UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETECÇÃO DE FATORES DE VIRULÊNCIA E RESISTÊNCIA ANTIMICROBIANA EM ESTIRPES DE *Campylobacter* spp. EM ISOLADOS HUMANOS E DE MATADOUROS-FRIGORIFICOS DE AVES NA REGIÃO SUL DO BRASIL.

Tese de Doutorado

Yuli Melisa Sierra Arguello

PORTO ALEGRE
2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Autora: Yuli Melisa Sierra Arguello

Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias na área de Medicina Veterinária Preventiva, na especialidade de Sanidade Avícola.

Orientador: Prof. Dr. Vladimir Pinheiro do Nascimento.

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A Dios,

A Yolanda, Milton y Julián Camilo,

A Johan, mi gran amor,

A mis queridas Oliva y Luz Mary.

A todos esos amigos incondicionales

Por su compañía inagotable

En mi mar de sueños.

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Charles Darwin.

DETECÇÃO DE FATORES DE VIRULÊNCIA E RESISTÊNCIA ANTIMICROBIANA EM ESTIRPES DE *Campylobacter* spp. EM ISOLADOS HUMANOS E DE MATADOUROS-FRIGORIFICOS DE AVES NA REGIÃO SUL DO BRASIL.

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Orientador: Vladimir Pinheiro do Nascimento

RESUMO

Campilobacteriose é uma zoonose de distribuição mundial, com repercussões importantes na saúde pública e um grande impacto socioeconômico. O objetivo deste estudo foi investigar a ocorrência, padrões de resistência antimicrobiana e sua relação fenotípica e genotípica, bem como a caracterização de marcadores de virulência em isolados de *Campylobacter* spp. obtidos a partir de fontes de origem aviária de diferentes pontos na linha do abate de matadouros-frigoríficos do Estado do Rio Grande do Sul. Um total de 141 amostras, incluindo fezes (n=8), água de *chiller* (n=18), carcaças de frango durante o processo do abate (n=26) e carne de frango pronta para o consumo (n=89) foram avaliadas. Todos os isolados foram confirmados pela técnica m-PCR baseados na detecção da região 16S rRNA e os genes *ceu*E e *map*A. Determinou-se a presença de *Campylobacter jejuni* em 140 amostras (99.2%), enquanto que *Campylobacter coli* foi identificado na amostra restante (0,7%). Cento e quarenta e uma cepas de *Campylobacter* spp. foram submetidas à analise de PCR para a detecção de marcadores de resistência e as 140 de *Campylobacter jejuni* para avaliar genes de patogenicidade. Em referência a *Campylobacter jejuni*, os resultaram indicaram que o gene *fla*A estava presente em 78.5% e o marcador *cad*F foi detectado em 77.8% dos isolados. Do total das amostras 85% (119/140) foram detectados para o gene *cdt*A, 80% (112/140) para o gene *cdt*B e 92.1% (129/140) para o gene *cdt*C. O operon (*cdt*ABC) associado com a expressão total da toxina citoletal estava presente em 74.2% (104/140) das amostras. O marcador genético associado à invasão (*iam*) não foi encontrado em nenhum dos isolados de *Campylobacter jejuni*, e a ocorrência dos genes *vir*B11 e *wla*N foi de 22.1% e 10.7%, respectivamente. Na pesquisa de resistência a antimicrobianos, uma alta porcentagem (65%) (91/141) dos isolados de *Campylobacter* spp. são resistentes a β-lactâmicos. Cinquenta cepas (35.5%) são resistentes a tetraciclinas e 26 (18.5%) tem a presença da bomba de efluxo. Neste contexto, 36 de 141 cepas de

Campylobacter (25.6%) são resistentes a dois diferentes marcadores de resistência (*bla*$_{\text{OXA-61}}$ e *tet*O). Realizou-se também outro estudo, para detectar a mutação no gene *gyr*A da região determinante de resistência a quinolonas (QRDR). Um total de 50 amostras de *Campylobacter jejuni* foram submetidas a testes de sensibilidade mediante ensaios genotípicos e fenotípicos. A Concentração Inibitória Mínima (CIM) foi determinada utilizando a técnica de microdiluição em caldo. Os resultados obtidos mostraram uma porcentagem de 98% sensíveis a eritromicina. Em contraste, 94% de *Campylobacter jejuni* isolados foram resistentes à ciprofloxacina (47/50) e quarenta e cinco cepas (90%) resistentes ao ácido nalidíxico. Em referência aos isolados resistentes à ciprofloxacina, 100% das estirpes apresentavam relação entre o fenótipo de resistência e uma mutação no aminoácido 86 do gene *gyr*A, sendo detectada pelo ensaio de PCR-RFLP e posteriormente confirmada por sequenciamento. Os resultados deste estudo mostram uma grande diversidade entre os isolados analisados. O esforço para reduzir as infecções por *Campylobacter* spp. em humanos está diretamente ligado a uma melhor compreensão dos aspectos biológicos deste microrganismo e, particularmente, dos seus mecanismos de virulência e resistência, que contribuem na patogênese da doença.

Palavras-chave: *Campylobacter,* resistência a antimicrobianos, genes de patogenicidade, MIC, PCR, RFLP.

DETECTION VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE PATTERNS IN *Campylobacter* spp. HUMANS ISOLATES AND FROM SLAUGHTERHOUSES IN SOUTHERN BRAZIL.

Yuli Melisa Sierra-Arguello

Orientador: Vladimir Pinheiro do Nascimento

ABSTRACT

Campylobacteriosis is a zoonosis of worldwide distribution, with important implications for public health and a significant socioeconomic impact. The aim of this study was to investigate the occurrence, antimicrobial resistance patterns and phenotypic and genotypic relationship of *Campylobacter* species. A total of 141 samples, including feces (n = 8), chiller tank processing water (n = 18), carcasses during the slaughter process (n = 26) and poultry meat (n = 89) were evaluated. All the isolates were confirmed by m-PCR based detection of 16SrRNA, *ceu*E and *map*A genes. The most ubiquitous of the thermotolerant *Campylobacter* spp. was *C. jejuni*. It was found in 140 of the contaminated samples (99.2%), whereas *C. coli* was identified in the remaining sample (0.7%). One hundred and forty-one strains of *Campylobacter* spp. analysis were subjected to PCR for the detection of resistance markers and 140 *Campylobacter jejuni* strains to evaluate pathogenic genes. In reference to *Campylobacter jejuni*, the obtained results showed that, the occurrence of *fla*A gene was 78.5% and *cad*F gene 77.8% in the isolates. The cytotoxin encoding cluster *cdt*ABC was detected in 74.2% isolates. The frequency rates found for *cdt*A, *cdt*B and *cdt*C was 85%, 80%, and 92.1% respectively. The invasion-associated marker (*iam*) gene was not found in any of the *Campylobacter* isolates, and the occurrence of plasmidial *vir*B11 and *wla*N genes were 22.1%, and 10.7%, respectively. The results obtained point to the high percentage (65%) of *Campylobacter* isolates resistant to β-lactam. Fifty strains (35.5%) were resistant to tetracycline and 26 (18.5%) to the efflux pump. Moreover, 36 out of the 141 *Campylobacter* strains (25.6%) were found to be resistant to at least two different antimicrobial resistance markers ($bla_{\text{OXA-61}}$ and *tet*O). Also, a total of 50 samples were screened for presence of antimicrobial sensibility for genotypic and phenotypic methods. Determination of Minimal Inhibitory Concentration (MIC) is tested using the standard microdilution method. The MICs results to 3

antimicrobial agents analyzed, showed that 98% of isolates were sensitive to erythromycin. In contrast, most isolates were resistant to ciprofloxacin (94%) and nalidixic acid (90%). Regarding ciprofloxacin-resistant isolates, 100% of the phenotype resistance strains had a mutation in the *gyr*A gene that was detected by the PCR-RFLP assay and sequencing. The results of this study show a great diversity among the isolates analyzed. The effort to reduce infections by *Campylobacter* spp. in humans is directly linked to a better understanding of the biological aspects of this microorganism and, particularly, its virulence and resistance mechanisms that contribute to the pathogenesis of the disease.

Keywords: *Campylobacter,* antimicrobial resistance, pathogenicity genes, MIC, PCR, RFLP.

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LISTA DE ABREVIATURAS – PORTUGUÊS

AAC	Acetiltransferases
AAD	Adeniltransferases
APH	Aminoglicosídeos fosfotransferases
β	Beta
CDC	Centro de Controle e Prevenção de Doenças
CDPA – UFRGS	Centro de Diagnóstico e Pesquisa Aviaria da Universidade Federal do Rio Grande do Sul
CMI	Concentração Mínima Inibitória
FTA	Cartões FTA - Flinders Technology Associates - FTA® Cards for DNA Analysis
DTA	Doenças transmitidas por alimentos
EFSA	Autoridade Europeia para a Segurança Alimentar
FQs	Fluoroquinolones
LOS	Lipo-oligossacarídeos
LPS	Lipo-polissacarídeos
KWL	Esquema Kauffmann-White-Le Minor
m-PCR	Multiplex-PCR
OIE	Organização Mundial de Saúde Animal
PCR	Reação em Cadeia da Polimerase
QRDR	Região Determinante de Resistência a Quinolonas
SGB	Síndrome de Guillain Barré
RFLP	Polimorfismo de Comprimentos dos Fragmentos de Restrição
UBABEF	União Brasileira de Avicultura
UE	União Europeia
USDA	Departamento de Agricultura dos Estados Unidos da América

LISTA DE ABREVIATURAS – INGLÊS

AMP	Ampicillin
ATCC	American Type Culture Collection
BA	Blood Agar
BPW	Buffered Peptone Water
CADF	Campylobacter Adhesion to Fibronectin F
CDPA–UFRGS	Centre for Diagnostics and Research in Avian Pathology
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
ERY	Erythromycin
FTA	Flinders Technology Associates - FTA® Cards for DNA Analysis
GSB	Guillain-Barre Syndrome
HUS	Hemolytic Uremic Syndrome
IAM	Invasion-associated Marker
ISO	International Standards Organization
KWL	Kauffmann-White-Le Minor
mCCDA	Modified Charcoal Cefoperazone Deoxycholate
MIC	Minimal Inhibitor Concentration
MFS	Miller Fisher Syndrome
NAL	Nalidixic Acid
NOR	Norfloxacin
RFLP	Restriction Fragment Length Polymorphism
UV	Ultraviolet
WHO	World Health Organization

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1. INTRODUÇÃO

O Brasil destaca-se mundialmente na produção e industrialização de alimentos de origem animal. O setor de carnes é uma das áreas do agronegócio brasileiro com maior dinâmica tecnológica e de conhecimento. O Brasil ocupa posição importante no cenário internacional. É o maior exportador de carne de frango (desde o ano de 2004), atingindo em 2013 aproximadamente 3,918 milhões de toneladas, seguido pelos Estados Unidos (EUA) e União Europeia (UE) com 3,354 milhões e 1,095 mil toneladas respectivamente, sendo que os dois primeiros países contribuem com 68% das exportações mundiais.

De acordo com o Departamento de Agricultura dos Estados Unidos da América (USDA), a produção norte-americana de carne de frango teria somado 16,958 milhões de toneladas em 2013, contra 13,500 milhões da produção chinesa. Com este desempenho, o Brasil é o terceiro maior produtor mundial de carne de frango alcançando 12,308 milhões de toneladas. Segundo a União Brasileira de Avicultura (UBABEF, 2014) a produção e consumo de carne de aves tem aumentado consideravelmente, uma vez que esta fonte proteica tornou-se a mais econômica entre as proteínas de origem animal. No entanto, em 2013 o consumo *per capita* de carne de frango foi de 41,80 kg/habitante/ano, o menor consumo observado nos últimos tempos, com diminuição de 7,11% em relação a 2012.

O desenvolvimento da avicultura, decorrente da evolução genética das aves, da nutrição e do sistema de manejo, propicia a produção avícola em grande escala. No entanto, o sistema de confinamento adotado favorece a introdução e disseminação de agentes patogênicos. Neste contexto, incluem-se as bactérias do gênero *Campylobacter* que por meio de produtos de origem avícola, podem causar doenças de origem alimentar em seres humanos.

Os microrganismos do gênero *Campylobacter* têm distribuição mundial, constituindo-se em potencial problema para a saúde pública. As infecções causadas por *Campylobacter* spp. têm sido relatadas como uma das mais frequentes causas de gastroenterite em vários países do mundo (KWAN et al., 2008). Dados publicados

durante o ano de 2013 pela Autoridade Europeia para a Segurança Alimentar (EFSA), apontam *Campylobacter* spp. como sendo o agente etiológico mais frequentemente isolado em patologia gastrointestinal aguda em seres humanos por toda a Europa e segundo agente mais isolado em infecções nos Estados Unidos (CDC, 2013). Para outras fontes bibliográficas, esta é uma problemática verificada em outros continentes (EFSA, 2013).

Nos últimos anos têm ocorrido avanços na compreensão sobre patógenos veiculados por alimentos e em tecnologias para controle de produtos e processos direcionados na inocuidade (MENDONÇA, 2003). As aves domésticas albergam *Campylobacter* spp. no intestino, que por meio de manipulação e operações de abate mal conduzidas e sem a observação de práticas higiênicas, contaminam a carcaça e as vísceras. Carne e miúdos de frango são fontes potenciais de zoonose (CARVALHO et al., 1996; CARVALHO et al., 2002). Embora várias espécies de *Campylobacter* estejam associadas à doença no homem, as espécies *Campylobacter jejuni (C. jejuni), Campylobacter coli (C. coli)*, *Campylobacter lari (C. lari)* e *Campylobacter upsaliensis (C. upsaliensis)* são as mais frequentes isoladas em casos de gastroenterite humana.

Nas últimas décadas temos assistido várias crises alimentares (dioxinas, encefalopatia espongiforme bovina, nitrofuranos em frangos, etc.) que coloca em risco a saúde dos consumidores, mas ao mesmo tempo despertam a atenção para várias questões relacionadas com a segurança dos alimentos consumidos. A alta incidência de Doenças Transmitidas por Alimentos (DTA) aliados a um consumidor cada vez mais exigente em relação à segurança dos produtos que adquire, gera uma maior preocupação com medidas para prevenir tais doenças. Com isto, os estudos sobre a qualidade alimentar são cada vez mais numerosos e de extrema relevância. Nos últimos anos, um aumento na prevalência de cepas de *Campylobacter* spp. multiresistentes tem sido um problema global (EFSA, 2013). Genes de resistência aos antimicrobianos em bactérias comensais ou estirpes patogênicas é um risco indireto para a saúde pública, porque uma vez que cepas patogênicas adquirem esses genes, promovem os mecanismos responsáveis da variabilidade genética (VEARRES et al., 2013). Assim, torna-se importante testar várias cepas de *Campylobacter* spp. e verificar sua

susceptibilidade a antimicrobianos utilizados para o tratamento de infecções mais graves.

A apresentação desta tese de Doutorado foi divida em 5 capítulos. A primeira parte corresponde à revisão de literatura sobre o gênero *Campylobacter*, seu potencial zoonótico, sua importância na cadeia produtiva avícola e sua resistência a agentes antimicrobianos, elucidando os principais mecanismos de patogenicidade e resistência envolvidos. Os capítulos 1, 2, 3, 4 e 5 foram redigidos na forma de artigos científicos, relatando os principais resultados obtidos com o presente estudo. O **capítulo I** aborda os principais genes de patogenicidade em uma coleção de isolados de *Campylobacter jejuni* provenientes de swabs (cloacal e caixas de transporte), água e carcaças de aves nas diferentes etapas da linha de abate no Sul do Brasil. No **capítulo II** e **capítulo III** e apresentaram as frequências de resistências aos agentes antimicrobianos comumente utilizados em avicultura e de importância no tratamento em humanos. Os isolados que apresentam perfis de multirresistência foram investigados para a presença dos principais genes de resistência encontrados em *Campylobacter* spp. O **capítulo IV** fez referência a novas metodologias moleculares na detecção de resistência a antimicrobianos que serviram como rotina no diagnóstico tanto na avicultura quanto para o processamento de amostras humanas. Finalmente, o **capítulo V** estabelece o uso de cartões FTA para o transporte sem risco biológico de amostras de DNA de *Campylobacter* spp. que possa servir como ferramenta para futuras pesquisas, especialmente em diagnóstico molecular.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo do presente estudo foi determinar as bases fenotípicas e genotípicas de resistência a agentes antimicrobianos e analisar os genes de patogenicidade de estirpes de *Campylobacter* spp. em isolados humanos e de matadouros-frigoríficos na Região Sul do Brasil.

2.2 Objetivos Específicos

- Determinar a frequência de cepas de *Campylobacter* spp. obtidos nas diferentes etapas da linha de abate de aves na Região Sul do Brasil.

- Realizar a análise microbiológica e identificação molecular de *Campylobacter jejuni* e *Campylobacter coli* mediante Multiplex-PCR em isolados humanos e de matadouros-frigoríficos da região sul do Brasil.

- Avaliar a presença dos principais genes de virulência e resistência encontrados nos isolados de *Campylobacter jejuni* de origem avícola mediante a técnica da Reação em Cadeia da Polimerase (PCR).

- Avaliar o perfil de sensibilidade de *Campylobacter jejuni* de origem avícola, mediante a técnica de Concentração Inibitória Mínima (CIM) das estirpes de *Campylobacter* spp. frente às fluoroquinolonas (ciprofloxacina e ácido nalidíxico) e macrolídeos (eritromicina).

- Estudar o principal mecanismo de resistência a ciprofloxacina em *Campylobacter* spp. por meio das técnicas PCR-RFLP, e sequenciamento da Região Determinante de Resistência às Quinolonas (QRDR) do gene *gyr*A.

- Estabelecer se o uso dos cartões FTA® é efetivo no transporte de amostras de DNA de *Campylobacter* spp. e se o DNA obtido tem uma concentração e pureza adequado para a realização de testes moleculares.

3. REVISÃO BIBLIOGRÁFICA

3.1 Histórico

Os primeiros relatos de *Campylobacter* foram feitos provavelmente na década de 1880, quando Theodor Escherich pela primeira vez observou bactérias com forma de espiral em cólons de crianças com diarréia que haviam morrido. Os primeiros isolamentos de espécies do gênero *Campylobacter* foram realizados na área de microbiologia veterinária em 1909 e 1913. McFadyean e Stockman (1913) e posteriormente Smith (1918) estabeleceram a participação de uma bactéria microaerófila no aborto do gado bovino e ovino denominando-a de *Vibrio fetus*. Mais tarde, Jones e Little (1931) isolaram, a partir de bovinos com distúrbio intestinal, um "*vibrion*" microaerófilo e denominaram de *Vibrio jejuni*.

Doyle (1944) obteve o isolamento de bactérias curvas microaerófilas a partir de casos de diarreia suína, cujas características fenotípicas não se assemelhavam totalmente ao *Vibrio fetus* nem ao *Vibrio jejuni*, denominando-o *Vibrio coli* e sugerindo-o como agente etiológico da disenteria suína. Levy (1946) descreveu um surto de diarreia em humanos, verificando em amostras de fezes e de sangue a presença de formas curvas e espiraladas de vibriões, sugerindo que a ingestão de leite cru teria sido a provável fonte de contaminação, estabelecendo assim, a primeira infecção humana relacionada a este grupo de bactérias.

