Universidade Estadual de Londrina

JULIANE ALVES

OTIMIZAÇÃO DE PCR TRADICIONAL E EM TEMPO REAL PARA DETECÇÃO DE *Campylobacter* spp. E *Salmonella* spp. EM ALIMENTOS

Londrina
2014

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Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, nível Doutorado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Ciência de Alimentos.

Orientadora: Profª Drª Tereza Cristina Rocha Moreira de Oliveira

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2014

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BANCA EXAMINADORA

____

Orientadora: Profa. Dra. Tereza Cristina Rocha Moreira de Oliveira
Universidade Estadual de Londrina - UEL

____

Profa. Dra. Jane Martha Graton Mitcha
Universidade Estadual de Maringá – UEM

____

Profa. Dra. Sueli Fumie Yamada Ogatta
Universidade Estadual de Londrina – UEL

____

Profa. Dra. Daniele Sartori
Universidade Estadual de Londrina – UEL

____

Profa. Dra. Elisa Yoko Hirooka
Universidade Estadual de Londrina – UEL

Londrina, 28 de agosto de 2014.

Dedico

Aos meus Pais, que com muito amor sempre me apoiaram e incentivaram em todos esses anos de estudos

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"***A mente que se abre a uma nova ideia nunca mais volta ao seu tamanho original***"

Albert Einstein

ALVES, Juliane. **Otimização de PCR tradicional e em tempo real para detecção de *Campylobacter* spp. e *Salmonella* spp. em alimentos.** 2014. 109f. Tese (Doutorado em Ciência de Alimentos) – Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

Salmonella spp. e *Campylobacter* spp. são as causas mais frequentes de doenças bacterianas transmitidas por alimentos no mundo. O monitoramento dessas bactérias em alimentos é essencial para o controle da doença humana. Os métodos convencionais de isolamento de *Campylobacter* e *Salmonella* em alimentos podem requerer de quatro a dez dias para a conclusão dos resultados. A reação em cadeia da polimerase (PCR) é empregada na área de alimentos devido a sua rapidez e especificidade na detecção de patógenos. Embora vários *kits* comerciais estejam disponíveis, o seu alto custo dificulta o uso nos laboratórios de diagnóstico e controle de qualidade, principalmente em países em desenvolvimento como o Brasil. A otimização de ensaios moleculares específicos e, principalmente, a detecção simultânea dessas duas bactérias patogênicas vem de encontro à necessidade desses laboratórios. O objetivo deste trabalho foi otimizar ensaios de PCR e extração de DNA para a detecção de *Salmonella* spp. e *Campylobacter* spp. em diferentes matrizes alimentares. Nas reações otimizadas utilizou-se o par de iniciadores Styinva-JHO, específico para *Salmonella* spp., que gerou um amplicom de 119 pares de bases. O par de iniciadores específico para *Campylobacter* spp. foi OT1559 e 18-1 gerando um amplicom de 287 pares de bases. A especificidade dos ensaios PCR otimizados foi de 100% e a sensibilidade foi de 1 UFC de *Campylobacter* ou *Salmonella* por mililitro da enxaguadura das diferentes amostras de alimentos testadas, após etapa de enriquecimento. A prevalência de *Campylobacter* spp., nas análises feitas em amostras de cortes de frango, foi de 60,9% (64/105). *C. jejuni* foi identificada em 28,1% (18/64) das amostras positivas para *Campylobacter* spp. e *C. coli* foi identificada na mesma proporção 28,1% (18/64). *C. jejuni* e *C. coli* foram identificadas simultaneamente em 12,5% (8/64) das amostras. Os ensaios PCR e as técnicas de extração de DNA otimizadas neste trabalho são uma alternativa rápida, barata e eficiente para detecção de *Samonella* spp. e *Campylobacter* spp. em alimentos, após o enriquecimento. A redução do tempo de análise e a eficiência na detecção destes patógenos são de grande importância para laboratórios de análises de alimentos na identificação do alimento contaminado e origem do surto.

Palavras-chave: Detecção de patógenos. Segurança de alimentos. Solução de lise TZ. Controle interno de amplificação. Método molecular.

ALVES, Juliane. **Development of traditional and real-time PCR assays for detection of *Campylobacter* spp. and *Salmonella* spp. in foods.** 2014. 109p. Thesis (Doctor Degree in Food Science) – Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Salmonella spp. and *Campylobacter s*pp. are the most frequent causes of food-borne diseases worldwide. The control of these bacteria in food is essential for the control of human disease. Conventional detection methods of *Campylobacter* and *Salmonella* in food demand four to ten days. Polymerase chain reaction (PCR) have been developed for detection, identification and quantification of those bacteria in foods due to their rapidity and specificity. Although there are several commercial kits available, their high cost makes routine use in quality control and diagnostic laboratories difficult, especially in developing countries such as Brazil. The development of a specific molecular assay and especially the simultaneous detection of both pathogenic bacteria would be essential for these laboratories. The aim of this study was to develop PCR assays and DNA extraction procedure for detection of *Salmonella* spp. and *Campylobacter spp.* in different food matrices. The PCR was developed using the primers Styinva-JHO specific for *Salmonella* spp. that amplified a 119 bp fragment. The primers specific for *Campylobacter* spp was OT1559 and 18-1 which amplified DNA fragments of 287 bp. The assay specificity was 100%. After the enrichment step, the PCR detected 1 CFU of *Campylobacter* spp. or *Salmonella* spp. per milliliter of rinse of samples from different foodstuffs. The prevalence of *Campylobacter* spp., in chicken cuts samples was 60.9% (64/105). *C. jejuni* was identified in 28.1% (18/64) of the positive samples for *Campylobacter* spp. and *C. coli* was identified in the same proportion 28.1% (18/64). *C. jejuni* and *C. coli* were identified simultaneously in 12.5% (8/64) of samples. The developed PCR assays and DNA extraction procedure are rapid, inexpensive and eficient alternative for *Samonella* spp. and *Campylobacter* spp. detection in foods after enrichment. The reduction in analysis time and the efficiency in detection of these pathogens have a great importance for food analysis laboratories identification of contaminated food and source of the outbreak.

Keywords: Pathogen detection. Food safety. TZ lysis solution. Internal amplification control. Molecular method.

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LISTA DE ABREVIATURAS E SIGLAS

%	Porcentagem
<	Menor
>	Maior
ºC	grau Celsius
ATCC	*American Type Culture Collection*
CDC	*Center for Disease Control and Prevention*
CDT	toxina distensora citoletal
cm	Centímetro
CO_2	dióxido de carbono
DMSO	Dimetilsulfóxido
DNA	ácido desoxirribonucleico
dNTPs	desoxonucleosídeos trifosfatados
EDTA	ácido etilenodiamino tetra-acético
FIOCRUZ	Fundação Oswaldo Cruz
g	Grama
g	força G (unidade de aceleração)
h	Horas
hab.	Habitantes
HCl	ácido clorídrico
IHF	fator de integração do hospedeiro
KCl	cloreto de potássio
kg	Quilogramas
LACEN	Laboratório Central do Estado
M	Molar
MAPA	Ministério da Agricultura Pecuária e Abastecimento
mg	Miligrama
$MgCl_2$	cloreto de magnésio
min	Minutos
mL	Mililitro
mM	Milimolar

mol	massa molar
mPCR	PCR multiplex
N_2	Nitrogênio
O_2	Oxigênio
pb	pares de base
PCR	*Polymerase Chain Reaction* (Reação em cadeia da polimerase)
pH	potencial hidrogeniônico
PR	Estado do Paraná
RJ	Estado do Rio de Janeiro
rRNA	ácido ribonucleico ribossomal
s	Segundos
SIF	Serviço de Inspeção Federal
SPI-1	Ilha de patogenicidade 1 de *Salmonella*
SPI-2	Ilha de patogenicidade 2 de *Salmonella*
Tris	Tris (hidroximetil)-aminometano
U	Unidade
UEL	Universidade Estadual de Londrina
UFC	unidades formadoras de colônias
UFC/g	unidades formadoras de colônias por grama
UFC/mL	unidades formadoras de colônias por mililitro
UV	Ultravioleta
V	Volt
WHO	*World Health Organization*
w/v	*Weight per volume*
XLD	Xilose Lisina Desoxicolato de Sódio
μg	micrograma
μL	Microlitro
μL/mL	microlitro por mililitro
μM	micromolar

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1 INTRODUÇÃO

Doenças de origem alimentar são um problema de saúde pública. *Salmonella* spp. e *Campylobacter* spp. são as causas mais frequentes de doença bacteriana de origem alimentar (WHO, 2009).

Os principais sintomas de salmonelose incluem náusea, vômito acompanhado de dor abdominal, diarreia e algumas vezes febre. Em 1 a 5% dos casos, a salmonelose pode causar doença sistêmica, a qual se não tratada pode ser fatal (MALORNY et al., 2009). Nos Estados Unidos, aproximadamente 42.000 casos de salmonelose são notificados a cada ano, com um número estimado de aproximadamente 400 óbitos (CDC, 2014b).

Campylobacter jejuni e *Campylobacter coli* são as espécies mais comuns que infectam humanos. Os sintomas mais comuns de campilobacteriose incluem náusea, cólicas abdominais e diarreia sanguinolenta. A maioria dos casos é branda e autolimitada, entretanto, complicações pós infecção podem ocorrer, tais como, artrite e síndromes de Reiter e Miller-Fisher. A Síndrome de Guillain-Barré, complicação mais frequente, é uma doença auto-imune que ataca o sistema nervoso periférico sendo esta considerada uma sequela da infecção causada especificamente por *C. jejuni* (LINTON et al., 1996; BLASER, 1997; YAN et al., 2005; SIMMONS et al., 2008). Segundo o CDC, a maioria dos casos de campilobacteriose ocorre esporadicamente e não como parte de surtos. Nos Estados Unidos, é estimado que ocorram aproximadamente 1,3 milhões de casos a cada ano, com uma média 76 óbitos (CDC, 2014a).

No Estado do Paraná, entre os anos de 1999 e 2008, 286 surtos de salmonelose foram notificados no Laboratório Central do Estado (LACEN), Curitiba, PR. Neste período 5.641 pessoas foram expostas a alimentos contaminados com *Salmonella* spp. sendo que 2.027 (35,9%) manifestaram os sintomas da doença e 881 (16,3%) foram hospitalizadas. Um óbito foi registrado em abril de 2000 devido a ingestão de bolo contaminado por *S.* Enteritidis (KOTTWITZ et al., 2010).

No Brasil, no período entre os anos 2000 a 2013 foram identificados três surtos por *Campylobacter* spp. e 1522 surtos por *Salmonella* spp. Os alimentos mistos foram apontados como a principal causa de ocorrência de doença transmitida

por alimentos. As residências, seguido por restaurantes e escolas foram os locais onde surtos ocorreram com maior frequência (BRASIL, 2013).

Os métodos convencionais de isolamento de *Campylobacter* spp. e *Salmonella* spp. em alimentos são demorados e podem requerer de quatro a dez dias para a conclusão dos resultados. Esses métodos utilizam meios de enriquecimento seletivo com posterior subcultivo em meios sólidificados seletivos. Após isolamento, a identificação fenotípica requer testes bioquímicos e sorológicos adicionais (CANDRIAN, 1995; OMICCIOLI et al., 2009).

Devido à rapidez e especificidade, métodos moleculares, como a PCR, têm sido otimizados para detecção, identificação e quantificação de *Campylobacter* spp. e *Salmonella* spp. em alimentos (OLIVEIRA et al., 2005; AMRI et al., 2007; DEBRETSION et al., 2007; KATZAV et al., 2008; O'REGAN et al., 2008; GÓMEZ-DUARTE et al., 2009; KANKI et al., 2009; MALORNY et al., 2009; OMICCIOLI et al., 2009; PEPE et al., 2009; CASARIL, 2010; FREITAS et al., 2010; PUI et al., 2010; ALVES et al., 2012; BARLETTA et al., 2013). É importante ressaltar que os *kits* comerciais disponíveis para detecção de *Campylobacter* spp. ou *Salmonella* spp. são importados e o custo elevado dificulta o uso nos laboratórios de diagnóstico e controle de qualidade. A otimização de um ensaio molecular específico e eficiente vem de encontro à necessidade desses laboratórios.

Dado o exposto, o objetivo deste trabalho foi otimizar ensaios de PCR, tradicional e em tempo real, para a detecção de *Salmonella* spp. e *Campylobacter* spp. em diferentes matrizes alimentares.

2 OBJETIVOS

Objetivo geral

Otimizar ensaios de PCR, tradicional e em tempo real, para a detecção de *Salmonella* spp. e *Campylobacter* spp. em diferentes matrizes alimentares.

Objetivos específicos

Otimizar ensaio PCR e extração de DNA para detecção de *Salmonella* spp. em oito matrizes alimentares complexas (gema de ovo, pizza, carne bovina moída, carne suína, linguiça suína, coxinha de frango, maionese e queijo).

Determinar a prevalência de *Campylobacter* spp., *C. jejuni* e *C. coli* isolados de cortes de frango refrigerados adquiridos em supermercados de Londrina, Paraná utilizando PCR.

Otimizar PCR uniplex e multiplex para detecção individual e simultânea de *Campylobacter* spp. e *Salmonella* spp. em leite.

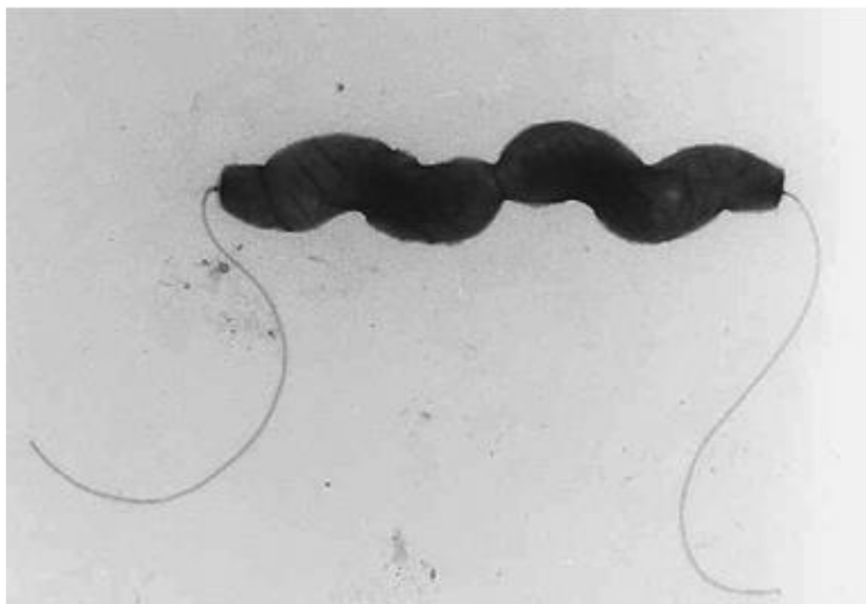
Otimizar PCR multiplex em tempo real, incluindo o controle interno de amplificação, para detecção simultânea de *Campylobacter* spp. e *Salmonella* spp. em carne de frango utilizando sondas de hidrólise.

3 REVISÃO BIBLIOGRÁFICA

3.1 Epidemiologia da campilobacteriose

O gênero *Campylobacter* é constituído por 24 espécies e oito subespécies (ON, 2013) de bacilos Gram-negativos, curvos ou espiralados podendo adquirir morfologia cocóide, correspondente às formas não cultiváveis. Apresentam um único flagelo polar, responsável pelo movimento em saca-rolha (figura 1) (FRANCO e LANDGRAF, 2002).

Figura 1. *Campylobacter jejuni* em processo de divisão



Fonte: Humphrey et al., (2007).

As espécies de *Campylobacter* são microaerófilas, multiplicam-se em atmosfera contendo aproximadamente 10% CO_2 e 5% O_2. As espécies patogênicas ao homem multiplicam-se em uma faixa de temperatura máxima de 46°C e mínima de 30°C, sendo classificadas como termofílicas (HUMPHREY et al., 2007).

C. jejuni e *C. coli* são os principais agentes etiológicos dos casos de campilobacteriose em humanos (WAGENAAR et al., 2013). Essas bactérias são

comensais do trato gastrointestinal de uma série de animais domésticos e silvestres (bois, porcos, gatos, cães, roedores e aves). As aves, especialmente o frango, são consideradas reservatórios primários de *C. jejuni* (FORSYTHE, 2002; PARK, 2002). Na maioria das vezes, o frango infectado por *Campylobacter* spp. não apresenta sintomas clínicos de doença, e isto representa um problema higiênico-sanitário importante na linha de produção, podendo ser encontrado até 10^9 UFC de *Campylobacter* por grama de fezes no trato intestinal destas aves (PARK, 2002).

A contaminação ambiental é apontada como a rota mais comum de transmissão de *Campylobacter spp*. para as aves. (MEAD, 2004). A colonização das aves por esse patógeno pode ocorrer, principalmente, por transmissão horizontal, tais como, água, ração e cama contaminadas, animais domésticos e silvestres presentes na granja, trânsito de trabalhadores nos galpões, limpeza e desinfecção inadequada dos galpões e caixas de transporte de aves para o abate contaminadas (NEWELL e FEARNLEY, 2003). A possibilidade de transmissão vertical não pode ser excluída, embora seja controversa e alvo de discussões (COX et al., 2012). A contaminação da carne pode ocorrer durante o abate e a bactéria aderida à pele forma um biofilme, que dificulta a sua remoção (ALTER et al., 2005).

O leite cru também é um possível veículo de transmissão de *Campylobacter* para humanos (OLIVER et al., 2009). O gado abriga *Campylobacter* frequentemente em seu trato gastrointestinal como comensais. A presença desta bactéria no leite cru deriva da contaminação fecal durante o processo de ordenha (HEUVELINK et al., 2009; REVEZ et al., 2014).

Os sintomas mais comuns de campilobacteriose incluem náusea, cólicas abdominais e diarreia sanguinolenta. A maioria dos casos é branda e autolimitada. Complicações pós infecção podem ocorrer, tais como, artrite, síndrome de Reiter e Síndrome de Guillain-Barré, uma doença auto-imune que ataca o sistema nervoso periférico, sendo esta considerada uma sequela da infecção causada especificamente por *C. jejuni* (LINTON et al., 1996; BLASER, 1997; YAN et al., 2005; SIMMONS et al., 2008).

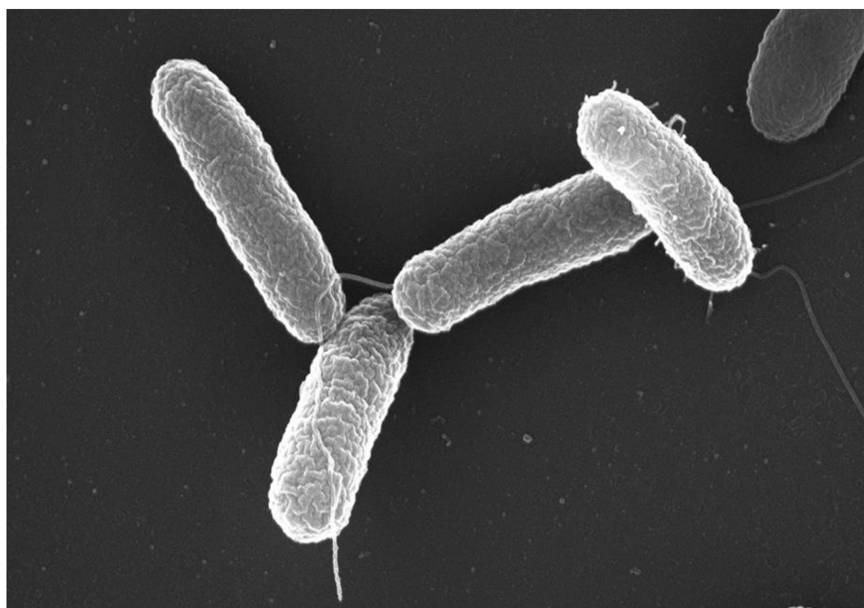
Nos Estados Unidos, é estimado que ocorram anualmente aproximadamente 1,3 milhões de casos de campilobacteriose. Apesar dessa infecção não ser uma causa comum de óbito, em média 76 pessoas morrem a cada ano (CDC, 2014a).

Segundo o CDC, (2014a), a maioria dos casos de campilobacteriose ocorre esporadicamente e não como parte de surtos. A estimativa é que *Campylobacter* seja responsável por 5% a 14% dos casos de diarreia em humanos no mundo, e que, um a cada 1000 casos evoluam para Síndrome de Guillain Barré (HUMPHREY et al., 2007; OLSEN et al., 2009a). No Brasil, os casos de campilobacteriose são subdiagnosticados e subnotificados e não há fácil acesso a dados epidemiológicos. No período entre os anos 2000 a 2013 foram identificados três surtos por *Campylobacter* spp. (BRASIL, 2013).

3.2 Epidemiologia da salmonelose

As bactérias do gênero *Salmonella,* família *Enterobacteriaceae,* compreende bacilos Gram-negativos (figura 2), anaeróbicos facultativos, redutores de nitrato a nitrito, fermentadores de glicose com ou sem produção de gás, oxidase negativa e geralmente móveis por flagelos peritríquios (exceto *S.* Gallinarrum e *S.* Pullorrum que são imóveis) (FRANCO e LANDGRAF, 2002).

Figura 2. *Salmonella* Typhimurium



Fonte: Volker Brinkmann, Max Planck Institute for Infection Biology, Berlin, Germany, DOI: 10.1371/journal.pbio.0030166.g001

Salmonella enterica é classificada, com base nos antígenos O (polissacarídeo de superfície) e H (flagelar), em mais de 2600 sorovares e aproximadamente 60% destes foram identificados a partir de isolados da subespécie *enterica* (I) (GRIMONT e WEILL, 2007; ARRACH et al., 2008; GUIBOURDENCHE et al., 2010). As formas antigênicas dos sorovares de *Salmonella* são definidas e mantidas pelo *World Health Organization* (WHO) *Collaborating Center for Reference and Research on Salmonella* no Instituto Pasteur, Paris, França, e novos sorovares são apresentados em atualizações do Esquema Kauffmann-White (BRENNER et al., 2000). O gênero *Salmonella* consiste em duas espécies, *S. bongori* e *S. enterica*, a qual é dividida em seis subespécies: *S. enterica* subesp. *enterica* (I), *S. enterica* subesp. *salamae* (II), *S. enterica* subesp. *arizonae* (IIIa), *S. enterica* subesp. *diarizonae* (IIIb), *S. enterica* subesp. *houtenae* (IV), e *S. enterica* subesp. *indica* (VI) (BRENNER et al., 2000; POPOFF et al., 2004; SU e CHIU, 2007; ARRACH et al., 2008) tabela 1. Uma terceira espécie, *S. subterranea,* foi identificada no ano de 2004, porém até o momento a sua inclusão como espécie de *Salmonela* não foi aceita por todos os órgãos internacionais (SHELOBOLINA et al., 2004; SU e CHIU, 2007).

Tabela 1 – Número de sorovares em cada espécie e subespécie de *Salmonella*

Espécies	Subespécies	Número de sorovares
S. enterica	*enterica* (I)	1547
	salamae (II)	513
	arizonae (IIIa)	100
	diarizonae (IIIb)	341
	houtenae (IV)	73
	indica (VI)	13
S. bongori	(V)	23
Total		**2610**

Fonte: Adaptado de Grimont; Weill (2007) e Guibourdenche et al., (2010).

A contaminação de alimentos por *Salmonella* continua sendo a maior causa de salmonelose e os sorovares Enteritidis e Typhimurium os

responsáveis pela maioria das infecções. Em vários países, os casos esporádicos e de surtos de salmonelose humana têm sido comumente associados a alimentos de origem animal, tais como, ovos, carne bovina, suína, de aves, e seus derivados e produtos de laticínios. Nos países em desenvolvimento, a contaminação fecal de água é a causa de infecção em humanos pelos sorovares Typhi e Paratyphi (ALCOCER et al., 2006; DUNKLEY et al., 2009; MALORNY et al., 2009).

As infecções por *Salmonella* em aves apresentam quadros clínicos graves, como a pulorose (*S.* Pullorum) e o tifo aviário (*S.* Gallinarum), que implicam na maioria das vezes na eliminação dos lotes infectados. O grande enigma desta zoonose reside nas formas clínicas inaparentes, denominadas na patologia aviária como infecções paratíficas. Essas infecções são responsáveis por uma queda da produtividade do plantel quando aparentes e propagam-se silenciosamente através da contaminação do ambiente (HOFER et al., 1997; CALIXTO et al., 2002).

Frangos podem ser reservatórios de *Salmonella* spp. e grande parte dos surtos em humanos têm sido associados a produtos de origem avícola (OLAH et al., 2005). Carne de frango e ovos são importantes veículos de transmissão de *Salmonella* spp., porém, um aumento na incidência de *Salmonella* spp. em frutas e vegetais frescos tem sido observado nos Estados Unidos. Esse aumento está relacionado a práticas agrícolas como irrigação com água contaminada e uso de adubo orgânico (ESPINOZA-MEDINA et al., 2006; LEE et al., 2006).

Apesar da carne de frango ser o principal veículo de transmissão o consumo de carne bovina e o leite cru contaminados, não podem ser excluídos como causa de salmonelose (NIELSEN et al., 2012).

Salmonelose em humanos pode ser caracterizada por gastroenterite autolimitada com sintomas brandos a moderados incluindo náusea, vômito, febre, dor abdominal e diarreia. Sintomas clínicos mais severos, podem ocorrer em casos de bacteremia ou febre entérica (tifoide), o qual é caracterizado por cefaléia severa, febre alta, porém com ausência de diarreia (LEADER et al., 2009; MALORNY et al., 2009).

Nos Estados Unidos, aproximadamente 42.000 casos de salmonelose são notificados a cada ano. É estimado que aproximadamente 400 pessoas morram a cada ano devido a salmonelose aguda (CDC, 2014b).

