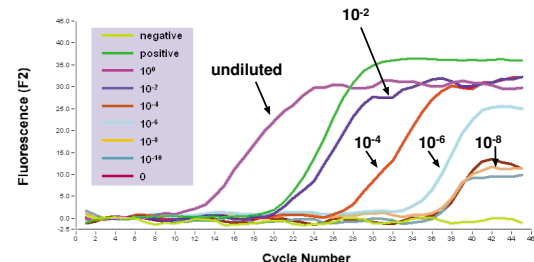


Figure 7. Detection of a serial dilution of *Campylobacter jejuni* into rinsate using R.A.P.I.D. real-time PCR.

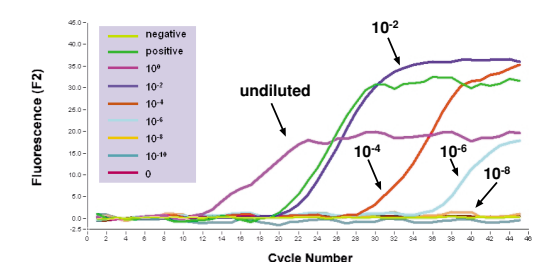


Figure 8. Detection of a serial dilution of *Campylobacter jejuni* into Butterfield's phosphate diluent using R.A.P.I.D. real-time PCR.

Discussion

These results suggest that real-time PCR using the R.A.P.I.D. System is an effective tool for assessing the presence of *Campylobacter* in overnight enrichment cultures of chicken carcass rinse samples. The limits-of-detection of this assay are 600 cfu/mL, which is equivalent to approximately 25 gene copies present in one PCR reaction (see Table 3). Lower concentrations of viable *Campylobacter* present in the rinse samples cannot be quantitatively determined because this is below the threshold of detection of the PCR assay. This is likely due in large part to the presence of low levels of non-viable *Campylobacter* in the rinsate, which contribute to a consistent background PCR signal with a cycle threshold of approximately 35 (Figure 5 and Table 2). Direct plating of carcass rinsate samples indicated that pre-chiller carcasses at 350 cfu/mL contain approximately ten-fold more viable *Campylobacter* than post-chiller carcasses at 40 cfu/mL (Table 2), both of which are well below the limits-of-detection for this assay. Although non-quantitative, an 18 hour enrichment of the rinsate in non-selective media followed by PCR analysis revealed that viable *Campylobacter* spp. were present in greater quantities in pre-chiller carcasses than post-chiller carcasses as indicated by the PCR cycle at which the target was detected in enriched samples (cycle 17 pre v. cycle 25 post) (compare Figures 5 and 6).

Strains Included (n=55)			
<i>Campylobacter jejuni</i> (ATCC 29428)	<i>Campylobacter</i> spp. field isolates C1-C50	Remaining isolates are <i>Campylobacter jejuni</i>	
<i>Campylobacter jejuni</i> (ATCC 33250)	C7 <i>Campylobacter</i> spp. isolated from poultry crate	24 isolated from chicken	
<i>Campylobacter coli</i> (ATCC 49941)	C14 <i>Campylobacter</i> spp. isolated from turkey	5 isolated from environmental	
<i>Campylobacter lari</i> (ATCC 43675)	C50 <i>Campylobacter</i> spp. isolated from insect	17 isolated from other	
<i>Campylobacter upsaliensis</i> (ATCC 43954)	C33 <i>Campylobacter coli</i> isolated from porcine		
Strains Excluded (n=67)			
<i>Aeromonas hydrophila</i>	<i>Clostridium perfringens</i>	<i>Helicobacter pylori</i> (ATCC 43504)	<i>Listeria grayi</i> (ATCC 70045)
<i>Acinetobacter baumannii</i>	<i>Enterobacter cloacae</i>	<i>Helicobacter pylori</i> (CDC clinical isolate 5001)	<i>Listeria innocua</i> (ATCC 51742)
<i>Anaerobaculum pyroaerum</i>	<i>Enterococcus faecium</i>	<i>Helicobacter pylori</i> (CDC clinical isolate 5002)	<i>Listeria ivanovi</i> (ATCC 49624)
<i>Arctobacter butzleri</i> (USDA field isolate 5365)	<i>Escherichia coli</i> Reference Collection 1	<i>Helicobacter pylori</i> (CDC clinical isolate 5003)	<i>Listeria monocytogenes</i> (isolate J1-225)
<i>Arctobacter butzleri</i> (USDA field isolate 5511)	<i>Escherichia coli</i> Reference Collection 27	<i>Helicobacter pylori</i> (CDC clinical isolate 5004)	<i>Listeria monocytogenes</i> (isolate C1-109)
<i>Arctobacter butzleri</i> (USDA field isolate 4097)	<i>Escherichia coli</i> Reference Collection 50	<i>Helicobacter pylori</i> (CDC clinical isolate 5005)	<i>Listeria seeligeri</i> (ATCC 51334)
<i>Arctobacter cryaerophilus</i> (ATCC 43158)	<i>Escherichia coli</i> Reference Collection 51	<i>Helicobacter pylori</i> (CDC clinical isolate 5006)	<i>Listeria wellfleetii</i> (ATCC 43551)
<i>Bacillus anthracis</i> , Sterne	<i>Escherichia coli</i> Reference Collection 60	<i>Helicobacter pylori</i> (CDC clinical isolate 5007)	<i>Micrococcus luteus</i>
<i>Bacillus cereus</i>	<i>Flavobacterium obovatum</i>	<i>Helicobacter pylori</i> (CDC clinical isolate 5008)	<i>Morganella morganii</i>
<i>Bacillus globigii</i>	<i>Flavobacterium johnsoniae</i> (ATCC 43968)	<i>Helicobacter pylori</i> (CDC clinical isolate 5009)	<i>Neisseria meningitidis</i>
<i>Bifidobacterium longum</i>	<i>Halobacter salinarum</i> (ATCC 51101)	<i>Helicobacter pylori</i> (CDC clinical isolate 5010)	<i>Neisseria meningitidis</i>
<i>Brevundimonas diminuta</i>	<i>Helicobacter acronychus</i> (ATCC 51101)	<i>Helicobacter pylori</i> (CDC clinical isolate 5011)	<i>Neisseria meningitidis</i>
<i>Campylobacter jejuni</i>	<i>Helicobacter bilis</i> (ATCC 51635)	<i>Helicobacter pylori</i> (CDC clinical isolate 5012)	<i>Neisseria meningitidis</i>
<i>Campylobacter jejuni</i>	<i>Helicobacter parvulus</i> (ATCC 51478)	<i>Helicobacter pylori</i> (CDC clinical isolate 5013)	<i>Neisseria meningitidis</i>
<i>Campylobacter coli</i>		<i>Helicobacter pylori</i> (CDC clinical isolate 5014)	<i>Neisseria meningitidis</i>
<i>Citrobacter freundii</i>		<i>Helicobacter pylori</i> (CDC clinical isolate 5015)	<i>Neisseria meningitidis</i>
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