Entretanto, apenas em 1963, Sebald e Véron propuseram a criação do gênero *Campylobacter* (do grego *campylo* = curvo e *bacter* = bacilo) para incluir as bactérias antes denominadas *Vibrio*. Com base em estudos filogenéticos, Véron e Chateleine (1966) propuseram a inclusão do gênero *Campylobacter* na família Spirillaceae. Apenas em 1991, o gênero *Campylobacter* foi incluído na família Campylobacteriaceae (VANDAMME et al., 1991).

Microbiologistas veterinários têm desempenhado um papel importante no desenvolvimento de métodos de cultura específicos. Martin Skirrow descreveu uma técnica relativamente simples para a cultura de *Campylobacter* em 1977: Agar sangue

contendo vancomicina, polimixina e trimetoprim e incubado a 43°C numa atmosfera de microaerofilia. A utilização desta nova técnica levou à compreensão de que as infecções que causam campilobacteriose em todo o mundo são um problema significativo de saúde, embora só no decorrer da década de 1980 fosse finalmente reconhecido como umas das causas mais frequentes de gastroenterite bacteriana no homem (MOORE et al., 2005).

3.2 Taxonomia

O gênero *Campylobacter* constitui assim os gêneros *Arcobacter*, *Dehalospirillum* e *Sulfurospirillum*, a família da *Campylobacteraceae* colocada na classe *Epsilonproteobacteria* (filo das "*Proteobacteria*", domínio ou império das "*Bacteria*" ou das "*Eubacteria*").

Atualmente, esse gênero conta com 25 espécies: 1) *Campylobacter avium*; 2)*Campylobacter canadensis*; 3) *Campylobacter coli*; 4) *Campylobacter concisus*; 5)*Campylobacter cuniculorum*; 6) *Campylobacter curvus*; 7) *Campylobacter fetus*; (*Campylobacter fetus* subsp *fetus*, *Campylobacter fetus* subsp *venerealis*), 8)*Campylobacter gracilis*; 9) *Campylobacter helveticus*; 10) *Campylobacter hominis*; 11)*Campylobacter hyoilei* 12)*Campylobacter hyointestinalis*; *(Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter hyointestinalis* subsp. *lawsonii*), 13) *Campylobacter insulaenigrae*; 14) *Campylobacter jejuni*; (*Campylobacter jejuni* subsp. *doylei*, *Campylobacter jejuni* subsp. *jejuni*), 15) *Campylobacter lanienae*; 16)*Campylobacter lari*; 17) *Campylobacter mucosalis*; 18) *Campylobacter peloridis*; 19) *Campylobacter rectus*; 20) *Campylobacter showae*; 21) *Campylobacter sputorum*; (*Campylobacter sputorum* subsp *bubulus*, *Campylobacter sputorum* subsp *sputorum*) 22) *Campylobacter subantarcticus*; 23) *Campylobacter upsaliensis*; 24) *Campylobacter ureolyticus*; 25) *Campylobacter volucris*. *(*www.bacterio.cict.fr/c/campylobacter).

O genoma de *Campylobacter* possui de 1600 a 1700 kb, embora a espécie *C. upsaliensis* tenha cerca de 2000 kb. Este genoma corresponde a um pouco mais de 30% do genoma de *E. coli*, que possui 4500 kb. Acredita-se que o pequeno tamanho de *Campylobacter* possa explicar o comportamento exigente e sua incapacidade de fermentar carboidratos (VANDAMME, 2000).

3.3 Características Bacteriológicas

3.3.1 Morfologia, Propriedades Físicas e Bioquímicas.

O gênero *Campylobacter* é constituído de bastonetes Gram-negativos, encurvados, em forma de S ou de forma espiralada, não esporulados com 0,2 a 0,5 μm de diâmetro de largura e 0,5 a 5,0 μm de comprimento e podem apresentar formas cocóides com retração citoplasmática quando as condições de desenvolvimento lhe são desfavoráveis, por envelhecimento das culturas (culturas com mais de 48 horas) ou por estresse oxidativo (KEENER et al., 2004). Segundo o estudo elaborado por Keum-LL et al. (2007), as células podem igualmente transitar para esta forma, quando sujeitas a diferentes temperaturas, nomeadamente de 4ºC e 25ºC, e quando as condições do meio se tornam novamente favoráveis, é possível a reversão para a forma espiral, bem como a respectiva multiplicação. Colônias de *Campylobacter* podem ser: lisas, convexas, brilhantes e com bordas perfeitas; ou ainda planas, translúcidas e lustrosas, com bordas irregulares e espalhadas. Geralmente são incolores, com tonalidades creme ou acinzentada (Figura 1).

Figura 1**-** Colônias de *Campylobacter jejuni* em ágar mCCDA.

Geralmente são muito móveis graças a um flagelo localizado em uma ou nas duas extremidades da célula; têm metabolismo respiratório; são incapazes de utilizar açúcares (nem oxidação, nem fermentação) (FEDERICK et al., 2011); obtendo energia a partir de aminoácidos ou ácidos intermediários como ácido tricarboxílico (HOLT et

al., 1994; FORSYTHE, 2002); não hidrolisam a gelatina nem a uréia (com exceção às linhagens atípicas de *C. lari* e linhagens de *Campylobacter sputorum* biovar *Paraureolyticus*) e são desprovidos de lipase (CARDOSO et al., 2011). São sensíveis ao pH ácido, abaixo de 4,9, e à desidratação, sendo a atividade da água (Aw) ideal de 0,997 (GERMANO et al., 2003). Apresentam oxidase positiva, catalase variáveis; que lhe conferem a capacidade de decomposição do peróxido de hidrogênio em oxigênio e água e a capacidade de catalisarem reações de oxidação/redução, envolvendo o oxigênio molecular como aceptor de elétrons, respectivamente (WASSENAAR et al., 2006; LEVIN, 2007).

As espécies *C. coli* e *C. jejuni* são praticamente idênticas, porém, a separação das duas espécies tem por base apenas o teste de hidrólise do hipurato, que é positivo para *C. jejuni* e negativo para *C. coli*. Apesar disso, não são conhecidas grandes diferenças entre as duas espécies com relação à patogenicidade, à composição antigênica e às características epidemiológicas relacionadas aos mecanismos de transmissão e sua distribuição em animais. Em relação a este último aspecto, *C. coli* é reconhecida em suínos como seu principal reservatório natural e em países desenvolvidos, é responsável por aproximadamente 3% dos casos de diarreia produzidos pelas espécies termotolerantes do gênero. Já nos países em desenvolvimento, esta frequência pode atingir até 25% (TRABULSI et al., 2005).

Campylobacter spp. são microrganismos de crescimento lento e extremamente exigente. A maioria das espécies são microaerófilas, com metabolismo respiratório complexo, necessitando de uma atmosfera com baixas concentrações de oxigênio para o seu crescimento, requerendo para isolamento incubação em atmosfera de 5% de oxigênio, 10% de dióxido de carbono e 85% de nitrogênio (ACHA et al., 2001). Contudo, algumas espécies como *Campylobacter gracilis*, *Campylobacter hyointestinalis*, *Campylobacter showae* e *Campylobacter sputorum* conseguem crescer em condições de anaerobiose (GUNTHER et al., 2009). Todas as espécies se desenvolvem a 37°C, ressaltando *C. jejuni, C. coli, C. lari e C. upsaliensis*, consideradas espécies termofílicas, cuja temperatura ótima de multiplicação oscila entre 42°C e 43°C (OIE, 2008).

3.4 Epidemiologia

Evidências epidemiológicas têm sugerido os produtos de origem animal, especialmente os produtos avícolas, como principal veículo para infecção humana (HARRIS et al., 1986; MACHADO et al., 1994; MOORE et al., 2005), uma vez que o trato intestinal das aves domésticas tem sido demonstrado como o principal reservatório de *Campylobacter jejuni* (SAHIN *et al.,* 2002; HERMANS et al., 2011) e que aproximadamente 30 a 100% das aves transportam este agente no intestino (CORRY et al., 2001; LOC CARRILLO et al., 2005).

A via horizontal é o mecanismo mais provável de transmissão (SAHIN et al., 2002) com as fontes possíveis incluindo roedores (RODRIGUES et al., 1998), insetos (CHOO et al., 2011), aves selvagens (CRAVEN et al., 2000; WALDENSTRÖM et al., 2010), animais domésticos (COLLES et al., 2003) e o homem (SHEPPARD et al., 2009). Embora o potencial de transmissão vertical de matrizes de frangos de corte exista (NEWELL et al., 2003), na prática, é uma ocorrência rara (SHANKER et al., 1986; VAN DE GIESSEN et al., 1992; CHUMA et al., 1997; PETERSEN et al., 2001), sem risco significativo para rebanhos comerciais (CALLICOTT et al., 2006).

Fatores responsáveis pela introdução e disseminação de *C. jejuni* em aves de produção comercial foram estudados por Kazwala et al. (1990) e Berndtson et al. (1996), sugerindo a cama, pés dos tratadores e água como os principais veiculadores do agente. O papel da cama na transmissão e no estabelecimento permanente da infecção por *C. jejuni* foi demonstrado por Montrose et al. (1985) em aves através da infecção artificial, detectando o agente pelo menos 63 dias após a infecção.

Desta maneira, durante o processo de abate, as carcaças e vísceras comestíveis podem se contaminar e o agente ser detectado no produto acabado e pronto para o consumo (SAKUMA et al., 1992; CARVALHO, 1998; HERMANS et al., 2011). *Campylobacter spp.* pode ser transmitido para seres humanos principalmente pela via fecal/ oral por contato direto, exposição à carne contaminada ou reservatórios de água e leite contaminados (YAN et al., 2005; CONLAN et al., 2007).

3.5 Patogenia em Humanos

Apesar dos mecanismos de patogenicidade ainda não se encontrarem completamente esclarecidos, são citados os potenciais fatores de virulência deste agente como motilidade, quimiotaxia, adesão, invasão e produção de toxinas (enterotoxinas e citotoxinas) (SNELLING et al., 2005; BHAVSAR et al., 2007) (Figura 2).

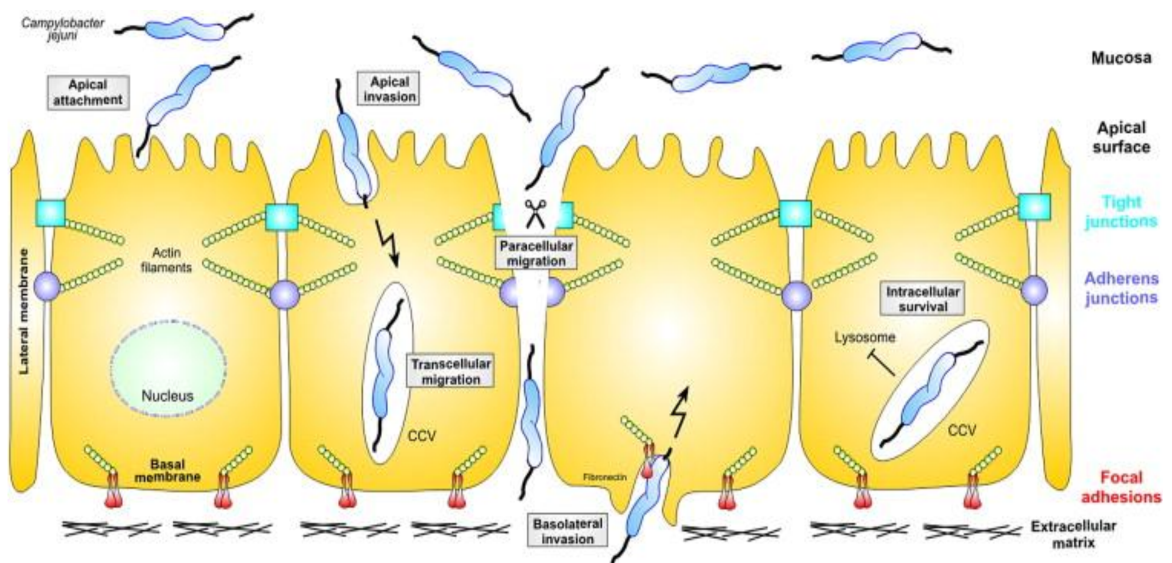

Figura 2**-** Modelo hipotético dos mecanismos de infecção. O patógeno pode interatuar e invadir, transmigrar e sobreviver polarizando as células intestinais (BACKERT et al., 2013).

Quimiotaxia: A quimiotaxia consiste num movimento de aproximação ou afastamento em relação a um estímulo químico, sendo um importante determinante na virulência deste agente (KONKEL et al., 2001). *Campylobacter* spp. apresenta uma forte resposta quimiotáctica ao carboidrato L-fucose, constituinte principal da mucina (STAHL et al., 2011).

Adesão: A etapa de adesão de *Campylobacter* é assegurada pela presença de adesinas, localizadas nos flagelos e outros componentes da superfície celular bacteriana, tais como Lipo-oligossacarídeos (LOS) (LEVIN, 2007). Um dos fatores que contribui para que este agente patogênico confunda o sistema imunitário são LOS da sua membrana, estruturas semelhantes aos Lipo-polissacarídeo (LPS), mas que não

possuem as cadeias laterais de polissacarídeos que se encontra nas enterobacterias. *C. jejuni* é uma das poucas bactérias que possui a capacidade de sintetizar de forma endógena o ácido siálico e incorporá-lo na constituição dos LOS (SCHWERER et al., 1995; SALLOWAY et al., 1996).

LOS são constituídos por um lipídeo A e uma região central "*core*" (oligossacarídeo), mas carece do polissacarídeo O presente nos LPS da maioria dos bacilos gram negativos. No "*core*" das estirpes de *Campylobacter jejuni* foi detectada a presença de ácido N-acetilneurâmico (acido siálico), que quando unido por ligações do tipo 2-3 a β-D galactosidase, assemelha-se à estrutura dos gangliosídeos. Este mimetismo molecular pode levar ao aparecimento de doenças autoimunes, como a Síndrome de Guillain-Barré ou Miller-Fischer, uma vez que são produzidos anticorpos contra a estrutura do LOS, ocorrendo uma reação cruzada entre estes e os gangliosídeos do hospedeiro (GUERRY et al., 2008), conduzindo à desmielinização e/ou degeneração axonal dos nervos periféricos (LEVIN, 2007).

De acordo com Konkel et al. (2001), LOS de *C. jejuni* são similares ao de *Haemophilus* spp. e *Neisseria* spp. A proteína de membrana externa e os LOS são fortemente imunogênicos, estimulando a produção de antissoros específicos em indivíduos que se recuperam da infecção (NACHAMKIN et al., 1998).

Segundo Jawets (1998), as espécies de *Campylobacter* possuem LOS e flagelos que atuam como estruturas de aderência e invasão sendo capazes de produzir citotoxinas e enterotoxinas (Figura 3). Recentemente, verificou-se que a ocorrência de mutações nos genes *cgt*B e *wla*N, que codificam ambos para uma β-1,3-galactosiltransferase, estão ligadas a diferenças na capacidade de colonizar o trato gastrointestinal de aves, e invadir de células Caco-2, similares às células epiteliais do intestino humano (MÜLLER et al., 2006).

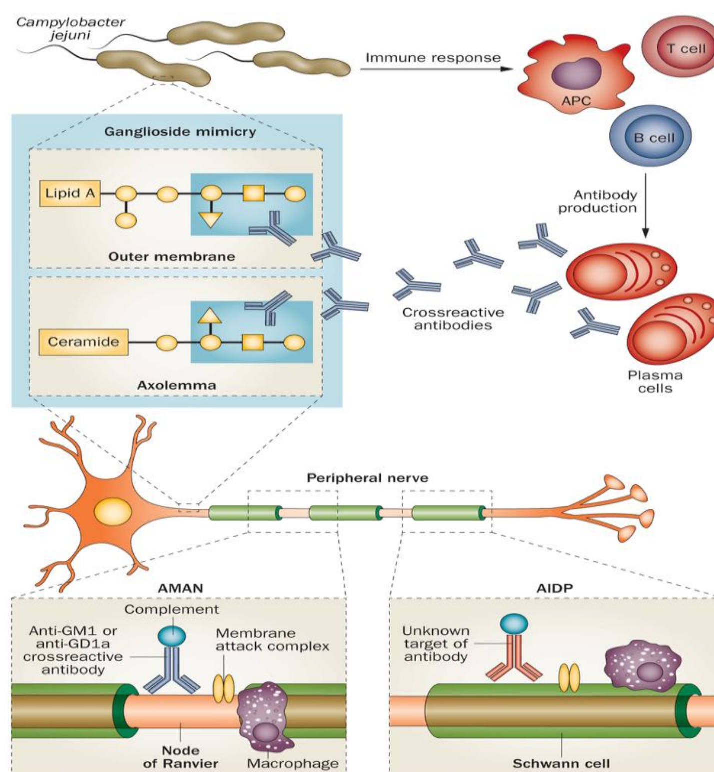

Figura 3 **-** Autoimunidade induzida por *C. jejuni*. LOS de estirpes de *C. jejuni* mimetizam a estrutura encontrada nos gangliosídeos humanos (VAN DEN BERG et al., 2014).

Invasão: A capacidade deste microorganismo de invadir as células epiteliais depende principalmente da estirpe envolvida (RIVERA-AMILL et al., 2001). A invasão por *Campylobacter* ocorre por endocitose (receptores de membrana). Estes receptores estão associados à proteína do citoplasma, clatrina, que forma uma depressão na membrana. Quando este receptor se liga a *Campylobacter*, esta depressão aumenta e transforma-se em vacúolos citoplasmáticos. Posteriormente, este vacúolo migra através da lamina própria e *Campylobacter* é liberado desencadeando o processo inflamatório (LEVIN, 2007).

Produção de Toxinas: As toxinas podem ser classificadas em duas classes, dependendo do mecanismo de ação: enterotoxinas e citotoxinas. As enterotoxinas são proteínas secretadas com capacidade de se ligarem a receptores celulares, penetrando na célula e aumentando os níveis de AMP cíclico intracelular. A invasão tecidual localizada associada à atividade tóxica parece ser responsável pela enterite (WASSENAAR, 1997).

C. jejuni é o principal causador de enterite aguda humana em muitos países desenvolvidos e em desenvolvimento. Possui a habilidade de aderir e colonizar células, como também invadir enterócitos e sintetizar uma ou mais toxinas (ROZYNEK et al., 2005), entre elas, a CDT, codificada por três genes adjacentes, *cdt*A*, cdt*B e *cdt*C, sendo que a expressão das três subunidades são requeridas para uma plena atividade da toxina (JEON et al., 2005). Evidências atuais indicam que o gene *cdt*B codifica a atividade e toxicidade dos componentes da toxina, enquanto que os genes *cdt*A e *cdt*C estão envolvidos na aderência e interiorização na célula hospedeira (ABUOUN et al., 2005). Sendo assim, a toxina CDT pode funcionar como um fator de virulência em patógenos que produzem essa toxina, desde que os genes estejam ativos (SMITH et al., 2006).