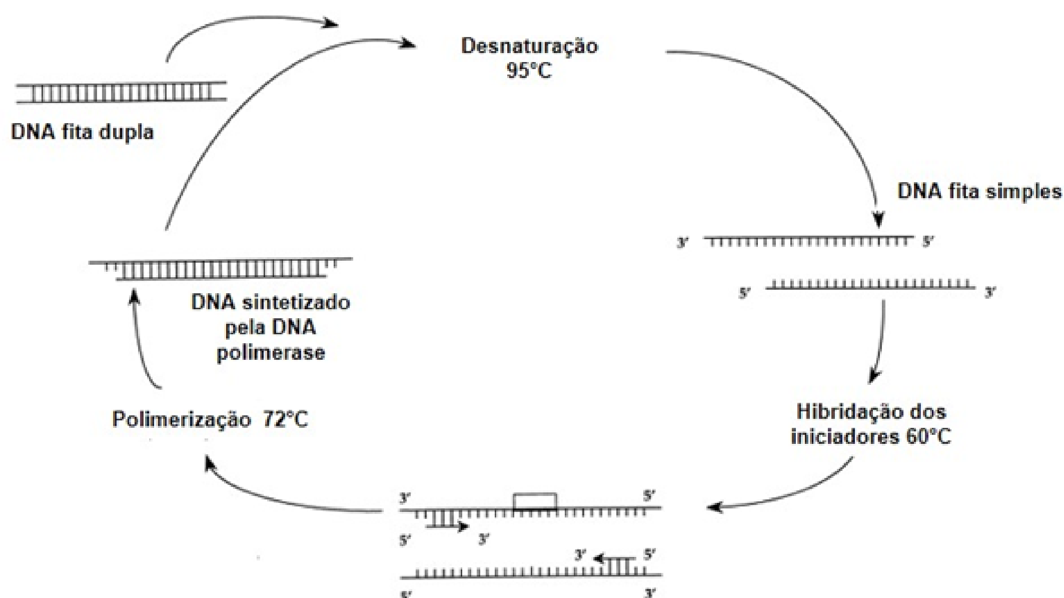
No Brasil, no período entre os anos 2000 a 2013 foram identificados 1522 surtos por *Salmonella* spp. Sendo este, apontado como a principal causa de doença transmitida por alimentos no país (BRASIL, 2013).

No Estado do Paraná, no período de janeiro de 1999 a dezembro de 2008, 52 municípios (13,0%) dos 399, notificaram surtos de salmonelose. Curitiba, município de maior população no Estado (1.797.408 habitantes), apresentou a maior incidência com 90 surtos (31,5%), seguida de Francisco Beltrão (72.409 hab.) com 17 surtos (6,0%) e Cascavel (285.784 hab.) com 15 (5,2%). Londrina notificou quatro surtos. Nesse período, 5.641 pessoas foram expostas a alimentos contaminados com *Salmonella* spp, 2.027 (35,9%) manifestaram os sintomas da doença e 881 (16,3%) foram hospitalizadas. Dos pacientes hospitalizados, um óbito foi registrado em abril de 2000, em Nova Cantu, provocado pela ingestão de bolo contaminado por *S.* Enteritidis. Dos alimentos associados aos surtos, 45,0% (84) eram à base de ovos, 34,8% carnes e derivados (65) e 20,2% classificados como alimentos variados, tais como queijos (1,0%), saladas (de tomate, repolho, couve, milho e ervilha) (4,8%), arroz cozido, extrato de tomate, fritas, mandioca, mousse, pudim, sorvetes, farofa, pavê e massas prontas (14,4%) (KOTTWITZ et al., 2010).

3.3 Técnicas de PCR e mPCR

A técnica de PCR (*Polymerase Chain Reaction*) foi criada em meados dos anos 1980 por Kary Mullis e constitui um procedimento cíclico *in vitro* com três passos: desnaturação do alvo, hibridação dos iniciadores e polimerização da sequência alvo. Esses passos se repetem de 30 a 40 vezes, gerando 2^n cópias da região de interesse do DNA, onde n é igual ao número de ciclos da reação (figura 3).

Figura 3 – Representação esquemática dos ciclos da PCR.



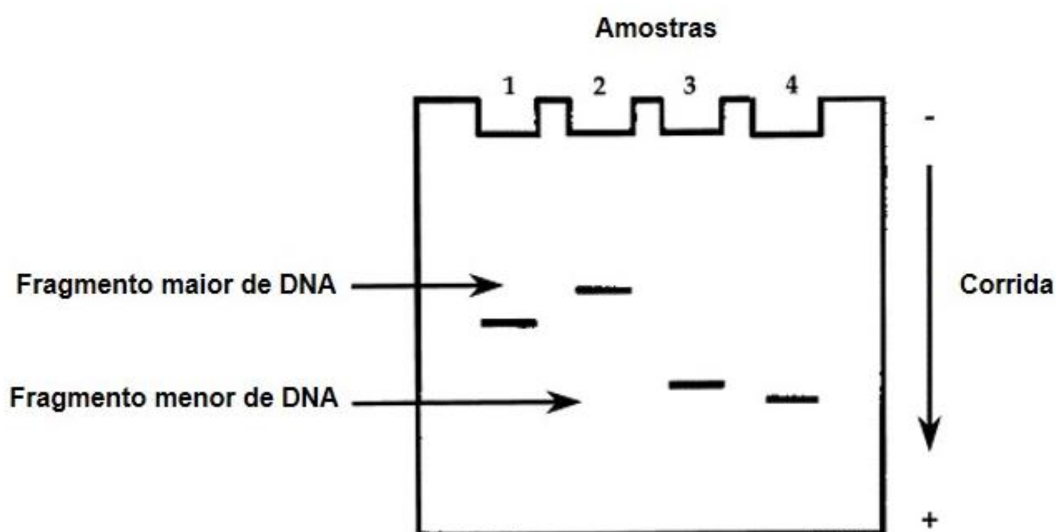
Fonte: Adaptado de Gachet (1998).

A PCR é baseada na habilidade da enzima DNA polimerase de replicar uma sequência alvo de DNA específica e na análise do produto de amplificação (MULLIS e FALOONA, 1987). Quando dois oligonucleotídeos iniciadores complementares em relação às extremidades da sequência que se deseja amplificar hibridam-se a uma das fitas do DNA, esta sequência é amplificada exponencialmente a cada ciclo. A escolha da sequência dos oligonucleotídeos iniciadores determina a especificidade e, consequentemente, o sucesso da PCR.

A mPCR foi descrita pela primeira vez em 1988 por Chamberlain *et al*. Essa técnica é uma variação da PCR e consiste na amplificação de várias sequências alvo, simultaneamente, devido ao uso de dois ou mais pares de oligonucleotídeos iniciadores em uma mesma reação. A mPCR reduz os gastos com reagentes, pois permite a detecção simultânea de diversos patógenos em uma única reação de amplificação (PERRY et al., 2007).

Os produtos da PCR (amplicons) são submetidos a eletroforese em gel de agarose (figura 4) para separação de acordo com sua massa molecular, os quais, são então visualizados sob luz UV, após coloração com moléculas intercalantes de DNA, tais como, brometo de etídio ou SYBR® safe (MALORNY et al., 2009).

Figura 4 – Representação esquemática dos amplicons após eletroforese em gel de agarose.



Fonte: Adaptado de Gachet (1998).

3.4 PCR em tempo real

A PCR convencional utiliza a eletroforese em gel de agarose para confirmar a presença do amplicon na reação, mas esta é laboriosa, demorada, carece de sensibilidade e especificidade, e sua interpretação é subjetiva (OLIVEIRA et al., 2005).

A segunda geração de tecnologia de PCR surgiu no início da década de 1990. O uso de moléculas fluorescentes intercalantes de DNA de fita dupla permitiu a realização do ensaio em uma única fase. A técnica de PCR em tempo real baseia-se na visualização do produto durante a amplificação da sequência alvo de ácidos nucleicos empregando um sistema computadorizado que detecta a emissão de fluorescência pelo fluoróforo. Logo, esta técnica não requer manipulação da amostra após amplificação, reduzindo o risco de resultados falso positivos devido a contaminação no laboratório (HEIN et al., 2006; OMICCIOLI et al., 2009). A eliminação da análise do produto de amplificação por eletroforese aumenta a confiabilidade e a reprodutibilidade do ensaio e diminui o tempo utilizado para realização da técnica (LUND et al., 2004). A visualização da amplificação em tempo

real, pode ser realizada pelo uso de vários métodos, sendo os dois citados a seguir os mais utilizados.

3.4.1 Métodos de monitoramento da amplificação em PCR em tempo real: sondas de hidrólise e corante intercalante de DNA de fita dupla.

O método de visualização de amplificação da PCR em tempo real baseado no emprego de sondas de hidrólise (figura 5) permite uma maior especificidade na detecção e identificação dos produtos de amplificação. As sondas são sequências de oligonucleótideos marcadas com dois fluoróforos (*reporter* e *quencher*) que hibridam com a sequência alvo. Os fluoróforos são moléculas com a capacidade de emissão de luz em um comprimento de onda específico A quantidade de fluorescência emitida é diretamente proporcional à quantidade de DNA alvo gerado durante a reação de amplificação. As sondas de hidrólise utilizam um oligonucleótido de 20-30 bases acoplado na extremidade 5' com um corante fluorescente ("*reporter*") e na extremidade 3' com o "*quencher*". O "*quencher*" absorve a fluorescência do "*reporter*", impedindo que haja emissão de fluorescência. Durante a amplificação, a atividade 5' nuclease da DNA polimerase hidrolisa a sonda ligada ao produto de amplificação do alvo. A florescência do "*reporter*" já não está neutralizada e, em seguida, poderá ser detectada. O emprego das sondas de hidrólise garante alta especificidade na detecção do alvo mas, com um maior valor agregado devido a utilização de uma sonda marcada com o fluoróforo, além dos oligonucleotídeos iniciadores habituais utilizados para PCR.

Um outro método utilizado consiste no uso de fluoróforo, com capacidade de se intercalar na cadeia dupla de DNA do produto amplificado (figura 5). O aumento da intensidade da fluorescência é proporcional ao aumento do produto amplificado. A especificidade dos produtos de amplificação é possível pela análise das curvas de temperatura de desnaturação (Tm). Cada produto de amplificação apresenta uma Tm específica, que será aquela na qual metade da fita dos produtos de amplificação está desnaturada e a outra metade se mantém em fita dupla. O uso de um corante intercalante de DNA, é mais barato porém apresenta a

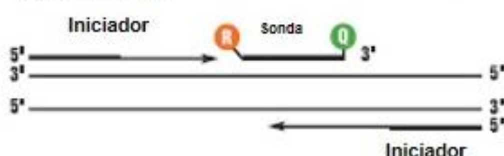
desvantagem de ser inespecífico e revelar qualquer dupla fita gerada na reação de amplificação, incluindo dímeros de iniciadores.

O primeiro método foi desenvolvido para detecção de sequências alvo múltiplas sendo possível o emprego de diferentes fluoróforos em única reação. O segundo geralmente é utilizado quando pretende-se detectar apenas uma sequência alvo (MALORNY et al., 2009; OMICCIOLI et al., 2009).

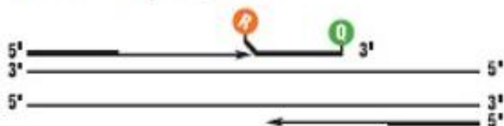
Figura 5 – Descrição dos mecanismos de emissão de fluorescência de sonda de hidrólise e corante intercalante de DNA.

Sondas de hidrólise

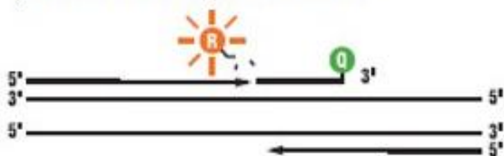
1- Polimerização: o corante fluorescente *reporter* (R) e o *quencher* (Q) estão, respectivamente, na porção 5' e 3' da sonda



2- Quando a sonda está intacta não há emissão de fluorescência pelo *reporter*.



3- Durante o ciclo de polimerização, a DNA polimerase cliva o *reporter* da sonda



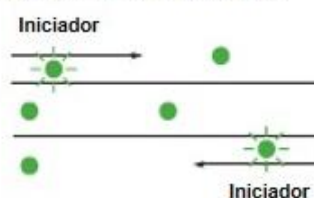
4- Ao final do ciclo de polimerização, uma vez separado do *quencher*, o corante *reporter* emite sua fluorescência característica.

Corante intercalante de DNA

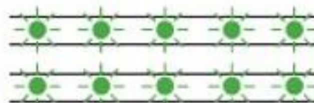
1- O corante emite fluorescência quando ligado ao DNA de fita dupla

2 – Desnaturação: Quando o DNA é desnaturado, o corante é liberado e a fluorescência é reduzida drasticamente.

3 – Polimerização: a fluorescência aumenta durante o pareamento dos iniciadores



4 – Final da Polimerização: o corante se liga ao DNA de fita dupla resultando no aumento significativo da fluorescência.



Fonte: Adaptado de http://www.lifetechnologies.com/br/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html

3.5 PCR para detecção de *Campylobacter* spp. e *Salmonella* spp.

Os métodos microbiológicos convencionais de detecção de *Salmonella* spp. e *Campylobacter* spp. em alimentos são laboriosos e requerem até sete dias para a conclusão dos resultados.

Apesar da disponibilidade de *kits* comerciais para detecção de patógenos em alimentos por técnica molecular, tais como, TaqMan® *Food Pathogen Detection Kits* (Applied Biosystems, Foster City, CA, USA), Roche *Diagnostics* (Mannheim, Germany), iQ-Check PCR *test kit* (Bio-Rad Laboratories, Hercules, CA, USA) e o sistema automatizado BAX® *system* Q7 (Du Pont Qualicon, Wilminggton, DE,USA), é desejável para os laboratórios de referência a otimização de ensaios não comerciais, os quais, possuem menor custo, além de informações sobre quais genes que estão sendo amplificados e as concentrações de reagentes utilizados (SCHODER et al., 2003).

O método de diagnóstico convencional de *Campylobacter* requer enriquecimento em caldo seletivo a 42°C sob condições de microaerofilia e posterior cultura em ágar seletivo sob as mesmas condições. Culturas com colônias morfologicamente características e oxidase positiva são consideradas *Campylobacter* spp. A identificação da espécie requer outros testes, incluindo crescimento em diferentes temperaturas, sensibilidade aos antibióticos cefalotina e ácido nalidíxico e testes bioquímicos. Esses métodos requerem quatro dias para a conclusão de um resultado negativo, e seis a sete dias para confirmar um resultado positivo (OLIVEIRA et al., 2005; AMRI et al., 2007).

Métodos de identificação fenotípica de *Campylobacter* spp. são limitados devido a existência de isolados bioquimicamente atípicos como também, pelo seu crescimento fastidioso e assacarolítico, o qual restringe o número de testes bioquímicos diferenciais. Além disso, os métodos convencionais de identificação apresentam interpretações subjetivas dos resultados (LINTON et al., 1996; LOGAN et al., 2001). Além de demorados e de alto custo, esses métodos são poucos sensíveis e não confiáveis para a detecção de pequenos números de células. Não detectam células viáveis e não cultiváveis e podem apresentar dificuldade na diferenciação das espécies devido a possibilidade de repressão, ou expressão

diferencial das características fenotípicas (JACKSON et al., 1996; WOLFFS et al., 2005; DEBRETSION et al., 2007).

As técnicas microbiológicas tradicionais para detecção de *Salmonella* spp. em alimentos levam até cinco dias para obtenção de um resultado positivo pois incluem: pré-enriquecimento em meio líquido não seletivo, enriquecimento em meio líquido seletivo, cultivo em ágar seletivo diferencial, identificação e confirmação por testes bioquímicos e sorológicos das colônias características. Além disso, a sorotipagem para determinação do sorovar é feita em laboratórios de referência. A sorotipagem é baseada na variabilidade antigênica do lipopolissacarídeo (antígeno O), das proteínas flagelares (antígeno H1 e H2) e do polissacarídeo capsular (antígeno Vi) quando presente (HEIN et al., 2006; MALORNY et al., 2009).

A detecção rápida de *Salmonella* spp. e *Campylobacter* spp. em alimentos, facilitaria a identificação das fontes de contaminação e auxiliaria a implementação efetiva de medidas de intervenção. A técnica de mPCR tem sido amplamente utilizada para detecção dessas bactérias em diferentes matrizes alimentares. PCR multiplex foi utilizada para detecção de *Salmonella* em amostras de solo e água (WAY et al., 1993), fezes (CHIU e OU, 1996; ALVAREZ et al., 2004), mexilhões (VANTARAKIS et al., 2000), ambiente de aviário e abatedouro (SOUMET et al., 1999; CORTEZ et al., 2006), pernil cozido (JOFRE´ et al., 2005), e carcaça e pele de frango (MAHON et al., 1994; FREITAS et al., 2010; SILVA et al., 2011). mPCR também foi otimizada para diferenciação de espécies de *Campylobacter* em etapas do abate de suínos (CLOAK e FRATAMICO, 2002), carne de frango (CASARIL, 2010; ALVES et al., 2012), como também, de amostras de fezes humanas e de frango (AMRI et al., 2007; SILVA et al., 2014).

Embora vários ensaios já tenham sido otimizados para detecção simultânea de *Salmonella* spp. e *Campylobacter* spp. (GILBERT et al., 2003; VOLLENHOFER-SCHRUMPF et al., 2005; GÓMEZ-DUARTE et al., 2009; PARK et al., 2011; WIEMER et al., 2011) apenas dois ensaios de PCR foram desenvolvidos para detecção exclusiva de *Campylobacter* e *Salmonella.* Alves et al., (2012) relataram a detecção simultânea de *Campylobacter* spp. e *Salmonella* spp. em água de enxaguadura de frango, utilizando mPCR. Wolffs et al. (2007) relataram a quantificação simultânea de *Campylobacter* spp. e *Salmonella* spp. em água de

enxaguadura de frango utilizando a técnica de flotação seguida por PCR multiplex em tempo real. Esse estudo, não incluiu um controle interno de amplificação. Além disso, a técnica de flotação utilizada não está disponível comercialmente para uso em laboratórios de análise de alimentos.

3.6 Gene *invA* e par de oligonucleotídeo iniciador específico para detecção de *Salmonella* spp.

A maioria dos ensaios de PCR tradicional e em tempo real usa parte do gene *invA* como sequência alvo (MALORNY et al., 2009). A sequência desse gene cromossomal é única para *Salmonella,* e está amplamente distribuída, possivelmente, entre todos os sorovares de *Salmonella* (OLAH et al., 2005) podendo ser utilizada como um marcador genético para o gênero.

Malorny et al. (2009) mostraram que um número pequeno de outros genes (tabela 2) foram utilizados para detecção de *Salmonella* spp., entre eles, a junção entre *sipB–sipC*, *sipC, spaO, fimC*, junção entre *invA–invE, himA e ttrA-ttrC.* Com exceção de *ttrA-ttrC, fimC e himA* os demais genes estão localizados na Ilha de patogenicidade 1 de *Salmonella* (SPI-1), que codifica o sistema de secreção tipo III, o qual exporta proteínas de membrana interna e externa em resposta a um contato bacteriano com células epiteliais (SALOMONSSON et al., 2005). Essa região tem um papel importante na patogenicidade de *Salmonella* e é altamente conservada nas espécies *S. enterica* e *S. bongori*. O locus *ttr*, o qual está localizado na SPI-2 também é altamente conservado nessas espécies de *Salmonella.* O locus *ttr* é necessário para respiração de *Salmonella* na presença de tetrationato. Esse locus consiste em cinco genes organizados como operon. Os genes *ttrA, ttrB* e *ttrC* codificam as proteínas estruturais da tetrationato redutase e os genes *ttrS* e *ttrR* codificam TtrS e TtrR, que correspondem ao sensor e os componentes da resposta regulatória de um sistema regulador de dois componentes, o qual é necessário para a transcrição do operon *ttrBCA* (HENSEL et al., 1999). O operon *fim* consiste em nove genes *fim* os quais codificam o pili tipo 1. O gene *himA* codifica a proteína IHF-alpha, capaz de se ligar ao DNA.

Tabela 2 - Genes alvos testados para detecção de *Salmonella* spp.

Gene	Características	Referência
invA	Localizados na Ilha de	(HOORFAR et al., 2000)
invA–invE	patogenicidade 1 de	(KUROWSKI et al., 2002)
sipB–sipC	*Salmonella* (SPI-1)	(ELLINGSON et al., 2004)
sipC	codificam o sistema de	(KUROWSKI et al., 2002)
spaO	secreção tipo III	(KUROWSKI et al., 2002)
fimC	Pili tipo 1	(KRASCSENICSOVÁ et al., 2008)
ttrA-ttrC	Localizados em SPI-2 codificam proteínas estruturais da tetrationato redutase	(MALORNY, PACCASSONI, et al., 2004)
himA	Proteína (IHF-alpha)	(CHEN et al., 2000)

Fonte: Adaptado de Malorny et al. (2009)

Uma característica comum da patogenicidade de todas as cepas de *Salmonella* é a sua habilidade de invasão de células do epitélio intestinal. O operon *inv* é composto por genes, os quais permitem que *Salmonella* spp. invada células epiteliais e está presente na maioria, se não em todos os sorovares de *Salmonella.* O gene *invA* é o primeiro gene do operon *inv* que é constituído de outros genes, *invB, C, D,* que codificam proteínas que participam do processo de invasão (GALÁN et al., 1992).

Rahn et al. (1992) publicaram um ensaio de PCR o qual utilizaram um par de oligonucleotideos iniciadores complementares ao gene *invA.* Nesse estudo, foram testadas 630 cepas de *Salmonella* e 142 cepas de outras bactérias. Apenas duas cepas de *S.* Senftenberg e duas cepas de *S.* Lichtfield não apresentaram produto de amplificação na reação (RAHN et al., 1992). A região do gene e os oligonucleotídeos iniciadores utilizados, apresentaram grande seletividade

em um estudo comparativo (MALORNY et al., 2003), e foram selecionados para participar de um estudo de validação internacional (MALORNY, COOK, et al., 2004).

Hoorfar et al. (2000) também utilizaram a sequência do gene *invA* para desenhar o par de oligonucleotídeos iniciadores Styinva-JHO-2-left e Styinva-JHO-2–right, os quais amplificam uma sequência de DNA de 119 pb. Nesse estudo, 110 cepas de *Salmonella* de diferentes sorovares foram avaliadas além de 120 cepas de outras bactérias. Todas as cepas de *Salmonella* foram detectadas pela PCR.

Outros dois estudos utilizaram o mesmo par de oligonucleotídeos iniciadores testados por Hoorfar et al. (2000). Rodríguez-Lázaro (2003) analisaram por PCR em tempo real 50 cepas de *Salmonella* spp. (incluindo sorovares de *S. enterica* e *S. bongori*) e 30 cepas de outras bactérias. Nesse estudo, a inclusividade (habilidade de detectar o alvo em uma grande gama de cepas) e exclusividade (interferência de um conjunto de cepas não alvo) foi de 100%. Nam et al.(2005) utilizaram esse mesmo par de oligonucleotídeos iniciadores para detecção de *Salmonella* spp. em amostras ambientais de uma fazenda produtora de leite. Um total de 124 cepas de *Salmonella* e 116 cepas de outras bactérias foram avaliadas. Todas as cepas de *Salmonella* testadas geraram produtos de amplificação específicos, e as cepas das outras bactérias não geraram produtos de amplificação.

3.7 Genes e par de oligonucleotídeo iniciador específico para detecção de *Campylobacter* spp.

Um número pequeno de genes (tabela 3) são utilizados para detecção de C*ampylobacter* spp., entre eles, o rRNA 23S e rRNA 16S. Os RNAs ribossomais (rRNA) são moléculas conservadas, distribuídas universalmente e apresentam sequências conservadas ao longo de distâncias filogenéticas. Assim, o grau de simililaridade entre sequências de rRNA de dois organismos indica uma relação evolucionária (GUTELL et al., 1994).

O gene *cdt* é um importante fator de virulência para *Campylobacter.* e codifica a CDT. Esta toxina afeta a camada de células epiteliais e causa a distensão progressiva e morte em várias linhagens celulares. A presença do gene

cdt tem sido sugerida nas espécies, *C. jejuni*, *C. coli* e *C. fetus, C. lari* e *C. upsaliensis*. Entretanto, a prevalência desse gene entre os isolados de *Campylobacter* de diferentes reservatórios e potenciais fontes de infecção não foi extensivamente investigada (WISESSOMBAT et al., 2009).

O gene de virulência *cadF* é conservado entre as espécies de *Campylobacter* de diversas origens. Este gene codifica uma proteína de membrana externa a qual promove a ligação da bactéria às células epiteliais do intestino (NAYAK et al., 2005; AMRI et al., 2007).

Tabela 3: Genes alvos testados para detecção de *Campylobacter* spp.

Gene	Produto	Espécies alvo	Referência
rRNA 23S	rRNA 23S	*C. jejuni, C. coli, C. lari*	(EYERS et al., 1993)
rRNA 16S	rRNA 16S	*C. jejuni, C. coli, C. lari*	(GIESENDORF et al., 1992; VANCAMP et al., 1993; LINTON et al., 1996; LÜBECK et al., 2003; LUND et al., 2004)
cdt	Toxina distensora citoletal (CDT)	*C. jejuni, C. coli, C. lari e C. upsaliensis*	(WISESSOMBAT et al., 2009)
cadF	Proteína de membrana externa	*Campylobacter* spp.	(NAYAK et al., 2005)

Fonte: o próprio autor

Lübeck et al. (2003) utilizaram diferentes combinações de variados pares de oligonucleotídeos iniciadores para desenvolver o melhor ensaio de PCR para detecção de *C. jejuni, C. coli* e *C. lari*. Nesse estudo foi mostrado que a

combinação mais seletiva de oligonucleotídeos iniciadores era a de OT1559 com 18-1, que amplificaram uma sequência de 287 pb do gene rRNA 16S. O oligonucleotídeo iniciador OT1559 foi utilizado primeiramente como sonda no sistema de amplificação de ácido nucleico NASBA (UYTTENDAELE et al., 1994) para identificação de *C. jejuni, C. coli* e *C. lari,* e 18-1 como oligonucleotídeo iniciador para detecção de *Campylobacter* enteropatogênica (VANCAMP et al., 1993). Em um total de 150 cepas foi mostrada uma inclusividade de 100% e exclusividade de 97%. A especificidade desse par de oligonucleotídeos também foi observada por Perelle et al. (2004).