O resultado da atividade da toxina citoletal distensiva pode diferir ligeiramente, dependendo do tipo de célula eucariótica afetada. A toxina CDT contribui para a patogênese através da inibição da imunidade humoral e celular, via apoptose de células de resposta imune, e pode gerar necrose do epitélio celular e fibroblastos envolvidos na reparação de lesões produzidas por patógenos, resultando em lenta cicatrização e indução dos sintomas da doença (SMITH et al., 2006). Portanto, interfere na divisão e diferenciação das células das criptas intestinais, contribuindo para o desenvolvimento da diarreia (WASSENAAR, 1997; PARK, 2002).

3.6 Manifestações Clínicas da Doença em Humanos

A dose infectante da infecção provocada por *Campylobacter* é bastante baixa, sendo suficiente a ingestão de 500 células deste microrganismo para o aparecimento de doença no homem (ROBINSON, 1981). Após a exposição, *Campylobacter* coloniza o trato intestinal baixo (íleo, jejuno e cólon), muitas vezes sem que se observe sintomatologia. Nos casos em que se observam sintomas, estes normalmente iniciam-se nos primeiros 2-3 dias, sendo a dor de cabeça, vômito e febre, os mais comuns. Posteriormente, observa-se o aparecimento de diarreia aquosa ou mesmo sanguinolenta, e dores abdominais, durante 3-7 dias. No entanto, na maior parte dos casos verifica-se uma evolução favorável do quadro clínico (SANTOS, 2011).

A ocorrência de doença extra-intestinal é quando á infecção por *Campylobacter* é baixa quando comparada com o aparecimento de doença entérica. A complicação pós-infecciosa mais observada em pacientes que foram infectados com *Campylobacter* é a síndrome de Guillain-Barré. Esta neuropatia consiste numa paralisia progressiva ascendente, que pode afetar os nervos periféricos e cranianos, podendo surgir à necessidade de ventilar mecanicamente o paciente uma vez que ocorre a paragem dos músculos respiratórios (KUWABARA, 2007). Nesta doença a inflamação aguda e desmielinização (perda da mielina, a membrana lipídica que envolve os nervos) são provocadas por uma reação autoimune que tem origem numa resposta cruzada dos anticorpos produzidos contra um determinado antígeno; no caso da infecção por *Campylobacter*, contra os LPS e seus os componentes da mielina, especificamente o ácido siálico (KOMAGAMINE et al., 2006).

3.7 *Campylobacter* spp. em Frangos

Bactérias do gênero *Campylobacter* encontram-se distribuídas mundialmente, em especial, nas regiões onde a criação comercial de frangos está estabelecida, sendo a alta densidade um fator facilitador para a disseminação do agente entre as aves (ZHANG, 2008). Como um organismo comensal em aves, *Campylobacter* spp. coloniza as células mucosas do intestino e suas criptas. Assim sendo, o número de bactérias nos cecos da ave pode alcançar uma contagem entre 10^6 e 10^8 UFC/g (MEADE et al., 2009). O comensalismo de *Campylobacter* em frangos é discutido em virtude da resposta imune inflamatória e capacidade de invadir e persistir em órgãos internos da ave (HUMPHREY et al., 2014). Existem outros mecanismos de interação entre o hospedeiro e a bactéria que auxiliam para que as aves não desenvolvam a infecção, porem estes mecanismos ainda não foram totalmente elucidados (GHAREEB et al., 2013).

Foi estimado que as doses mínimas de *Campylobacter* para colonização em frangos de corte estão aproximadamente entre 35 UFC (STERN et al., 1988; HERMANS, 2011). Seguidamente, a bactéria atinge o ceco e multiplica-se, resultando na colonização em aproximadamente 24 horas após a ingestão do microrganismo (COWARD et al., 2008). Acredita-se que a temperatura da ave favoreça esta relação comensal, pois a predileção destas bactérias pelo trato intestinal das aves pode estar

relacionada ao fato das mesmas possuírem temperatura maior que 40º C, tornando esse local um nicho para seu desenvolvimento e multiplicação (YOUNG, 2007).

A colonização dos frangos está relacionada com a idade. A maior parte das aves são negativas para este microrganismo nas primeiras duas semanas de idade (JACOBS-REITMA et al., 1995; VAN GERWE et al., 2009), provavelmente devido à presença de anticorpos maternos que conferem proteção contra a colonização nos primeiros dias de vida (SAHIM et al., 2003). Uma vez ocorrida a colonização por *Campylobacter* a transmissão por coprofagia é extremadamente rápida, podendo um 100% dos frangos ser colonizados num período de 72 horas (STERN, 2001) e ficar assim até o abate (COWARD et al., 2008; STERN et al., 2008). Os frangos são colonizados por *Campylobacter jejuni* (65 a 95%), menos frequentemente por *Campylobacter coli* e raramente por outras estirpes de *Campylobacter* (OIE, 2008).

Assim como no meio externo, no intestino do frango é provável encontrar fatores estressores que comprometem o ótimo crescimento bacteriano (MURPHY et al., 2006). A habilidade de permanecer viável no ambiente durante logos períodos e a capacidade de colonização persistente no trato-gastrointestinal do frango indica que a bactéria possui sistemas regulatórios que conferem proteção tanto no hospedeiro quanto fora dele, garantindo o crescimento e aporte de nutrientes em ambientes onde as condições não são favoráveis (HERMANS et al., 2011). O mecanismo pelo qual a bactéria se adapta a esse ambiente "hostil" é bem sucedido, mas é pouco compreendido. No entanto é reconhecido que pode deber-se a um processo multifatorial (NEWELL et al., 2002), em que os genes de *C. jejuni* envolvidos em todas as áreas do processo de colonização desempenham um papel decisivo na supervivência da bactéria (HERMANS et al., 2011).

3.8 *Campylobacter* spp. e Resistência a Antimicrobianos

A resistência de vários tipos de bactérias patogênicas a agentes antimicrobianos frequentemente utilizados constitui uma problemática atual que é alvo de discussão tanto no contexto de medicina veterinária quanto de medicina humana. As principais consequências do aparecimento de resistências em bactérias que causam doenças em humanos incluem o aumento da frequência de falhas terapêuticas e a severidade das

infecções, o que pode ser revelado pelo aumento da duração da doença, incremento do número de manifestações sistêmicas, maior número de hospitalização e altas taxas de mortalidade.

São necessárias duas condições para que uma bactéria desenvolva resistência a antimicrobianos. Por um lado, o microrganismo tem que entrar em contacto com esta substância e por outro, tem que desenvolver um mecanismo de resistência ao mesmo antimicrobiano e transmiti-lo ou às células que o rodeiam ou às células filhas afetando as gerações futuras. De um modo geral, a rápida evolução da resistência antimicrobiana é uma resposta das bactérias às importantes alterações no seu ambiente introduzido pelo uso de antimicrobianos (EFSA, 2005).

As infecções por *Campylobacter* são leves, auto-limitadas e geralmente desaparecem dentro de alguns dias, não sendo necessário tratamento específico na maioria dos casos. No entanto, a terapia com antimicrobianos tem sido recomendada principalmente para o tratamento de espécies susceptíveis. Na terapia clínica, a eritromicina é a primeira opção de tratamento da campilobacteriose em humanos, seguido pelas fluoroquinolonas (FQs) (ciprofloxacina), sendo estas últimas frequentemente usadas devido ao seu amplo espectro de atividade contra patógenos entéricos (ENGBERG et al., 2001).

Nos últimos anos tem havido avanços na compreensão de patógenos e tecnologias de origem alimentar para o controle de produtos e processos direcionados na segurança. Os alimentos de origem animal podem constituir um meio para a transmissão de *Campylobacter* resistentes aos seres humanos, particularmente de estirpes resistentes à eritromicina e quinolonas (PEZZOTTI et al., 2003). No caso das bactérias do gênero *Campylobacter* spp., há estirpes que são resistentes naturalmente a um determinado tipo de antimicrobiano e outras que adquirem essa resistência através de mutações ou aquisição de genes que codificam vários tipos de mecanismos de resistência. *C. coli* e *C. jejuni* eram até alguns anos atrás, caracterizadas por ser invariavelmente susceptíveis ao ácido nalidíxico, um antimicrobiano pertencente ao grupo das fluoroquinolonas, sendo esta característica muitas vezes utilizada em nível laboratorial para caracterizar este tipo de bactéria (AARESTRUP et al., 2001).

A resistência microbiana pode ser associada a um aumento na virulência da cepa, resultante da aquisição de genes com fatores de resistência e também pela escolha do agente de tratamento, antes do teste de susceptibilidade (TRAVERS et al., 2002). O uso indiscriminado de antimicrobianos, sub-dosagem, adição de promotores de crescimento à dieta, que expõe os animais por longos períodos a pequenas concentrações de antimicrobianos, podem colaborar para a seleção e manutenção de organismos resistentes (McEWEN et al., 2002).

3.8.1 Resistência a Fluoroquinolonas

Existem três mecanismos que podem conferir o fenótipo de resistência a fluoroquinolonas em bactérias Gram-negativas: alteração no gene que codifica a região de ligação do antimicrobiano na bactéria; redução do acúmulo do produto no interior da célula bacteriana (ou por diminuição da permeabilidade da membrana externa ou por aumento do efluxo do fármaco) e pela proteção do alvo do antimicrobiano mediada por uma proteína *Qnr*.

Em bactérias Gram-negativas, a DNA girase (também conhecida como topoisomerase II) e a topoisomerase IV são, respectivamente, os alvos primário e secundário das fluoroquinolonas. Essas enzimas são estruturas protéicas quaternárias de grandes dimensões e estão constituídas por duas subunidades: DNA girase (codificada pelos genes *gyr*A e *gyr*B) e a topoisomerase IV (codificada pelos genes *par*C e *par*E) (BACHOUAL *et al.,* 2001).

Para que uma estirpe bacteriana se torne resistente a fluoroquinolonas basta uma mutação no gene que codifica para uma das subunidades das topoisomerases II e IV. No entanto, mutações adicionais no gene que codifica para *gyr*A, *gyr*B ou *par*C podem ir aumentando o nível de resistência da cepa. No caso das cepas de *Campylobacte*r spp. o alvo secundário das fluoroquinolonas (topoisomerase IV) parece estar ausente. Esta ausência faz com que tanto para *C. jejuni* quanto para *C. coli*, uma mutação na QRDR da subunidade *gyr*A seja suficiente para conferir o fenótipo de resistência a fluoroquinolonas (LUANGTONGKUM et al., 2009; PAYOT et al., 2006).

Em *C. jejuni*, o sequenciamento do gene *gyr*A demonstrou que a substituição do aminoácido Treonina por Isoleucina na posição 86 (Thr-86-Ile) estava associado a um alto nível de resistência às fluoroquinolonas e a troca da Asparagina por Alanina na posição 90 (Asp-90-Ala) e Ala-70-Thr foram associados com moderado nível de resistência aos mesmos antimicrobianos (GRIGGS et al., 2005). Gibreel et al. (1998) sugeriu que a combinação da substituição do aminoácido Thr-86-Ile na proteína GyrA e de Arginina por Glutamina na posição 139 (Arg-139-Gln) na proteína ParC leva a um alto nível de resistência às fluoroquinolonas. Outro mecanismo de FQ-resistência que parece funcionar no desempenho com mutações *gyr*A é através do mecanismo da bomba de efluxo (LIN et al., 2002; PUMBWE et al., 2002; IOVINE, 2013). Estes dois mecanismos (mutação e bomba de efluxo) trabalham em conjunto de forma sinérgica.

3.8.2 Resistência a Macrolídeos

Os macrolídeos são antimicrobianos que se caracterizam pela presença de um anel lactâmico macrocíclico em sua estrutura básica, ao qual se ligam um ou mais açúcares. Em C. *jejuni*, os macrolídeos aderem à parte 50S do ribossomo bacteriano impedindo a transferência dos aminoácidos conduzidos pelo RNA transportador para a cadeia polipeptídica em formação, passo conhecido como translocação (GIBREEL et al., 2006; IOVINE, 2013). Essa resistência em macrolídeos ocorre através de quatro mecanismos gerais: a inibição da ligação do fármaco ao ribossomo, bombas de efluxo, por inativação do fármaco e modificação enzimática. Os dois primeiros mecanismos agem sinergicamente para conferir resistência de alto nível (CAGLIERO et al., 2006; LIN et al., 2007). O quarto é chamado de modificação enzimática dos macrolídeos, mas não está bem caracterizado em *Campylobacter*. A bomba de efluxo *cme*ABC também contribui para a resistência macrolídeos (PAYOT et al., 2006).

3.8.3 Resistência a β-lactâmicos

Algumas estirpes pertencentes ao gênero *Campylobacter* spp. são intrinsecamente resistentes a alguns antimicrobianos, como é o caso dos β-lactâmicos (penicilinas e cefalosporinas). A produção de β-lactamases por algumas destas cepas permite que a fragmentação do anel β-lactâmico impeça o efeito do antimicrobiano sobre a bactéria (YAN et al., 2005). Além da produção das enzimas β-lactamases, outros mecanismos

de resistência como a alteração das proteínas de ligação das penicilinas e a impermeabilidade da bactéria, poderão ser responsáveis pelo aparecimento de linhagens resistentes a este grupo de antimicrobianos. No entanto, a produção de β-lactamases parece ser o principal mecanismo responsável pela resistência das estirpes a β-lactâmicos como amoxicilina, ampicilina e ticarcilina (GRIGGS et al., 2005).

3.8.4 Resistência a Tetraciclinas

As tetraciclinas se ligam à subunidade 30S do ribossomo e inibem a tradução bacteriana, pois bloqueiam a ligação do RNA transportador ao complexo ribossomo-RNA mensageiro (DASTI et al., 2007). A resistência às tetraciclinas poderá ser mediada por quatro mecanismos: efluxo do fármaco para o exterior da bactéria, modificação das tetraciclinas, alteração do local de ligação deste antimicrobiano e mutação da subunidade 16S do rDNA. No entanto, níveis altos de resistência a este antimicrobiano geralmente estão associados com a presença de um gene, designado *tet*(O), que codifica uma proteína envolvida na proteção do sítio de ligação do ribossomo. Na maior parte das estirpes de *Campylobacter spp*. o gene *tet*(O) é codificado em plasmídeos, mas foram já detectadas estirpes onde uma cópia do gene se encontrava no cromossoma. A resistência a tetraciclinas em *C. jejuni* está também associada à bomba de efluxo *cme*ABC (PRATT et al., 2005; LUANGTONGKUM et al., 2009).

3.8.5 Resistência a Aminoglicosídeos

Os aminoglicosídeos inibem a síntese de proteínas, se ligando à subunidade 30S do ribossomo bacteriano, impedindo a leitura correta do RNA mensageiro e síntese da proteína correspondente (IOVINE, 2013). A resistência de *Campylobacter* spp. aos aminoglicosídeos está relacionada com a presença de enzimas que modificam a estrutura destes fármacos. Essas enzimas são divididas em três diferentes grupos, as aminoglicosídeos fosfotransferases (APH), adeniltransferases (AAD) e acetiltransferases (AAC) (AARESTRUP et al., 2001). A resistência a este grupo farmacológico foi inicialmete detectada em *E. coli*, sendo mediada pela presença da enzima aminoglicosídeo 3-fosfotransferase II (codificada por *aph*A-3) que previamente

conferia resistência a kanamicina em estreptococos e estafilococos (IOVINE, 2013). Estes genes são adquiridos por transferência horizontal entre bactérias (AARESTRUP et al., 2001; YAN et al., 2005).

3.8.6 Bomba de Efluxo

Sistemas de exportação dos antimicrobianos através de bombas de efluxo (CmeABC) estão também implicados nos mecanismos de resistência às fluoroquinolonas conferindo resistência intrínseca a um extenso grupo de antimicrobianos incluindo fluoroquinolonas, macrolídeos, ampicilina e tetraciclinas. Payot et al. (2006) verificaram que este sistema atuando sinergicamente com as mutações nos genes *gyr*A e 23S rRNA conferem alto nível de resistência a fluoroquinolonas e macrolídeos, respectivamente. Esse mecanismo de resistência bacteriana envolve a expressão de bombas de efluxo associadas às membranas e que conferem resistência a múltiplos antimicrobianos, pois bombeiam ativamente o antimicrobiano para fora da célula bacteriana (ENDTZ, 1991).

A bomba de efluxo *cme*ABC é codificada por três genes localizados no cromossomo bacteriano, e está constituída de três componentes: uma proteína de fusão periplasmática (CmeA), um transportador de substâncias da membrana plasmática (CmeB) e uma proteína da membrana externa (CmeC). O complexo de proteínas funciona como uma bomba ejetora, expulsando da célula uma grande variedade de componentes, como detergentes, corantes e antimicrobianos (LIN et al., 2002; PUMBWE et al., 2002; GIBREEL et al., 2007; IOVINE, 2013). Estudos têm fornecido evidências de que bomba de efluxo em *C. jejuni* reduz a concentração intracelular de FQs e vários outros antimicrobianos (LUANGTONGKUM et al., 2009; IOVINE, 2013). Esta bomba de efluxo também contribui para a resistência aos ácidos biliares garantindo a colonização do microorganismo (LIN et al., 2003).

3.9 Potencial impacto na Saúde Pública por *Campylobacter* spp.

Campylobacter spp. é uma bactéria cosmopolita e a maioria das infecções possuem caráter zoonótico. No entanto, há apenas duas décadas a campilobacteriose foi reconhecida como zoonose. Desde 2005, as infecções por *Campylobacter* termófilos, particularmente por *Campylobacter jejuni* e *Campylobacter coli*, tornaram-se a causa mais importante de gastroenterite bacteriana humana em muitos países desenvolvidos. No entanto, muitas infecções não são diagnosticadas, não são declaradas ou simplesmente não existem programas de vigilância. Nos Estados Unidos, se estima que por cada 100.000 habitantes existam 14 casos de pacientes afetados com a doença (CDC, 2013). Segundo dados do CDC (2013), um em cada mil casos de campilobacteriose relatados conduz à síndrome de Guillain-Barré. Além disso, 40% dos casos reportados com a síndrome tem relação com um episódio de campilobacteriose prévio (CDC, 2013).

Por outro lado, na União Europeia o cenário é ainda mais alarmante, sendo reportados 64.8 casos de campilobacteriose por cada 100.000 habitantes. Com mais de 214.779 casos humanos no ano em 2013, esta doença é a enfermidade transmitida por alimentos mais frequentemente relatada na União Europeia. A EFSA estima que o custo de campilobacteriose nos sistemas de saúde pública e à perda de produtividade na UE está em torno aos 2,4 bilhões de euros por ano (EFSA, 2013). Foi estimado que uma porcentagem de 50-80% das estirpes de infectam seres humanos são procedentes de produtos de origem avícola. A manipulação, preparação e consumo de carne de frango, em particular, é responsável por 20-30% dos casos humanos de campilobacteriose (EFSA, 2013). Neste contexto, a aquisição da resistência antimicrobiana em bactérias isoladas de aves comerciais é considerada na atualidade como um grave problema de saúde pública.

CAPITULO I

Identification of Pathogenic Genes in *Campylobacter jejuni* isolated from Broiler Carcasses and Broiler Slaughterhouses

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ABSTRACT. *Campylobacter jejuni* is one of the most common causes of human gastroenteritis worldwide and is a significant cause of foodborne disease. Other clinical presentations of *Campylobacter* infection are meningitis, septicaemia, localized extraintestinal infections, reactive arthritis and immune-reactive complications such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS). The pathogenesis of *Campylobacter jejuni* is poorly understood compared to other enteric pathogens. In this study 140 strains of *Campylobacter jejuni* were isolated from samples of broilers of twelve slaughterhouses in the state of Rio Grande do Sul, Brazil; and identified by phenotypic and genotypic methods. PCR was used to confirm the specie and the presence of eight virulence markers of *Campylobacter jejuni*. The *fla*A gene was present in 78.5% and *cad*F marker was detected in 77.8% of the samples. The toxin encoded by an operon (*cdt*ABC) associated with cytolethal distending toxin was present in 74.2% of the isolates. The invasion-associated marker (*iam*) gene was not found in any of the *Campylobacter* isolates, and the identification of plasmidial *vir*B11 and *wla*N genes were 22.1%, and 10.7%, respectively. These results may reveal pathogenic potential in *Campylobacter jejuni* from poultry, which suggest their high capacity of causing disease in humans.

Keywords: *Campylobacter jejuni*, slaughterhouses, virulence markers, PCR.

RESUMEN. *Campylobacter jejuni* es una de las más comunes causas de gastroenteritis humana a nivel mundial e importante enfermedad transmitida por alimentos. Otras presentaciones clínicas de la infección por *Campylobacter* son: meningitis, septicemia, infecciones extraintestinales localizadas, artritis reactiva y complicaciones inmune reactivas tales como el síndrome de Guillain-Barré (SGB) y síndrome de Miller Fisher (MFS). La patogénesis de *Campylobacter jejuni* es poco conocida en comparación con otros patógenos entéricos. En este trabajo fueron aisladas e identificadas genotípica y fenotípicamente 140 cepas de *Campylobacter jejuni* de muestras de pollo de engorde provenientes de doce plantas de beneficio del estado de Rio Grande del Sul, Brasil. Utilizando la técnica de PCR fue confirmada la especie y fueron identificados ocho marcadores de virulencia. El gen *fla*A estaba presente en 78.5% y el marcador *cad*F se detectó en 77.8% de las muestras. El operón (*cdt*ABC) asociado con la toxina citoletal distensiva está presente en 74.2% de los aislamientos. El gen asociado a invasión (*iam*) no se encuentra en ninguna de las muestras analizadas. La ocurrencia del gen plasmidial *vir*B11 y gen *wla*N fue de 22.1% y 10.7%, respectivamente. Estos resultados pueden revelar potencial patogénico en *Campylobacter jejuni* procedente de aves, lo que sugiere una alta capacidad de causar enfermedad en humanos.

Palabras-clave*: Campylobacter jejuni*, genes de virulencia, PCR.

INTRODUCTION

Campylobacteriosis is a worldwide distributed disease with significant impact on public health. *Campylobacter jejuni* (*C. jejuni*) infections are one of the most common causes of foodborne diarrheal illness in humans and it is the most common bacterium causing gastroenteritis around the world. Other clinical presentations of *Campylobacter* infection are meningitis, septicaemia, localized extraintestinal infections, reactive arthritis or Reiter's syndrome (40, 42) and immune–reactive complications such as Guillain–Barré syndrome (GBS) and its variant, Miller Fisher syndrome (MFS) (47, 48).

Campylobacter is widespread in nature and lives as a commensal organism in the gut of many birds and domestic animals. It can enter the food chain of humans through

different ways (3). Poultry products are considered the most important source of *C. jejuni* infections in humans (21). The development of the poultry industry, due to the genetic improvement of avian, nutrition and management system, allows large–scale poultry production. However, high density and confinement system favors the introduction and spread of pathogens. Transmission to humans, probably occurs through consumption and handling of chicken meat contaminated during slaughter and carcass processing (7, 17, 21). The slaughter stages considered as critical contamination points are: scalding, de–feathering, evisceration, washing and chilling (17).

Potential virulence factors and mechanisms of *Campylobacter jejuni* pathogenesis remain poorly understood. However, the most of known virulence genes related to pathogenesis are associated with abilities of adherence and colonization of intestinal epithelial cells, invasion and translocation capabilities, production of toxins and secreted proteins (9). Others pathogenic mechanisms like production of enterotoxin and cytotoxin and the ability to adhere and invade epithelial cells have been proposed play role in enteritis (13, 43). The CDT holotoxin consists of three subunits encoded by the genes, *cdt*A, *cdt*B, and *cdt*C, which are genetically arranged as an operon (2, 38), and expression of all three *cdt* genes is required for the maximum toxin activity (31). Current evidence suggested that *cdt*B encodes the active/toxic component of the toxin, while *cdt*A and *cdt*C are involved with binding to and internalization into the host cell (1).

Campylobacter exhibits bipolar flagella composed by two structural proteins FlaA and FlaB. The *fla*A gene seems to be highly conserved among *Campylobacter* isolates and its transcription is usually higher than that of *fla*B (20, 21). Thus, flagella are crucial for the approaching attachment sites on intestinal epithelial cells and they are involved not only with motility and chemotaxis but also with the secretion of virulence proteins, autoagglutination, microcolony formation and avoidance of the innate immune response (18). The *cad*F (*Campylobacter* Adhesion to Fibronectin F) gene is an adhesion and fibronectin–binding protein involved in the process of invasion, influencing microfilament organization in host cells (34). Another virulence gene linked with *Campylobacter* invasiveness is the invasion–associated marker (*iam*) gene (51). It has been suggested that the pVir plasmid encodes for proteins of a type IV secretion

system, and the mutation of the plasmid *vir*B11 gene results in reduced adherence and invasion potential *in vitro* as well as less severe symptoms *in vivo* (4); and *wla*N was selected as gene that is presumably involved in the expression of ganglioside mimics in Guillain–Barré syndrome and may encode for β–1,3–galactosyltransferases with identical enzymatic activities (14, 26).

Thus, as *Campylobacter* may be transferred from animals to human, it is important to know whether all *Campylobacter* isolates obtained from different sources are equally virulent. The purpose of this study was to determine if 9 genes previously identified coding for pathogenic markers in *Campylobacter* were present in the 140 samples obtained from poultry sources collected at slaughter using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Between January 2012 and December 2013, a total of 140 *Campylobacter* strains were isolated from broiler slaughterhouses in the state of Rio Grande do Sul, Brazil.

Campylobacter strains were isolated from swabs (cloacal and boxes of transport), carcasses through slaughter line, and from water collected from the chiller tank. The fecal and water samples were collected in sterile clean containers and carcasses into sterile polyethylene bags; later they were shipped to the laboratory for further analysis. The carcasses were rinsed inside sterile polyethylene bags containing 400 mL of Buffered Peptone Water (BPW 1%) (CM1049 Oxoid®). An aliquot of one milliliter of each sample (carcasses, feces and water) was homogenized in 9 mL of Bolton broth supplemented with antimicrobials (CM0983 Oxoid®, supplement SR0183) and incubated in microaerophilic conditions using a gas tank with a mixture (10% CO_2, 2% H_2, 5% O_2, and 85% N_2) for 48 hours at a temperature of 41.5°C. After incubation, 100μL of the suspension was filtered on an acetate membrane with a 0.65 μm pore, this membrane was spread on the surface of a modified charcoal cefoperazone deoxycholate agar (mCCDA) plate (CM739, Oxoid®, with cefoperazone selective supplement SR

155E) for 30 minutes. The plate was incubated in microaerophilic conditions at 41.5°C for 48h. *Campylobacter* spp. colonies suspected were examined for cell morphology by phase–contrast microscopy (Olympus B201). Subsequently, the isolates were purified using blood agar plates (BA; Blood Agar Base N.2, Oxoid®, supplemented with sterile defibrinated sheep blood 5%). Single colonies were picked and streaked onto wet BA plates, and characterized by specie level using their catalase reaction ability to hydrolyse hippurate and indoxyl acetate. The colonies were collected and resuspended in 1 mL of ultrapure water, transferred to microtubes, and frozen at –20°C. Isolates were stored with glycerol 15% at –80°C until required for further researches.

DNA Extraction

Template DNAs for PCR were extracted using an adapted protocol described by Borsoi et al. (10). One milliliter of bacterial culture was boiled at 95°C for 10 min. After centrifugation at 12.000 r.p.m for 2 min, the supernatants were stored at –20°C and used as template DNA. The isolates were confirmed by PCR based in the detection of 16S rRNA and *map*A gene (15).

PCR primer design and amplification

The confirmed *Campylobacter jejuni* isolates were screened for the presence of 8 pathogenic genes: *fla*A, *cad*F, *iam*, *vir*B, *wla*N, *cdt*A, *cdt*B, *cdt*C. Primers, PCR conditions and lengths of products generated in this study are listed in Table 1.

TABLE 1. List of primers and PCR conditions used in this study.

Target gene	Primers	Sequence (5'→3')	PCR conditions	Product (bp)	Reference
16S rRNA	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	95°C/10 min, 35 cycles: 95°C/30s, 59°C/90 s, 72°C/1 min, and 72°C/10 min	857 for *Campylobacter* genus identification	(15, 25)
***map*A**	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA		589 for *C. jejuni* species identification	(15)
***fla*A**	flaAF flaAR	GGATTTCGTATTAACACAAATGGTGC CTGTAGTAATCTTA AACATTTTG	94°C/5 min, 30 cycles: 94°C/1 min, 48°C/1 min, 72°C/1 min, and 72°C/5 min	1700	http://campynet.vetinst.dk/Fla.htm
***cad*F**	F2B R1B	TGGAGGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	94°C/5 min, 30 cycles: 94°C/1 min, 54°C/1 min, 72°C/1 min, and 72°C/5 min	400	(30)
iam	IAMF IAMR	GCGCAAAATATTATCACCC TTCACGACTACTATGCGG	94°C/5 min, 30 cycles: 94°C/1 min, 55°C/1 min, 72°C/1 min, and 72°C/5 min	518	(11)
***vir*B11**	virBF virBR	GAACAGGAAGTGGAAAAACTAGC TTCCGCATTGGGCTATATG	95°C/5 min, 35 cycles: 95°C/30s, 53.5°C/30s, 72°C/30s, and 72°C/5 min	708	(4)
***wla*N**	wlaN-DL39 Cj1139cF	TTAAGAGCAAGATATGAAGGTG TGCTGGGTATACAAAGGTTGTG	95°C/10 min, 25 cycles: 95°C/30s, 60°C/30s, 72°C/1 min, and 72°C/5 min	434	(26)
***cdt*A**	cdtAF cdtAR	CCTTGTGATGCAAGCAATC ACACTCCATTTGCTTTCTG	94°C/5 min, 30 cycles: 94°C/1 min, 49°C/30 s, 72°C/1 min, and 72°C/5 min	370	(22)
***cdt*B**	cdtBF cdtBR	CAGAAAGCAAATGGAGTGTT AGCTAAAAGCGGTGGAGTAT	94°C/5 min, 30 cycles: 94°C/1 min, 51°C/30 s, 72°C/1 min, and 72°C/5 min	620	(14)
***cdt*C**	cdtCF cdtCR	CGATGAGTTAAAACAAAAAGATA TTGGCATTATAGAAAATACAGTT	94°C/5 min, 30 cycles: 94°C/1 min, 47°C/30 s, 72°C/1 min, and 72°C/5 min	182	

The PCR conditions were adapted. All PCR amplifications were performed in a mixture (25μL) consisting of 3 μL of 10X PCR Buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 0.3 μL (5U/μL) of *Taq* thermostable DNA polymerase (Invitrogen®), 1.2 μmol 1^{-1} of $MgCl_2$ (25 mM), 2.5μL dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 2 μL extracted template DNA and 0.5μL (10 pmole 1^{-1}) of each primer.

Sterile Milli–Q water was added q.s.p 25 μL. All amplification reactions are performed in thermal cycler (Peltier Thermal Cycler Biocycler–MJ96+/MJ96G). The cycles were performed as described in Table 1. For visualization of PCR products, 10 μL aliquots were subjected to electrophoresis in a 2% agarose gel (Invitrogen®) in Tris–Acetated EDTA (TAE) buffer. DNA bands were stained with ethidium bromide for 2h at 100V, viewed under Ultraviolet (UV) transilluminator (ATTO®) and photographed (Fig. 1). The size of the PCR amplicons was compared to the 100 bp DNA ladder (Invitrogen®).

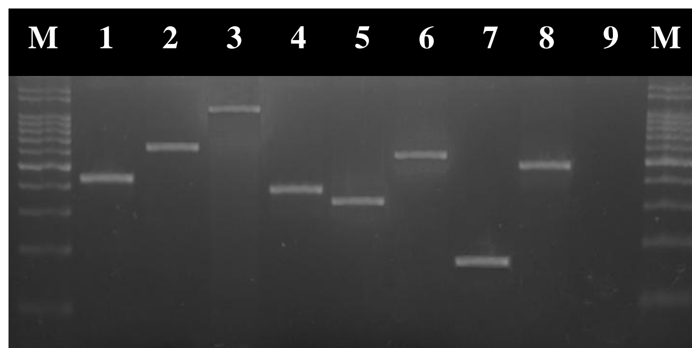

FIG. 1. Agarose gel electrophoresis of PCR products of 8 pathogenic genes of *C. jejuni*. Lanes: M, 100 bp marker; 1, *wla*N (434 bp); 2, *vir*B (708 bp); 3, *fla*A (1700 bp); 4, *cad*F (400 bp); 5, *cdt*A (370 bp); 6, *cdt*B (620 bp) ; 7, *cdt*C (182 bp); 8; *iam* (518 bp); 9, reaction control.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v18 (IBM). Discrete variables were expressed as percentages, and proportions were compared using the Chi–square test with the significance level defined at P value <0.05.

RESULTS

The isolates were confirmed and identified using genus– and species–specific PCR assays. Out of total 140 samples screened, 140 (100%) revealed belong to *Campylobacter jejuni* specie. The isolates were confirmed by PCR based detection of 16SrRNA and *map*A gene.

The results of PCR detection of 8 virulence associated genes are summarized in Table 2. The results showed that, the occurrence of *fla*A gene was 78.5% and *cad*F gene 77.8% in the isolates. The cytotoxin encoding cluster *cdt*ABC was detected in 74.2% isolates. The frequency rates found for *cdt*A, *cdt*B and *cdt*C was 85%, 80%, and 92.1% respectively. The plamidial virulence marker determining of invasiveness in *C. jejuni*, *vir*B11 gene, was examined. This gene was present in 22.1% of isolates. Moreover, another virulence gene linked with *Campylobacter* invasiveness *iam* was not detected in any of the investigated strains and the *wla*N gene was detected in 10.7% of isolates tested.

The occurrence of the virulence factor genes *fla*A, *cad*F, *iam*, *vir*B, *wla*N, and the gene cluster *cdt*ABC was analyzed. The obtained data showed that 27.9% *Campylobacter jejuni* strains possess four virulence determinant (n = 39), and 49 isolates (35%) presents three virulence–associated genes. A percentage 17.8% of *C. jejuni* presents two virulence markers (n = 25), and the presence of one gene was detected in (9.3%) (n = 13) of the isolates. No virulence markers were found in 14 isolates (9.9%).

The identification and frequency of *Campylobacter* species throughout the food chain revealed a high potential virulence marker emphasizes the importance of broilers as a potential reservoir (Table 2).

TABLE 2. Distribution of virulence markers among *Campylobacter jejuni* tested according to the source of isolation.

Number (%) of strains Found positive by PCR for:									
Sources	*fla*A	*cad*F	*cdt*A	*cdt*B	*cdt*C	*cdt*ABC	*iam*	*vir*B	*wla*N
Cloacal swab (n=5)	60 (3/5)	80 (4/5)	80 (4/5)	100 (5/5)	100 (5/5)	80 (4/5)	0	40 (2/5)	0
Swab of broiler transportation cage (n=3)	100 (3/3)	100 (3/3)	100 (3/3)	33.3 (1/3)	100 (3/3)	33.3 (1/3)	0	0	33.3 (1/3)
Broiler carcasses through slaughter process (n=115)									
Scalding	40 (2/5)	20 (1/5)	20 (1/5)	20 (1/5)	20 (1/5)	0	0	0	0
Defeathering	100(10/10)	100(10/10)	100(10/10)	100(10/10)	100(10/10)	100(10/10)	0	30 (3/10)	30 (3/10)
Evisceration	100 (5/5)	80 (4/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	0	40 (2/5)	0
Spray-washing	71.5 (5/7)	57.1 (4/7)	86 (6/7)	86 (6/7)	100 (7/7)	86 (6/7)	0	0	29 (2/7)
Cooling	78.6 (70/89)	79.7 (71/89)	87.6 (78/89)	80.8 (72/89)	93.25 (83/89)	77.52 (69/89)	0	23.5 (21/89)	6.7 (6/89)
Chiller tank processing water (n=17)	70.5 (12/17)	70.5 (12/17)	70.5 (12/17)	70.5 (12/17)	88.23 (15/17)	52.9 (9/17)	0	17.6 (3/17)	17.6 (3/17)
Total	78.5 (110/140)	77.8 (109/140)	85 (119/140)	80 (112/140)	92.1 (129/140)	74.2 (104/140)	0	22.1 (31/140)	10.7 (15/140)

DISCUSSION

The purpose of this study was to determine the presence of *C. jejuni* virulence markers indicated the potential role of these strains in the pathogenesis of human diseases, established the need for vigilance and control during slaughtering process to protect the health of the consumer. An understanding of the epidemiology of all the food–borne zoonotic agents is essential to the implementation of control strategies and interventions (49).

The bacterial flagellum and the virulence–associated injectisome are complex, structurally related to nanomachines, that bacteria use for locomotion or the translocation of virulence factors into eukaryotic host cells (16). It has been suggested that *Campylobacter* can survive to mild and strong acid–shock conditions, and this is linked with increased transcription of a subset of flagellar biosynthetic genes and stress responses, as well as a down–regulation in genes involved in cell division and metabolism (23). The flagellum does not only have a distinct function in bacterial motility and cell binding, but also acts as a type III secretion system (T3SS) (3, 30).

The flagellar filament consists of multimers of the protein flagellin. The flagellin locus contains two adjacent genes, *fla*A (encoding the major flagellin) and *fla*B (encoding a minor flagellin) (21). The most common virulence determinant was the *fla*A gene, present in 78.5% (n = 110) of isolates. Flagellin is a potent activator of a broad range of host cell types involved in innate and adaptive immunity (33). Rizal et al. (43) examined the presence of *fla*A factor in *C. jejuni* and *C. coli* derived from chicken and human isolates and obtained results of 100% of prevalence. Other authors, who determined virulence markers in *C. jejuni* from feces and broiler carcasses (51) and a study, including 56 isolates from human clinical samples, 21 from poultry meat, 21 from broiler feces and 13 from bovine feces (14), obtained similar date. In this study, in 30 samples (21.5%) was not detected the *fla*A gene.