Wolffs et al. (2005) utilizaram a técnica de flotação, a qual se baseia na centrifugação de soluções com diferentes densidades, seguida de PCR em tempo real para detecção de *Campylobacter* spp. em amostras de enxágue de frango sem enriquecimento empregando OT1559 e 18-1. A especificidade nesse caso não foi extensivamente testada pois os autores basearam-se na especificidade previamente validada por Lübeck et al. (2003). Outro trabalho usando a técnica de flotação foi publicado por Wolffs et al. (2007) para detecção simultânea de *Campylobacter* e *Salmonella* em água de enxágue de frangos utilizando-se PCR em tempo real. O mesmo par de oligonucleotídeos iniciadores foram utilizados. Apesar da especificidade já ter sido testada por Lübeck et al. (2003) a especificidade desse par de oligonucleotideos iniciadores foi confirmada por novo teste com 73 cepas as quais não apresentaram nenhuma detecção cruzada (WOLFFS et al., 2007).

Krause et al. (2006) validaram um ensaio PCR utilizando TaqMan® e o par de iniciadores OT1559 e 18-1 como ferramenta para produção certificada de frangos livres de *Campylobacter* spp. Olsen et al. (2009a) utilizaram o par de iniciadores OT1559 e 18-1 para detecção de *Campylobacter* spp. em amostras de ar durante o monitoramento contínuo da colonização de *Campylobacter* spp. em frangos.

3.8 Diferenciação das espécies *C. jejuni* e *C. coli*

Campylobacter spp. são bactérias assacarolíticas e fastidiosas. Estas características limitam os testes fenotípicos pelos quais os isolados podem ser

diferenciados (LINTON et al., 1996; LOGAN et al., 2001). A identificação das espécies é dificultada por variações na metodologia, interpretação subjetiva dos resultados dos testes bioquímicos e existência de isolados com características fenotípicas atípicas.

A diferenciação de *C. jejuni* e *C. coli* baseia-se na capacidade de *C. jejuni* hidrolisar hipurato mas cerca de 10% das cepas de *C. jejuni* são incapazes de realizar esta reação (BURNETT et al., 2002). Assim, isolados hipurato-negativos podem pertencer a outras espécies de *Campylobacter*, ou pode ser, de fato estirpes hipurato-negativas de *C. jejuni.*

Devido a essas limitações, laboratórios clínicos frequentemente reportam este enteropatógeno como *Campylobacter* spp. A capacidade de diferenciar *C. jejuni* e *C. coli* é importante pois tratamentos à base de eritromicina não são adequados se a enterite for causada por *C. coli*, que são mais resistentes a este antibiótico (GONZALEZ et al., 1997).

Neste sentido, os ensaios PCR de diferenciação são particularmente úteis devido as características já descutidas anteriormente.

Neste trabalho para diferenciação das espécies foram utilizados iniciadores para a amplificação do gene que codifica hippuricase (*Hip*) (LINTON et al., 1997), que está presente apenas nas cepas de *C. jejuni*. E iniciadores específicos para detecção de *C. coli,* complementares ao gene *ceuE* (GONZALEZ et al., 1997) o qual codifica uma lipoproteína de 34,5-36,2 kDa componente de um sistema de transporte do sideróforo enteroquelina.

4 REFERÊNCIAS

ALCOCER, I.; OLIVEIRA, K. M. P. D.; VIDOTTO, M. C.; OLIVEIRA, T. C. R. M. Discriminação de sorovares de *Salmonella* spp. isolados de carcaças de frango por REP e ERIC-PCR e fagotipagem do sorovar Enteriditis. **Ciência e Tecnologia de Alimentos,** v. 26, n. 2, p. 414-420, 2006.

ALTER, T.; GAULL, F.; FROEB, A.; FEHLHABER, K. Distribution of *Campylobacter jejuni* strains at different stages of a turkey slaughter line. **Food Microbiology,** v. 22, n. 4, p. 345-351, 8// 2005.

ALVAREZ, J.; SOTA, M.; VIVANCO, A. B. N.; PERALES, I.; CISTERNA, R. N.; REMENTERIA, A.; GARAIZAR, J. Development of a Multiplex PCR Technique for Detection and Epidemiological Typing of *Salmonella* in Human Clinical Samples. **Journal of Clinical Microbiology,** v. 42, n. 4, p. 1734-1738, 2004.

ALVES, J.; MARQUES, V. V.; PEREIRA, L. F. P.; HIROOKA, E. Y.; DE OLIVEIRA, T. C. R. M. Multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. **Journal of Food Safety,** v. 32, n. 3, p. 345-350, 2012.

AMRI, A. A.; SENOK, A. C.; ISMAEEL, A. Y.; AL-MAHMEED, A. E.; BOTTA, G. A. Multiplex PCR for direct identification of *Campylobacter* spp. in human and chicken stools. **Journal of Medical Microbiology,** v. 56, p. 1350-1355, 2007.

ARRACH, N.; PORWOLLIK, S.; CHENG, P.; CHO, A.; LONG, F.; CHOI, S.-H.; MCCLELLAND, M. Salmonella Serovar Identification Using PCR-Based Detection of Gene Presence and Absence. **Journal of Clinical Microbiology,** v. 46, n. 8, p. 2581-2589, 2008.

BARLETTA, F.; MERCADO, E. H.; LLUQUE, A.; RUIZ, J.; CLEARY, T. G.; OCHOA, T. J. Multiplex real-time PCR for detection of *Campylobacter, Salmonella,* and *Shigella*. **Journal of Clinical Microbiology,** v. 51, n. 9, p. 2822-9, Sep 2013.

BLASER, M. J. Epidemiologic and Clinical Features of *Campylobacter jejuni* Infections. **The Journal of Infectious Diseases,** v. 176, n. 2, p. S103-5, 1997.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Coordenação Geral de Doenças Transmissíveis. Vigilância Epidemiologica das Doenças Transmitidas por Alimentos. 2013. Disponível em: < http://www2.camara.leg.br/atividade-legislativa/comissoes/comissoes-permanentes/cmads/audiencias-publicas/audiencia-publica-2013/crueldade-a-que-os-animais-de-producao-sao-expostos-em-abatedouros-municipais/apresentacoes >. Acesso em: 20 julho 2014.

BRENNER, F. W.; VILLAR, R. G.; ANGULO, F. J.; TAUXE, R.; SWAMINATHAN, B. *Salmonella* Nomenclature. **Journal of Clinical Microbiology,** v. 38, n. 7, p. 2465-2467, 2000.

BURNETT, T. A.; HORNITZKY, M. A.; KUHNERT, P.; DJORDJEVIC, S. P. Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays. **FEMS Microbiology Letters,** v. 216, n. 2, p. 201-209, 2002.

CALIXTO, A. E. R.; SERAFINI, A. B.; KIPNIS, A.; ANDRÉ, M. C. D. P. B. Prevalencia de Salmonella e ocorrência de cepas resistentes a antimicrobianos, em insumos de rações para aves preoduzidos por um matadouro - frigorífico com fiscalização permenente, em Goiânia, GO. **Revista Higiene Alimentar,** v. 16, n. 101, p. 56-61, 2002.

CANDRIAN, U. Polimerase chain reaction in food microbiology **Journal of Microbiological Methods,** v. 23, p. 89-103, 1995.

CASARIL, K. B. P. B. **Padronização de PCR tradicional e em tempo real para detecção de *Campylobacter jejuni* e *Campylobacter coli* em alimentos**. 2010. 100 f. Tese Doutorado em Ciência de Alimentos, Universidade Estadual de Londrina, Londrina.

CDC. Centers for Disease Control and Prevention. *Campylobacter* General Information. 2014a. Disponível em: < http://www.cdc.gov/nczved/dfbmd/disease_listing/campylobacter_gi.html#2 >. Acesso em: 12 julho 2014.

______. *Salmonella*. 2014b. Disponível em: < http://www.cdc.gov/salmonella/general/ >. Acesso em: 20 jul 2014.

CHEN, W.; MARTINEZ, G.; MULCHANDANI, A. Molecular Beacons: A Real-Time Polymerase Chain Reaction Assay for Detecting *Salmonella*. **Analytical Biochemistry,** v. 280, n. 1, p. 166-172, 2000.

CHIU, C.-H.; OU, J. T. Rapid Identification of *Salmonella* Serovars in Feces by Specific Detection of Virulence Genes, *invA* and *spvC*, by an Enrichment Broth Culture-Multiplex PCR Combination Assay. **Journal of Clinical Microbiology,** v. 34, n. 10, p. 2619-2622, 1996.

CLOAK, O. M.; FRATAMICO, P. M. A Multiplex Polymerase Chain Reaction for the Differentiation of *Campylobacter jejuni* and *Campylobacter coli* from a Swine Processing Facility and characterization of Isolates by Pulsed-Field Gel Electrophoresis and Antibiotic Resistance Profiles. **Journal of Food Protection,** v. 65, n. 2, p. 266-273, 2002.

CORTEZ, A. L. L.; CARVALHO, A. C. F. B.; IKUNO, A. A.; BÜRGER, K. P.; VIDAL-MARTINS, A. M. C. Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex-PCR. **Research in Veterinary Science,** v. 81, p. 340-344, 2006.

COX, N. A.; RICHARDSON, L. J.; MAURER, J. J.; BERRANG, M. E.; FEDORKA-CRAY, P. J.; BUHR, R. J.; BYRD, J. A.; LEE, M. D.; HOFACRE, C. L.; O'KANE, P. M.; LAMMERDING, A. M.; CLARK, A. G.; THAYER, S. G.; DOYLE, M. P. Evidence

for Horizontal and Vertical Transmission in *Campylobacter* Passage from Hen to Her Progeny. **Journal of Food Protection,** v. 75, n. 10, p. 1896–1902, 2012.

DEBRETSION, A.; HABTEMARIAM, T.; WILSON, S.; NGANWA, D.; YEHUALAESHET, T. Real-time PCR assay for rapid detection and quantification of *Campylobacter jejuni* on chicken rinses from poultry processing plant. **Molecular and Cellular Probes,** v. 21, p. 177-181, 2007.

DUNKLEY, K. D.; CALLAWAY, T. R.; CHALOVA, V. I.; MCREYNOLDS, J. L.; HUMEB, M. E.; DUNKLEY, C. S.; KUBENA, L. F.; NISBET, D. J.; RICKE, S. C. Foodborne *Salmonella* ecology in the avian gastrointestinal tract. **Anaerobe,** v. 15, p. 26-35, 2009.

ELLINGSON, J. L. E.; ANDERSON, J. L.; CARLSON, S. A.; SHARMA, V. K. Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. **Molecular and Cellular Probes,** v. 18, p. 51-57, 2004.

ESPINOZA-MEDINA, I. E.; RODRÍGUEZ-LEYVA, F. J.; VARGAS-ARISPURO, I.; ISLAS-OSUNA, M. A.; ACEDO-FÉLIX, E.; MARTÍNEZ-TÉLLEZ, M. A. PCR identification of *Salmonella*: potential contamination sources from production and postharvest handling of cantaloupes. **Journal of Food Protection,** v. 69, n. 6, p. 1422-1425, 2006.

EYERS, M.; CHAPELLE, S.; CAMP, G. V.; GOOSSENS, H.; WACHTER, R. D. Discrimination among Thermophilic *Campylobacter* Species by Polymerase Chain Reaction Amplification of 23S rRNA Gene Fragments. **Journal of Clinical Microbiology,** v. 31, n. 12, p. 3340-3343, 1993.

FORSYTHE, S. J., Ed. **Microbiologia da segurança alimentar**. São Paulo: Artmed Editora, p.424, 1ª ed. 2002.

FRANCO, B. D. G. D. M.; LANDGRAF, M., Eds. **Microbiologia dos Alimentos**. São Paulo: Atheneu, p.182ed. 2002.

FREITAS, C. G. D.; SANTANA, Â. P.; SILVA, P. H. C. D.; GONÇALVES, V. S. P.; BARROS, M. D. A. F.; TORRES, F. A. G.; MURATA, L. S.; PERECMANIS, S. PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat **International Journal of Food Microbiology,** v. 139, n. 1-2, p. 15-22, 2010.

GACHET, E.; MARTIN, G. G.; VIGNEAU, F.; MEYER, G. Detection of genetically modified organisms (GMOs) by PCR: a brief review of methodologies available. **Trends in Food Science & Technology,** v. 9, n. 11–12, p. 380-388, 11// 1998.

GALÁN, J. E.; GINOCCHIO, C.; COSTEAS, P. Molecular and Functional Characterization of the *Salmonella* Invasion Gene *invA*: Homology of InvA to Members of a New Protein Family. **Journal of Bacteriology,** v. 174, n. 13, p. 4338-4349, 1992.

GIESENDORF, B. A. J.; QUINT, W. G. V.; HENKENS, M. H. C.; STEGEMAN, H.; HUF, F. A.; NIESTERS, H. G. M. Rapid and Sensitive Detection of *Campylobacter* spp. in Chicken Products by Using the Polymerase Chain Reaction. **Applied and Enviromental Microbiology,** v. 58, n. 12, p. 3804-3808, 1992.

GILBERT, C.; WINTERS, D.; O'LEARY, A.; SLAVIK, M. Development of a triplex PCR assay for the specific detection of *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia coli* O157:H7. **Molecular and Cellular Probes,** v. 17, p. 135-138, 2003.

GÓMEZ-DUARTE, O. G.; BAI, J.; NEWELL, E. Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. **Diagnostic Microbiology and Infectious Disease,** v. 63, p. 1-9, 2009.

GONZALEZ, I.; GRANT, K. A.; RICHARDSON, P. T.; PARK, S. F.; COLLINS, M. D. Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceuE* gene encoding a putative virulence determinant. **Journal of Clinical Microbiology,** v. 35, n. 3, p. 759-63, March 1, 1997 1997.

GRIMONT, P. A. D.; WEILL, F.-X. **Antigenic formulae of the *Salmonella* serovars**. Instituto Pasteur. Paris, p.166. 2007

GUIBOURDENCHE, M.; ROGGENTIN, P.; MIKOLEIT, M.; FIELDS, P. I.; BOCKEMÜHL, J.; GRIMONT, P. A. D.; WEILL, F.-X. Supplement 2003–2007 (No. 47) to the White-Kauffmann-Le Minor scheme. **Research in Microbiology,** v. 161, n. 1, p. 26-29, 1// 2010.

GUTELL, R. R.; LARSEN, I.; WOESE, C. R. Lessons from an Evolving rRNA: 16S and 23S rRNA Structures from a Comparative Perspective. **Microbiological Reviews,** v. 58, n. 1, p. 10-26, 1994.

HEIN, I.; FLEKNA, G.; KRASSNIG, M.; WAGNER, M. Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. **Journal of Microbiological Methods,** v. 66, p. 538-547, 2006.

HENSEL, M.; HINSLEY, A. P.; NIKOLAUS, T.; SAWERS, G.; BERKS, B. C. The genetic basis of tetrathionate respiration in *Salmonella* Typhimurium. **Molecular Microbiology,** v. 32, n. 2, p. 275-287, 1999.

HEUVELINK, A. E.; VAN HEERWAARDEN, C.; ZWARTKRUIS-NAHUIS, A.; TILBURG, J. J. H. C.; BOS, M. H.; HEILMANN, F. G. C.; HOFHUIS, A.; HOEKSTRA, T.; DE BOER, E. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. **International Journal of Food Microbiology,** v. 134, n. 1–2, p. 70-74, 2009.

HOFER, E.; FILHO, S. J. S.; REIS, E. M. F. Prevalência de sorovares de *Salmonella* isolados de aves no Brasil. **Pesquisa Veterinária Barasileira,** v. 17, n. 2, p. 55-62, 1997.

HOORFAR, J.; AHRENS, P.; RÅDSTRÖM, P. Automated 5' Nuclease PCR Assay for Identification of *Salmonella enterica*. **Journal of Clinical Microbiology,** v. 38, n. 9, p. 3429-3435, 2000.

HUMPHREY, T.; O'BRIEN, S.; MADSEN, M. Campylobacters as zoonotic pathogens: A food production perspective. **International Journal of Food Microbiology,** v. 117, p. 237-257, 2007.

JACKSON, C. J.; FOX, A. J.; JONES, D. M. A novel polymerase chain reaction assay for the detection and speciation of thermophilic *Campylobacter* spp. **Journal of Applied Bacteriology,** v. 81, p. 467-473, 1996.

JOFRE´, A.; MARTIN, B.; GARRIGA, M.; HUGAS, M.; PLA, M.; RODRÍGUEZ-LÁZARO, D.; AYMERICH, T. Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. **Food Microbiology** v. 22, p. 109-115, 2005.

KANKI, M.; SAKATA, J.; TAGUCHI, M.; KUMEDA, Y.; ISHIBASHI, M.; KAWAI, T.; KAWATSU, K.; YAMASAKI, W.; INOUE, K.; MIYAHARA, M. Effect of sample preparation and bacterial concentration on *Salmonella enterica* detection in poultry meat using culture methods and PCR assaying of preenrichment broths. **Food Microbiology,** v. 26, p. 1-3, 2009.

KATZAV, M.; ISOHANNI, P.; LUND, M.; HAKKINEN, M.; LYHS, U. PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. **Food Microbiology,** v. 25, p. 908-914, 2008.

KOTTWITZ, L. B. M.; OLIVEIRA, T. C. R. M.; ALCOCER, I.; FARAH, S. M. S. S.; ABRAHÃO, W. S. M.; RODRIGUES, D. P. Avaliação epidemiológica de surtos de salmonelose ocorridos no período de 1999 a 2008 no Estado do Paraná, Brasil **Acta Scientiarum,** v. 32, n. 1, p. 9-15, 2010.

KRASCSENICSOVÁ, K.; PIKNOVÁ, L.; KACLÍKOVÁ, E.; KUCHTA, T. Detection of *Salmonella enterica* in food using two-step enrichment and real-time polymerase chain reaction. **Letters in Applied Microbiology,** v. 46, p. 483-487, 2008.

KRAUSE, M.; JOSEFSEN, M. H.; LUND, M.; JACOBSEN, N. R.; BRORSEN, L.; MOOS, M.; STOCKMARR, A.; HOORFAR, J. Comparative, Collaborative, and On-Site Validation of a TaqMan PCR Method as a Tool for Certified Production of Fresh, *Campylobacter*-Free Chickens. **Applied and Enviromental Microbiology,** v. 72, n. 8, p. 5463-5468, 2006.

KUROWSKI, P. B.; TRAUB-DARGATZ, J. L.; MORLEY, P. S.; GENTRY-WEEKS, C. R. Detection of *Salmonella* spp in fecal specimens by use of real-time polymerase chain reaction assay. **American Journal of Veterinary Research,** v. 63, n. 9, p. 1265-1268, 2002.

LEADER, B. T.; FRYE, J. G.; HU, J.; FEDORKA-CRAY, P. J.; BOYLE, D. S. High-throughput Molecular Determination of *Salmonella enterica* Serovars using Multiplex

PCR and Capillary Electrophoresis Analysis. **Journal of Clinical Microbiology**, 2009.

LEE, S. Y.; OH, S. S. W.; CHUNG, H. J.; REYES-DE-CORCUERA, J. I.; POWERS, J. R.; KANG, D. H. Reduction of *Salmonella enterica* serovar Enteritidis on the surface of raw shelled almonds by exposure to steam. **Journal of Food Protection,** v. 69 n. 3, p. 591-595, 2006.

LINTON, D.; LAWSON, A. J.; OWEN, R. J.; STANLEY, J. PCR Detection, Identification to Species Level, and Fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* Direct from Diarrheic Samples. **Journal of Clinical Microbiology,** v. 35, n. 10, p. 2568–2572, 1997.

LINTON, D.; OWEN, R. J.; STANLEY, J. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. **Res. Microbiol.,** v. 147, p. 707-718, 1996.

LOGAN, J. M. J.; EDWARDS, K. J.; SAUNDERS, N. A.; STANLEY, J. Rapid Identification of *Campylobacter* spp. by Melting Peak Analysis of Biprobes in Real-Time PCR. **Journal of Clinical Microbiology,** v. 39, n. 6, p. 2227-2232, 2001.

LÜBECK, P. S.; WOLFFS, P.; ON, S. L. W.; AHRENS, P.; RADSTRÖM, P.; HOORFAR, J. Toward an International Standard for PCR-Based Detection of Food-Borne Thermotolerant Campylobacters: Assay Development and Analytical Validation. **Applied and Enviromental Microbiology,** v. 69, n. 9, p. 5664-5669, 2003.

LUND, M.; NORDENTOFT, S.; PEDERSEN, K.; MADSEN, M. Detection of *Campylobacter* spp. in Chicken Fecal Samples by Real-Time PCR. **Journal of Clinical Microbiology,** v. 42, n. 11, p. 5125-5132, 2004.

MAHON, J.; MURPHY, C. K.; JONES, P. W.; BARROW, P. A. Comparison of multiplex PCR and standard bacteriological methods of detecting *Salmonella* on chicken skin. **Letters in Applied Microbiology,** v. 19, p. 169-172, 1994.

MALORNY, B.; COOK, N.; D'AGOSTINO, M.; MEDICI, D. D.; CROCI, L.; ABDULMAWJOOD, A.; FACH, P.; KARPISKOVA, R.; AYMERICH, T.; KWAITEK, K.; KUCHTA, T.; HOORFAR, J. Multicenter Validation of PCR-Based Method for Detection of *Salmonella* in Chicken and Pig Samples. **Journal of AOAC International,** v. 87, n. 4, p. 861-866, 2004.

MALORNY, B.; HOORFAR, J.; BUNGE, C.; HELMUTH, R. Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. **Applied and Enviromental Microbiology,** v. 69, n. 1, p. 290-296, 2003.

MALORNY, B.; HUEHN, S.; DIECKMANN, R.; KRÄMER, N.; HELMUTH, R. Polymerase Chain Reaction for the Rapid Detection and Serovar Identification of *Salmonella* in Food and Feeding Stuff. **Food Analytical Methods,** v. 2, p. 81-95, 2009.

MALORNY, B.; PACCASSONI, E.; FACH, P.; BUNGE, C.; MARTIN, A.; HELMUTH, R. Diagnostic Real-Time PCR for Detection of *Salmonella* in Food. **Applied and Enviromental Microbiology,** v. 70, n. 12, p. 7046-7052, 2004.

MEAD, G. *Campylobacter* update - the challenge. **International Poultry Production,** v. 12, n. 4, p. 26-29, 2004.

MULLIS, K. B.; FALOONA, F. A. Specific synthesis of DNA in vitro via a polymerasecatalyzed chain reaction. **Methods in enzymology,** v. 155, p. 335-350, 1987.

NAM, H.-M.; SRINIVASAN, V.; GILLESPIE, B. E.; MURINDA, S. E.; OLIVER, S. P. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. **International Journal of Food Microbiology,** v. 102, p. 161-171, 2005.

NAYAK, R.; STEWART, T. M.; NAWAZ, M. S. PCR identification of *Campylobacter coli* and *Campylobacter jejuni* by partial sequencing of virulence genes. **Molecular and Cellular Probes,** v. 19, p. 187-193, 2005.

NEWELL, D. G.; FEARNLEY, C. Sources of Campylobacter Colonization in Broiler Chickens. **Applied and Environmental Microbiology,** v. 69, n. 8, p. 4343-4351, August 1, 2003 2003.

NIELSEN, T. D.; GREEN, L. E.; KUDAHL, A. B.; ØSTERGAARD, S.; NIELSEN, L. R. Evaluation of milk yield losses associated with *Salmonella* antibodies in bulk tank milk in bovine dairy herds. **Journal of Dairy Science,** v. 95, n. 9, p. 4873-4885, 9// 2012.

O'REGAN, E.; MCCABE, E.; BURGESS, C.; MCGUINNESS, S.; BARRY, T.; DUFFY, G.; WHYTE, P.; FANNING, S. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology,** v. 8, p. 156, 2008.

OLAH, P. A.; SHERWOOD, J. S.; LOGUE, C. M. Molecular analysis of *Salmonella* isolates recovered from processed turkey carcasses. **Journal of Food Protection,** v. 68, p. 845-849, 2005.

OLIVEIRA, T. C. R. M.; BARBUT, S.; GRIFFITHS, M. W. Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. **International Journal of Food Microbiology,** v. 104, p. 105-111, 2005.

OLIVER, S. P.; BOOR, K. J.; MURPHY, S. C.; MURINDA, S. E. Food Safety Hazards Associated with Consumption of Raw Milk. **Foodborne Pathogens and Disease,** v. 6, n. 7, p. 793-806, 2009.

OLSEN, K. N.; LUND, M.; SKOV, J.; CHRISTENSEN, L. S.; HOORFAR, J. Detection of *Campylobacter* Bacteria in Air Samples for Continuous Real-Time Monitoring of

Campylobacter Colonization in Broiler Flocks. **Applied and Enviromental Microbiology,** v. 75, n. 7, p. 2074–2078 2009a.

______. Towards real-time monitoring of broiler flocks: detection of *Campylobacter* in air samples for continuous monitoring of *Campylobacter* colonization in broiler flocks. **Applied and Enviromental Microbiology**, 2009b.

OMICCIOLI, E.; AMAGLIANI, G.; BRANDI, G.; MAGNANI, M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiology,** v. 26, p. 615-622, 2009.

ON, S. L. Isolation, identification and subtyping of *Campylobacter:* where to from here? **Journal of Microbiological Methods,** v. 95, n. 1, p. 3-7, Oct 2013.

PARK, S. F. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. **International Journal of Food Microbiology,** v. 74, n. 3, p. 177-188, 2002.

PARK, S. H.; HANNING, I.; JARQUIN, R.; MOORE, P.; DONOGHUE, D. J.; DONOGHUE, A. M.; RICKE, S. C. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. **FEMS Microbiology Letters,** v. 316, n. 1, p. 7-15, 2011.

PEPE, T.; DE DOMINICIS, R.; ESPOSITO, G.; VENTRONE, I.; FRATAMICO, P. M.; CORTESI, M. L. Detection of *Campylobacter* from Poultry Carcass Skin Samples at Slaughter in Southern Italy **Journal of Food Protection,** v. 72, n. 8, p. 1718-1721, 2009.

PERELLE, S.; JOSEFSEN, M.; HOORFAR, J.; DILASSER, F.; GROUT, J.; FACH, P. A LightCycler real-time PCR hybridization probe assay for detecting food-borne thermophilic *Campylobacter*. **Molecular and Cellular Probes,** v. 18, p. 321–327, 2004.

PERRY, L.; HEARD, P.; KANE, M.; KIM, H.; SAVIKHIN, S.; DOMÍNGUEZ, W.; APPLEGATE, B. Application of multiplex Polimerase Chain Reaction to the detection of pathogens in food. **Journal of Rapid Methods & Automation in Microbiology,** v. 15, p. 176-198, 2007.