Mutation of *fla*A can results in the production of a truncated flagellar filament composed of *fla*B with a severe reduction in motility (18, 19). It has been hypothesized that a second copy of *fla*A serves as a potential donor for reassortment and

recombination of the DNA as a mechanism for creating new antigenic variants for immune avoidance (32). Wassenaar et al. (50) supported this hypothesis by selecting a variant in which *fla*B apparently replaced a defective *fla*A. Research has determined that flagellum of *C. jejuni* has been found to be variable (36). Phase variation of surface structures occurs in diverse bacterial species due to stochastic, high frequency and reversible mutations (6). Some strains of *C. jejuni* have the capacity to spontaneously undergo bi–directional transition, called transition phase variation between flagellated and no flagellated phenotypes (20).

Other virulence gene examined in this study was *cad*F, one of the markers determining the adherence in *Campylobacter*. This gene was present in 109 of 140 (77.8%) isolates analyzed and is an adhesion factors used by *C. jejuni* to attach and eventually invade mammalian cells by binding to fibronectin, a component of the extracellular matrix (29, 30). Investigations reported that Δ*cad*F mutant strains were not able to colonize chicken guts (53). Monteville et al. (34) verified that Δ*cad*F mutant strain reduced in 50% adhesion and transmigration to human INT–407 cells compared to a wild–type strain. Thus, the *cad*F gene which appears to be essential for chicken gut colonization may presumably have a similar role in the pathogenesis of human infection (44).

Previous reports have indicated that *cad*F is present in 100% of *C. jejuni* isolates from poultry samples (14, 51). The high occurrence of the *cad*F gene in this study was consistent with previous results using identical PCR assays. Our study is in accordance with the results of Rizal et al. (43) which showed the presence of this marker (88.33%) in *C. jejuni* isolated from chicken. These studies suggest that geographical differences may be an important consideration for the assessment of the presence of these genes. This gene is also an essential mediator of the material and information transference between cells and their environment, and between compartments within cells. Not only is highly conserved in *C. jejuni* strains, but these surface–exposed proteins are also highly immunogenic in chicks (45).

Toxin–Producing bacteria CDT may potentially plays role in the disease development. This cytotoxin arrests eukaryotic cell to G_2 phase of the cell cycle,

preventing them from entering mitosis, and leading to cell death. The toxin was named according to the morphological changes associated with its action, cytoplasmic distension (52). The function of this cytotoxin is well documented (8, 24, 39). The *cdt* gene cluster was detected in 74.2% (104/140) of isolates. 85% of the samples tested (119/140) were positive for *cdt*A, 80% (112/140) positive for *cdt*B, and 92.1% (129/140) positive for *cdt*C. These results differ of literature, which reports frequencies usually near to 100% for the three genes. Datta et al. (14) reported a frequency of 100% for the genes in their studied samples, whose included chicken feces, cattle feces and human clinical samples. Rozynek et al. (44) found 100% of prevalence in isolates from broiler carcasses. Wieczorek et al. (51) reported in samples isolated from different sources, 76.6% of positivity for *cdt*A, 85.3% and 83.2% for *cdt*B and *cdt*C, respectively.

In Brazil, there are few studies about CDT in *C. jejuni*. Carvalho et al. (12) searching *Campylobacter jejuni* in broiler carcasses reported CDT complex in only 36.4% of the samples. Martinez et al. (31) determined that essentially all strains of *C. jejuni* have the *cdt* genes, and most have toxin activity. However, there are exceptions of isolates that mutate and do not express the toxin activity. Asakura et al. (2) observed that some mutations, such as deletion, insertion, and substitution, were identified in the nucleotide sequence of the *cdt* gene clusters when compared sequences of reference and test strains, and suggest that these mutations may affect the activity of the toxin. AbuOun et al. (1) showed that toxin production is low or absent when mutations in regions of the *cdt* gene occur. However, since certain strains still retain some toxigenic activity when Δ*cdt*–negative mutant have been analyzed (38, 41). It is possible that additional toxigenic activities are present in some strains of *C. jejuni*. Consequently, it is apparent that different strains may manifest different potentials to cause disease (37).

A significantly percentage of *C. jejuni* carry the *vir*B11 gene, localised on pVir plasmid. The pVir plasmid encodes several genes homologous to a type IV secretion system and contributes to ability of *Campylobacter jejuni* subsp. *jejuni* 81–176 to invade INT–407 cells *in vitro*, a marker that correlates it with virulence in the ferret diarrheal disease model (4, 5). Moreover, other investigations showed both: an association (46) and a lack of association (27) between the pVir plasmid and bloody diarrhea in *C. jejuni* enteritis.

In previous studies *vir*B11 was present in 18.5 % strains of *C. jejuni* (51). These results were, similar to the present study where this gene was detected in 31 of 140 (22.1%) isolates. Other authors showed a small percentage of this marker in poultry samples; 9.5% (14); 11.66% (43). The reasons underlying this variation are not known. Is possible that this divergence might be consequence of different targets sequences and amplifications conditions useful in marker identification.

The *iam* gene is a genetic marker that was preferentially associated with adherence and invasion – on Hep–2 cells *in vitro.* There is a good correlation between the clinical presentation of diarrhea and the isolation of *Campylobacter* strains that adhere to and invade HEp–2 cells. However, the involvement and function of the *iam* marker in the process of campylobacteriosis has not been precisely explained yet (11). Rozynek et al. (44) detected this gene in 53.8% of *Campylobacter jejuni* isolated from chicken. The presence of this molecular marker is not restricted to *C. jejuni* but is also present in *C. coli* and *C. lari*. Wieczorek et al. (51), found that the marker was predominant in *C. coli* (89.9% positive isolates), whereas only 46.7% *C. jejuni* strains have this gene. The variable occurrence depends not only on the origin but the species of *Campylobacter* isolates. In this study, the *iam* sequence was not detected in any of the investigated isolates. The fact that in some invasive strains the gene were not identified by PCR, supports the existence of important polymorphism and high heterogeneity in the *iam* locus or suggests that, may exist other genetic marker of invasion in different loci (11).

The *wla*N gene is involved in variations of LOS structure facilitating the avoidance of *C jejuni* to the host immune system. Our results showed that this marker was present in 10.7% (15/140) of *C. jejuni* strains, tested. According investigation by Datta et al. (14), this gene was present in 4.7% of isolates from broiler feces and 23.8% of poultry meat isolates. The presence of the gene can vary depending on the research area and the high rate of polymorphism that has *C. jejuni* strain. Koga et al. (28) reported that the genetic polymorphism of *C. jejuni* determines the reactivity of autoantibodies and clinical presentation of Guillain–Barré syndrome, possibly through the modification of the host molecule mimic. Importantly, these positive isolates may have a higher pathogenic potential, therefore, higher capacity to induce autoimmune

disease in its host. A previous study showed a correlation between increased capacity of cell invasion, (*in vitro* and *in vivo*), and the presence of the *wla*N gene, since 80% of the strains which lack this gene presented lower or total lack of invasiveness (35).

In conclusion, this study revealed that eight pathogenic genes of *C. jejuni* circulate in all slaughter line among poultry sources in Rio Grande do Sul state, Brazil. On the other hand, the high frequencies of *fla*A and *cad*F genes and cluster *cdt*ABC in *C. jejuni* show that these pathogenic markers may be important for the survival of *C. jejuni* in poultry. Each ecosystem requires its inhabitants a certain evolutionary adaptations according to the circumstances and possibilities. These differentially genes are located in hypervariable regions of the *C. jejuni* genome and may contribute to the bacterial survival in different environments and hosts (21). Our understanding of the ecology of pathogenic genes producing by bacterial species has greatly expanded by molecular biology. Adhesion and invasion are critical steps that must occur before the disease develops. Then, the identification of virulence factors is a key to developing effective treatments as well as researching on disease process. Reducing the proportion of *Campylobacter* infected poultry flocks and/or reducing the numbers of *Campylobacter* on broiler carcasses in the slaughterhouses will decrease considerably the risk to consumers.

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CAPITULO II

Resistance to β–lactam and Tetracycline in *Campylobacter* spp. Isolated from Broiler Slaughterhouses in Southern Brazil

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ABSTRACT.– Sierra–Arguello Y.M., Perdoncini G., Morgan R.B., Lima L.M. & Nascimento V.P. 2015. **Resistance to β–lactam and Tetracycline in *Campylobacter* spp. isolated from Broiler Slaughterhouses in southern Brazil.** *Pesquisa Veterinária Brasileira 00(0):00–00.* Centro de Diagnóstico e Pesquisa em Patologia Aviária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 8824, Porto Alegre, RS 91540–000, Brazil. E–mail: yuli_melisasierra@yahoo.com.

In the present research, we evaluated *Campylobacter* spp. contamination on poultry sources and phenotypic and genotypic profiles resistance to β–lactam and tetracycline of the isolated strains. Resistance to two different antimicrobials was assessed using the disk diffusion method. In addition, all the strains were tested for ampicillin ($bla_{\mathrm{OXA-61}}$), tetracycline *tet*(O), and the energy–dependent multi–drug efflux pump (*cme*B) resistance genes using polymerase chain reaction. Between January 2012 and December 2013 a total of 141 samples of *Campylobacter* from broiler slaughterhouses in the state of Rio Grande do Sul, Brazil were analyzed. *Campylobacter jejuni* was the most ubiquitous, its presence was determined in 140 samples out of 141 (99.3%), whereas *Campylobacter coli* was found in one of the contaminated samples (0.70%). The results obtained showed high percentage (65%) of *Campylobacter* isolates resistant to β–lactams. Fifty strains (35.5%) were resistant to tetracycline and 26 (18.5%) to the efflux pump. Moreover, 36 out of the 141 *Campylobacter* strains

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(25.6%) were found to be resistant to at least two different antimicrobial resistance markers (β–lactams and tetracyclines).

INDEX TERMS: *Campylobacter*, Tetracycline, β–lactam, efflux pump, resistance genes, PCR.

RESUMO.– [Resistência a β–lactâmicos e Tetraciclina em *Campylobacter* spp. isolados de matadouros–frigoríficos de aves no sul do Brasil]. Na presente pesquisa, foi avaliada a contaminação de *Campylobacter* spp. em produtos avícolas e os perfis fenotípicos e genotípicos de resistência a β–lactâmicos e tetraciclinas das cepas isoladas. A resistência a dois diferentes grupos de antimicrobianos foi avaliada pelo método de difusão em disco. Adicionalmente, todas as estirpes de *Campylobacter* foram testadas para detectar os marcadores de resistência à ampicilina ($bla_{\text{OXA–61}}$), tetraciclina *tet*(O), e a bomba de efluxo (*cme*B), usando a reação em cadeia da polimerase. Entre janeiro de 2012 a dezembro de 2013, um total de 141 amostras de *Campylobacter* isolados em matadouros–frigoríficos de aves do estado do Rio Grande do Sul, Brazil, foram analisados. *Campylobacter jejuni* foi à espécie mais isolada, sua presença foi determinada em 140 amostras de 141 (99,3%), e *Campylobacter coli* foi encontrada em uma única amostra (0,70%). Os resultados obtidos mostraram alta percentagem (65%) de *Campylobacter* resistentes a β–lactâmicos. Cinquenta amostras (35,5%) eram resistentes à tetraciclina e 26 (18,5%) para a bomba de efluxo. Neste contexto, 36 dos 141 das amostras (25,6%) foram consideradas resistentes a dois grupos diferentes de antimicrobianos (β–lactâmicos e tetraciclinas).

TERMOS DE INDEXAÇÃO: *Campylobacter*, Tetraciclinas, β–lactâmicos, bomba de efluxo, genes de resistência, PCR.

INTRODUCTION

Campylobacter is recognized as the leading causes of bacterial foodborne diarrheal disease throughout the worldwide (Park 2002, Silva et al. 2011). Campylobacteriose is estimated to cause approximately 1.3 million infections, 13,000 hospitalizations and 120 deaths each year in the United States (CDC 2013). It is also

the most commonly reported antecedent infection in the development of Guillain Barré syndrome (GBS) and Miller Fisher syndrome (MFS) (Hardy et al. 2011, Godschalk et al. 2004, Van den Berg et al. 2014). A risk factor for human disease is the consumption of contaminated poultry products (Conlan et al. 2007, Ellström et al. 2014).

Transmission to man usually results in sporadic infection, and is often associated with improper handling or cooking of food (Moore et al. 2005). *Campylobacter jejuni (C. jejuni)* and *Campylobacter coli (C. coli)* are considered to be zoonotic pathogens, antimicrobial resistance among isolates in the animal reservoir has serious implications for the treatment in humans (Moore et al. 2006, EFSA 2011). The majority of cases of clinical *Campylobacter* enteritis are generally mild or self–limiting disease not to require of antimicrobial chemotherapy (Moore et al. 2005). However, antimicrobial therapy may be used in a subset of patients with severe, prolonged or systemic infections or to control infection (Avrain et al. 2003, Janssen et al. 2008). Currently, macrolides and fluoroquinolones are the antimicrobial agents of choice when therapeutic intervention is warranted (Engberg et al. 2001, Moore et al. 2005). Bacterial populations can respond to the threat of an antimicrobial agent by evolving some type of resistance mechanism(s) (Rowe–Magnus et al. 2002, Luangtongkum et al. 2009). These resistant bacteria may be transferred to humans either through the food supply or by direct contact with animals (Khachatourians 1998, Angulo et al. 2004). Ampicillin and tetracycline have activity against *Campylobacter*, but in general, are not recommended for the treatment of *Campylobacter* infections (Blaser 1995, Dasti et al. 2007). However, the recent increment in resistance to antibiotics in this genus makes it necessary to consider alternatives therapies as well as the search of easy and reliable methods to study antimicrobials susceptibility. The aim of the present study was to determine the occurrence of *Campylobacter* spp. strains carrying *resistance* genes (tetracycline, β–lactam and the energy–dependent multi–drug efflux pump) through phenotypic analysis and molecular analyses in poultry sources from slaughterhouses in Rio Grande do Sul state, Brazil.

MATERIALS AND METHODS

Sample collection

Since January 2012 to December 2013, a total of 141 isolates: carcasses through slaughter line (n=115); water collected from the chiller tank (n=18); and from swabs (cloacal and boxes of transport) (n=8) were obtained from broiler slaughterhouses of Rio Grande do Sul state, Brazil.

Antibiotic susceptibility screening

Isolation was performed in accordance with the International Standards Organization guidelines (ISO 10272–1:2006). The *Campylobacter* isolates were analyzed for antimicrobial resistance using the agar disk diffusion method. The suspension was adjusted to match the 0.5 McFarland turbidity standards as recommended by the Clinical and Laboratory Standards Institute (CLSI 2010). Isolated cultures were analyzed for antimicrobial resistance using the disk diffusion assay on Mueller–Hinton agar plates (CM0337 *Oxoid*®, containing 5% sheep blood) incubated under microaerophilic conditions using a *gas tank with* a mixture (10% CO_2, 2% H_2, 5% O_2, and 85% N_2) for 48 hr at 41.5°C. Sheep blood agar plates were inoculated, and disks (Oxoid®) including tetracycline (30 µg), and ampicillin (10 µg) were added. Plates were incubated as described above. In view of lack of interpretative CLSI criteria for *Campylobacter* strains, the criteria used for the Enterobacteriaceae family were employed as breakpoints for *Campylobacter* resistance (CLSI 2012). *C jejuni* ATCC 33560 strains was used as control throughout the testing period.

DNA Extraction

Genomic DNA was extracted using an adapted protocol described by Borsoi et al. (2009). Stored *Campylobacter* isolates were cultured on 5% sheep blood agar plates and incubated at 41.5°C for 48 hrs in microaerophilic conditions (10% CO_2, 2% H_2, 5% O_2, and 85% N_2). One milliliter of bacterial culture was centrifuged at 12.000 r.p.m. for 2 min (5415C Microcentrifuge, Eppendorf, Hamburg, Germany) and the supernatant was discarded. The pellet was suspended in 800 µL of sterile distilled water and the resulting mixture was centrifuged at 12.000 r.p.m. for 2 min. The pellet was again suspended in 200 µL of sterile distilled water. The sample was placed on a thermal

block (Multi–Block Heater, Baxter, USA) at 95°C for 10 min. The mixture was centrifuged as describe above and the supernatants were transferred into fresh Eppendorf tubes to serve as a DNA template for subsequent processing.

Multiplex–PCR assay

The isolates were confirmed by Multiplex–PCR based detection of 16S rRNA, *ceu*E and *map*A genes (Denis et al. 1999).

Genotypic antimicrobial resistance

The confirmed *C. jejuni* isolates were screened for the presence of three resistance genes: tetracycline (*tet*O), β–lactam (bla_{OXA-61}), and the energy–dependent multi–drug efflux pump (*cme*B). Primers, PCR conditions and lengths of products generated in this study are listed (Table 1). The PCR conditions were adapted (Pratt & Korolik et al. 2005, Obeng et al. 2012). All PCR amplifications were performed in a mixture (25 μL) consisting of 5 μL of 10X PCR Buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 0.25 μL (5U/μL) of *Taq* thermostable DNA polymerase (Invitrogen®), 2 μmol 1^{-1} of $MgCl_2$ (25 mM), 2 μL dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 2 μL extracted template DNA and 0.5 μL (10 pmole 1^{-1}) of each primer. Sterile Milli–Q water was added q.s.p 25 μL. All amplification reactions are performed in thermal cycler (Peltier Thermal Cycler Biocycler–MJ96+/MJ96G). The cycles were performed as described in Table 1. For visualization of PCR products, 10–μL aliquots were subjected to electrophoresis in a 2% agarose gel (Invitrogen®) in Tris–Acetated EDTA (TAE) buffer. DNA bands were stained with ethidium bromide for 2h at 100V, viewed under Ultraviolet (UV) transilluminator (ATTO®) and photographed (Fig. 1). The size of the PCR amplicons was compared to the 100 bp DNA ladder (Invitrogen®).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v18 (IBM). Discrete variables were expressed as percentages, and proportions were compared using the Chi–square test with the significance level defined at *P* value <0.05.

Table 1. List of primers and PCR conditions used in this study.

Target gene	Primers	Sequence (5’→3’)	PCR conditions	Product (bp)	Reference
16S rRNA	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	95°C/10 min, 35 cycles: 95°C/30s, 59°C/90 s, 72°C/1 min, and 72°C/10 min.	857 for *Campylobacter* genus identification	Linton et al. 1997 Denis et al. 1999
*map*A	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA		589 for *C. jejuni* species identification	Denis et al. 1999
ceuE	col3 MDcol2	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG		462 for *C. coli* species identification	
*tet*O	tetO1 tetO2	GCGTTTTGTTTATGTGCG ATGGACAACCCGACAGAAG	94°C/5 min, 30 cycles: 94°C/30s, 54°C/30s, 72°C/1 min, and 72°C/7 min.	559	Pratt & Korolik et al. 2005
bla_{OXA-61}	$bla_{OXA-61}F$ $bla_{OXA-61}R$	AGAGTATAATACAAGCG TAGTGAGTTGTCAAGCC		372	Obeng et al. 2012
*cme*B	cmeB1 cmeB2	TCCTAGCAGCACAATATG AGCTTCGATAGCTGCATC		241	

RESULTS AND DISCUSSION

Antimicrobial resistance in both medicine and agriculture is documented by the World Health Organization (WHO), along with other various national authorities as a major emerging problem of public health importance (Moore et al. 2006, Silva et al. 2011). *Campylobacter* are recognized as reservoirs for antimicrobial resistance genes that potentially can be exchanged between other pathogenic and commensal bacteria (Anderson et al. 2003, Epps et al. 2013). Antimicrobial therapy, generally macrolides, tetracycline and fluoroquinolones, are reserved for more severe cases. However, the increasing resistance to fluoroquinolones, tetracycline and erythromycin of *C. coli* and *C. jejuni* strains, might compromise the efficacy of this treatment (Aarestrup & Engberg 2001, Engberg et al. 2001, Gibreel & Taylor 2006; Alfredson & Korolik 2007, Silva et al. 2011).

All the isolates were confirmed by Multiplex–PCR based detection of 16SrRNA, *ceu*E and *map*A genes. The most ubiquitous of the thermotolerant *Campylobacter* spp. was *C. jejuni.* It was found in 140 of the contaminated samples (99.2%), whereas *C. coli* was identified in the remaining sample (0.7%). The PCR–amplified products of *Campylobacter* species and three resistance associated genes in agarose gel are summarized in Figure 1.

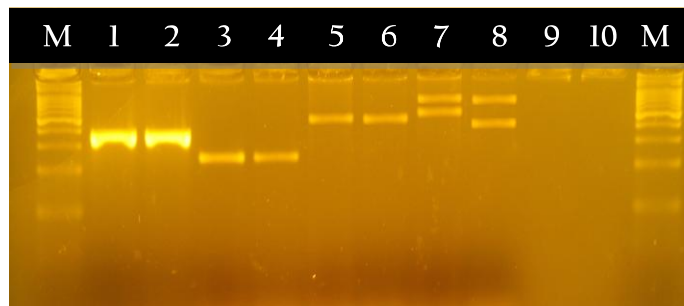

Figure 1. Agarose gel electrophoresis of PCR products of 4 resistance genes of *C. jejuni*. Lanes: M, 100 bp marker; 1-2, bla_{OXA-61} (372 bp); 3-4, *cme*B (241bp); 5, *tet*O (559 bp); 7, *map*A (589 bp); 8, *ceuE* (462 bp); 9-10, Reaction control.

An initial screening was performed to identify the antimicrobial susceptibility of 141 *Campylobacter* strains against ampicillin and tetracycline by using the disk diffusion test. Resistance of *Campylobacter* isolates among this method to ampicillin and tetracycline was detected in 65% and 35.5%, respectively (Fig. 2). The resistance we found to β–lactam among both our *Campylobacter* spp. isolates was at the lower range of that reported for amoxicillin *(*87.5%) in the prior Brazilian study (de Moura et al. 2013). Expression of a penicillinase–type of β–lactamase in *Campylobacter* confers resistance to amoxicilin, ampicillin and ticarcilin (Iovine 2013). This enzyme provided resistance to penicillins but not to cefotaxime and imipenem (Alfredson & Korolik 2005). The level of resistance to *Campylobacter* spp. to tetracycline by phenotypic method was similar to that reported in other studies in Brazil (Kuana et al. 2008). However, in contrast high presence of resistance (93.75%) was found by de Moura et al. (2013).

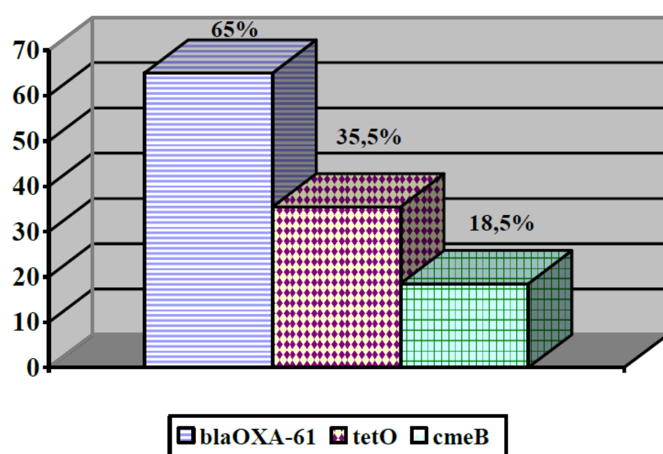

Figure 2. The presence of the antimicrobial resistance in *Campylobacter* spp. isolates.

The results of phenotypic and genetic analyses of antimicrobial susceptibility were fully concordant. We showed that ampicillin resistance by disk diffusion in campylobacters isolated was associated with the presence of the $bla_{OXA–61}$ gene carried on the chromosome. This study found a high level of resistance to β –lactam gene in 65% (91/141) of isolates. Our results are similar to those described by Obeng et al. (2012), who observed resistance of 59–65.4% for *C. jejuni*. In Brazilian studies, Hungaro et al. (2015) was found 100% resistant isolates to ampicillin. β–lactam

(ampicillin) is not recommended for the treatment of *Campylobacter* infections. However, in severe cases of *Campylobacter* resistant to both erythromycin and ciprofloxacin the therapy is required (Blaser 1995, Griggs et al. 2009). Several major β–lactam resistance mechanisms have been characterized, including the production of β–lactamases (Lachance et al. 1991, Griggs et al. 2009). A majority of *C. jejuni* and *C. coli* isolates are able to produce β–lactamases, which inactivate the β–lactam molecule by hydrolysis the structural lactam ring (Tajada et al. 1996, Wieczorek & Osek 2013).

An increase in tetracycline resistance in *C. jejuni* and *C.coli* strains has been observed in recent years (Luangtongkum et al. 2008). The *tet*O gene was detected in 35.5% (50/141) of isolates. Previous reports have indicated that *tet*O is present in 40% of *C. jejuni* isolates from chicken carcasses (Hungaro et al. 2015), and of *C. jejuni* from chickens 19.2–40.7% (Obeng et al. 2012). Tetracyclines have been suggested as an alternative choice in the treatment of clinical campylobacterosis, but in practice are rarely used (Moore et al. 2006). The *tet*(O) gene in *Campylobacter* encodes the Tet(O) protein that protects the ribosome from the inhibitory effect of tetracycline (Connell et al. 2003). Its primary antimicrobial effect takes place by direct steric hindrance by binding to the A site in the 30S subunit, thus hindering the movement of transfer RNA and inhibits peptide elongation (Wieczorek & Osek 2013). Tet(O) is coded by a gene located on plasmids of different sizes and in some strains chromosomally (Lee et al. 1994, Gibreel et al. 2004, Dasti et al. 2007). However, studies provide evidence that tetracycline–resistance *Campylobacter* have been recovered from organic and other production systems in which no antimicrobial have been used, which indicates that prior or current use may not be a defining attribution to resistance (Piddock et al. 2000, Luangtongkum et al. 2008, Cox et al. 2009).

In *Campylobacter* is described a multidrug efflux system (CmeABC), belonging to the Resistance Nodulation Division (RND) family of transporters that conferring intrinsic resistance to various antimicrobials and toxic compounds (Pumbwe & Piddock 2002, Lin et al. 2002, Guo et al. 2010). In addition, Lin et al. (2003) showed that this efflux pump is essential for the growth and survival of *C. jejuni* in chicken intestinal extracts, and their report indicates that, through mediating resistance to bile salts in the intestinal tract, CmeABC allows *C. jejuni* to colonize chickens successfully. This pump

is widely distributed in *Campylobacter*, including *C. coli*, and is constitutively expressed (Payot et al. 2002).

In this study, twenty six isolates (18.5%) presents the gene that encode a multidrug efflux pump. The CmeABC efflux pump may also contribute to β–lactam resistance (Iovine 2013). Gibreel et al. (2007) reported that inactivation of the *cmeB* gene in the resistant isolates examined led to a 16 to 128–fold decrease in tetracycline *minimum inhibitory concentration* (MIC), resulting in the complete restoration of tetracycline susceptibility. Studies also suggested that when both CmeABC and *tet*O are functional, the impact on tetracycline resistance is synergistic (Lin et al. 2002, Pumbwe & Piddock 2002).

The obtained data revealed that 7.1% *Campylobacter* strains possess three resistance determinant (n = 10), and 43 isolates (30.5%) presents two *resistance–* associated *genes*. A percentage 35.5% of isolates presents one markers (n = 50). No resistance markers were found in 38 isolates (27%). Moreover, 36 out of the 141 *Campylobacter* strains (25.6%) were found to be resistant to at least two different antimicrobial *resistance groups* (*bla*$_{OXA-61}$ and *tet*O). In our study the resistance of isolated thermotolerant strains of *Campylobacter* spp. to antimicrobial agents was estimated. The results are summarized in Table 2.

Table 2. Distribution of virulence markers among *Campylobacter* spp. tested according to the source of isolation.

	Number (%) of strains		
	Found positive by PCR for:		
Sources	***bla*$_{OXA-61}$**	***tet*O**	***cme*B**
Cloacal swab (n=5)	60 (3/5)	60 (3/5)	40 (2/5)
Swab of broiler transportation cage (n=3)	66.6 (2/3)	0	33.3 (1/3)
Broiler carcasses through slaughter process(n=115)			
Scalding	25 (1/4)	50 (2/4)	0
Defeathering	80 (8/10)	30 (3/10)	40 (4/10)
Evisceration	60 (3/5)	40 (2/5)	60 (3/5)
Spray–washing	57.1 (4/7)	57.1 (4/7)	28.5 (2/7)
Cooling	66.3(59/89)	30.33 (27/89)	11.2 (10/89)
Chiller tank processing water (n=18)	61.11(11/18)	50 (9/18)	22.2 (4/18)
Total	65 (91/141)	35.5 (50/141)	18.5 (26/141)

Antimicrobial resistance in *Campylobacter* spp. is an important problem of public health. Bacteria have developed multiple ways of becoming resistant to antimicrobials; in most cases bacteria are exposed to these substances, but have found a way to evade or resist the antimicrobial agent. Further research in understanding the antimicrobial resistance mechanisms will facilitate the selection of antimicrobials for clinical treatment and the formulation of diagnostic media for various *Campylobacter* spp. To our knowledge, this is the first study to determine the frequency of the bla_{OXA-61}, *tet*O and *cme*B genes in campylobacters from poultry origin of Rio Grande do Sul state, Brazil. It is also a study of *Campylobacter* spp. from correlate of disk diffusion assay and presence of resistance genes for tetracycline, β–lactam and the energy–dependent multi–drug efflux pump. Our results also emphasize the need for a surveillance and monitoring system and risk analyzes for the prevalence and resistances of *Campylobacter* in poultry and other food animals.

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CAPITULO III

Fluoroquinolone and Macrolide Resistance in *Campylobacter jejuni* isolated from Poultry in Slaughterhouses

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ABSTRACT. *Campylobacter jejuni,* have been recognized as a major cause of acute bacterial gastroenteritis in humans. The emergence of antimicrobial resistance among *Campylobacter* species has increased dramatically. The aim of this study was to isolate and antimicrobial susceptibility patterns to Fluoroquinolones and Macrolides of *Campylobacter jejuni* isolated from poultry slaughterhouses in Rio Grande do Sul State, Brazil. The antimicrobial susceptibilities of *C. jejuni* strains were tested and analyzed using the MIC assay as well as molecular biological method (RFLP) in the case of fluoroquinolones. In addition, all the strains were tested for the presence of the energy-dependent multi-drug efflux pump (*cme*B) resistance gene using polymerase chain reaction. A total of 50 samples from different points of poultry slaughterhouses were screened for presence of *Campylobacter jejuni* and determining antimicrobial sensibility for genotypic and phenotypic methods. Minimal inhibitory concentrations were determined for 3 antimicrobial agents, Ciprofloxacin, Nalidixic Acid and Erythromycin. The MICs results showed 98% of isolates were sensitive to erythromycin. In contrast, most isolates were resistant to ciprofloxacin (94%) and nalidixic acid (90%). Regarding ciprofloxacin-resistant isolates, 100% of the phenotype resistance strains had a mutation in the *gyr*A gene that was detected by the PCR-RFLP assay. Finally, the *cme*B gene that is responsible for multidrug resistance was detected in 16 isolates out the 50 strains (32%).

Keywords: *Campylobacter*, Fluoroquinolones, Macrolides, resistance genes, PCR, RFLP.

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) infections are distributed worldwide and recognized as potential problem for public health. The human infection can occur from a variety of sources, commonly associated with consumption of undercooked poultry and cross–contamination of other foods with drippings from raw poultry (Quinn *et al*., 2007). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler showed that higher count on carcasses is strongly associated with bacterial colonization of the flock. However, contaminated carcasses could also derive from non–colonized broiler flocks, suggesting a potential cross–contamination in the slaughterhouse environment (EFSA, 2010). Meat becomes contaminated during the slaughtering process, and *C. jejuni* survives in the crevices of animal carcasses where oxygen tension is low (Iovine, 2013), increasing their capacity to adapt and disseminate.

Commonly, *Campylobacter* contaminations are mild, self–limiting and usually resolve within a few days, no specific treatment is required for most cases. Antimicrobial treatment is necessary for systemic infections, immune–suppressed patients and severe or long lasting infections (Aarestrup & Engberg, 2001; Gibreel & Taylor, 2006). For clinical therapy of campylobacteriosis, erythromycin (Ery) is considered the treatment choice, but fluoroquinolone (FQ) (ciprofloxacin) are also frequently used due to their broad spectrum of activity against enteric pathogens (Engberg *et al*., 2001). The foodborne route is the major transmission pathway for resistant bacteria and resistance genes from food animals to humans, but other routes of transmission exist (FAO, 2003).

Relatively recent advances in pathogen testing technology, based on the biotechnology tools underlying modern medical diagnostics, are changing the ability of food producers to measure food safety. Molecular techniques to study resistance mechanisms are available and are useful for monitoring programs. The genetic basis of ciprofloxacin resistance in *Campylobacter* isolates was determined by PCR amplification of the Fluoroquinolone Resistance Determining Region (QRDR) of the *gyr*A gene as previously described by Wang *et al*. (1993). Macrolides such as erytromycin and tylosin are bacteriostatic, act by binding to the 50S ribosomal

subunit and inhibits peptide chain elongation (Gibreel *et al.*, 2005). Resistance can occur through target modification by mutation or methylation, antibiotimicrobial inactivation or efflux (Leclercq, 2002). The present study was aimed to provide information on the current status of the antimicrobial resistance pattern in *C. jejuni* isolated from broiler slaughterhouses in Rio Grande do Sul state, Brazil. The antimicrobial susceptibilities of *C. jejuni* strains were tested and analyzed using the minimum inhibitory concentration (MIC) test as well as PCR-restriction fragment length polymorphism (PCR-RFLP) technique in the case of fluoroquinolones. In addition, all the strains were tested for the presence of the energy–dependent multi–drug efflux pump (*cme*B) resistance gene by PCR assay.

MATERIAL AND METHODS

Bacterial Strains

A total of fifty isolates (carcasses: n=37; chiller tank processing water n=9; cloacal swab n=4) obtained from broiler slaughterhouses in Rio Grande do Sul state, during Jan–Nov 2012 were used in this study.

Isolation was performed in accordance with the International Standards Organization guidelines (ISO, 2006). Previously, to carcass was added 400 mL of 0.1% buffered peptone water (BPW) (CM1049 Oxoid®) and vigorously shaken by hand for 1 min. One mililliter of each sample (carcasses, swabs and water) was removed and homogenized in 9 mL of Bolton broth supplemented with antimicrobial (CM0983 Oxoid®, supplement SR0183) followed by incubation for 48 hr at 41.5^{o}C under an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Briefly, a quantity of 10 µL of the suspension after incubation were streaked on the surface of selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (CM739, Oxoid®, with cefoperazone selective supplement SR 155E). The plates were incubated in microaerophilic conditions at 41.5°C for 48 hr. Colonies with suspicious morphology were picked and streaked onto wet BA plates (BA; Blood Agar Base N.2, Oxoid®, supplemented with sterile defibrinated sheep blood 5%) and incubated for 48 hrs under the above mentioned conditions. These colonies were confirmed by the microscopic

morphology, motility, microaerobic growth at 25°C and oxidase test. The colonies were collected and resuspended in 1mL of ultrapure water, transferred to microtubes, and later frozen at –20°C until DNA extraction. Isolates were stored with glycerol 15% at –80°C until required for further researches.

DNA extraction and species confirmation

Genomic DNA was extracted using an adapted protocol as previously described by Borsoi *et al*. (2009). The isolates were confirmed by Multiplex–PCR based detection of 16S rRNA and *map*A gene (Denis *et al*., 1999).

Minimal Inhibitory Concentration –MIC

Broth microdilution method was employed in this study followed the procedure according to the Clinical and Laboratory Standard guidelines (CLSI) (CLSI, 2008; 2010). In this method, susceptibility panel in 96–well microtiter plates containing serial concentrations of antimicrobial agents was performed. The antimicrobial were tested in a 2–fold concentration series: ciprofloxacin (Sigma, St Louis, MO, USA) 0.125–64 µg/mL, nalidixic acid (Sigma) 0.25–128 µg/mL and erythromycin (Sigma) 0.125–64 µg/mL. The microtiter plates were incubated for 24 hrs at 41.5°C under microaerophilic conditions. The MIC was defined as the lowest concentration of an antimicrobial agent that completely inhibits visible growth.

The organisms designed as reference strains for quality control procedures included type strains obtained from the American Type Culture Collection (ATCC): *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Campylobacter jejuni* ATCC 33560. Interpretive criteria for *Campylobacter* susceptibility testing are recommended by the CLSI (CLSI, 2008; 2010). The following CLSI breakpoints for resistance were used: for ciprofloxacin MIC ≥4 mg/L. Isolates were considered resistant to erythromycin with a MIC ≥32 mg/L and to nalidixic acid with an MIC of ≥32 mg/L. MIC_{50} and MIC_{90} provide an estimation of the antimicrobial concentration that inhibits 50% and 90% growth, respectively.

Molecular methods for detection of cmeB gene

Fifty *Campylobacter jejuni* strains were tested for the presence of the energy–dependent multi–drug efflux pump resistance gene (*cme*B). The PCR conditions were performed using methods previously described (Obeng *et al*., 2012). The PCR was carried out in 25 µL of reaction mixture containing: 5 µL of 10X PCR Buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 0.3 µL (5U/µL) of *Taq* thermostable DNA polymerase (Invitrogen®), 2 µmol 1^{-1} of $MgCl_2$ (25 mM), 2 µL dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 2 µL extracted template DNA and 1 µL (25 pmole 1^{-1}) of each primer. Sterile Milli–Q water was added q.s.p 25 µL. The sequences of primers and PCR conditions are listed in Table 1. All amplification reactions are performed in thermal cycler (Peltier Thermal Cycler Biocycler–MJ96+/MJ96G). The cycles were performed as follows (Table 1). For visualization of PCR products, 10 µL aliquots were electrophoresed in 1.5% agarose gels (Invitrogen UltrapureTM Agarose®–Carlsbad, USA), stained with ethidium bromide, and the amplified products were visualized in an ultraviolet light transilluminator (Pharmacia LKB Macro–Vue®) (Figure 1).