POPOFF, M. Y.; BOCKEMÜHL, J.; GHEESLING, L. L. Supplement 2002 (no. 46) to the Kauffmann–White scheme. **Research in Microbiology,** v. 155, p. 568-570, 2004.

PUI, C. F.; WONG, W. C.; CHAI, L. C.; NILLIAN, E.; GHAZALI, F. M.; CHEAH, Y. K.; NAKAGUCHI, Y.; NISHIBUCHI, M.; RADU, S. Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. **Food Control,** v. 22, p. 337-342, 2010.

RAHN, K.; GRANDIS, S. A. D.; CLARKE, R. C.; MCEWEN, S. A.; GALÁN, J. E.; GINOCCHIO, C.; III, R. C.; GYLES, C. L. Amplification of an *invA* gene sequence of

Salmonella typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. **Molecular and Cellular Probes,** v. 6, p. 271-279, 1992.

REVEZ, J.; ZHANG, J.; SCHOTT, T.; KIVISTÖ, R.; ROSSI, M.; HÄNNINEN, M.-L. Genomic Variation between *Campylobacter jejuni* Isolates Associated with Milk-Borne-Disease Outbreaks. **Journal of Clinical Microbiology,** v. 52, n. 8, p. 2782-2786, August 1, 2014 2014.

RODRÍGUEZ-LÁZARO, D.; HERNÁNDEZ, M.; ESTEVE, T.; HOORFAR, J.; PLA, M. A rapid and direct real time PCR-based method for identification of *Salmonella* spp. **Journal of Microbiological Methods,** v. 54, p. 381-390, 2003.

SALOMONSSON, A. C.; ASPÁN, A.; JOHANSSON, S.; HEINO, A.; HÄGGBLOM, P. E. R. *Salmonella* detection by polymerase chain reaction after pre-enrichment of feed samples. **Journal of Rapid Methods and Automation in Microbiology,** v. 13, p. 96-110, 2005.

SCHODER, D.; SCHMALWIESER, A.; SCHAUBERGER, G.; KUHN, M.; HOORFAR, J.; WAGNER, M. Physical characteristics of six new thermocycles. **Clinical Chemistry,** v. 49, p. 960-963, 2003.

SHELOBOLINA, E. S.; SULLIVAN, S. A.; O'NEILL, K. R.; NEVIN, K. P.; LOVLEY, D. R. Isolation, Characterization, and U(VI)-Reducing Potential of a Facultatively Anaerobic, Acid-Resistant Bacterium from Low-pH, Nitrate- and U(VI)-Contaminated Subsurface Sediment and Description of *Salmonella subterranea* sp. nov. **Applied and Enviromental Microbiology,** v. 70, n. 5, p. 2959-2965, 2004.

SILVA, D. S. P.; CANATO, T.; MAGNANI, M.; ALVES, J.; HIROOKA, E. Y.; OLIVEIRA, T. C. R. M. D. Multiplex PCR for simultaneous detection of *Salmonella* spp. and *Salmonella* Enteritidis in food. **International Journal of Food Science & Technology,** v. 46, p. 1502-1507, 2011.

SILVA, D. T.; TEJADA, T. S.; CUNHA, C. C.; LOPES, N. A.; AGOSTINETTO, A.; COLLARES, T.; LEON, P. M. M.; TIMM, C. D. Ocorrência de *Campylobacter* em carne de frango, fezes de frango e humanas e pesquisa dos genes *cdt*. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia,** v. 66, p. 297-304, 2014.

SIMMONS, M.; HIETT, K. L.; STERN, N. J.; FRANK, J. F. Comparison of poultry exudate and carcass rinse sampling methods for the recovery of *Campylobacter* spp. subtypes demonstrates unique subtypes recovered from exudate. **Journal of Microbiological Methods,** v. 74, p. 89-93, 2008.

SOUMET, C.; ERMEL, G.; ROSE, V.; ROSE, N.; DROUIN, P.; SALVAT, G.; COLIN, P. Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains from environmental swabs of poultry houses. **Letters in Applied Microbiology,** v. 29, p. 1-6, 1999.

SU, L.-H.; CHIU, C.-H. *Salmonella*: Clinical importance and evolution of nomenclature. **Chang Gung Medical Journal,** v. 30, n. 3, p. 210-219, 2007.

UYTTENDAELE, M.; SCHUKKINK', R.; GEMEN', B. V.; DEBEVERE, J. Identification of *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBAR. **Journal of Applied Bacteriology,** v. 77, p. 694-701, 1994.

VANCAMP, G.; FIERENS, H.; VANDAMME, P.; GOOSENS, H.; HUYGHEBAERT, A.; WACHTER, R. D. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chain reaction based on 16s rRNA genes. **Systemic and Applied Microbiology,** v. 16, p. 30-36, 1993.

VANTARAKIS, A.; KOMNINOU, G.; VENIERI, D.; PAPAPETROPOULOU, M. Development of a multiplex PCR detection of *Salmonella* spp. and *Shigella* spp. in mussels. **Letters in Applied Microbiology,** v. 31, p. 105-109, 2000.

VOLLENHOFER-SCHRUMPF, S.; BURESCH, R.; UNGER, G.; STAHL, N.; FRÄNZL, G.; SCHINKINGER, M. Detection of *Salmonella s*pp., *Escherichia Coli* O157, *Listeria Monocytogenes a*nd *Campylobacter* spp. in Chicken Samples by Multiplex Polymerase Chain Reaction and Hybridization Using the Genegen Major Food Pathogens Detection Kit **Journal of Rapid Methods and Automation in Microbiology,** v. 13, p. 148-176, 2005.

WAGENAAR, J. A.; FRENCH, N. P.; HAVELAAR, A. H. Preventing *Campylobacter* at the source: why is it so difficult? **Clinical Infectious Diseases** v. 57, n. 11, p. 1600-6, Dec 2013.

WAY, J. S.; JOSEPHSON, K. L.; PILLAI, S. D.; ABBASZADEGAN, M.; GERBA, C. P.; PEPPER, I. L. Specific Detection of *Salmonella* spp. by Multiplex Polymerase Chain Reaction. **Applied and Enviromental Microbiology,** v. 59, n. 5, p. 1473-1479, 1993.

WHO. World Health Organization. FAO/WHO Expert Meeeting on *Salmonella* and *Campylobacter* in chicken meat, 4 – 8 May 2009. 2009. Disponível em: < http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html >. Acesso em: 18 jul. 2014.

WIEMER, D.; LODERSTAEDT, U.; VON WULFFEN, H.; PRIESNITZ, S.; FISCHER, M.; TANNICH, E.; HAGEN, R. M. Real-time multiplex PCR for simultaneous detection of Campylobacter jejuni, Salmonella, Shigella and Yersinia species in fecal samples. **International Journal of Medical Microbiology,** v. 301, n. 7, p. 577-584, 2011.

WISESSOMBAT, S.; INTHAGARD, J.; KITTINIYOM, K.; SRIMANOTE, P.; WONGLUMSOM, W.; VORAVUTHIKUNCHAI, S. P. Multiplex PCR for direct identification of thermophilic *Campylobacter, C. jejuni, C. coli, C. lari* and *C. upsaliensis* and simultaneous detection of *cdtB* gene. **Journal of Rapid Methods & Automation in Microbiology,** v. 17, p. 438-454, 2009.

WOLFFS, P.; NORLING, B.; HOORFAR, J.; GRIFFITHS, M.; RÅDSTRÖM, P. Quantification of *Campylobacter* spp. in Chicken Rinse Samples by Using Flotation prior to Real-Time PCR. **Applied and Enviromental Microbiology,** v. 71, n. 10, p. 5759-64, 2005.

WOLFFS, P. F. G.; GLENCROSS, K.; NORLING, B.; GRIFFITHS, M. W. Simultaneous quantification of pathogenic *Campylobacter* and *Salmonella* in chicken rinse fluid by a flotation and real-time multiplex PCR procedure. **International Journal of Food Microbiology,** v. 117, p. 50-54, 2007.

YAN, S. S.; PENDRAK, M. L.; FOLEY, S. L.; POWERS, J. H. *Campylobacter* infection and Guillain–Barré syndrome: public health concerns from a microbial food safety perspective. **Clinical and Applied Immunology Reviews,** v. 5, p. 285-305, 2005.

5 RESULTADOS E DISCUSSÃO

Os resultados e discussão deste trabalho serão apresentados em quatro artigos, organizados da seguinte forma:

- Artigo 1 – Detection of *Salmonella* spp. in eight complex food matrices using polymerase chain reaction assay
- Artigo 2 – Prevalence of *Campylobacter* spp., *C. jejuni* and *C. coli* from chicken meat in Paraná, Brazil
- Artigo 3 – Evaluation of uniplex and multiplex PCR assays for detection of *Campylobacter* spp. and *Salmonella* spp. in milk
- Artigo 4 – Development of a multiplex real-time PCR assay with an internal amplification control for detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat

5.1 Artigo 1 – Detection of *Salmonella* spp. in eight complex food matrices using polymerase chain reaction assay

Running title: Detection of *Salmonella* spp. in complex food matrices

Juliane Alves*, Natália Harumi Niguma and Tereza Cristina Rocha Moreira de Oliveira

Department of Food Science and Technology, State University of Londrina, Brazil. Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86057-970, Londrina, Paraná, Brasil.

***Corresponding author:**

Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86.057-970, Londrina, Paraná, Brasil.
Tel:+55-43-3371-5968; Fax:+55-43-3371-4080;
E-mail: julianealves@yahoo.com.br

Abstract

Conventional methods for the detection of *Salmonella* based on culturing are time consuming and laborious. The aim of this study was to develop a template preparation method and polymerase chain reaction (PCR) assay for the detection of *Salmonella* spp. in egg yolk, pizza, ground beef, pork, pork sausage, chicken drumsticks, mayonnaise and Minas cheese. Four aliquots of 50 mL of each food in buffered peptone water (BPW) were spiked with *S*. Enteritidis ATCC 13076 to achieve the concentration range of 1 to 10^3 UFC/mL. After an incubation at 37 °C for 18 h, DNA extraction was performed with thermal lysis followed by nucleic acid purification with phenol-chloroform. No inhibitory substances in these eight complex food matrices were observed. The PCR technique detected bacteria with a sensitivity of 1 UFC/mL of food in BPW. This method is relatively inexpensive and can be used in food microbiological analyses and for epidemiological studies.

Keywords: Food-borne pathogens. PCR. Pathogen detection. *Salmonella.* Food matrices.

1 Introduction

Salmonella remains one of the most common cause of bacterial foodborne disease worldwide (WHO, 2009). Poultry products are the main vehicles of transmission to humans, however raw or undercooked meat sausages, dairy products and other industrialised foods can also be contaminated with *Salmonella* spp.

Traditional methods for the detection of *Salmonella* spp. in foods involve non-selective and selective enrichments, followed by subculture to selective media and subsequent phenotypic and serological identification (HEIN et al., 2006). Those methods are time consuming and laborious, and the food industry and public health services demand faster methods (RATHNAYAKA and RAKSHIT, 2010; ZHANG et al., 2013). The Polymerase Chain Reaction (PCR) is an alternative method that has several advantages over the conventional methods, such as speed, sensitivity and the ability to work with fastidious bacteria. An enrichment step is normally necessary, and although enrichment increases the analysis time, it provides essential benefits, such as the dilution of inhibitor effects, the differentiation of viable

from non-viable cells and the repair of cells stressed or injured during food processing (MALORNY et al., 2009).

DNA extraction methods are crucial for the quality and quantity of DNA. Methods to extract and purify DNA from foods frequently follow four steps: a) mechanical homogenisation, b) treatment with buffers, detergents and/or enzymes, c) the application of mechanical lysis and d) organic DNA extraction (JANY e BARBIER, 2008; QUIGLEY et al., 2012). The PCR inhibitors generally act by interfering with nucleic acid degradation or capture or by inhibiting the ability of the polymerase to amplify the DNA target. The common inhibitors in foods are organic and phenolic compounds, glycogen, fats and calcium (WILSON, 1997).

Several PCR methods for the detection of *Salmonella* spp. have been published (MALORNY et al., 2004; BOHAYCHUK et al., 2007; O'REGAN et al., 2008; LÖFSTRÖM et al., 2009; PUI et al., 2010; MARATHE et al., 2012; RIYAZ-UL-HASSAN et al., 2013; RODRIGUEZ-LAZARO et al., 2014). Testing with different food matrices is an important criterion for the use of a method in food analyses.

The aim of this study was to develop a template preparation method and a PCR assay for the detection of *invA* gene of *Salmonella* spp. in eight complex food matrices (egg yolk, pizza, ground beef, pork, pork sausage, chicken drumsticks, mayonnaise and Minas cheese).

2 Material and methods

2.1 Preparation of *S.* Enteritidis ATCC 13076 suspension

Egg yolk, pizza, ground beef, pork, pork sausage, chicken drumsticks, mayonnaise and Minas cheese samples were spiked using bacterial suspensions prepared as described. A serial dilution was prepared in 1% (w/v) sterile buffered peptone water (Acumedia, Lansing, Michigan, USA) to obtain suspensions containing between 1 and 10^3 CFU of *S.* Enteritidis ATCC 13076 *per* mL. The bacterial density was estimated using the drop plate method for counting bacteria (MILES et al., 1938). The bacterial suspensions of *S*. Enteritidis ATCC 13076 were

subcultured on Xylose Lysine Sodium Deoxycholate (XLD) plates (Acumedia) and were incubated at 37 °C for 24 h. Experiments were carried out in duplicate.

2.2 Contamination of food matrices

The eight food samples were previously analysed to rule out prior contamination with *Salmonella* spp. A portion of 25 g of each uncontaminated food was added to 225 mL of sterile buffered peptone water (BPW) (Acumedia). Four aliquots of 50 mL each were spiked with *S.* Enteritidis ATCC 13076 to achieve the concentration range between 1 and 10^3 CFU/mL of food in BPW and this enrichment was incubated at 37 ° C for 18 h. This experiment was performed in two independent replicates.

2.3 DNA extraction

Aliquots of 1 mL of non-selective enrichments of *Salmonella* spp., obtained from spiked food samples were centrifuged at 16.000 × *g* for 10 min. The sediment was washed with 1 mL of 1.0% (w/v) BPW (Acumedia) and centrifuged for 10 min at 16.000 × *g*. The pellet was resuspended in 300 µL of TZ lysis solution (ABOLMAATY et al., 2000). The suspensions were heated at 100 °C for 10 min, cooled on ice, and centrifuged at 16.000 × *g* for 5 min. The sediment was discarded and the supernatant containing DNA was used in PCR and purification step.

2.4 DNA purification

For DNA purification, 300 µL of a solution of phenol, chloroform and isoamyl alcohol (25:24:1) (v/v) were added to the supernatant. The mixture was shaken via inversion and centrifuged at 13.000 x *g* for 10 min. After the aqueous phase was transferred to a new tube, 30 µL of sodium acetate (3 M) and 270 µL of

etanol were added. The tubes were kept for 12 min at -80 °C, and centrifuged at 13.000 x *g* for 10 min. The pellet was washed with 70% ethanol, and centrifuged again as described above. The DNA was dried at 37°C and dissolved in 50 µL of sterile ultrapure water. This experiment step was performed in two independent replicates.

2.5 PCR for the detection of *Salmonella* spp.

The PCR assay was carried out in two replicates. All reactions were performed with negative (ultrapure water) and positive controls (*S.* Enteritidis ATCC 13076).

Styinva-JHO-2-left (5'-TCGTCATTCCATTACCTACC-3') and Styinva-JHO-2-right (5'-AAACGTTGAAAAACTGAGGA-3') primers (HOORFAR et al., 2000), which are specific for the genus *Salmonella* and amplify a 119 bp sequence of the *InvA* gene, were used for the PCR.

The reaction contained 4 µL of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen Brazil, São Paulo, SP, Brazil), 5.0 mM of $MgCl_2$ (Invitrogen™), 0.2 mM of dNTP (Invitrogen™), 0.3 µM each of Styinva-JHO-2-left and Styinva-JHO-2-right primers, and 1 U of *Taq* DNA Polymerase (Invitrogen™) in a total volume of 20 µL.

The PCR assays were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 30 s at 60 °C, 45 s at 72 °C and 5 min of final extension at 72 °C.

The PCR amplification products were visualised on a 1.5% (w/v) agarose gel containing 0.02 µL/mL of SYBR® Safe 10,000x in DMSO (Invitrogen™), viewed under ultraviolet light (UV) and photo-documented. A DNA fragment of 100 bp (Invitrogen™) was used as a molecular size marker.

3 Results and discussion

The PCR assay tested in this study was based on the *inv*A primer set reported by Rahn et al. (1992). The primer set for the detection of *Salmonella* spp. based on this gene was previously tested by others (HOORFAR et al., 2000; RODRÍGUEZ-LÁZARO et al., 2003; NAM et al., 2005).

All samples spiked with *Salmonella* generated the specific amplification, thus indicating that the PCR inhibitors from food matrices were efficiently removed via the DNA extraction procedure.

The PCR assay detected 1 CFU of *Salmonella* per mL of food suspension, after 18 h of non-selective enrichment. Thermal lysis with the TZ solution was sufficient for the detection of *Salmonella* spp. in egg yolk, pork, pork sausage and Minas cheese. DNA purification with phenol/ chloroform was necessary for pizza, ground beef, chicken drumsticks and mayonnaise, most likely due to the interference of food matrices in the template amplification. According to Abolmaaty *et al.* (2000), the addition of sodium azide to the lysis solution was more efficient for the DNA extraction of *S*. Enteritidis than extraction using only Triton X-100. Other studies have also shown the same efficiency of the TZ lysis solution (ABOLMAATY et al., 2000; DUODU et al., 2009; LEE e LEVIN, 2009; LUO et al., 2010; ALVES et al., 2012), however, the mechanism by which sodium azide enhances DNA extraction has not been elucidated (ABOLMAATY et al., 2000).

The present study has shown that PCR can be used to detect microorganisms in complex biological materials such as clinical, environmental or food samples but that PCR is limited in part by the presence of substances that inhibit or reduce the reaction efficiency of amplification. The most common inhibitors present in food are proteases, DNases, polysaccharides, fats, high concentration of calcium ions, as well as low pH or high microbial loads (MALORNY et al., 2009). These PCR inhibitors can act by one or more mechanisms such as interference in cell lysis, degraded or binding with nucleic acids and inactivation of the DNA polymerase (WILSON, 1997; ABOLMAATY et al., 2007). Many techniques have been used to reduce the effects of PCR inhibitors or to separate the target microorganism from inhibitors reaction such as a two aqueous phases, density

gradient centrifugation, dilution, filtration and enrichment techniques immunocapture systems (GU and LEVIN, 2006).

For all foods analysed, thermal lysis with the TZ solution and phenol/ chloroform purification resulted in higher quality DNA amplification than the use of only the TZ lysis solution. The template preparation method was very cost-effective and rapid.

Because of the difficulty in recovering bacteria from foods, the enrichment of food samples is required to increase the number of cells and facilitate the DNA extraction. A count of approximately 10^4 CFU of bacteria *per* mL or *per* g of food obtained by non-selective (or selective) enrichment is sufficient to ensure the sensitivity of PCR (MALORNY et al., 2009). Therefore, enrichment steps have been included in several studies (JOFRÉ et al., 2005; CORTEZ et al., 2006; KATZAV et al., 2008; GERMINI et al., 2009; FREITAS et al., 2010; RAHIMI et al., 2010; ALVES et al., 2012).

The PCR and the template preparation tested in the present study detected *Salmonella* spp. in eight complex food matrices directly after 18 hours of non-selective enrichment. The PCR assay was relatively inexpensive and can be used in food laboratories and for epidemiological studies.

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References

ABOLMAATY, A.; GU, W.; WITKOWSKY, R.; LEVIN, R. E. The use of activated charcoal for the removal of PCR inhibitors from oyster samples. **Journal of Microbiological Methods,** v. 68, n. 2, p. 349-352, 2007.

ABOLMAATY, A.; VU, C.; OLIVER, J.; LEVIN, R. E. Development of a new lysis solution for releasing genomic DNA from bacterial cells for DNA amplification by polymerase chain reaction. **Microbios,** v. 101, n. 400, p. 181-189, 2000.

ALVES, J.; MARQUES, V. V.; PEREIRA, L. F. P.; HIROOKA, E. Y.; DE OLIVEIRA, T. C. R. M. Multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. **Journal of Food Safety,** v. 32, n. 3, p. 345-350, 2012.

BOHAYCHUK, V. M.; GENSLER, G. E.; MCFALL, M. E.; KING, R. K.; RENTER, D. G. A Real-Time PCR Assay for the Detection of *Salmonella* in a Wide Variety of Food and Food-Animal Matrices. **Journal of Food Protection,** v. 70, n. 5, p. 1080-1087, 2007.

CORTEZ, A. L. L.; CARVALHO, A. C. F. B.; IKUNO, A. A.; BÜRGER, K. P.; VIDAL-MARTINS, A. M. C. Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex-PCR. **Research in Veterinary Science,** v. 81, p. 340-344, 2006.

DUODU, S.; MEHMETI, I.; HOLST-JENSEN, A.; LONCAREVIC, S. Improved Sample Preparation for Real-Time PCR Detection of *Listeria monocytogenes* in Hot-Smoked Salmon using Filtering and Immunomagnetic Separation Techniques. **Food Analytical Methods,** v. 2, p. 23-29, 2009.

FREITAS, C. G. D.; SANTANA, Â. P.; SILVA, P. H. C. D.; GONÇALVES, V. S. P.; BARROS, M. D. A. F.; TORRES, F. A. G.; MURATA, L. S.; PERECMANIS, S. PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat **International Journal of Food Microbiology,** v. 139, n. 1-2, p. 15-22, 2010.

GERMINI, A.; MASOLA, A.; CARNEVALI, P.; MARCHELLI, R. Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. **Food Control,** v. 20, p. 733-738, 2009.

GU, W.; LEVIN, R. E. Quantitative detection of *Plesiomonas shigelloides* in clam and oyster tissue by PCR. **International Journal of Food Microbiology,** v. 111, n. 1, p. 81-86, 8/15/ 2006.

HEIN, I.; FLEKNA, G.; KRASSNIG, M.; WAGNER, M. Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. **Journal of Microbiological Methods,** v. 66, p. 538-547, 2006.

HOORFAR, J.; AHRENS, P.; RÅDSTRÖM, P. Automated 5' Nuclease PCR Assay for Identification of *Salmonella enterica*. **Journal of Clinical Microbiology,** v. 38, n. 9, p. 3429-3435, 2000.

JANY, J.-L.; BARBIER, G. Culture-independent methods for identifying microbial communities in cheese. **Food Microbiology,** v. 25, n. 7, p. 839-848, 10// 2008.

JOFRÉ, A.; MARTIN, B.; GARRIGA, M.; HUGAS, M.; PLA, M.; RODRÍGUEZ-LÁZARO, D.; AYMERICH, T. Simultaneous detection of *Listeria monocytogenes* and

Salmonella by multiplex PCR in cooked ham. **Food Microbiology,** v. 22, p. 109-115, 2005.

KATZAV, M.; ISOHANNI, P.; LUND, M.; HAKKINEN, M.; LYHS, U. PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. **Food Microbiology,** v. 25, p. 908-914, 2008.

LEE, J.-L.; LEVIN, R. E. Discrimination of viable and dead Vibrio vulnificus after refrigerated and frozen storage using EMA, sodium deoxycholate and real-time PCR. **Journal of Microbiological Methods,** v. 79, n. 2, p. 184-188, 2009.

LÖFSTRÖM, C.; KRAUSE, M.; JOSEFSEN, M.; HANSEN, F.; HOORFAR, J. Validation of a same-day real-time PCR method for screening of meat and carcass swabs for Salmonella. **BMC Microbiology,** v. 9, n. 1, p. 85, 2009.

LUO, J.-F.; LIN, W.-T.; GUO, Y. Method to detect only viable cells in microbial ecology. **Applied Microbiology and Biotechnology,** v. 86, n. 1, p. 377-384, 2010.

MALORNY, B.; HUEHN, S.; DIECKMANN, R.; KRÄMER, N.; HELMUTH, R. Polymerase Chain Reaction for the Rapid Detection and Serovar Identification of *Salmonella* in Food and Feeding Stuff. **Food Analytical Methods,** v. 2, p. 81-95, 2009.

MALORNY, B.; PACCASSONI, E.; FACH, P.; BUNGE, C.; MARTIN, A.; HELMUTH, R. Diagnostic Real-Time PCR for Detection of *Salmonella* in Food. **Applied and Enviromental Microbiology,** v. 70, n. 12, p. 7046-7052, 2004.

MARATHE, S. A.; CHOWDHURY, R.; BHATTACHARYA, R.; NAGARAJAN, A. G.; CHAKRAVORTTY, D. Direct detection of *Salmonella* without pre-enrichment in milk, ice-cream and fruit juice by PCR against *hilA* gene. **Food Control,** v. 23, n. 2, p. 559-563, 2012.

MILES, A. A.; MISRA, S. S.; IRWIN, J. O. The estimation of the bactericidal power of the blood. **Journal of Hygiene,** v. 38, n. 6, p. 732-49, Nov 1938.

NAM, H.-M.; SRINIVASAN, V.; GILLESPIE, B. E.; MURINDA, S. E.; OLIVER, S. P. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. **International Journal of Food Microbiology,** v. 102, p. 161-171, 2005.

O'REGAN, E.; MCCABE, E.; BURGESS, C.; MCGUINNESS, S.; BARRY, T.; DUFFY, G.; WHYTE, P.; FANNING, S. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology,** v. 8, p. 156, 2008.

PUI, C. F.; WONG, W. C.; CHAI, L. C.; NILLIAN, E.; GHAZALI, F. M.; CHEAH, Y. K.; NAKAGUCHI, Y.; NISHIBUCHI, M.; RADU, S. Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. **Food Control,** v. 22, p. 337-342, 2010.

QUIGLEY, L.; O'SULLIVAN, O.; BERESFORD, T. P.; PAUL ROSS, R.; FITZGERALD, G. F.; COTTER, P. D. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. **Journal of Applied Microbiology,** v. 113, n. 1, p. 96-105, 2012.

RAHIMI, E.; MOMTAZ, H.; BONYADIAN, M. PCR detection of *Campylobacter* sp. from turkey carcasses during processing plant in Iran. **Food Control,** v. 21, n. 5, p. 692-694, 2010.