Restriction Fragment Length Polymorphism-RFLP

Thr–86–Ile mutations in the QRDR of gene *gyr*A were identified by PCR–RFLP. Analysis of the *gyr*A gene mutation was started with the amplification of a 179–bp fragment by PCR assay using a pair of primers: *cjgyr*AM1 and *cjgyr*A2 (Table 1). The PCR assay was carried out as previously described (Wardak *et al*., 2005). The amplified PCR products were digested with *Rsa*I enzyme (PROMEGA®) resulting in 125–bp and 54–bp fragments. The DNA segments were separated using 3% agarose gel (Invitrogen®). DNA bands were stained with ethidium bromide for 2h at 100V and viewed under UV light (Figure 2).

Table 1. List of primers and PCR conditions used to *Campylobacter* species confirmation, detection of *cme*B and RFLP-PCR for ciprofloxacin-resistance.

Target gene	Primers	Sequence (5'→3')	PCR conditions	Product (bp)	Reference
16S rRNA	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	95°C/10 min, 35 cycles: 95°C/30s, 59°C/90s, 72°C/1 min, and 72°C/10 min.	857 for *Campylobacter* genus identification	(Denis *et al*., 1999; Linton et al., 1997)
*map*A	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA		589 for *C. jejuni* species identification	(Denis *et al*., 1999)
*cme*B	*cme*B1 *cme*B2	TCCTAGCAGCACAATATG AGCTTCGATAGCTGCATC	94°C/5 min, 30 cycles: 94°C/30 s, 54°C/30s, 72°C/1 min, and 72°C/7 min.	241 for *cme*B detection	(Obeng *et al*., 2012)
PCR-RFLP (*gyr*A)	cjgyrAM1 cjgyrA2	AAATCAGCCCGTATAGTGGGTGCTG TTATAGGTCGTTATCACCCACACAT GGAGGT TCAGTATAACGCATCGCAGC	94°C/5 min, 30 cycles: 94°C/1 min, 51°C/1 min, 72°C/45s, and 72°C/7 min.	179 for *gyr*A detection	(Wardak *et al*., 2005)

Accession numbers

The GenBank accession numbers for the sequences of resistance genes are: *cme*B (JN003413) and *gyr*A (L04566).

Statistical Analysis

Data study of susceptibility was analyzed using the software WHONET version 5.4 (Stelling & O'Brien, 1997; WHO, 1999). Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) v18 (IBM). (SPSS Inc., Chicago, USA). Comparisons of association between phenotypic resistance and presence of specific resistance genes in *Campylobacter* isolates were compared using the Chi–square with *P*–values below 0.05 considered significant.

RESULTS

Bacterial identification and differentiation

The isolates were confirmed and identified using genus and species specific PCR assays. All 50 *Campylobacter* isolates were identified as *C. jejuni* by Multiplex–PCR method (Figure 1).

Antimicrobial susceptibility profile

The results of antimicrobial susceptibility testing and the resistance rate to each antimicrobial agent were calculated. The MICs results for three antimicrobial agents, as well as MIC_{50} and MIC_{90}, are shown in the Table 2. A percentage of 98% isolates were sensitive to erythromycin. In contrast, most isolates were resistant to ciprofloxacin (94%) and nalidixic acid (90%).

Molecular detection of antimicrobial resistance

Regarding ciprofloxacin–resistant isolates, 100% of the phenotype resistance strains had a mutation in the *gyr*A gene that was detected by the PCR–RFLP assay. The *cme*B gene responsible for contribute in multidrug resistance was identified in 16 isolates out the 50 strains (32%) isolates. Furthermore, this study found that in 16 isolates out 47 (34%) were phenotypically FQ–resistant and in one sample with Ery–resistant values (MIC≥32 µg/mL) the *cme*B gene was present.

DISCUSSION

A rapid increase in the proportion of *Campylobacter* strains resistant to antimicrobial agents, particularly to FQs and macrolides, has been reported in many countries worldwide, concerning to public health (Engberg *et al*., 2001; Alfredson & Korolik, 2007; Ge *et al*., 2013). Several laboratory methods including disk diffusion, broth microdilution, agar dilution and the Epsilometer–test (E–test) have been employed to determine the antimicrobial susceptibility (Luangtongkum *et al*., 2007). The use of molecular techniques such as the LightCycler–based PCR–hybridization *gyr*A mutation assay (GAMA) (Dionisi *et al*., 2004) and the Mismatch Amplification Mutation Assay (MAMA–PCR) (Zirnstein *et al*., 1999), offers fast alternatives, as well as the possibility of direct detection from a sample, screening a large numbers of organisms within a single assay. However, they may not detect resistance profile if a new or unexpected resistance mechanism is present (Moore *et al*., 2006). For these reasons, could be beneficial to combine phenotypic and genotypic methods of susceptibility testing.

Table 2. Results of Minimal Inhibitory Concentrations to Ciprofloxacin, Nalidixic acid and Erytromicin from 50 *C. jejuni* isolates.

Antimicrobials (μg/mL)	<0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	MIC $_{50}$	MIC $_{90}$	R*%
Ciprofloxacin				2	1	7	11	16	13					>16	>32	94
Nalidixic acid						1	2	2	27	18				>32	>64	90
Erythromycin			6	13	13	7	8	2	1					>2	>8	2

Breakpoint values, MIC values, and resistance percentage for 50 *C. jejuni* strains. A thick black line indicates the break point between clinically sensitive and resistant strains. Gray shadowed area indicates the test range (μg/mL) of each antimicrobial agent. MIC_{50}= (n χ 0.5); MIC_{90}= (n χ 0.9); R*= resistance rate.

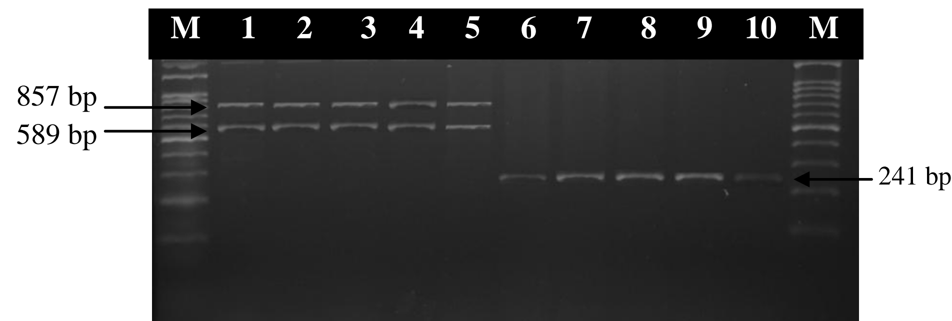

Figure 1. Agarose gel electrophoresis of PCR products. M= 100 bp marker; Lanes 1 to 5= 16S rRNA (857bp) and *map*A (589 bp), 6 to 10= *cme*B (241 bp).

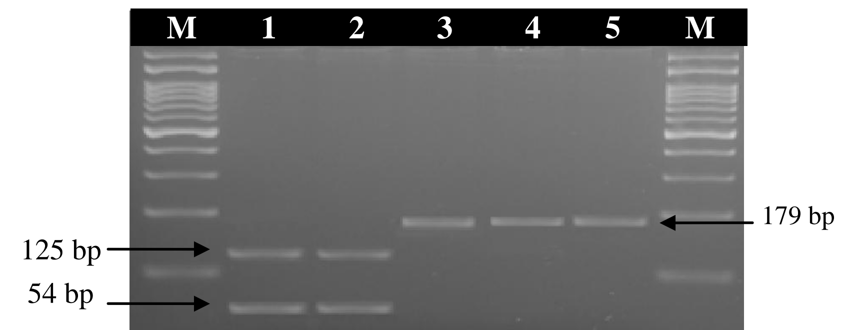

Figure 2. Agarose gel of 5 *C. jejuni* PCR-RFLP pattern obtain after digestion with *Rsa*I. Lanes: M= 100-bp marker; 1, ATCC 33560; 2, DNA from ciprofloxacin-sensitive strain; lanes 3 through 5= DNA from resistance strains.

In this study, the identification method for *C. jejuni* as previously described by Denis *et al*. (1999) was applied successfully. Multiplex–PCR represents an important tool for the diagnosis and study of a large number of samples, reducing the amount of reagents used and time required to obtain the results. In the present research, the results obtained showed a high percentage (94%) of *Campylobacter jejuni* isolates resistant to ciprofloxacin. Forty five strains (90%) were resistant to nalidixic acid. We observed a high level of resistance to ciprofloxacin, which was similar to that reported in other Brazilian studies by using the disk diffusion test (95%–100%) (de Moura *et al*., 2013; Hungaro *et al*., 2015). In China 98% out of 275 *Campylobacter* strains isolated from broiler were resistant to quinolones (Chen *et al*., 2010). Moreover, higher resistance rates to ciprofloxacin of *Campylobacter* isolated from humans have been reported in Thailand and India, 84% and 71.4%, respectively (Hoge *et al*., 1998; Jain *et al*., 2005). The relatively high fluoroquinolone resistance rates among *Campylobacter* isolates are most probably caused by the broad use of this class of antimicrobials in veterinary medicine (especially in poultry) (Iovine & Blaser, 2004). In contrast, low ciprofloxacin resistance has been reported in the USA (19%) (Gupta *et al*., 2004), and Europe with14.9% of resistance in *C. jejuni* and 39.6% in *C. coli* isolates (Bywater *et al*., 2004).

In *Campylobacter*, there are two well–described mechanisms that underlie resistance to FQs: inactivation of the target of FQ and efflux pump. These two mechanisms work together synergistically (Ge *et al*., 2005; Iovine, 2013). In general, the two intracellular enzymatic mechanism of resistance appears to be due often to mutations in the genes encoding subunits of DNA gyrase (encoded by *gyr*A and *gyr*B) and occasionally to topoisomerase IV (encoded by *par*C and *par*E) (Engberg *et al*., 2001). Fluoroquinolones form a stable complex with these enzymes and traps them onto DNA, leading to decreased DNA replication, transcription, and ultimately, to cell death (Shea & Hiasa, 1999). Cloning and sequencing in the *C. jejuni gyr*A gene, demonstrate that mutations at positions Thr–86, Asp–90 and Ala–70 were responsible for resistance (Wang *et al*., 1993; Ruiz *et al*., 1998). Mutations at Thr–86 are associated with higher level of resistance to nalidixic acid (MIC 64–128 µg/mL) and ciprofloxacin (MIC 16–64 µg/mL) than mutations at Asp–90 or Ala–70 (Engberg *et al*., 2001).

The macrolides are the most frequently used for therapeutics purpose in treatment of severe human campylobacteriosis. In this study, erythromycin resistance (MIC≥32 µg/mL) was found in one isolate (2%). Microbiological resistance to erythromycin was detected at very low to moderate levels in *Campylobacter* isolates from broilers (*Gallus gallus*) and broiler meat (0.4 %−16.5 %) (EFSA, 2014). There is recent evidence that the continuous use of a macrolide at sub–therapeutic level in chickens results in the development of Ery–resistance in *Campylobacter* (Ladely *et al*., 2007).

Macrolides inhibit protein synthesis by binding reversibly to the P site on the subunit 50S of the bacterial ribosome (Iovine, 2013). This resistance occurs through four general mechanisms: target modification, efflux altered membrane permeability and enzymatic modification. The first two mechanisms act synergistically to confer high–level resistance (Cagliero *et al*., 2006; Lin *et al*., 2007). A fourth is named enzymatic modification of macrolides, and has not been described in *Campylobacter* (Payot *et al*., 2006).

Studies have provided evidence of the presence of efflux pump in *C. jejuni* which reduces the intracellular concentration of detergents, FQ and several other antimicrobials, such as macrolides (Luangtongkum *et al*., 2009; Iovine, 2013). This efflux pump also contributes to bile salts tolerance in the intestinal tract, as required for successful colonization of *C. jejuni* in chickens (Lin *et al*., 2003).

Multidrug efflux pumps are chromosomally encoded by genetic elements capable of mediating resistance to toxic compounds in several forms of life. The efflux pump resistance was attributed to the presence of *cme*ABC operon. The best–described multidrug efflux pump in *Campylobacter* is CmeABC, consisting of three components: an outer membrane protein (encoded by *cme*C), an inner membrane drug transporter (encoded by *cme*B), and a periplasmic protein (encoded by *cme*A) that bridges CmeB and CmeC (Lin & Zhang, 2002; Pumbwe & Piddock, 2002; Iovine, 2013). By PCR the *cme*B presence was screened, 16 strains (32%) had a positive result.

In contrast to the results observed for FQ–resistant, it is noteworthy that 15 isolates were *cme*B positive of 16 samples which showed sensitivity to Ery (MIC<32 μg/mL), suggesting that just the presence of *cme*B gene is not sufficient for Ery–resistance. This observation confirmed previous findings that CmeABC works synergistically with other mechanisms to maintain high–level and low–level of Ery resistance in *C. jejuni* (Cagliero *et al*., 2006; Lin *et al*., 2007). *C. jejuni* utilizes complex and different mechanisms to develop Ery resistance *in vitro* and *in vivo*. Emergence of a specific mutation or modification that confers Ery resistance is dependent on the environment in which the mutation is selected, the genetic features of a strain, and/or the specific macrolide agent used for selection (Caldwell *et al*., 2008).

This study found correlation between molecular tool for detection of a point mutation at position Thr–86 on the *gyr*A gene, and the MICs of ciprofloxacin and nalidixic acid, at 100% level in the investigation carried out by MIC versus PCR–RFLP. The results showed that PCR–RFLP is a rapid and simple method for the detection of high–level fluoroquinolone resistance in *C. jejuni.*

In conclusion, this research provides information about antimicrobial resistance of thermophilic *C. jejuni* isolated from poultry slaughterhouse in Brazil. The results of present study emphasize the need to enhance the hygienic–sanitary control strategies during slaughter processing. A combination of phenotypic and genotypic methods for resistance characterization provided optimal results. The PCR–based methods of characterizing FQ–associated mutations (RFLP) offer viable alternatives for screening large numbers of isolates proving to be a useful option, fast and economic for clinical diagnostic and routine assays.

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CAPITULO IV

PCR-Restriction Fragment Length Polymorphism Assay in *Campylobacter jejuni* and *Campylobacter coli* from Poultry and Human samples

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ABSTRACT. The objective was to determine the fluoroquinolones resistance of *Campylobacter* from slaughter process and human origin samples in Brazil. In this study 52 *Campylobacter* strains; 41 *C. jejuni* isolates (30 of poultry origin and 11 of human origin) and 11 *C. coli* isolates (10 of human origin and 1 of poultry source), were examined for ciprofloxacin, norfloxacin and nalidixic acid susceptibility by MIC method. Following, were analyzed by PCR-Restriction Fragment Length Polymorphism (RFLP) assay for detection of Thr-86 mutation. Finally, was realized sequencing as confirmation for detection of *gyr*A gene mutation. A complete correlation was observed between the MICs, PCR-RFLP assay and sequencing. The results revealed high quinolone-resistance rates for *C. jejuni* (100%) and *C. coli* (100%) of isolates obtained from poultry and moderate resistance *C. jejuni* (9.1%) and *C. coli* (40%) in samples of human origin. A mutation in codon 86 of the *gyr*A gene with a Thr to Ile substitution is reported to be the main cause of high-level resistance to quinolones. This mutation can be analyzed using PCR-RFLP assay, showed the possibility use this prove as a simple and fast method for the detection of fluoroquinolone resistance in *Campylobacter* spp.

Keywords: *Campylobacter*, Fluoroquinolones, resistance genes, PCR-RFLP.

INTRODUCTION

Campylobacteriosis is one of the most important bacterial food-borne illnesses in humans worldwide and, therefore, a major target for the public health. Two thermotolerant species, *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are responsible for the vast majority of human infections, among which 80–90% are due to *C. jejuni* (7, 17, 28). This bacterium is transmitted to humans via contaminated foods of animal origin, especially undercooked poultry meat and unpasteurized milk/dairy products (24). *Campylobacter* species are able to induce gastrointestinal and systemic infections. Serious long-term sequelae of infection that may occur in humans include Guillain-Barré syndrome (GBS), Miller Fisher syndrome (MFS), reactive arthritis, Reiter's syndrome, haemolytic uremic syndrome (HUS) and septicaemia (10, 15, 21, 30, 33, 35).

When patients suffer from recurrent or systemic *Campylobacter* infection, antimicrobial treatment is indicated. Fluoroquinolones are one of two families of antimicrobials used to treat *Campylobacter* infections in humans (2, 16, 31). In *C. jejuni* and *C. coli*, fluoroquinolone resistance is primarily associated with a single threonine at position 86 to isoleucine (Thr-86 to Ile) mutation in *gyr*A gene in isolates from both humans and animals (3, 4, 29, 36). Due to the importance of fluoroquinolones in clinical therapy of human campylobacteriosis, development of resistance in *Campylobacter* has become a concern for public health. The purpose of the present work was to determine the fluoroquinolones resistance of *Campylobacter* from slaughter process and human origin samples in Brazil. The antimicrobial susceptibilities of *Campylobacter* spp. strains were tested and analyzed using broth microdilution test as well as molecular biological methods: a PCR- based restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing as confirmation for detection of *gyr*A gene mutation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Human clinical isolates were obtained from the Culture Collection of the Oswaldo Cruz Institute (IOC) in Rio de Janeiro, Brazil, (n=21). Of these, 11 isolated had been previously identified as *Campylobacter jejuni* and 10 as *Campylobacter coli*. Poultry isolates (carcasses: n=30; chiller water samples n=1); were obtained in poultry slaughterhouses of federal inspection from Rio Grande do Sul, in Jan–Dec 2012.

Sampling of broiler carcasses

Fresh disposable gloves were worn to remove each carcass from the processing line. Each carcass was placed in a sterile plastic bag, and carcasses were transported to the laboratory in insulated boxes with ice packs. Immediately upon arrival at the laboratory, rinse samples were collected by shaking carcasses for 1 min after the addition of 400 mL of Buffered Peptone Water (BPW 1%) (CM1049 Oxoid®). Subsequent to shaking, was immediately transferred 1 mL of each sample to 9 mL of Bolton broth supplemented with antimicrobial (CM0983 Oxoid®, supplement SR0183) and incubated at 41.5^{o}C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) for 48 hrs.

Isolation was performed in accordance with the International Standards Organization guidelines (20). Next, 10μL were streaked on the modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (CM739, Oxoid®, with cefoperazone selective supplement SR 155E) and further incubated for 48 hrs in microaerophilic conditions at 41.5°C. Presumptive *Campylobacter* colonies were subcultivated on BA plates (BA; Blood Agar Base N.2, Oxoid®, supplemented with sterile defibrinated sheep blood 5%) and incubated for 48 hrs under the above mentioned conditions. Preliminary identification of *Campylobacter* species from primary culture is by colonial appearance, Gram stain, growth in oxygen and oxidase test. The colonies were collected and resuspended in 1mL of ultrapure water, transferred to microtubes, and later frozen at -20°C until DNA extraction. All isolated strains were stored in cryovials at -80°C.

Sampling and examination of chiller water

Chiller water samples (100mL) were collected by immersing sterile plastic containers in it. The water was transported to the laboratory, on ice in an insulated container or portable refrigerator, for enrichment culture and enumeration of campylobacters as described above.