RAHN, K.; GRANDIS, S. A. D.; CLARKE, R. C.; MCEWEN, S. A.; GALÁN, J. E.; GINOCCHIO, C.; III, R. C.; GYLES, C. L. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. **Molecular and Cellular Probes,** v. 6, p. 271-279, 1992.

RATHNAYAKA, U. S. K.; RAKSHIT, S. K. Evaluation of metal hydroxide immobilization and DNA extraction methods on detection of *Salmonella enterica* from pork sausage by nested polymerase chain reaction. **Journal of Muscle Foods,** v. 21, n. 4, p. 801-812, 2010.

RIYAZ-UL-HASSAN, S.; VERMA, V.; QAZI, G. N. Real-time PCR-based rapid and culture-independent detection ofSalmonellain dairy milk - addressing some core issues. **Letters in Applied Microbiology,** v. 56, n. 4, p. 275-282, 2013.

RODRIGUEZ-LAZARO, D.; GONZALEZ-GARCÍA, P.; DELIBATO, E.; DE MEDICI, D.; GARCÍA-GIMENO, R. M.; VALERO, A.; HERNANDEZ, M. Next day Salmonella spp. detection method based on real-time PCR for meat, dairy and vegetable food products. **International Journal of Food Microbiology,** v. 184, n. 0, p. 113-120, 8/1/ 2014.

RODRÍGUEZ-LÁZARO, D.; HERNÁNDEZ, M.; ESTEVE, T.; HOORFAR, J.; PLA, M. A rapid and direct real time PCR-based method for identification of *Salmonella* spp. **Journal of Microbiological Methods,** v. 54, p. 381-390, 2003.

WHO. World Health Organization. FAO/WHO Expert Meeeting on *Salmonella* and *Campylobacter* in chicken meat, 4 – 8 May 2009. 2009. Disponível em: < http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html >. Acesso em: 18 jul. 2014.

WILSON, I. G. Inhibition and Facilitation of Nucleic Acid Amplification. **Applied and Enviromental Microbiology,** v. 63, n. 10, p. 3741-3751, 1997.

ZHANG, Q.; CHEN, T.; YANG, S.; WANG, X.; GUO, H. Response surface methodology to design a selective enrichment broth for rapid detection of *Salmonella* spp. by SYBR Green I real-time PCR. **Applied Microbiology and Biotechnology** v. 97, n. 9, p. 4149-4158 2013.

5.2 Artigo 2 – Prevalence of *Campylobacter* spp., *C. jejuni* and *C. coli* from chicken meat in Paraná, Brazil

Running title: Prevalence of *Campylobacter* from chicken meat

Juliane Alves[*] and Tereza Cristina Rocha Moreira de Oliveira

Department of Food Science and Technology, State University of Londrina, Brazil. Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86057-970, Londrina, Paraná, Brazil.

***Corresponding author:**

Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86.057-970, Londrina, Paraná, Brasil.
Tel:+55-43-3371-5968; Fax:+55-43-3371-4080;
E-mail: julianealves@yahoo.com.br

Abstract

Campylobacter spp. is a common cause of bacterial food-borne illness. *C. jejuni* and *C. coli* are recognized as important agents of acute human diarrheal diseases worldwide. The main source of human infection is undercooked chicken, raw milk, and cross-contamination from the environment, notably in kitchens. The aim of this study was to determine the prevalence of *Campylobacter* spp., *C. jejuni* and *C. coli* isolated from chicken cuts. Confirmation of the presence of *Campylobacter* spp., *C. jejuni* and *C. coli* in chicken samples was performed by using the polymerase chain reaction. From March to June 2014, 105 chilled chicken cuts in their original packaging were randomly purchased from retail supermarket in Londrina, Paraná State, Brazil. In the present study, *Campylobacter* spp. were found in 60.9% (64/105) of commercial chicken cuts samples. *C. jejuni* were found in 28.1% (18/64) of positive samples and *C. coli* were also found in 28.1% (18/64). The results of this study showed that PCR-based detection and identification to the species level of *Campylobacters* directly from chicken meat enrichment is possible for food analysis laboratories or epidemiological studies. Furthermore, highlighted the importance of chicken meat as a potential source of *Campylobacter* transmission to humans, due to the significant number of positive samples found.

Keywords: *Campylobacter* spp. Chilled chicken cuts. Detection. Food safety. PCR

Highlights

Campylobacter spp. were found in 60.9% of chicken cuts samples.
C. jejuni or *C.coli* were found in the same concentraton (28.1%) of positive samples.
C. jejuni and *C.coli* were found simultaneously in 12.5% of positive samples.
PCR-based identification of *Campylobacters* directly from enrichment is possible.
Chicken meat is a potential source of *Campylobacter* transmission to humans.

1 Introduction

Campylobacter is a spiral formed, Gram-negative, essentially microaerophilic, growing best in an atmosphere containing approximately 10% CO_2 and approximately 5% O_2. Human pathogenic species are classified as thermophilic campylobacters with ability to grown in maximum temperature of 46 °C and a minimum of 30 °C (HUMPHREY et al., 2007).

Campylobacteriosis in humans is mainly caused by *Campylobacter jejuni* and *Campylobacter coli*. Other *Campylobacter* species (eg, *C. lari, C. upsaliensis, C. fetus*) are also reported to cause disease in humans, but the number of these non-jejuni/coli infections worldwide is a small fraction of all *Campylobacter* infections (WAGENAAR et al., 2013). The disease is usually self-limiting, and antimicrobial treatment is only indicated in severe cases. Infection with *C. jejuni* is the most common predisposing fator to the peripheral neuropathies Guillain–Barré (GBS) and Miller Fisher Syndromes (HUMPHREY et al., 2007).

Horizontal transmission is generally considered the most significant cause of *Campylobacter* infection in broiler flocks (BULL et al., 2006). The possibility of vertical transmission can not be excluded, although it is controversial and subject of discussion (COX et al., 2012). Contamination of poultry carcasses by *Campylobacter* during processing occurs directly via intestinal contents or indirectly from bird to bird, via equipment and water (ZENDEHBAD et al., 2013).

The main sources of human infection by *Campylobacter* is the consumption of contaminated food, water, undercooked chicken, unpasteurized milk, direct contact with infected animals, and cross-contamination from the environment, notably in kitchens (BURNETT et al., 2002; GARIN et al., 2012).

Chicken meat is widely consumed worldwide as it supplies high quality protein with low price. In Brazil the *per capita* consumption of chicken is 41.8kg *per* person (UBA, 2014). Although the great production and consumption there are limited data available on *Campylobacter* occurrence in Brazil.

The demanding growth requirements of campylobacters make their microbiological detection and identification a time-consuming and delicate task (GONZALEZ et al., 1997). *Campylobacter* are asaccharolytic, fastidious bacteria, and this limits the available phenotypic tests by which isolates may be differentiated. Identification to species level is hindered by variations in methodology and the subjective interpretation of biochemical test results. There are also isolates with atypical phenotypes. The differentiation of *C. jejuni* from *C. coli* relies on the ability of *C. jejuni* to hydrolyze hippurate, but certain atypical *C. jejuni* strains fail to do so, rendering identification based on this single test unreliable (LINTON et al., 1997). *C. jejuni* and *C. coli* share many clinical characteristics, and thus the precise identification and differentiation between these thermophilic enteropathogens represents a crucial step for the definition of their disease spectrum, as well as for detection and epidemiological purposes (BURNETT et al., 2002).

Limitations in *Campylobacter* culture and differentiation might be overcome by the use of PCR-based method. On this basis, the aim of this study was to identify *Campylobacter* spp., *C. jejuni* and *C. coli* in chicken meat purchased from retail supermarket in Londrina, Paraná State, Brazil, directly from enrichment by the use of PCR-based methods avoiding the need of phenotypic identification.

2 Material and methods

2.1 Samples collection

From March to June 2014, 105 chilled chicken cuts samples in their original packaging were randomly purchased from retail supermarket in Londrina, Paraná State, Brazil. Each sample was produced at slaughterhouses with Federal Inspection Service (SIF) of the Ministry of Agriculture, Livestock and Supply (MAPA, Brazil) and belonged to different production batch number. The samples were within

the validity period and stored under refrigeration at temperatures between 2 °C to 6 °C. It was transported to the laboratory in a cooler with ice packs and kept in a cold chamber at 4 °C until analysis, which did not exceed 24 h after purchase.

2.2 Detection of *Campylobacter* in chicken samples

All samples were processed by using aseptic techniques. For the detection of thermophilic *Campylobacter* spp., a 25 g aliquots of chicken skin cuts were placed in separate sterile plastic bags and 25 mL of buffered peptone water broth (Acumedia Lansing, Michigan, USA) was added. The mixture was then massaged by hand for 30 s. Selective enrichment for *Campylobacter* were carried out by adding aliquots of 5 mL of chicken rinses to 45 mL of Bolton Broth (CM 0983, Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with Modified Bolton Broth Selective Supplement SR0208E (Oxoid) (cefoperazone, 10 mg/500 mL; vancomycin, 10 mg/500 mL, trimethoprim 10 mg/500 mL and amphotericin B, 5 mg/500 mL) and incubated at 42 °C for 48 h under microaerobic conditions created with the Microaerobac system (Probac, São Paulo, SP, Brazil). Then, aliquots of 0.1 mL of selective enrichment were inoculated on Bolton (Oxoid) Agar supplemented with Modified Bolton Broth Selective Supplement SR0208E (Oxoid) (cefoperazone, 10 mg/500 mL, vancomycin, 10 mg/500 mL, trimethoprim 10 mg/500 mL and amphotericin B, 5 mg/500 mL). Plates were incubated at 42 °C for 44 h under microaerobic conditions created by the Microaerobac system (Probac). Presumptive identification of *Campylobacter* was performed using PCR assay.

2.3 DNA extraction and purification

Aliquots of 1 mL of selective enrichment of *Campylobacter* spp. were centrifuged at 16.000 × *g* for 10 min. The sediment was washed with 1 mL of 1.0% (w/v) buffered peptone water (Acumedia) and centrifuged for 10 min at 16.000 × *g*. The suspect colonies from Bolton agar plates were transferred to microtube with 1 mL of 1.0% (w/v) buffered peptone water (Acumedia) and centrifuged for 10 min at

16.000 × *g*. The pellets ware resuspended in 300 µL of solution of Triton X-100 (Nuclear) 1%. The suspensions were heated at 100 °C for 10 min, cooled in ice, and centrifuged at 16,000 × *g* for 5 min. The sediment was discarded and the supernatant containing DNA purified. For DNA purification, 300 µL of a solution of phenol, chloroform and isoamyl alcohol (25:24:1) was added to the supernatant. The mixture was shaken by inversion and centrifuged at 13,000 x *g* for 10 min. The aqueous phase was transferred to a new tube, to which was added 30 µL of sodium acetate (3M) and 270 µL of absolute ethanol. The tubes were kept for 12 min at -80 °C, and centrifuged at 13,000 x *g* for 10 min. The pellet was washed with 70% ethanol, and centrifuged again as described above. The DNA was dried at 37°C and dissolved in 50 µL of sterile ultrapure water. This experiment step was performed in two independent genuine replicates

2.4 PCR for detection of *Campylobacter* spp.

The OT1559 (5'-CTGCTTAACACAAGTTGAGTAGG-3') (UYTTENDAELE et al., 1994; LÜBECK et al., 2003) and 18-1 (5'-TTCCTTAGGTACCGTCAGAA-3') (VANCAMP et al., 1993; DOCHERTY et al., 1996; LÜBECK et al., 2003) primers, which are specific for the genus *Campylobacter* and amplify a 287 bp sequence of the 16S rRNA gene, were used for the detection of *Campylobacter* by PCR.

The final volume of the reaction was 20 µL, containing 5 µl of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen Brazil, São Paulo, SP, Brazil), 5.0 mM of $MgCl_2$ (Invitrogen^{TM} Life Technologies, Alameda, CA, USA), 0.2 mM of dNTP (Invitrogen^{TM}), 0.25 µM each of OT1559 (Invitrogen^{TM}) and 18-1 (Invitrogen^{TM}) primers, and 1 U of *Taq* DNA Polymerase (Invitrogen^{TM}).

The PCR reactions were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 30 s at 60 °C, 45 s at 72 °C and 5 min of final extension at 72 °C.

Samples positives for *Campylobacter* spp. this assay, were used for differentiation of *C. jejuni* and *C.coli* by specific PCR assays described at 2.5 and 2.6 items.

PCR reactions were carried out in two replicates. All reactions were performed with negative (ultrapure water) and positive controls (*C. jejuni* ATCC 33291 or *C. coli* CCAMP 1008, strain obtained from the Oswaldo Cruz Foundation (FIOCRUZ) Collection, Rio de Janeiro, Brazil).

2.5 PCR for detection of *Campylobacter jejuni*

The HIP400 (5'-GAAGAGGGTTTGGGTGGTG-3') and HIP1134 (5'-AGCTAGCTTCGCATA ATAACTTG-3') primers (LINTON et al., 1997), which are specific for *Campylobacter jejuni* and amplify a 735 bp sequence of the *hipO* gene, were used for the PCR.

The final volume of the reaction was 20 µL, containing 5 µl of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen^{TM}), 2.5 mM of $MgCl_2$ (Invitrogen^{TM}), 0.2 mM of dNTP (Invitrogen^{TM}), 0.4 µM each of HIP400/HIP1134 (Invitrogen^{TM}) primers, and 0.5 U of *Taq* DNA Polymerase (Invitrogen^{TM}).

The PCR reactions were carried out with the following conditions: an initial denaturation step at 94 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 1min at 66 °C, 1min at 72 °C and 5 min of final extension at 72 °C.

2.6 PCR protocols for detection of *Campylobacter coli*

The COL1 (5'-ATGAAAAAATATTTAGTTTTTGCA-3') and COL2 (5'-TTATTATTTGTAGCAGCG-3') primers (GONZALEZ et al., 1997), which are specific for *Campylobacter coli* and amplify a 984 bp sequence of the *ceuE* gene, were used for the PCR.

The final volume of the reaction was 20 µL, containing 5 µl of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen^{TM}), 3.5 mM

of $MgCl_2$ (Invitrogen™), 0.2 mM of dNTP (Invitrogen™), 1.0 µM each of COL1/COL2 (Invitrogen™) primers, and 0,5 U of *Taq* DNA Polymerase (Invitrogen™).

The PCR reactions were carried out in with the following conditions: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 30 s at 57 °C, 1 min at 72 °C and 5 min of final extension at 72 °C.

2.7The analysis of PCR amplification products

The PCR amplification products were visualized on a 1.5% (w/v) agarose gel containing 0.02 µL/mL of SYBR® Safe 10,000x in DMSO (Invitrogen™), viewed under ultraviolet light (UV) and photo-documented. A DNA fragment of 100 bp (Invitrogen™) was used as a molecular size marker.

3 Results and discussion

In the present study, *Campylobacter* spp. were detected in 60.9% (64/105) of commercial chicken cuts samples analyzed. The prevalence of *C. jejuni* in 64 chicken meat samples positive to *Campylobacter* spp. was 28.1% (18/64). The same percentage of positivity 28.1% (18/64) was obtained for *C. coli. C. jejuni* and *C.coli* were found simultaneously in 12.5% (8/64) of samples contaminated to *Campylobacter* spp. Identification to the species level is an important step in epidemiological studies of *Campylobacter* prevalence in chicken meat.

Although Brazil is a major worldwide exporter of chicken (UBA, 2014) few information about contamination of this product by *Campylobacter* spp. is available. In the period between the years 2000 to 2013, *Campylobacter* spp. were identified in only three outbreaks in Brazil (BRAZIL, 2013b). The prevalence of *Campylobacter* spp. in chicken meat obtained in this study is higher than in previous studies conducted in different regions of Brazil which reported percentages of contamination ranging from 27% to 56% (AZEREDO et al., 2010; MAZIERO e OLIVEIRA, 2010; ALVES e OLIVEIRA, 2013; OLIVEIRA e OLIVEIRA, 2013).

Campylobacter it is the most common couse form of food poisoning in the UK, affecting an estimated 280,000 people a year. The majority of these cases come from contaminated poultry. The Food Standards Agency (FSA) is making a survey, running from February 2014 to February 2015, looking at the prevalence and levels of *Campylobacter* contamination on fresh whole chilled chickens and their packaging. The survey will test 4,000 samples of whole chickens bought from UK retail outlets and smaller independent stores and butchers. In August 2014, the FSA has published the first set of quarterly results (representing 853 samples) from a new survey of *Campylobacter* on fresh shop-bought chickens. The results show 59% (501/853) of birds tested positive for the presence of *Campylobacter* with 16% (137/853) of birds tested with the highest level of contamination (>1000 CFU/g). Tests of the packaging showed 4% (37/853) of samples were positive for the presence of *Campylobacter* (FSA, 2014).

It has been shown that the avian intestinal tract is a favorable environment for *Campylobacter* colonization. This may lead to contamination of carcasses during processing, especially at the defeathering, evisceration, and chilling stages (RAHIMI e AMERI, 2011). Commercial chicken meat has been identified as one of the most important food vehicles of *Campylobacter* to humans (WHO, 2009).

Attempts to reduce the presence of *Campylobacter* in chicken have shown limited results because the biosecurity procedures used, for the control of *Campylobacter,* on farms are deficient and difficult to maintain. Despite the Brazilian Legislation which demands hygienic-sanitary control and biosecurity in poultry farms (BRAZIL, 2013a). Standards for *Campylobacter* in food has been not already established (BRAZIL, 2001).

Cross-contamination due to improper food handling at home is considered an important source of infection (MATTICK et al., 2003). National Agency for Sanitary Surveillance (ANVISA) edited a RDC Resolution nº 13 on January, 2001 with guidelines about preparation and storage which must appear on the labels of these products. Following these guidelines is considered a critical control point, which can help to prevent human infection with *Campylobacter* spp.

Diferentiation of *C. jejuni* from other *Campylobacter* spp. has relied on its ability to hydrolyze hippurate, although about 10% of *C. jejuni* isolates do not hydrolyze this metabolite (BURNETT et al., 2002). The ability to differentiate *C. jejuni*

from *C. coli* is particularly important because erythromycin-based treatments are not adequate if the enteritis is caused by *C. coli*, which may be more likely to be resistant to this antibiotic. Discriminatory assays would be particularly useful in outbreak situations in which large numbers of specimens need to be handled quickly so that the appropriate therapy can be instituted (GONZALEZ et al., 1997).

In conclusion, the results of this study showed that PCR-based detection and identification to the species level of *Campylobacters* directly from chicken meat enrichment is possible and also highlighted the importance of chicken meat as potential food vehicles of *Campylobacter jejuni e C. coli*. The data presented here are promising enough to warrant further evaluation of the assay in routine food analysis laboratories or epidemiological studies.

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References

ALVES, J.; OLIVEIRA, T. C. R. M. D. Presença de Campylobacter spp. em cortes refrigerados de frango. **Semina: Ciências Agrárias,** v. 34, n. 6, p. 2829-2835, 2013.

AZEREDO, L. I.; LUCHESE, R. H.; LAURIA-FILGUEIRAS, A. L. *Campylobacter* spp em carne de ave crua: avaliação da etapa de resfriamento. . **Revista do Instituto Adolfo Lutz,** v. 4, n. 69, p. 518-524, 2010.

BRAZIL. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 12, de 02 de janeiro de 2001. Regulamento Técnico sobre padrões microbiológicos para alimentos. Anvisa - Agência Nacional de Vigilância Sanitária. Diário Oficial da União. Poder Executivo, Seção I, p. 45-53., 2001.

______. Ministério da Agricultura, Pecuária e Abastecimento. Plano de contingência para Influenza Aviária e Doença de Newcastle, versão 1.4. Brasília: Ministério da Agricultura, Pecuária e Abastecimento., 2013a. Disponível em: <

http://www3.servicos.ms.gov.br/iagro_ged/pdf/1906_GED.pdf >. Acesso em: 30 maio 2013.

______. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Coordenação Geral de Doenças Transmissíveis. Vigilância Epidemiologica das Doenças Transmitidas por Alimentos. 2013b. Disponível em: < http://www2.camara.leg.br/atividade-legislativa/comissoes/comissoes-permanentes/cmads/audiencias-publicas/audiencia-publica-2013/crueldade-a-que-os-animais-de-producao-sao-expostos-em-abatedouros-municipais/apresentacoes >. Acesso em: 20 julho 2014.

BULL, S. A.; ALLEN, V. M.; DOMINGUE, G.; JØRGENSEN, F.; FROST, J. A.; URE, R.; WHYTE, R.; TINKER, D.; CORRY, J. E. L.; GILLARD-KING, J.; HUMPHREY, T. J. Sources of Campylobacter spp. Colonizing Housed Broiler Flocks during Rearing. **Applied and Environmental Microbiology,** v. 72, n. 1, p. 645-652, January 1, 2006 2006.

BURNETT, T. A.; HORNITZKY, M. A.; KUHNERT, P.; DJORDJEVIC, S. P. Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays. **FEMS Microbiology Letters,** v. 216, n. 2, p. 201-209, 2002.

COX, N. A.; RICHARDSON, L. J.; MAURER, J. J.; BERRANG, M. E.; FEDORKA-CRAY, P. J.; BUHR, R. J.; BYRD, J. A.; LEE, M. D.; HOFACRE, C. L.; O'KANE, P. M.; LAMMERDING, A. M.; CLARK, A. G.; THAYER, S. G.; DOYLE, M. P. Evidence for Horizontal and Vertical Transmission in *Campylobacter* Passage from Hen to Her Progeny. **Journal of Food Protection,** v. 75, n. 10, p. 1896–1902, 2012.

DOCHERTY, L.; ADAMS, M. R.; PATEL, P.; MCFADDEN, J. The magnetic immuno-polymerase chain reaction assay for the detection of *Campylobacter* in milk and poultry. **Letters in Applied Microbiology,** v. 22, p. 288-292, 1996.

FSA. Food Standards Agency. *Campylobacter* survey results published. 2014. Disponível em: < http://food.gov.uk/news-updates/news/2014/aug/campylobacter-survey#.U-HG6vldWb8 >. Acesso em: 3 agosto 2014.

GARIN, B.; GOUALI, M.; WOUAFO, M.; PERCHEC, A.-M.; THU, P. M.; RAVAONINDRINA, N.; URBÈS, F.; GAY, M.; DIAWARA, A.; LECLERCQ, A.; ROCOURT, J.; POUILLOT, R. Prevalence, quantification and antimicrobial resistance of *Campylobacter* spp. on chicken neck-skins at points of slaughter in 5 major cities located on 4 continents. **International Journal of Food Microbiology,** v. 157, n. 1, p. 102-107, 2012.

GONZALEZ, I.; GRANT, K. A.; RICHARDSON, P. T.; PARK, S. F.; COLLINS, M. D. Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceuE* gene encoding a putative virulence determinant. **Journal of Clinical Microbiology,** v. 35, n. 3, p. 759-63, March 1, 1997 1997.

HUMPHREY, T.; O'BRIEN, S.; MADSEN, M. Campylobacters as zoonotic pathogens: A food production perspective. **International Journal of Food Microbiology,** v. 117, p. 237-257, 2007.

LINTON, D.; LAWSON, A. J.; OWEN, R. J.; STANLEY, J. PCR Detection, Identification to Species Level, and Fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* Direct from Diarrheic Samples. **Journal of Clinical Microbiology,** v. 35, n. 10, p. 2568–2572, 1997.

LÜBECK, P. S.; WOLFFS, P.; ON, S. L. W.; AHRENS, P.; RADSTRÖM, P.; HOORFAR, J. Toward an International Standard for PCR-Based Detection of Food-Borne Thermotolerant Campylobacters: Assay Development and Analytical Validation. **Applied and Enviromental Microbiology,** v. 69, n. 9, p. 5664-5669, 2003.

MATTICK, K.; DURHMAN, K.; DOMINIGUE, G.; JØRGENSEN, F.; SEN, M.; SCHAFFNER, D. W.; HUMPHREY, T. The survival of foodborne pathogens during domestic washing up and subsequent transfer onto washing-up sponges, kitchen surfaces, and food. **International Journal of Food Microbiology,** v. 85, n. 3, p. 213-226, 2003.

MAZIERO, M. T.; OLIVEIRA, T. C. R. M. Efect of refrigeration and frozen storage on the *Campylobacter jejuni* recovery from naturally contaminated broiler carcasses. **Brazilian Journal of Microbiology** v. 41, p. 501-505, 2010.

OLIVEIRA, A. D. L.; OLIVEIRA, R. B. P. D. Enumeração de Campylobacter spp. e presença de Campylobacter jejuni em carcaças de frango no estado de Minas Gerais. **Ciência Rural,** v. 43, p. 480-484, 2013.

RAHIMI, E.; AMERI, M. Antimicrobial resistance patterns of *Campylobacter* spp. isolated from raw chicken, turkey, quail, partridge, and ostrich meat in Iran. **Food Control,** v. 22, n. 8, p. 1165-1170, 2011.

UBA. União Brasileira de Avicultura. Annual Report - Brazilian Poultry Association. 2014. Disponível em: < http://www.ubabef.com.br/files/publicacoes/8ca705e70f0cb110ae3aed67d29c8842.pdf >. Acesso em: 20 maio 2014.

UYTTENDAELE, M.; SCHUKKINK', R.; GEMEN', B. V.; DEBEVERE, J. Identification of *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBAR. **Journal of Applied Bacteriology,** v. 77, p. 694-701, 1994.

VANCAMP, G.; FIERENS, H.; VANDAMME, P.; GOOSENS, H.; HUYGHEBAERT, A.; WACHTER, R. D. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chain reaction based on 16s rRNA genes. **Systemic and Applied Microbiology,** v. 16, p. 30-36, 1993.

WAGENAAR, J. A.; FRENCH, N. P.; HAVELAAR, A. H. Preventing *Campylobacter* at the source: why is it so difficult? **Clinical Infectious Diseases** v. 57, n. 11, p. 1600-6, Dec 2013.

WHO. World Health Organization. FAO/WHO Expert Meeeting on *Salmonella* and *Campylobacter* in chicken meat, 4 – 8 May 2009. 2009. Disponível em: < http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html >. Acesso em: 18 jul. 2014.

ZENDEHBAD, B.; ARIAN, A. A.; ALIPOUR, A. Identification and antimicrobial resistance of Campylobacter species isolated from poultry meat in Khorasan province, Iran. **Food Control,** v. 32, n. 2, p. 724-727, 2013.

5.3 Artigo 3 – Evaluation of uniplex and multiplex PCR assays for detection of *Campylobacter* spp. and *Salmonella* spp. in milk

Running title: PCR assays for *Campylobacter* spp. and *Salmonella* spp.

Juliane Alves* **and Tereza Cristina Rocha Moreira de Oliveira**

Department of Food Science and Technology, State University of Londrina, Brazil. Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86057-970, Londrina, Paraná, Brazil.

***Corresponding author:**

Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86.057-970, Londrina, Paraná, Brasil.
Tel:+55-43-3371-5968; Fax:+55-43-3371-4080;
E-mail: julianealves@yahoo.com.br

Abstract

Conventional methods for the detection of *Campylobacter* and *Salmonella* based on culturing are time consuming and laborious. The control of these pathogens in milk is essential for preventing human disease. The aim of this study was to develop PCR assay for the detection of *Campylobacter* spp. and *Salmonella* spp. in milk. Boiling was used for DNA extraction followed by nucleic acid purification with phenol-chloroform. The specificity of the assay was 100%. After a 24 hours of enrichment the PCR assay detected 1 CFU of *Campylobacter* spp. and *Salmonella* spp. *per* 5 mL and 25 mL aliquots respectively. The developed assay is relatively inexpensive and could be suitable for a rapid routine testing of these two pathogenic bacteria in milk.

Keywords: Food-borne pathogens. PCR. Pathogen detection. Milk. *Campylobacter. Salmonella.*

1 Introduction

Campylobacter spp. and *Salmonella* spp. are the most common causes of bacterial foodborne disease in several countries (WHO, 2009). Although chicken and pork are the major animal sources of *Salmonella*, milk and beef can not be excluded as an important cause of human salmonellosis (NIELSEN et al., 2012). Cattle frequently harbour *Campylobacter* as commensals in their gastrointestinal tract and campylobacters in raw milk most commonly derive from secondary faecal contamination during the milking process (HEUVELINK et al., 2009).

Conventional methods for the detection of *Campylobacter* and *Salmonella,* based on culturing, are time consuming and laborious (CANDRIAN, 1995; OMICCIOLI et al., 2009). The use of a rapid and specific method to detect these pathogens is essential considering the perishable nature of milk.

Recently developed molecular methods to detect and quantify pathogenic microorganisms in foods are faster, more sensitive and more specific than classical microbiological techniques (BOYER e COMBRISSON, 2013). However, matrix-based PCR inhibitors can cause false negative results and remain an issue to overcome when analyzing most food samples. Dairy products are a notoriously complex matrix containing lipids and proteins that can influence the outcome of PCR (LUSK et al., 2013).

The rapid detection of *Campylobacter* spp. and *Salmonella* spp. in milk allows the identification of sources of contamination and facilities dairy industry to take appropriate measures to prevent the distribution of contaminated milk and foodborne outbreaks.

The aim of this study was to develop PCR protocols for the individual and simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in milk using TZ lises solution and boiling for DNA extraction followed by nucleic acid purification with phenol-chloroform.

2 Material and Methods

2.1 Preparation of *S.* Enteritidis ATCC 13076 and *C. jejuni* ATCC 33291 suspensions

Pasteurized milk sample was spiked using bacterial suspensions prepared as described following. A serial dilution was prepared in 1% (w/v) sterile buffered peptone water (Acumedia) to obtain suspensions containing 1 to 10^6 CFU of *S.* Enteritidis ATCC 13076 or *C. jejuni* ATCC 33291 *per* mL. The bacterial density were estimated by the drop plate method for counting bacteria (MILES et al., 1938). The bacterial suspensions of *S.* Enteritidis ATCC 13076 were subcultured on Xylose Lysine Sodium Deoxycholate (XLD) plates (Acumedia) and were incubated at 37 °C for 24 h. The suspensions of *C. jejuni* ATCC 33291 were subcultured on Bolton Agar [Bolton Broth (CM 0983, Oxoid) plus 2.0% (w/v) agar-agar (HiMedia, Mumbai, India)] and incubated at 42 °C for 48 h under microaerobic conditions created with the Microaerobac system (Probac). Experiments were conducted in duplicate.

2.2 Contamination of milk

Pasteurized milk samples were previously analyzed to rule out prior contamination with *Campylobacter* spp. and *Salmonella* spp. Aliquots of 25 mL of milk were inoculated with suspensions of *S.* Enteritidis ATCC 13076 and aliquots of 5 mL of milk were inoculated with suspensions of *C. jejuni* ATCC 33291 (prepared as described in item 2.1) to obtain approximately 1 to 10^6 CFU per samples. The 25 mL of milk spiked with different concentration of *S.* Enteritidis ATCC 13076 were added to 225 mL of sterile buffered peptone water (Acumedia) and incubated at 37 ° C for 24 h. The 5 mL of milk spiked with different concentrations of *C. jejuni* ATCC 33291 were added to 45 mL of Bolton Broth (Oxoid) supplemented with Modified Bolton Broth Selective Supplement SR0208E (Oxoid) (cefoperazone, 10 mg/500 mL; vancomycin, 10 mg/500 mL, trimethoprim 10 mg/500 mL and amphotericin B, 5 mg/500 mL) and incubated at 42 °C for 24h under microaerobic conditions created

with the Microaerobac system (Probac). This experiment was performed in two independent genuine replicates.

2.3 DNA extraction

Aliquots of 1 mL of selective enrichments of *Campylobacter* spp. and 1 mL of non-selective enrichments of *Salmonella* spp., obtained from spiked milk samples, were centrifuged at 16,000 ×*g* for 10 min. The sediment was washed with 1 mL of 1.0% (w/v) buffered peptone water (Acumedia) and centrifuged for 10 min at 16,000 × *g*. the pellet was resuspended in 300 µL of TZ lysis solution (ABOLMAATY et al., 2000). The suspensions were heated at 100 °C for 10 min, cooled in ice, and centrifuged at 16.000 × *g* for 5 min. The sediment was discarded and the supernatant containing DNA was used in PCR and purification step.

2.4 DNA purification

For DNA purification, 300 µL of a solution of phenol, chloroform and isoamyl alcohol (25:24:1) was added to the supernatant. The mixture was shaken by inversion and centrifuged at 13.000 x *g* for 10 min. The aqueous phase was transferred to a new tube, to which was added 30 µL of sodium acetate (3M) and 270 µL of ethanol. The tubes were kept for 2 h at -20 °C, and centrifuged at 13.000 x *g* for 10 min. The pellet was washed with 70% ethanol, and centrifuged again as described above. The DNA was dried at 37°C and dissolved in 50 µL of sterile ultrapure water. This experiment step was performed in two independent genuine replicates

2.5 PCR identification

The PCR assays were carried out in two replicates. All reactions were performed with negative (ultrapure water) and positive controls (*C. jejuni* ATCC 33291 or *S.* Enteritidis ATCC 13076).

2.5.1 PCR for detection of *Campylobacter* spp.

The OT1559 (5'-CTGCTTAACACAAGTTGAGTAGG-3') (UYTTENDAELE et al., 1994; LÜBECK et al., 2003) and 18-1 (5'-TTCCTTAGGTACCGTCAGAA-3') (VANCAMP et al., 1993; DOCHERTY et al., 1996; LÜBECK et al., 2003) primers, which are specific for the genus *Campylobacter* and amplify a 287 bp sequence of the 16S rRNA gene, were used for the PCR.

The final volume of the reaction was 20 µL, containing 4 µl of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen Brazil, São Paulo, SP, Brazil), 5.0 mM of $MgCl_2$ (Invitrogen™ Life Technologies, Alameda, CA, USA), 0.2 mM of dNTP (Invitrogen™), 0.25 µM each of OT1559 (Invitrogen™) and 18-1 (Invitrogen™) primers, and 1 U of *Taq* DNA Polymerase (Invitrogen™).

2.5.2 PCR for detection of *Salmonella* spp.

The Styinva-JHO-2-left (5'-TCGTCATTCCATTACCTACC-3') and Styinva-JHO-2-right (5'-AAACGTTGAAAAACTGAGGA-3') primers (HOORFAR et al., 2000), which are specific for the genus *Salmonella* and amplify a 119 bp sequence of the *InvA* gene, were used for the PCR.

The final volume of the reaction was 20 µL, containing 4 µL of DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen Brazil, São Paulo, SP, Brazil), 5.0 mM of $MgCl_2$ (Invitrogen™), 0.2 mM of dNTP (Invitrogen™), 0.3 µM each of Styinva-JHO-2-left and Styinva-JHO-2-right primers, and 1 U of *Taq* DNA Polymerase (Invitrogen™).

2.5.3 mPCR assay for detection of *Campylobacter* spp. and *Salmonella* spp.

The OT1559 and 18-1 primers for the genus *Campylobacter* and the Styinva-JHO-2-left and Styinva-JHO-2-right primers for the genus *Salmonella* were used for the mPCR.

The final volume of the reaction was 20 µL, containing 4 µl of each DNA template, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; (Invitrogen™),

10 mM of $MgCl_2$ (Invitrogen™), 0.4 mM of dNTP (Invitrogen™), 0.3 µM each of Styinva-JHO-2-left and Styinva-JHO-2-right primers (Invitrogen™) 0.25 µM each of OT1559 primers (Invitrogen™), and 2.0 U of *Taq* DNA Polymerase (Invitrogen™).

2.5.4 The PCR amplification conditions

The PCR reactions were carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 30 s at 60 °C, 45 s at 72 °C and 5 min of final extension at 72 °C.

2.5.5 The analysis of PCR amplification products

The PCR amplification products were visualized on a 1.5% (w/v) agarose gel containing 0.02 µL/mL of SYBR® Safe 10,000x in DMSO (Invitrogen™), viewed under ultraviolet light (UV) and photo-documented. A DNA fragment of 100 bp (Invitrogen™) was used as a molecular size marker.

3 Results and discussion

In this study, the uniplex and multiplex PCR assays were able to detect 1 CFU of each of these pathogens in 5 mL and 25 mL aliquots of milk after the 24 hours of the selective enrichment of *Campylobacter* spp. and non-selective enrichment of *Salmonella* spp., respectively. The multiplex reaction was tested efficiently even when was used high counts (10^6 CFU) of a target and low (1 CFU) of the other.

Milk samples contaminated with *Salmonella* and *Campylobacter* were also analyzed without enrichment. However, in these conditions, it was possible to detect *Campylobacter* by PCR only in milk samples contaminated with 10^6 CFU/5mL. *Salmonella* and *Campylobacter* were detect by PCR in all samples using

only the TZ lysis solution followed by boiling after 24 hours of enrichment. However, the quality of amplification was not satisfactory, probably due to the food matrix interference in the amplification of template. Therefore, the purification of DNA samples with phenol / chloroform was performed. The extraction using TZ lysis solution follow by purification allowed the amplification of DNA samples in uniplex and multiplex reaction with high quality.

The PCR assay used has high specificity (ALVES et al., 2012), and there are no cross-reactions occurred with background flora. All the samples spiked with *Salmonella* and *Campylobacter* cells generated the specific amplification, thus indicating that PCR inhibitors from milk were efficiently removed by the DNA extraction procedure. The template preparation method from milk, developed in this study, was very effective for obtaining high yields of DNA, suitable for the PCR assay. Further, this method is cost-effective and rapid.

TZ lysis solution (ABOLMAATY et al., 2000), followed by boiling, was used in this study for DNA extraction. TZ lysis solution was efficient for the extraction of DNA from *Campylobacter* spp. and *Salmonella* spp. in contaminated milk samples. According to Abolmaaty *et al.* (2000), the addition of sodium azide in the lysis solution was more efficient for the DNA extraction of *S*. Enteritidis than extraction using only Triton X-100. Other studies showed the same efficiency of TZ lysis solution (ABOLMAATY et al., 2000; DUODU et al., 2009; LEE e LEVIN, 2009; LUO et al., 2010); however, the mechanism by which sodium azide enhances DNA extraction has not been elucidated (ABOLMAATY et al., 2000).

PCR use for detection of pathogenic microorganisms in food is an alternative that has several advantages over conventional methods, such as speed and the possibility of working with fastidious bacteria. However, the sensitivity of this technique to direct analysis is not sufficient and a sample enrichment is mandatory. Several studies (JOFRÉ et al., 2005; CORTEZ et al., 2006; KATZAV et al., 2008; GERMINI et al., 2009; FREITAS et al., 2010; RAHIMI et al., 2010; ALVES et al., 2012) have observed the need of enrichment use. Although enrichment increases the analyse time, it provides essential benefits, such as diluting the effects of inhibitors, allowing the differentiation of viable from non-viable cells and allowing the repair of cells stressed or injured during food processing, increasing the number of cells (MALORNY et al., 2009).

The developed uniplex and multiplex PCR method and the template preparation method are relatively inexpensive and specific method to detect *Salmonella* spp. and *Campylobacter* spp. after a 24 hours of enrichment, which significantly reduced the detection time of these two bacteria in milk. The results suggest that the method has the potencial to be use in routine diagnosis of those bacteria in dairy industry.

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References

ABOLMAATY, A.; VU, C.; OLIVER, J.; LEVIN, R. E. Development of a new lysis solution for releasing genomic DNA from bacterial cells for DNA amplification by polymerase chain reaction. **Microbios,** v. 101, n. 400, p. 181-189, 2000.

ALVES, J.; MARQUES, V. V.; PEREIRA, L. F. P.; HIROOKA, E. Y.; DE OLIVEIRA, T. C. R. M. Multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. **Journal of Food Safety,** v. 32, n. 3, p. 345-350, 2012.

BOYER, M.; COMBRISSON, J. Analytical opportunities of quantitative polymerase chain reaction in dairy microbiology. **International Dairy Journal,** v. 30, n. 1, p. 45-52, 2013.

CANDRIAN, U. Polimerase chain reaction in food microbiology **Journal of Microbiological Methods,** v. 23, p. 89-103, 1995.

CORTEZ, A. L. L.; CARVALHO, A. C. F. B.; IKUNO, A. A.; BÜRGER, K. P.; VIDAL-MARTINS, A. M. C. Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex-PCR. **Research in Veterinary Science,** v. 81, p. 340-344, 2006.

DOCHERTY, L.; ADAMS, M. R.; PATEL, P.; MCFADDEN, J. The magnetic immuno-polymerase chain reaction assay for the detection of *Campylobacter* in milk and poultry. **Letters in Applied Microbiology,** v. 22, p. 288-292, 1996.

DUODU, S.; MEHMETI, I.; HOLST-JENSEN, A.; LONCAREVIC, S. Improved Sample Preparation for Real-Time PCR Detection of *Listeria monocytogenes* in Hot-Smoked Salmon using Filtering and Immunomagnetic Separation Techniques. **Food Analytical Methods,** v. 2, p. 23-29, 2009.

FREITAS, C. G. D.; SANTANA, Â. P.; SILVA, P. H. C. D.; GONÇALVES, V. S. P.; BARROS, M. D. A. F.; TORRES, F. A. G.; MURATA, L. S.; PERECMANIS, S. PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat **International Journal of Food Microbiology,** v. 139, n. 1-2, p. 15-22, 2010.

GERMINI, A.; MASOLA, A.; CARNEVALI, P.; MARCHELLI, R. Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. **Food Control,** v. 20, p. 733-738, 2009.

HEUVELINK, A. E.; VAN HEERWAARDEN, C.; ZWARTKRUIS-NAHUIS, A.; TILBURG, J. J. H. C.; BOS, M. H.; HEILMANN, F. G. C.; HOFHUIS, A.; HOEKSTRA, T.; DE BOER, E. Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. **International Journal of Food Microbiology,** v. 134, n. 1–2, p. 70-74, 2009.

HOORFAR, J.; AHRENS, P.; RÅDSTRÖM, P. Automated 5' Nuclease PCR Assay for Identification of *Salmonella enterica*. **Journal of Clinical Microbiology,** v. 38, n. 9, p. 3429-3435, 2000.

JOFRÉ, A.; MARTIN, B.; GARRIGA, M.; HUGAS, M.; PLA, M.; RODRÍGUEZ-LÁZARO, D.; AYMERICH, T. Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. **Food Microbiology,** v. 22, p. 109-115, 2005.

KATZAV, M.; ISOHANNI, P.; LUND, M.; HAKKINEN, M.; LYHS, U. PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. **Food Microbiology,** v. 25, p. 908-914, 2008.

LEE, J.-L.; LEVIN, R. E. Discrimination of viable and dead Vibrio vulnificus after refrigerated and frozen storage using EMA, sodium deoxycholate and real-time PCR. **Journal of Microbiological Methods,** v. 79, n. 2, p. 184-188, 2009.

LÜBECK, P. S.; WOLFFS, P.; ON, S. L. W.; AHRENS, P.; RADSTRÖM, P.; HOORFAR, J. Toward an International Standard for PCR-Based Detection of Food-Borne Thermotolerant Campylobacters: Assay Development and Analytical Validation. **Applied and Enviromental Microbiology,** v. 69, n. 9, p. 5664-5669, 2003.

LUO, J.-F.; LIN, W.-T.; GUO, Y. Method to detect only viable cells in microbial ecology. **Applied Microbiology and Biotechnology,** v. 86, n. 1, p. 377-384, 2010.

LUSK, T. S.; STRAIN, E.; KASE, J. A. Comparison of six commercial DNA extraction kits for detection of Brucella neotomae in Mexican and Central American-style cheese and other milk products. **Food Microbiology,** v. 34, n. 1, p. 100-105, 2013.

MALORNY, B.; HUEHN, S.; DIECKMANN, R.; KRÄMER, N.; HELMUTH, R. Polymerase Chain Reaction for the Rapid Detection and Serovar Identification of *Salmonella* in Food and Feeding Stuff. **Food Analytical Methods,** v. 2, p. 81-95, 2009.

MILES, A. A.; MISRA, S. S.; IRWIN, J. O. The estimation of the bactericidal power of the blood. **Journal of Hygiene,** v. 38, n. 6, p. 732-49, Nov 1938.

NIELSEN, T. D.; GREEN, L. E.; KUDAHL, A. B.; ØSTERGAARD, S.; NIELSEN, L. R. Evaluation of milk yield losses associated with *Salmonella* antibodies in bulk tank milk in bovine dairy herds. **Journal of Dairy Science,** v. 95, n. 9, p. 4873-4885, 9// 2012.

OMICCIOLI, E.; AMAGLIANI, G.; BRANDI, G.; MAGNANI, M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiology,** v. 26, p. 615-622, 2009.

RAHIMI, E.; MOMTAZ, H.; BONYADIAN, M. PCR detection of *Campylobacter* sp. from turkey carcasses during processing plant in Iran. **Food Control,** v. 21, n. 5, p. 692-694, 2010.

UYTTENDAELE, M.; SCHUKKINK', R.; GEMEN', B. V.; DEBEVERE, J. Identification of *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBAR. **Journal of Applied Bacteriology,** v. 77, p. 694-701, 1994.

VANCAMP, G.; FIERENS, H.; VANDAMME, P.; GOOSENS, H.; HUYGHEBAERT, A.; WACHTER, R. D. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chain reaction based on 16s rRNA genes. **Systemic and Applied Microbiology,** v. 16, p. 30-36, 1993.

WHO. World Health Organization. FAO/WHO Expert Meeeting on *Salmonella* and *Campylobacter* in chicken meat, 4 – 8 May 2009. 2009. Disponível em: < http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html >. Acesso em: 18 jul. 2014.

5.4 Artigo 4 – Development of a multiplex real-time PCR assay with an internal amplification control for detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat

Running title: Multiplex real-time PCR for *Campylobacter* spp. and *Salmonella* spp.

Juliane Alves[*], **Elisa Yoko Hirooka and Tereza Cristina Rocha Moreira de Oliveira**

Department of Food Science and Technology, Londrina State University, Brazil. Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86057-970, Londrina, Paraná, Brazil.

***Corresponding author:**
Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380, Campus Universitário, Caixa Postal 10.011 CEP: 86.057-970, Londrina, Paraná, Brasil.
Tel:+55-43-3371-5968; Fax:+55-43-3371-4080;
E-mail: julianealves@yahoo.com.br

Abstract

Conventional methods for the detection of *Campylobacter* and *Salmonella* based on culturing are time consuming and laborious. The aim of this study was to develop a multiplex real-time PCR assay with an internal amplification control for the simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. Boiling was used for DNA extraction, followed by nucleic acid purification with phenol-chloroform. Assay specificity was 100%, and the detection limit was 10^3 CFU of *Campylobacter* spp. and 10^6 CFU of *Salmonella* spp. per milliliter of spiked chicken meat rinse without an enrichment step. After 24 hours of the selective enrichment of *Campylobacter* spp. and the non-selective enrichment of *Salmonella* spp., the assay sensitivity was 1 CFU of each of these pathogens per milliliter of rinse. To our knowledge, the present study is the first multiplex real-time PCR assay developed for the simultaneous detection of these pathogens with the inclusion of an internal amplification control to monitor PCR inhibitors. The developed assay is a relatively inexpensive and efficient means to detect *Campylobacter* spp. and *Salmonella* spp. in chicken meat after enrichment, and can be a useful alternative in food processing to prevent the distribution of contaminated food.

Keywords: Multiplex real-time PCR. Internal amplification control. *Campylobacter* spp. *Salmonella* spp. Foodborne pathogens. Chicken meat

Highlights

The control of *Salmonella* and *Campylobacter* is essential for food safety.
The developed PCR assay can detect both foodborne bacteria in a chicken meat rinse.
This assay can assist in the implementation of preventive measures for contamination.
It can become an alternative in microbiological analysis with an enrichment step.

1 Introduction

Campylobacter spp. and *Salmonella* spp. are the most common causes of bacterial foodborne disease in several countries, and poultry products are the main vehicles of transmission to humans (WHO, 2009). Brazil is the third largest producer of chicken meat (behind the United States and China) and is the largest exporter of this product (followed by the United States and the European Union). In 2013, Brazil produced 12.30 million tons of chicken, and 31.6% of this was exported. In Brazil, the *per capita* consumption of chicken increased from 29.91 kg in 2000 to 41.8 kg in 2013 (UBA, 2014).

Conventional methods for the detection of *Salmonella* and *Campylobacter,* based on culturing, are time consuming and laborious (CANDRIAN, 1995; OMICCIOLI et al., 2009). A rapid and specific detection method for these pathogens is essential due to the perishable nature of raw chicken meat. Multiplex PCR allow simultaneous amplification of more than one target sequence in a single PCR reaction. A multiplex PCR assay has the potential to produce considerable savings of time, reagent costs and effort, without compromising the results (PERRY et al., 2007; OMICCIOLI et al., 2009). The elimination of postamplification steps

increases the reliability and reproducibility of the assay and decreases the time of analyses (LUND et al., 2004).

Real-time PCR is based on fluorescence measurements during the PCR run. Hydrolysis probes use a short oligonucleotide of 20–30 bases coupled on the 5′ end with a fluorescent reporter dye and on the 3′ end with a quencher dye. The quencher dye absorbs the fluorescence from the reporter, preventing the light signal from reaching the detector. During amplification, the 5′ nuclease activity of the DNA polymerase hydrolyzes the probe bound to the target amplification product. The released reporter dye is no longer quenched and then can be detected (MALORNY et al., 2009).

A problem in real-time PCR is DNA amplification failure due to the presence of inhibitory substances in the samples that can produce false negative results. The European Standardization Committee, in collaboration with International Standard Organization, has proposed a general guideline for PCR testing that requires the presence of an internal amplification control (IAC) in each PCR reaction. Thus, an IAC must be included when a PCR assay is to be validated through a multicenter collaborative trial (HOORFAR et al., 2003; LUND e MADSEN, 2006).

The rapid detection of *Campylobacter* spp. and *Salmonella* spp. in food can identify sources of contamination and help food supply monitors to take appropriate measures to prevent the distribution of contaminated food. The aim of this study was to develop a multiplex real-time PCR assay for the simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat using a hydrolysis probe assay and an IAC.

2 Material and Methods

2.1 Bacterial strains and growth conditions

Salmonella serovars, *Campylobacter* strains and other bacterial strains used in this study are listed in Table 1. *Campylobacter* strains were grown in Bolton Broth (CM 0983, Oxoid, Basingstoke, Hampshire, United Kingdom) at 42 °C for 48 hours (h) under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2)

created with the Microaerobac system (Probac, São Paulo, SP, Brazil). *Salmonella* and other bacterial strains were grown in brain and heart infusion broth (BHI, Acumedia, Lansing, Michigan, USA) at 37 °C for 24 h.

Table 1 - *Salmonella* serovars*, Campylobacter* and other bacterial strains used in the development and evaluation of the multiplex real-time PCR assay

Serovar	**Origin**	**Strains[a]**	**Origin**
Salmonella. Anatum	LACEN[d]	*Bacillus cereus*	UEL[c]
S. Agona	UEL[c]	*Campylobacter coli* CCAMP 1003	FIOCRUZ[b]
S. Bredeney	LACEN[d]	*C. coli* CCAMP 1008	FIOCRUZ[b]
S. Dublin	LACEN[d]	*C. coli* CCAMP 595	FIOCRUZ[b]
S. Derby	LACEN[d]	*Campylobacter jejuni* ATCC 33291	FIOCRUZ[b]
S. Enteritidis ATCC 13076	UEL[c]	*C. jejuni* CCAMP 971	FIOCRUZ[b]
S. Infantis	UEL[c]	*C. jejuni* CCAMP 594	FIOCRUZ[b]
S. Johannesburg	LACEN[d]	*C. jejuni* CCAMP 1014	FIOCRUZ[b]
S. Kentucky	IB[e]	*Campylobacter lari*	LACEN[d]
S. London	LACEN[d]	*Citrobacter freundii*	UEL[c]
S. Montevideo	UEL[c]	*Enterobacter aerogenes*	UEL[c]
S. Muenchen	LACEN[d]	*E. cloacae*	UEL[c]
S. Newport	UEL[c]	*Escherichia coli*	UEL[c]
S. Panama	LACEN[d]	*Klebsiella pneumoniae*	UEL[c]
S. Senftenberg	LACEN[d]	*Morganella morganii*	UEL[c]
S. Typhi	UEL[c]	*Proteus mirabilis*	UEL[c]
S. Typhimurium ATCC 14028	UEL[c]	*Shigella sonnei*	UEL[c]
		Staphylococcus aureus	UEL[c]
		S. saprophyticus	UEL[c]

[a] One isolate of each strain was tested.
[b] Strain obtained from the Oswaldo Cruz Foundation Collection (FIOCRUZ), Rio de Janeiro, Brazil.
[c] Strain belongs to the Food Microbiology Laboratory Collection, Londrina State University, Londrina, Paraná, Brazil.
[d] Central Laboratory of Paraná State (LACEN), Paraná, Brazil.
[e] São Paulo Biological Institute (IB), São Paulo, Brazil.

2.2 DNA extraction and purification

Aliquots of 1 mL of bacterial suspensions were centrifuged at 16,000 × *g* for 10 minutes (min). The pellet was washed twice with 1 mL buffered peptone water (Acumedia, Lansing, Michigan, USA), centrifuged at 16,000 ×*g* for 10 min and resuspended in a 300 µL of solution of Triton X-100 (Nuclear) 1%. Suspensions were heated at 100 °C for 10 min, cooled in ice, and centrifuged at 16,000 × *g* for 5 min.

For DNA purification, 300 µL of a solution of phenol, chloroform and isoamyl alcohol (25:24:1) (v/v) was added to the supernatant. The mixture was shaken by inversion, centrifuged at 13,000 x *g* for 10 min and the aqueous phase transferred to a new tube, to which 30 µL of sodium acetate (3 M) and 270 µL of absolute ethanol were added. Tubes were kept for 12 min at -80 °C, and centrifuged at 13,000 x *g* for 10 min. The pellet was washed with 70% ethanol, and centrifuged again as described above. DNA was dried at 37°C and ressuspended in 50 µL of ultrapure water.

The extracted DNA concentration and purity was determined by measuring the absorbance at 260 and 280 nm (GeneQuant Tm Amersham Biosciences, USA). The DNA samples were diluted in sterile nuclease free water to a stock concentration of 20 ng/µL stored at -20 °C and used in the optimization of the multiplex real-time PCR assay.

2.3 Primers and fluorogenic probes

Primers, sequences and the size of the amplified DNA sequences are listed in Table 2. The internal amplification control, TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, USA) was used according to manufacturer's recommendation.

Table 2 - Primers and probes used in the multiplex real-time PCR assay for the detection of *Salmonella* spp. and *Campylobacter* spp.

Bacteria	Target gene	Primer/ probe	Sequence (5' to 3')	Amplicon size (bp)
Salmonella	*Inv*A	Styinva-JHO-2-left[a]	TCGTCATTCCATTACCTACC	119
		Styinva-JHO-2-right[a]	AAACGTTGAAAAACTGAGGA	
		Salmonella Probe [a]	[6-FAM™]TCTGGTTGATTTCCTGATCGCA[BHQ-1]*	
Campylobacter	rRNA 16S	OT1559[b]	CTGCTTAACACAAGTTGAGTAGG	287
		18-1[c]	TTCCTTAGGTACCGTCAGAA	
		Campylobacter Probe [d]	[Cy5®]TGTCATCCTCCACGCGGCGTTGCTGC[BHQ-3]*	

[a] Hoofar, (2000)
[b] Uyttendaele et al.(1994); Lübeck et al.(2003)
[c] Vancamp et al.(1993); Docherty et al.(1996); Lübeck et al.(2003)
[d] Josefsen et al.(2004)
*6-FAM™ (*6-carboxyfluorescein*); BHQ-1(*Black-hole quencher* 1); Cy5® (*Cyanine-5*); BHQ-3 (*Black-hole quencher 3*)

Specific probes for *Campylobacter, Salmonella* and IAC were labeled at the 5'-end with Cy5, 6-FAM and VIC fluorophores, respectively. Fluorophores were selected taking into account their maximum emission wavelengths (Cy5, 651; 6-FAM, 494; VIC, 550 nm), and the filters provided in the ABI 7500 Fast Real-time PCR System (Applied Biosystems) were used to reduce spectral overlapping. All probes were also labeled at the 3'-end with non-fluorescent quenchers (dark quencher).

2.4 Development of multiplex real-time PCR assay

Multiplex real-time PCRs were carried out in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.). A 100 ng concentration of DNA from *Salmonella* and *Campylobacter* was used to optimize the concentrations of primers, probes, and $MgCl_2$. The following concentrations were assayed for simultaneous amplification of the three amplicons: 200, 250, and 300 nM of primers OT1559 and 18-1 (Sigma-Aldrich, St Louis, MO, USA); 600, 700, 800, and 900 nM of primers Styinva-JHO (Sigma-Aldrich); 100, 150 and 200 nM of probes (Sigma-Aldrich) and 3, 4, 5, 6 mM of $MgCl_2$.

Multiplex real-time PCRs were carried out in duplicate, with a final volume of 25 µL. All reactions contained 12,5 µL of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, San Diego, CA, USA), 50 nM of ROX (Invitrogen), 2.5 µL of 10 × Exo IPC Mix and 0.5 µL of 50 × Exo IPC DNA (Applied Biosystems) as an internal amplification control. A negative amplification control was included in all assays by replacing the DNA with 5 µL of ultrapure water.

The amplification conditions were 50°C for 2 min, 95 ° C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 sec. A fluorescence reading was taken at the end of each extension step and the threshold cycle (Ct), which is the intersection between each fluorescence curve and the threshold line, was calculated using the 7500 Software v2.0.4 (Applied Biosystems).

2.5 Determination of multiplex real-time PCR efficiency

Evaluation of uniplex efficiency was done by using serial dilutions (1:10), of known concentrations of pure DNA of *S.* Enteritidis ATCC 13076 or *C. jejuni* ATCC 33291, ranging between 10^3 and 10^{-1} ng per reaction. Efficiency was determined using the mixture, without the IAC, with all primers and probes but only with the DNA of *Salmonella* or *Campylobacter*.

Ct values (the intersection between each curve and the fluorescence threshold) and standard deviation of duplicates were calculated for each dilution. Standard curves were generated for each pathogen by plotting the Ct from each dilution versus DNA concentration.

Efficiency was calculated from the slope of the line through the following formula: efficiency (%) = $(10^{(-1/slope)}-1)*$ 100. All data were calculated automatically using the 7500 Software v2.0.4 program (Applied Biosystems).

2.6 Specificity of primers and probes

PCR specificity assays were performed using all bacterial strains listed in Table 1. Bacterial suspensions, DNA extraction and purification, and the PCR assay were carried out as described previously. *S.* Enteritidis ATCC 13076 and *C. jejuni* ATCC 33291 were used in all reactions as positive controls.

2.7 PCR sensitivity in spiked chicken skin samples

Portions of skin (25 g) from chicken carcass samples were previously analyzed to rule out prior contamination with *Campylobacter* spp. and *Salmonella* spp. Portions of uncontaminated chicken skin (25 g) were placed in a sterile plastic bag and 225 mL of buffered peptone water broth (Acumedia) was added. The mixture was then massaged by hand for 30 s. Aliquots of 50 mL and 5 mL of rinse were inoculated, respectively with suspensions of *S.* Enteritidis ATCC 13076 and *C.*

jejuni ATCC 33291 to obtain approximately 1 to 10^7 CFU/mL. Cell bacterial concentrations were estimated by the drop plate method for counting bacteria (MILES et al., 1938). Bacterial suspensions of *S.* Enteritidis were subcultured on Xylose Lysine Sodium Deoxycholate (XLD) plates (Acumedia) and were incubated at 37 °C for 24 h. Suspensions of *C. jejuni* ATCC 33291 were subcultured on Bolton Agar [Bolton Broth (CM 0983, Oxoid) plus 2.0% (w/v) agar-agar (HiMedia, Mumbai, India)] and incubated at 42 °C for 48 h under microaerobic conditions created with the Microaerobac system (Probac). Chicken skin rinses (50 mL) spiked with different concentrations of *S.* Enteritidis ATCC 13076 were incubated at 37 °C for 24 h. Chicken rinses (5 mL) spiked with different concentrations of *C. jejuni* ATCC 33291 were added to 45 mL of Bolton Broth (Oxoid) supplemented with Modified Bolton Broth Selective Supplement SR0208E (Oxoid) (cefoperazone, 10 mg/500 mL; vancomycin, 10 mg/500 mL, trimethoprim 10 mg/500 mL and amphotericin B, 5 mg/500 mL) and incubated at 42 °C for 24 h under microaerobic conditions created with the Microaerobac system (Probac). This experiment was performed in two independent replicates.

2.8 DNA extraction of chicken skin samples spiked with *Salmonella* and *Campylobacter* and evaluation of the developed multiplex real-time PCR assay

Aliquots of 1 mL of each spiked rinse before and after enrichment were used for DNA extraction. First, aliquots of 1 mL of each spiked rinse before the enrichment step were used for DNA extraction. Aliquots of 1 mL of chicken skin rinse spiked with *Salmonella* spp. and 1 mL of chicken skin rinse spiked with *Campylobacter* spp., were centrifuged at 16,000 × *g* for 10 min separately. Another aliquot (1 mL) of each chicken skin rinse spiked with *Salmonella* spp. and *Campylobacter* spp. were pooled and centrifuged at 16,000 × *g* for 10 min. Then, the same steps were performed with the samples after the enrichment step. Aliquots of 1 mL of non-selective enrichments of *Salmonella* spp. and 1 mL of selective enrichments of *Campylobacter* spp., were centrifuged at 16,000 × *g* for 10 min separately. Aliquots (1 mL) of each chicken skin rinse spiked with *Salmonella* spp.

and *Campylobacter* spp. after the enrichment step were pooled and centrifuged at 16,000 ×*g* for 10 min.

The sediment obtained was washed with 1 mL of 1.0% (w/v) buffered peptone water (Acumedia) and centrifuged for 10 min at 16,000 ×*g*. The pellet was resuspended in 300 µL of solution of Triton X-100 (Nuclear) 1% and DNA extraction was performed as described previously for item 2.2. For use in the developed multiplex real-time PCR assay, the DNA extracted from the samples without enrichment were resuspended in 50 µL of ultrapure water and those from enriched samples were resuspended in 250 µL of ultrapure water.

DNA samples obtained as described above were used as target DNA for the developed multiplex real-time PCR assay.

3 Results and Discussion

3.1 Set up of multiplex real-time PCR

Optimized reaction conditions for the multiplex real-time PCR were obtained by using 5,0 µL of DNA, added to a reaction mixture (20 µL) composed of 5 mM of $MgCl_2$, 300 nM of primer pair OT1559 and 18-1, 900 nM of primer pair Styinva-JHO-2, 100 nM of the specific probe for *Campylobacter,* 150 nM of the specific probe for *Salmonella,* 12,5 µL of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, San Diego, CA, USA), 50 nM of ROX (Invitrogen), 2,5 µL of 10 × Exo IPC Mix and 0,5 µL of 50 × Exo IPC DNA (Applied Biosystems), as an internal amplification control.

The assay developed in the present study, to our knowledge, is the first multiplex real-time PCR assay for the detection of *Campylobacter* spp. and *Salmonella* spp. with an internal amplification control.

Previously, only two PCR assays have been developed for the exclusive detection of *Campylobacter* and *Salmonella* in foods. Alves et al. (2012) reported the simultaneous detection of *Campylobacter* spp. and *Salmonella* spp. in a chicken meat rinse by using a traditional multiplex PCR. Wolffs et al. (2007) reported simultaneous quantification of *Campylobacter* spp. and *Salmonella* spp. in a chicken

rinse by using real-time multiplex PCR. However, an internal amplification control was not included in the assay, and flotation technique used for sample concentration is not routinely available for use in food analysis laboratories.

A negative response in the PCR assay (no band or signal) can mean that there was no target sequence present in the reaction. However, it could also mean that the reaction was inhibited due to a malfunction of the thermal cycler, incorrect PCR mixture, poor polymerase activity, and, not least, the presence of inhibitory substances in the sample matrix. An IAC in the real-time PCR differentiates negative reactions from possible false negatives. An IAC signal will always be produced when there is no target sequence present, however if neither the IAC signal nor the target signal is produced, the PCR has failed (HOORFAR et al., 2003).

3.2 Efficiency and specificity

Efficiency of the optimized assay was 97.64% for *Salmonella* and 96.92% for *Campylobacte*r with high correlation coefficients of 1.0 and 0.995, respectively (Table 3).

Table 3.- Amplification efficiency of the multiplex real-time PCR for pure DNA from *Salmonella* Enteritidis ATCC 13076 or *Campylobacter jejuni* ATCC 33291

DNA concentrations (ng)	**Ct[a]**	
	Salmonella	***Campylobacter***
10^3	12.52 ± 0.15	11.43 ± 0.03
10^2	15.70 ± 0.00	13.99 ± 0.08
10^1	19.18 ± 0.00	17.5 ± 0.19
10^0	22.54 ± 0.00	21.05 ± 0.00
10^{-1}	26.00 ± 0.04	24.88 ± 0.07
r^2	1	0.995
Standard curve slope	-3.38	-3.398
Efficiency (%)	97.64	96.92

[a] Values are the averages and standard deviation of duplicates

Specificity of the uniplex and multiplex real-time PCR assays developed in this study, checked with 36 bacteria listed in Table 1, was 100%. This specificity test was conducted because the use of two primer pairs, probes, and the inclusion of an internal amplification control in the same reactive mixture of the PCR could generate nonspecific amplification products and primer-dimer. However, in the present study, only the IAC produced fluorescence when other strains, different from *Salmonella* and *Campylobacter*, were tested. Absence of nonspecific amplification products and primer-dimer was observed by analysis of the amplification products by agarose gel (data not shown). Specificity of 100% was previously reported in uniplex PCR assays developed with primers OT1559 and 18-1 (LÜBECK et al., 2003; PERELLE et al., 2004; WOLFFS et al., 2007) and with primers Styinva-JHO-2 (HOORFAR et al., 2000; RODRÍGUEZ-LÁZARO et al., 2003; NAM et al., 2005). Alves et al. (2012) also obtained a 100% specificity in a traditional mPCR developed with those two pair of primers for the simultaneous detection of *Campylobacter* and *Salmonella* in chicken meat.

3.3 Sensitivity of the uniplex real-time PCR compared to the multiplex real-time PCR

The ability of the multiplex real-time PCR assay to co-amplify a high concentration of one specific DNA (10^3 ng) and a very low concentration of another DNA target (10^{-1} ng) in a single reaction was tested (Table 4). In this study, when DNA of one of the target pathogens was present at a high concentration (10^3 ng), the DNA detection of the other pathogen was inhibited when it was at a low concentration (10^{-1} ng). However, the sensitivity of the assay was not affected if equal amounts of DNA from both pathogens were tested. In addition, the Ct values of the amplified target in the multiplex real-time PCR were the same as those obtained in the uniplex real-time PCR (Table 4). Amplification failure possibly occurred due to the competition of both primers pairs and probe sets for the same pool of resources. In other studies, similar results were also reported showing that a high concentration of one target gene could interfere with the amplification of a low amount of the other target gene (ELIZAQUÍVEL e AZNAR, 2008; HYEON et al., 2010).

Table 4 - Comparison of Ct values obtained by uniplex and multiplex real-time PCR assays using a purified template DNA of *Salmonella* Enteritidis ATCC 13076 or *Campylobacter jejuni* ATCC 33291

Uniplex reaction without IAC		**Ct[a] values**		
DNA concentration (ng) from *Salmonella* or *Campylobacter*		***Salmonella***	***Campylobacter***	
10^3		12.52 ± 0.15	11.43 ± 0.03	
10^2		15.70 ± 0.00	13.99 ± 0.08	
10^1		19.18 ± 0.00	17.50 ± 0.19	
10^0		22.54 ± 0.00	21.05 ± 0.00	
10^{-1}		26.00 ± 0.04	24.88 ± 0.07	
Multiplex reaction with IAC		**Ct[a] values**		
DNA concentration (ng) from *Salmonella* and *Campylobacter*		***Salmonella***	***Campylobacter***	**IAC**
10^3	10^3	13.84 ± 0.06	13.19 ± 0.16	indeterminate
10^2	10^2	16.51 ± 0.02	13.99 ± 0.04	37.08 ± 0.19
10^1	10^1	20.00 ± 0.06	17.38 ± 0.00	30.99 ± 0.33
10^0	10^0	23.43 ± 0.01	21.21 ± 0.06	28.97 ± 0.14
10^{-1}	10^3	indeterminate	11.24 ± 0.07	indeterminate
10^0	10^2	indeterminate	13.90 ± 0.00	35.91 ± 0.16
10^1	10^1	19.91 ± 0.12	17.52 ± 0.10	31.26 ± 0.03
10^2	10^0	15.89 ± 0.03	indeterminate	33.04 ± 0.42
10^3	10^{-1}	12.43 ± 0.20	indeterminate	28.83 ± 0.14

[a] Values are the averages and standard deviation of duplicates

3.4 Detection limits of the multiplex real-time PCR assay of artificially inoculated chicken samples with and without a enrichment step

Results of the detection of *Salmonella* and *Campylobacter* in chicken meat rinse samples without enrichment are presented in Table 5. The detection limit of the multiplex real-time PCR assay was 10^5 CFU/mL of *Salmonella* and 10^3 CFU/mL of *Campylobacter.* The influence of target concentration and food matrix in the assay were tested. DNA extraction by boiling and purification with phenol-chloroform, was efficient to remove PCR inhibitors from the food matrix.

Table 5 - Evaluation of the developed multiplex real-time PCR assay in chicken meat samples spiked with *Salmonella* Enteritidis ATCC 13076 or *Campylobacter jejuni* ATCC 33291

***Salmonella* (CFU/mL) without enrichment**	**Ct[a] values**	
	Salmonella	**IAC**
10^0	indeterminate	29.90 ± 0.12
10^1	indeterminate	29.93 ± 0.09
10^2	indeterminate	29.99 ± 0.04
10^3	indeterminate	30.12 ± 0.09
10^4	indeterminate	29.94 ± 0.07
10^5	38.40 ± 0.00	29.90 ± 0.08
10^6	28.72 ± 0.01	30.03 ± 0.14
10^7	20.50 ± 0.03	31.86 ± 0.29
***Campylobacter* (CFU/mL) without enrichment**	**Ct[a] values**	
	Campylobacter	**IAC**
10^0	indeterminate	29.86 ± 0.20
10^1	indeterminate	29.97 ± 0.26
10^2	indeterminate	29.95 ± 0.11
10^3	34.00 ± 0.00	29.98 ± 0.08
10^4	24.64 ± 0.10	29.94 ± 0.14
10^5	21.17 ± 0.08	30.55 ± 0.01
10^6	16.18 ± 0.02	34.78 ± 1.73
10^7	11.69 ± 0.22	indeterminate

[a] Values are the averages and standard deviation of duplicates

There was no detection of *Salmonella* (10^5 CFU/mL) in the presence of *Campylobacter* in concentrations of 10^3 to 10^6 CFU/mL (Table 6). At the concentration of 10^6 CFU/mL of *Salmonella*, only the highest concentrations of *Campylobacter* (10^5 to 10^6 CFU/mL) inhibited the detection. *Salmonella* at a concentration of 10^7 CFU/mL was detected independent of the *Campylobacter* concentration. The amplification of *Salmonella* in concentrations of 10^3 to 10^6 CFU/mL did not interfere with the detection of *Campylobacter*, except when the concentration of *Salmonella* was 10^7 CFU/ mL, and *Campylobacter* was 10^3 to 10^4 CFU/mL.

Amplification of the IAC, with Ct values between 29 and 31, in all reactions indicated that there was no interference of the food matrix. The inhibition of the IAC in the reaction with 10^7 CFU/mL of *Salmonella* and 10^6 CFU/mL of *Campylobacter* possibly occurred due to excess DNA and competition for the PCR reactive mixture. When one of the bacteria was present in concentrations ranging

from 10^5 to 10^7 CFU/mL of rinse (Table 6), the sensitivity of the method decreased. In this case, there was no inhibition of the IAC but the Ct value was higher (37-39) than in samples with low concentrations of both targets. Similar studies have demonstrated inhibition in detection when the concentration of a target is far superior to another (ELIZAQUÍVEL e AZNAR, 2008; HYEON et al., 2010).

Table 6 - Evaluation of the developed multiplex real-time PCR assay in chicken meat samples spiked with different concentrations of *Salmonella* Enteritidis ATCC 13076 and *Campylobacter jejuni* ATCC 33291 without enrichment

Concentration (CFU/mL) of the mixture of non-enriched sample of *Salmonella* and *Campylobacter*		**Ct[a] values**		
Salmonella	***Campylobacter***	***Salmonella***	***Campylobacter***	**IAC**
10^5	10^3	indeterminate	26.82 ± 0.00	29.99 ± 0.06
10^5	10^4	indeterminate	24.62 ± 0.03	30.03 ± 0.19
10^5	10^5	indeterminate	20.81 ± 0.02	31.01 ± 0.15
10^5	10^6	indeterminate	15.25 ± 0.05	37.91 ± 1.15
10^6	10^3	30.69 ± 0.12	26.89 ± 0.05	30.01 ± 0.01
10^6	10^4	24.64 ± 0.16	32.69 ± 0.33	30.29 ± 0.35
10^6	10^5	indeterminate	20.95 ± 0.00	30.76 ± 0.07
10^6	10^6	indeterminate	15.38 ± 0.12	38.27 ± 0.03
10^7	10^3	21.34 ± 0.14	indeterminate	31.49 ± 0.14
10^7	10^4	21.27 ± 0.08	indeterminate	31.91 ± 0.23
10^7	10^5	20.87 ± 0.29	20.97 ± 0.06	32.95 ± 0.13
10^7	10^6	24.55 ± 0.43	15.43 ± 0.24	indeterminate

[a] Values are the averages and standard deviation of duplicates

Detection of *Salmonella* and *Campylobacter* was possible in samples contaminated with 1 to 10^5 CFU/mL, after non-selective and selective enrichments, respectively (Table 7). Purified DNA samples were resuspended in 250 μl of ultrapure water. Different volumes of ultrapure water were tested (not demonstrated in this work), and, in smaller volumes than 250 μL, inhibition of the IAC was observed, possibly due to the high amount of DNA in the reaction. There was no inhibition of the IAC in the spiked samples (Table 7).

Table 7 - Evaluation of the developed multiplex real-time PCR assay in chicken meat samples spiked with *Salmonella* Enteritidis ATCC 13076 or *Campylobacter jejuni* ATCC 33291 after enrichment

Initial concentration (CFU/mL) of *Salmonella* followed by 24 h of enrichment	**Ct[a] values**	
	Salmonella	**IAC**
10^0	17.33 ± 0.03	32.83 ± 0.06
10^1	17.16 ± 0.04	33.29 ± 0.15
10^2	17.59 ± 0.00	31.97 ± 0.35
10^3	17.09 ± 0.02	32.88 ± 0.66
10^4	16.86 ± 0.08	33.98 ± 0.00
10^5	16.52 ± 0.04	35.11 ± 0.83
Initial concentration (CFU /mL) of *Campylobacter* followed by 24 h of enrichment	**Ct[a] values**	
	Campylobacter	**IAC**
10^0	18.95 ± 0.01	30.85 ± 0.23
10^1	20.29 ± 0.19	30.26 ± 0.07
10^2	19.55 ± 0.13	30.42 ± 0.01
10^3	18.38 ± 0.00	31.58 ± 0.13
10^4	18.16 ± 0.01	31.22 ± 0.04
10^5	16.03 ± 0.02	32.49 ± 0.55

[a] Values are the averages and standard deviation of duplicates

Absence of these bacteria in 25 g cannot be confirmed by the standard PCR reaction without enrichment. The difficulty of separating the microorganisms from the food matrix, and consequently the DNA extraction, requires enrichment of the samples. The enrichment for *Salmonella* and *Campylobacter* allowed the detection of these pathogens in samples with a contamination of 1 CFU/mL and has other benefits, such as the dilution of inhibitors, the differentiation between viable and non-viable cells and the repair of injured cells, despite being a limitation because enrichment increases the time of the analysis (MALORNY et al., 2009).

Phenotypic identification of *Campylobacter* is limited due to isolates with atypical biochemical characteristics. Furthermore, this bacterium has fastidious and asaccharolytic growth, which restricts the use of differential biochemical tests. The conventional microbiological techniques for the detection of *Salmonella* are time consuming, and serotyping can only be performed in reference laboratories. Therefore, the detection of these pathogens, in just a few hours, allows food supply

monitors to take appropriate measures to prevent the distribution of contaminated food.

4 Conclusion

The multiplex real-time PCR assay developed in this study was the first assay for exclusive and simultaneous detection of *Salmonella* spp. and *Campylobacter* spp. in chicken meat with an IAC. Using this multiplex real-time PCR system, *Salmonella* spp. and *Campylobacter* spp. can be detected in a single reaction after 24 h, reducing time and labor costs. In addition, the method is a useful diagnostic tool for the rapid detection of these two pathogenic bacteria in perishable products such as chicken meat and in other food products.

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References

ALVES, J.; MARQUES, V. V.; PEREIRA, L. F. P.; HIROOKA, E. Y.; DE OLIVEIRA, T. C. R. M. Multiplex PCR for the detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat. **Journal of Food Safety,** v. 32, n. 3, p. 345-350, 2012.

CANDRIAN, U. Polimerase chain reaction in food microbiology **Journal of Microbiological Methods,** v. 23, p. 89-103, 1995.

DOCHERTY, L.; ADAMS, M. R.; PATEL, P.; MCFADDEN, J. The magnetic immuno-polymerase chain reaction assay for the detection of *Campylobacter* in milk and poultry. **Letters in Applied Microbiology,** v. 22, p. 288-292, 1996.

ELIZAQUÍVEL, P.; AZNAR, R. A multiplex RTi-PCR reaction for simultaneous detection of Escherichia coli O157:H7, Salmonella spp. and Staphylococcus aureus on fresh, minimally processed vegetables. **Food Microbiology,** v. 25, n. 5, p. 705-713, 2008.

HOORFAR, J.; AHRENS, P.; RÅDSTRÖM, P. Automated 5' Nuclease PCR Assay for Identification of *Salmonella enterica*. **Journal of Clinical Microbiology,** v. 38, n. 9, p. 3429-3435, 2000.

HOORFAR, J.; MALORNY, B.; WAGNER, M.; ABDULMAWJOOD, A.; FACH, P. Making Internal Amplification Control Mandatory for Diagnostic PCR. **Journal of Clinical Microbiology,** v. 41, n. 12, p. 5835, 2003.

HYEON, J.-Y.; PARK, C.; CHOI, I.-S.; HOLT, P. S.; SEO, K.-H. Development of multiplex real-time PCR with Internal amplification control for simultaneous detection of *Salmonella* and *Cronobacter* in powdered infant formula. **International Journal of Food Microbiology,** v. 144, n. 1, p. 177-181, 2010.

JOSEFSEN, M. H.; JACOBSEN, N. R.; HOORFAR, J. Enrichment Followed by Quantitative PCR both for Rapid Detection and as a Tool for Quantitative Risk Assessment of Food-Borne Thermotolerant Campylobacters. **Applied and Enviromental Microbiology,** v. 70, n. 6, p. 3588-3592, 2004.

LÜBECK, P. S.; WOLFFS, P.; ON, S. L. W.; AHRENS, P.; RADSTRÖM, P.; HOORFAR, J. Toward an International Standard for PCR-Based Detection of Food-Borne Thermotolerant Campylobacters: Assay Development and Analytical Validation. **Applied and Enviromental Microbiology,** v. 69, n. 9, p. 5664-5669, 2003.

LUND, M.; MADSEN, M. Strategies for the inclusion of an internal amplification control in conventional and real time PCR detection of *Campylobacter* spp. in chicken fecal samples. **Molecular and Cellular Probes,** v. 20, p. 92-99, 2006

LUND, M.; NORDENTOFT, S.; PEDERSEN, K.; MADSEN, M. Detection of *Campylobacter* spp. in Chicken Fecal Samples by Real-Time PCR. **Journal of Clinical Microbiology,** v. 42, n. 11, p. 5125-5132, 2004.

MALORNY, B.; HUEHN, S.; DIECKMANN, R.; KRÄMER, N.; HELMUTH, R. Polymerase Chain Reaction for the Rapid Detection and Serovar Identification of *Salmonella* in Food and Feeding Stuff. **Food Analytical Methods,** v. 2, p. 81-95, 2009.

MILES, A. A.; MISRA, S. S.; IRWIN, J. O. The estimation of the bactericidal power of the blood. **Journal of Hygiene,** v. 38, n. 6, p. 732-49, Nov 1938.

NAM, H.-M.; SRINIVASAN, V.; GILLESPIE, B. E.; MURINDA, S. E.; OLIVER, S. P. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. **International Journal of Food Microbiology,** v. 102, p. 161-171, 2005.

OMICCIOLI, E.; AMAGLIANI, G.; BRANDI, G.; MAGNANI, M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiology,** v. 26, p. 615-622, 2009.

PERELLE, S.; JOSEFSEN, M.; HOORFAR, J.; DILASSER, F.; GROUT, J.; FACH, P. A LightCycler real-time PCR hybridization probe assay for detecting food-borne thermophilic *Campylobacter*. **Molecular and Cellular Probes,** v. 18, p. 321–327, 2004.

PERRY, L.; HEARD, P.; KANE, M.; KIM, H.; SAVIKHIN, S.; DOMÍNGUEZ, W.; APPLEGATE, B. Application of multiplex Polimerase Chain Reaction to the detection of pathogens in food. **Journal of Rapid Methods & Automation in Microbiology,** v. 15, p. 176-198, 2007.

RODRÍGUEZ-LÁZARO, D.; HERNÁNDEZ, M.; ESTEVE, T.; HOORFAR, J.; PLA, M. A rapid and direct real time PCR-based method for identification of *Salmonella* spp. **Journal of Microbiological Methods,** v. 54, p. 381-390, 2003.

UBA. União Brasileira de Avicultura. Annual Report - Brazilian Poultry Association. 2014. Disponível em: < http://www.ubabef.com.br/files/publicacoes/8ca705e70f0cb110ae3aed67d29c8842.pdf >. Acesso em: 20 maio 2014.

UYTTENDAELE, M.; SCHUKKINK', R.; GEMEN', B. V.; DEBEVERE, J. Identification of *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBAR. **Journal of Applied Bacteriology,** v. 77, p. 694-701, 1994.

VANCAMP, G.; FIERENS, H.; VANDAMME, P.; GOOSENS, H.; HUYGHEBAERT, A.; WACHTER, R. D. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chain reaction based on 16s rRNA genes. **Systemic and Applied Microbiology,** v. 16, p. 30-36, 1993.

WHO. World Health Organization. FAO/WHO Expert Meeeting on *Salmonella* and *Campylobacter* in chicken meat, 4 – 8 May 2009. 2009. Disponível em: < http://www.who.int/foodsafety/micro/jemra/meetings/may09/en/index.html >. Acesso em: 18 jul. 2014.

WOLFFS, P. F. G.; GLENCROSS, K.; NORLING, B.; GRIFFITHS, M. W. Simultaneous quantification of pathogenic *Campylobacter* and *Salmonella* in chicken rinse fluid by a flotation and real-time multiplex PCR procedure. **International Journal of Food Microbiology,** v. 117, p. 50-54, 2007.

6 CONCLUSÃO GERAL

A extração de DNA por fervura seguida de purificação com fenol-clorofórmio associada à PCR tradicional ou em tempo real foi suficiente para a detecção simultânea ou individual de *Salmonella* e *Campylobacter* em diferentes matrizes alimentares complexas.

A PCR e a metodologia de extração de DNA utilizadas neste trabalho são mais baratas que os *Kits* comerciais. O ensaio PCR foi capaz de detectar *Salmonella* spp. diretamente do enriquecimento das amostras das oito matrizes alimentares complexas testadas, detectar *Campylobacter* spp. e identificar *Campylobacter jejuni e C. coli* diretamente do enriquecimento de carne de frango e detectar *Salmonella* spp. e *Campylobacter* spp. em leite.

O ensaio PCR em tempo real otimizado neste trabalho foi o primeiro ensaio de detecção exclusiva de *Salmonella* spp. e *Campylobacter* spp. em carne de frango, após 24 h de enriquecimento, utilizando o controle interno de reação, reduzindo custos e tempo de análise.

A redução do tempo de análise e a eficiência na detecção destes patógenos são de grande importância para laboratórios de análises de alimentos na identificação do alimento contaminado e origem do surto.

APÊNDICES

APÊNDICE A

Artigo 1 – Detection of *Salmonella* spp. in eight complex food matrices using polymerase chain reaction assay

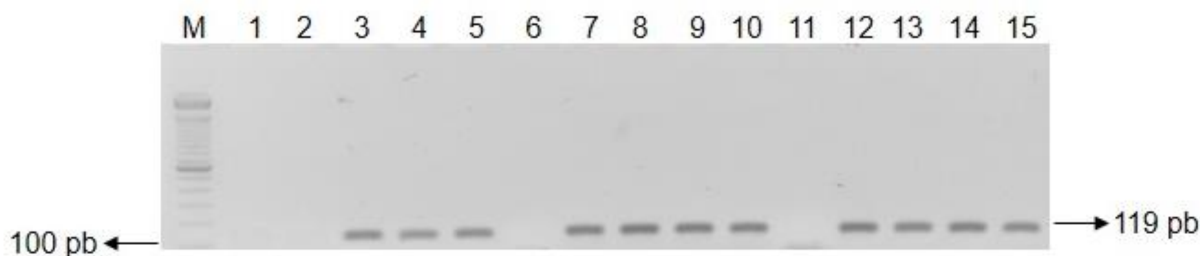

Figura 1: Análise de amostras de gema de ovo e pizza através de PCR, para detecção de *Salmonella* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2) representativo de controle negativo; 3-5) produto da PCR utilizando-se os oligonucleotídeos iniciadores Styinva-JHO-2, específicos para detecção do gênero *Salmonella*.; 6) controle negativo da amostra de gema de ovo; 7-10) amostra de gema de ovo contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. 11) controle negativo da amostra de pizza; 12-15) amostra de pizza contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

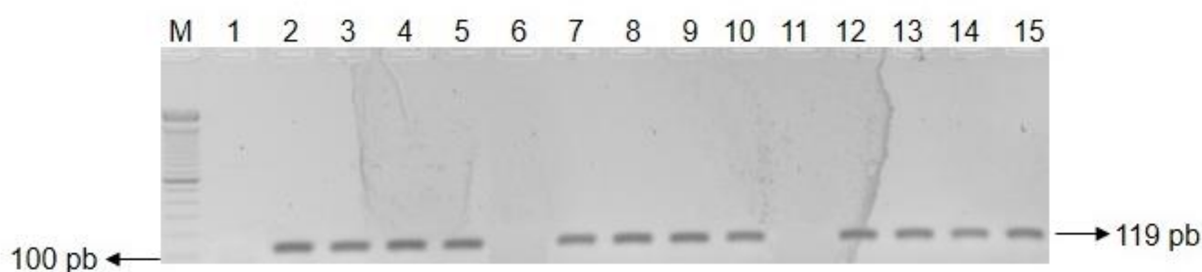

Figura 2: Análise de amostras de carne bovina moída, carne suína e linguiça suína através de PCR, para detecção de *Salmonella* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1) controle negativo da amostra de carne bovina moída; 2-5) amostra de carne bovina moída contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076; 6) controle negativo da amostra de carne suína; 7-10) amostra de carne suína contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. 11) controle negativo da amostra de linguiça suína; 12-15) amostra de linguiça suína contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

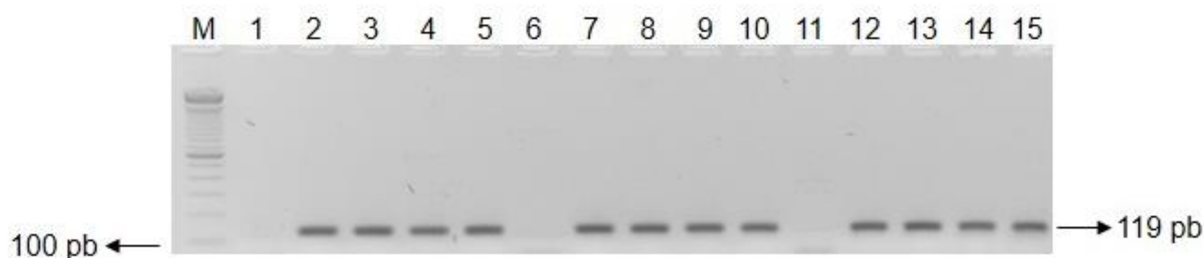

Figura 3: Análise de amostras de coxinha de frango, maionese e queijo minas através de PCR, para detecção de *Salmonella* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1) controle negativo da amostra de coxinha de frango; 2-5) amostra de coxinha de frango contaminadas com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076; 6) controle negativo da amostra de maionese; 7-10) amostra de maionese contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. 11) controle negativo da amostra de queijo minas; 12-15) amostra de queijo minas contaminada com suspensão bacteriana contendo entre 1 a 10^3 UFC/mL de *S.* Enteritidis ATCC 13076. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

APÊNDICE B

Artigo 2 – Prevalence of *Campylobacter* spp., *C. jejuni* and *C. coli* from chicken meat in Paraná, Brazil

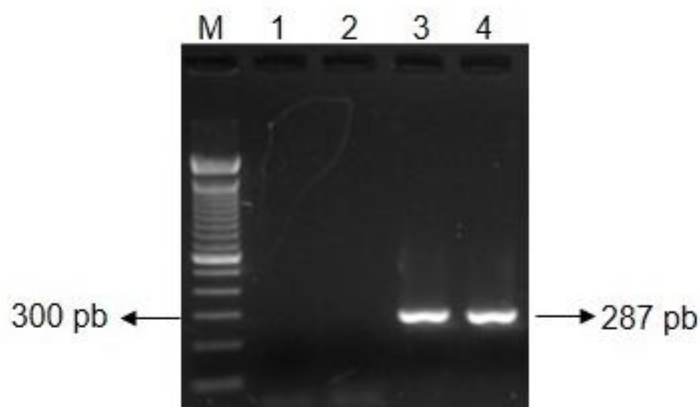

Figura 4: Produto do ensaio PCR para detecção de *Campylobacter* spp. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2 representativo de controle negativo; 3-4) produto da PCR utilizando-se os oligonucleotídeos iniciadores OT1559 e 18-1, específicos para o detecção do gênero *Campylobacter;* Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

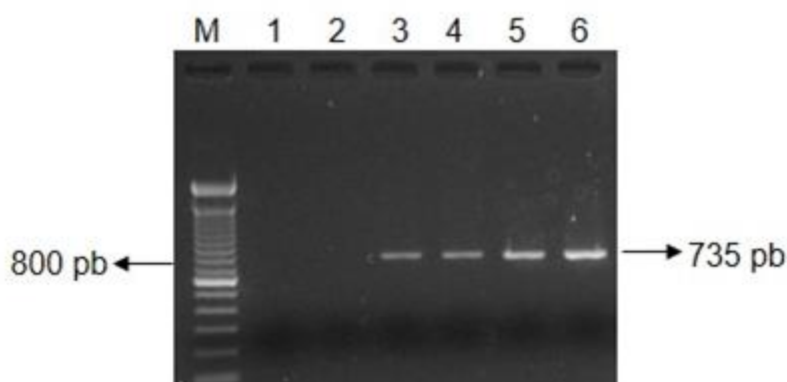

Figura 5: Produto do ensaio PCR para detecção de *Campylobacter jejuni*. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2 representativo de controle negativo; 3-6) produto da PCR utilizando-se os oligonucleotídeos iniciadores HIP400 e HIP1134, específicos para o detecção de *Campylobacter jejuni*. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

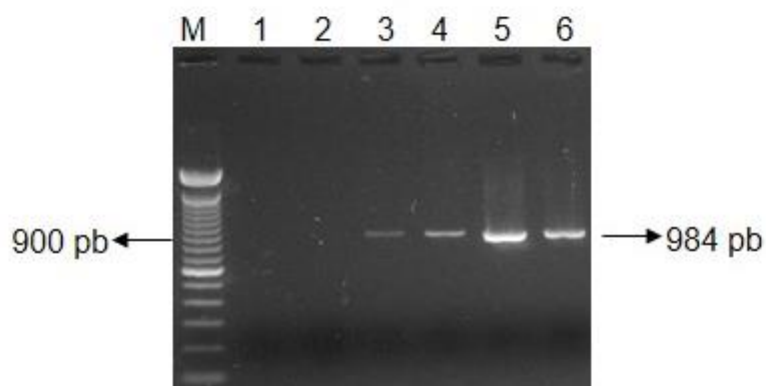

Figura 6: Produto do ensaio PCR para detecção de *Campylobacter coli*. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2 representativo de controle negativo; 3-4) produto da PCR utilizando-se os oligonucleotídeos iniciadores COL1 e COL2, específicos para o detecção de *Campylobacter coli*. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

APÊNDICE C

Artigo 3 – Evaluation of uniplex and multiplex PCR assays for detection of *Campylobacter* spp. and *Salmonella* spp. in milk

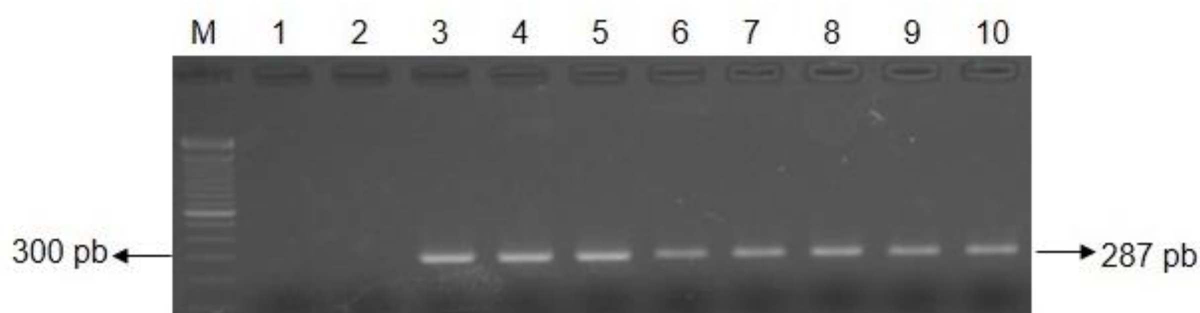

Figura 7: Análise de amostra de leite através de PCR, para detecção de *Campylobacter* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2) controle negativo da amostra de leite; 3) produto da PCR utilizando-se os oligonucleotídeos iniciadores OT1559 e 18-1, específicos para o detecção do gênero *Campylobacter;* 4-10) amostra de leite contaminada com suspensão bacteriana contendo entre 1 a 10^6 UFC de *C. jejuni* ATCC 33291. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

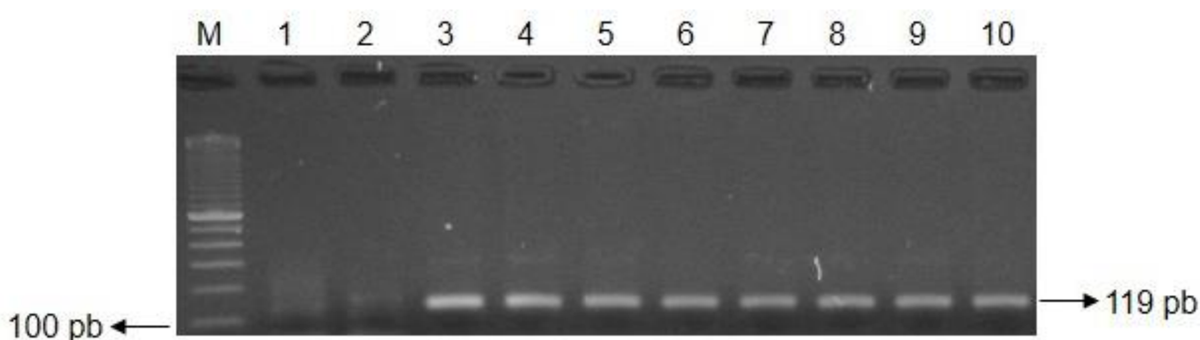

Figura 8: Análise de amostra de leite através de PCR, para detecção de *Salmonella* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2) controle negativo da amostra de leite; 3) produto da PCR utilizando-se os oligonucleotídeos iniciadores Styinva-JHO-2, específicos para detecção do gênero *Salmonella.*; 4-10) amostra de leite contaminada com suspensão bacteriana contendo entre 1 a 10^6 CFU de *S.* Enteritidis ATCC 13076. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

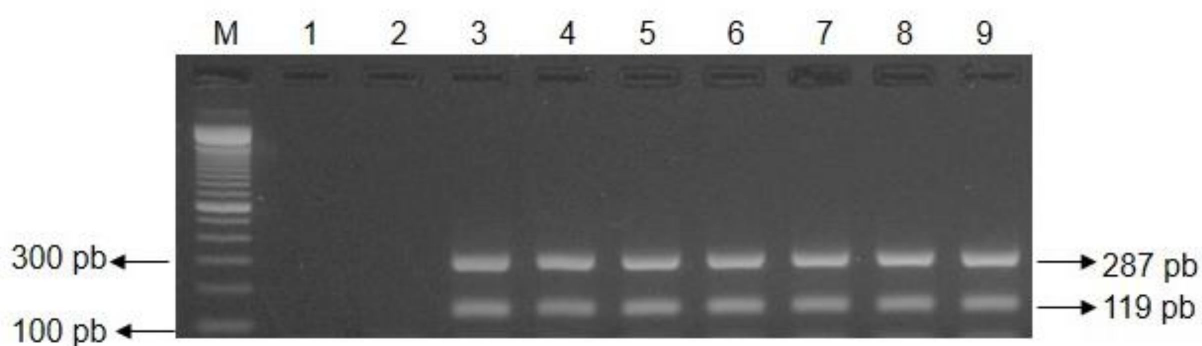

Figura 9: Análise de amostra de leite através de PCR multiplex, para detecção de *Salmonella* spp. e *Campylobacter* spp. após enriquecimento, utilizando solução de lise TZ e purificação com fenol/clorofórmio/álcool isoamílico. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2) controle negativo da amostra de leite; 3-9) amostra de leite contaminada com suspensão bacteriana contendo entre 1 a 10^6 UFC de *S.* Enteritidis ATCC 13076 e 10^6 a 1 UFC de *C. jejuni* ATCC 33291 respectivamente. Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

APÊNDICE D

Artigo 4 – Development of a multiplex real-time PCR assay with an internal amplification control for detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat

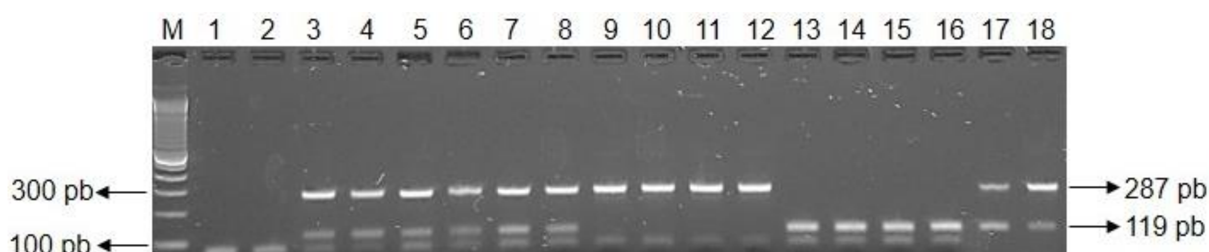

Figura 10: Análise da ausência de dímeros de iniciadores e bandas inespecíficas com as condições otimizadas para o ensaio PCR multiplex em tempo real otimizado. M) Marcador de massa molecular 100 pb (Invitrogen); 1 e 2) produto da PCR representativo do controle interno de amplificação; 3-8) produtos da PCR representativos do controle interno de amplificação, *Salmonella* spp. e *Campylobacter*; 9-12) produtos da PCR representativos do controle interno de amplificação e *Campylobacter;* 13-16) produtos da PCR representativos do controle interno de amplificação e *Salmonella* spp.; 17 e 18) produtos da PCR representativos de *Salmonella* spp. e *Campylobacter.* Gel de agarose 1,5% corado com 0,02 µL/mL de SYBR® Safe 10,000x em DMSO, (Invitrogen).

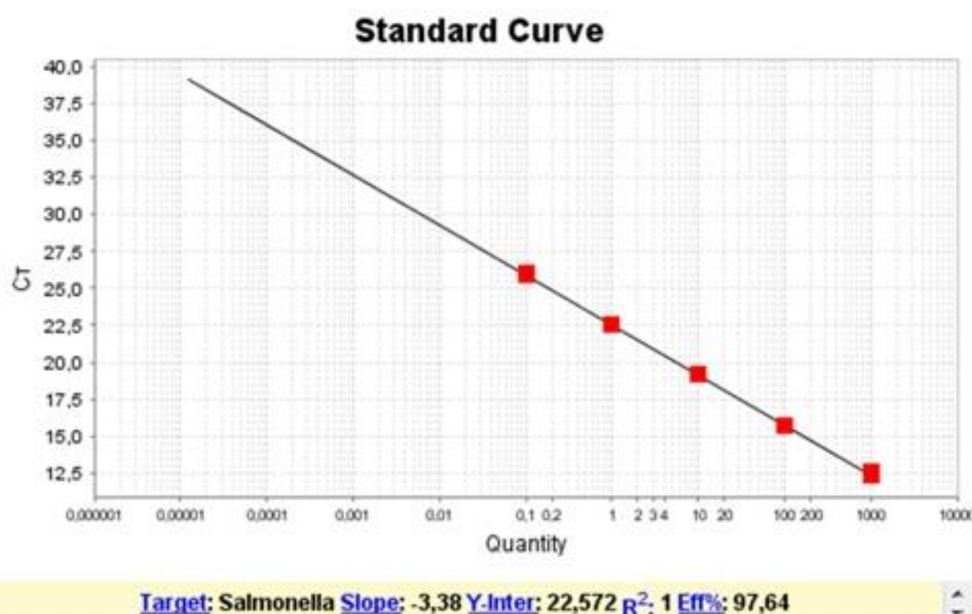

Figura 11: Curva padrão de *Salmonella* spp., da reação de PCR multiplex em tempo real otimizada, gerada pelo 7500 Software v2.0.4 (Applied Biosystems).

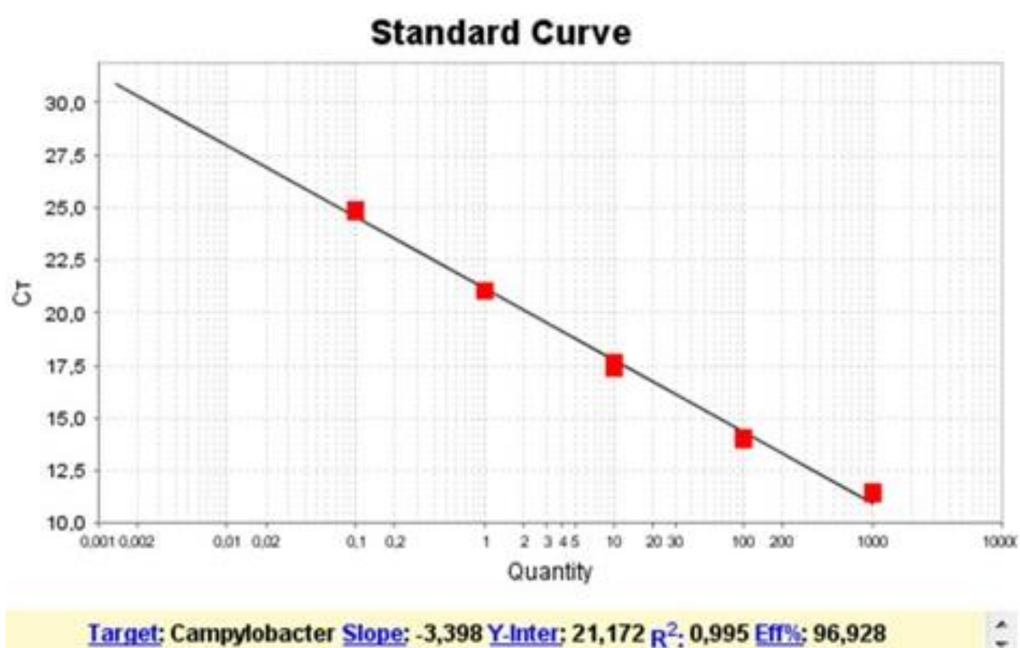

Figura 12: Curva padrão de *Campylobacter* spp., da reação de PCR multiplex em tempo real otimizada, gerada pelo 7500 Software v2.0.4 (Applied Biosystems).

ANEXOS

ANEXO A

Certificado da revisão da língua inglesa do artigo 1, intitulado: "Detection of *Salmonella* spp. in eight complex food matrices using polymerase chain reaction assay".

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ANEXO B

Certificado da revisão da língua inglesa do artigo 4, intitulado: “Development of a multiplex real-time PCR assay with an internal amplification control for detection of *Campylobacter* spp. and *Salmonella* spp. in chicken meat”.

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Authors:

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