DNA extraction and species confirmation

Genomic DNA was extracted using a modified protocol described by Borsoi et al. (5). Briefly, isolated colonies were picked from blood agar plates and suspended in 1 ml of distilled water in a microfuge tube. Samples were boiled for 10 min at 95°C before being added to PCR mix with specific primers selected from *map*A and *ceu*E genes for simultaneous detection of the species *C. jejuni* and *C. coli,* respectively. All isolates were identified by Multiplex-PCR according to a method previously developed by Denis et al. (11) with some modifications.

Minimal Inhibitory Concentration -MIC

The minimal inhibitory concentrations (MICs) of ciprofloxacin (CIP), norfloxacin (NOR) and nalidixic acid (NAL) for all isolates were determined by broth microdilution method according to the Clinical and Laboratory Standard guidelines (CLSI) (8, 9). The antimicrobial were tested in a 2-fold concentration series: ciprofloxacin (Sigma, St Louis, MO, USA) 0.125-64μg/mL, nalidixic acid (Sigma) 0.25-128 μg/mL and norfloxacin (Sigma) 0.125-64 μg/mL. The microtiter plates were incubated for 24 hrs at 41.5°C under microaerophilic conditions. The phenotype clusters were established by considering isolates that were not susceptible as resistant. The MIC breakpoints used for resistance were those recommended by the CLSI for non-Enterobacteriaceae (8, 9), to those fluoroquinolones for which such recommendations are available: ciprofloxacin (MIC ≥4 mg/L); norfloxacin (MIC ≥16 mg/L); and nalidixic acid (≥32 mg/L). The organisms designed as reference strains for quality control procedures included type strains obtained from the American Type Culture Collection (ATCC); *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Campylobacter jejuni* ATCC 33560.

Analysis of the quinolone resistance- determining region (QRDS) of gyrA

The resistance of *C. jejuni* and *C. coli* to quinolones mainly depend on mutations in the quinolone resistance determining region (QRDR) of gene *gyr*A and was identified by PCR-RFLP.

A PCR-RFLP assay using the common restriction enzyme *Rsa*I is described to distinguish a point mutation at position Thr-86 on the *gyr*A gene product, involving the replacement of Thr-86 by Ile. Analysis of the *gyr*A gene mutation was started with the amplification of a 179-bp fragment.

The PCR conditions were adapted (3, 37). The PCR was carried out in 25 μL of reaction mixture containing: 2.5 μL of 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.5 μL (5U/μL) of *Taq* thermostable DNA polymerase (Invitrogen®), 1 μmol 1^{-1} of $MgCl_2$ (25 mM), 2μL dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 1 μL extracted template DNA and 1 μL (10 pmole 1^{-1}) of each primer. Sterile Milli-Q water was added q.s.p 25 μL. The sequences of primers and PCR conditions are listed in Table 1. All amplification reactions are performed in thermal cycler (Peltier Thermal Cycler Biocycler-MJ96+/MJ96G). The cycles were performed as follows (Table 1). For visualization of PCR products, 10 μL aliquots were electrophoresed in 1.5% agarose gels (Invitrogen UltrapureTM Agarose®-Carlsbad, USA), stained with ethidium bromide, and the amplified products were visualized in an ultraviolet light transilluminator (Pharmacia LKB Macro-Vue®). Amplification products of the expected size (179 bp) were obtained for all strains, whether they had been resistant or susceptible to the ciprofloxacin.

Finally, the PCR products were digested with *Rsa*I (PROMEGA®) to screen for mutations at position Thr-86. Enzyme digestion was performed in a 20 μL mixture containing 2μL of the PCR product and 1 μL of enzyme (10U/μL) following the manufacturer's instructions. The amplified PCR products were digested with *Rsa*I enzyme resulting in 125-bp and 54-bp fragments. The DNA segments were separated using 3% agarose gel (Invitrogen®). DNA bands were stained with ethidium bromide for 2h at 100V and viewed under UV light.

TABLE 1. List of primers and PCR conditions used in this study.

Target gene	Primers	Sequence (5’→3’)	PCR conditions	Product (bp)	References
16S rRNA	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	95°C/10 min, 35 cycles: 95°C/30s, 59°C/90 s, 72°C/1 min, and 72°C/10 min.	857 for *Campylobacter* genus identification.	(11, 23)
***map*A**	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA		589 for *C. jejuni* species identification.	(11)
ceuE	col3 MDcol2	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG		462 for *C. coli* species identification.	(11)
PCR-RFLP (gyr*A) *C. jejuni	cjgyrAM1 cjgyrA2	AAATCAGCCCGTATAGTGGGTGCTGTTA TAGGTCGTTATCACCCACACATGGAGGT TCAGTATAACGCATCGCAGC	94°C/5 min, 30 cycles: 94°C/1 min, 51°C/1 min, 72°C/45 s, and 72°C/7 min.	179 detection *gyr*A *(C. jejuni).*	(29, 37)
PCR-RFLP (gyr*A) *C. coli	colgyrA colgyrA2	AAATCTGCTCGTATAGTAGGGGATGTTA TCGGTAAGTATCATCCACATGGCGGT TCAGTATAACGCATCGCAGC	94°C/5 min, 30 cycles: 94°C/1 min, 55°C/1 min,72°C/45 s, and 72°C/7 min.	179 detection *gyr*A *(C.coli).*	(3, 29)

DNA sequencing for mutation gyr*A *gene

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) to use in sequencing reactions. Both strands were sequenced with a reaction contain 80 ng of target DNA and five pmol of forward and reverse primers. Product sequences were resolved on an automatic sequencer (ABI.PRISM 3100 Genetic Analyzer, Applied Biosystems, CA, USA). The resulting sequences were assembled and analyzed using the BioEdit software package (19).

Accession numbers

On the basis of the DNA sequence of the *gyr*A gene of *C. coli* (GenBank accession numbers AF092101) and *C. jejuni* (GenBank accession numbers L04566).

Analysis of results

Data analyses of susceptibility were analyzed using the software WHONET version 5.4 (32). For the analysis the statistical package SPSS (version 18) was used. The chi-square test was the statistical method used (as a distribution goodness of fit test).

RESULTS

All 52 *Campylobacter* isolates were identified and confirmed as *C. jejuni* (n=41) and *C. coli* (n=11) by Multiplex-PCR method. The results of antimicrobial susceptibility testing and the rate of resistance to each antimicrobial agent were calculated. MIC_{50} and MIC_{90} values, as well as rates of resistances, were calculated and presented (Table 2).

The resistance rate for *C. coli* and *C. jejuni* was variable depending to the source. A percentage of 100% the isolates from poultry sources were resistant to fluoroquinolones. In contrast, most of *Campylobacter jejuni* and *C. coli* isolates from human origin were sensitive to fluoroquinolones, with a percent of 89% and 60% respectively.

TABLE 2. Distribution of MICs for the *Campylobacter* spp. isolated from poultry and human origins.

Source	Antimicrobials (μg/mL)	<0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	MIC_{50}	MIC_{90}	R*%	Geom. mean
C. jejuni Poultry origin (n=30)	Ciprofloxacin						4	6	12	8					16	32	100	13,929
	Nalidixic acid									18	12				32	64	100	42,224
	Norfloxacin								10	20					32	32	100	25,398
C. jejuni Human origin (n=11)	Ciprofloxacin	6	3		1			1							0.125	1	9,1	0,26
	Nalidixic acid						3	4	3	1					8	16	9,1	9,075
	Norfloxacin	5	2	3					1						0.25	0.5	9,1	0,316
C. coli Human origin (n=10)	Ciprofloxacin	6						2	2						0.125	16	40	0,74
	Nalidixic acid							3	3	1	3				16	64	40	21,112
	Norfloxacin	2	3	1					4						0.25	16	40	1,221
C. coli Water chiller (n=1)	Ciprofloxacin								1						16	16	100	16
	Nalidixic acid										1				64	64	100	64
	Norfloxacin								1						16	16	100	16

Breakpoint values, MIC values, and resistance percentage for 52 *Campylobacter* spp. strains. A thick black line indicates the break point between clinically sensitive and resistant strains. Gray shadowed area indicates the test range (μg/mL) of each antimicrobial agent. MIC_{50}= (n χ 0.5); MIC_{90}= (n χ 0.9); R*= resistance rate.

A complete correlation was observed between the PCR-RFLP results and the MICs for the corresponding samples. Analysis of restriction patters after digestion with *Rsa*I showed that all resistance strains had the same RFLP, the 179-bp fragment. These strains were assumed to have mutation at Thr-86. The susceptible strains had two fragments of 54 bp and 125 bp, respectively, produced by *Rsa*I digestion. These samples were assumed to have no mutation at Thr-86 (Fig. 1, lane 6-10). Thr-86 on the *gyr*A gene product and the PCR-RFLP assays was 100% in the investigation carried out by DNA sequencing. The highest rates of resistance of *Campylobacter* spp. were among poultry sources (100%).

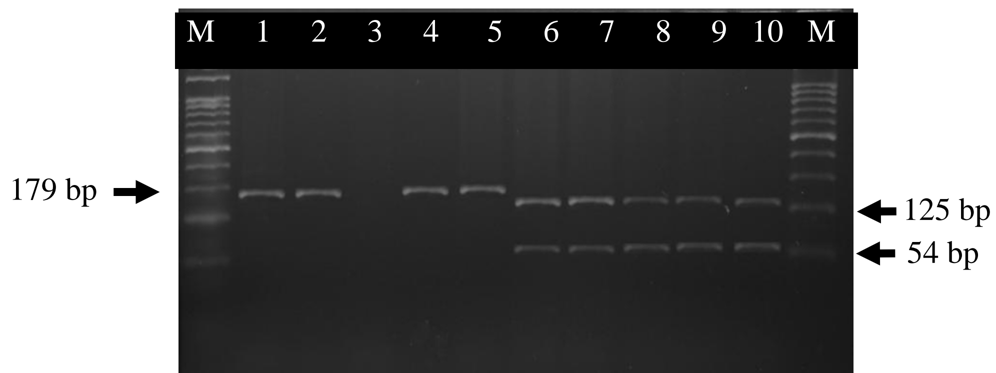

FIG.1. PCR-Restriction Fragment Length Polymorphism patterns obtained after digestion with *Rsa*I in 10 *C. coli* strains. Lanes: M= 100-bp DNA Ladder (Invitrogen®); 1 to 2, undigested PCR product of *gyr*A gene; 3, negative control; 4 to 5, ciprofloxacin-resistance; 6 to 10, ciprofloxacin-sensitive strains.

DISCUSSION

Microorganisms have an inherent ability to evolve to mutate and adapt to environmental stressors allowing them to survive otherwise lethal conditions (13). The imposed selective pressure results in the development of a corresponding resistance determinant, either through direct acquisition or intrinsically by modification of a host gene target, designed to facilitate evasion of the inhibitory substance (27).

In the present investigation, the isolates showed various degrees of susceptibility to antimicrobial agent depend to origin. In general, high percentage fluoroquinolone-resistance rates (100%) for *C. jejuni* and *C. coli* of isolates obtained from poultry and moderate resistance *C. jejuni* (9.1%) and *C. coli* (40%) in samples of human origin. Resistance to fluoroquinolones in human samples was more often in *C. coli* (40%) that in *C. jejuni* strains (9.1%). Several studies have emphasized that *C. coli* isolates are more likely to acquire resistance that *C. jejuni* isolates (6, 34).

For *Campylobacter* spp., alteration of codon 86 from ACA to ATA in the *gyr*A of *C. jejuni*, and ACT to ATT of the *gyr*A of *C. coli* has been reported to be the main mechanism of CIP resistance. It has been shown that factors other than *gyr*A QRDR mutations, such as efflux pumps, may contribute to the resistance phenotype (18, 22). In this study, the correlation between the molecular tool results for detection of a point mutation at position Thr-86 at the *gyr*A gene product, and the MICs of ciplofloxacin, norfloxacin and nalidixic acid, was 100% in the investigation carried out by DNA sequencing. This result was similar at showed by Alonso et al. (3) and El-Adawy et al. (14). The high prevalence of quinolone-resistance could be related to the introduction of these medicaments in the industry (25).

Concerns regarding the appearance of resistant bacterial pathogens as a consequence of antimicrobial use in food animals, and the potential transfer of resistant strains from those food products to humans have led to changes in antimicrobial use in food animal production systems worldwide (1, 26). Although direct sequencing is the more accurate technique for the detection of nucleotide mutations, DNA sequencing cannot be used as diagnosis because the protocols usually are expensive and time-consuming, making it impractical for routine use in many laboratories (3). Molecular techniques offer an alternative means of assessing antimicrobial resistance among bacterial isolates (3, 12, 37, 38). This study is important for the understanding of the frequency of resistance fluoroquinolone from *Campylobacter* spp. in Brazil. The results showed that PCR-RFLP assay is a simple method for the detection of resistance in *Campylobacter* spp. It also emphasizes the need for a more strict strategy in the use of antimicrobial agents in food animals.

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CAPITULO V

The use of FTA cards for transport and detection of *gyr*A mutation of *Campylobacter jejuni* from poultry

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ABSTRACT. The purpose of the present study was to evaluate a technique involving the use of commercially available FTA Classic card (Whatman) for transporting and detection of DNA to use in PCR analysis and genetic sequencing of *Campylobacter jejuni* from poultry origin. Fifty isolates of *Campylobacter jejuni* were obtained from broiler carcasses in Rio Grande do Sul state, Brazil. Antimicrobial susceptibility testing to ciprofloxacin revealed that all 50 isolates were resistance to ciprofloxacin. Each isolate was transferred to *Brucella* broth tubes and incubated overnight at 41.5°C. Cell cultures were diluted to match a McFarland Turbidity Standard 0.5, and 110 µL of the cell suspension were applied to one circle on Whatman FTA classic cards. The samples were then covered and allowed to dry at room temperature. Cards were identified and stored at room temperature until further use (3 months after collection). FTA cards were shipped for analysis to the Department of Poultry Science, University of Arkansas. Amplification of *Campylobacter gyr*A gene was successful and demonstrated strong bands for a large amplicon for all of 50 samples preserved in FTA cards. Mutations present in each gene were confirmed by DNA sequencing. Then, seven samples were chosen for the sequencing. The detection of a mutation regarding ciprofloxacin-resistant isolates revealed that 7 samples had a mutation in the *gyr*A gene. In conclusion, the characteristics of the profiles suggest that the DNA has maintained its integrity after 3 months storage at room temperature and is a suitable template for PCR and sequencing from *Campylobacter* samples. The application of this technology has potential in numerous methodologies, especially when working in remote areas and in developing countries where access to laboratory facilities and equipment is limiting.

Keywords: *Campylobacter jejuni*, DNA, FTA cards, *gyr*A, sequencing.

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) is recognized as leading food-borne pathogens causing acute bacterial enteritis worldwide. Fluoroquinolones are commonly used to treat gastroenteritis caused by *Campylobacter* species. However, the number of antimicrobial-resistant *Campylobacter* serotypes has increased drastically in recent years (Engberg et al., 2001; Alfredson and Korolik 2007; Ge et al., 2013). Resistance to these antimicrobial may be due to a single base mutation (C-257 to T) in codon 86 of the quinolone resistance determining region (QRDR) of the gy*rA* gene substitution the amino acid sequence from threonine at position 86 to isoleucine (Thr-86 to Ile) (Moore et al., 2006; Iovine, 2013). The utility of molecular technical to detect base changing in the *gyr*A gene mediated resistance to ciprofloxacin is extensively studied (Zirnstein et al., 1999; Piddock et al., 2003; Dionisi et al., 2004).

The strongest epidemiological risk factor for campylobacteriosis identified is the consumption of poultry products, becoming a problem direct concern to public health. In contrast to its pathogenic importance, relatively little is known about of biology and the mechanism of virulence. Actually, the interest in *C. jejuni* has increased as a result of the growing appreciation of its importance as a zoonotic agent, antimicrobial resistance and the availability of new model systems and advances in genomic technologies. Presently molecular biology offers a wide repertoire of techniques and innovation of these analytical tools. Nucleic acid analysis based procedures have become a routine and highly valued component of laboratory diagnosis, providing increased efficacy and facility for detection of a wide range of pathogens. Integrity, quantity, purity and transfer of DNA will affect the results of all subsequent applications, so highest quality of DNA is desirable for diagnosis and research. The purpose of the present work was to evaluate a technique involving the use of commercially available FTA Classic card (Whatman) for transporting and detection of DNA to use in PCR analysis and genetic sequencing of *Campylobacter jejuni* from poultry origin.

MATERIALS AND METHODS

Sample collection

Fifty 50 isolates of *Campylobacter jejuni* were obtained from carcasses of broiler slaughterhouses of Rio Grande do Sul state, Brazil. Isolation was performed in accordance with the International Standards Organization guidelines (ISO 10272–1:2006). Carcasses were placed in sterile bags and 400 mL of 0.1% buffered peptone water (BPW) (CM1049 Oxoid®) were added to each bag and vigorously shaken by hand for 1 min. One mililliter of each sample (carcasses) was removed and homogenized in 9 mL of Bolton broth supplemented with antimicrobial (CM0983 Oxoid®, supplement SR0183) followed by incubation for 48 hr at 41.5°C under an atmosphere of 5% O_2, 10% CO_2 and 85% N_2. Briefly, a quantity of 10 µL of the suspension after incubation were streaked on the surface of selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (CM739, Oxoid®, with cefoperazone selective supplement SR 155E). The plates were incubated in microaerophilic conditions at 41.5°C for 48 hr. Colonies with suspicious morphology were picked and streaked onto wet BA plates (BA; Blood Agar Base N.2, Oxoid®, supplemented with sterile defibrinated sheep blood 5%) and incubated for 48 hrs under the above mentioned conditions. These colonies were confirmed by the microscopic morphology, motility, microaerobic growth at 25°C and oxidase test. The colonies were collected and resuspended in 1mL of ultrapure water, transferred to microtubes, and later frozen at –20°C until DNA extraction. Isolates were stored with glycerol 15% at –80°C until required for further researches.

Antimicrobial susceptibility screening

Broth microdilution method was employed in this study followed the procedure according to the Clinical and Laboratory Standard guidelines (CLSI, 2008; 2010). The antimicrobial was tested in a 2–fold concentration series: ciprofloxacin (Sigma, St Louis, MO, USA) 0.125–64 µg/mL. The organisms designed as reference strains for quality control procedures included type strains obtained from the American Type Culture Collection (ATCC): *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Campylobacter jejuni* ATCC 33560.

Multiplex–PCR assay

Genomic DNA was extracted using an adapted protocol described by Borsoi et al. (2009). The isolates were confirmed by Multiplex–PCR based detection of 16S rRNA and *map*A genes (Denis et al. 1999).

Inoculation FTA cards

Campylobacter jejuni strains were revived from freezer stocks in 5 mL of Bolton (CM0983 Oxoid) by transferring one 10 µL loop of frozen cells in the media and incubated in microaerophilic conditions (10% CO_2, 2% H_2, 5% O_2, and 85% N_2) for 24 hours at a temperature of 41.5°C. Cultures were streaked on the surface of selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (CM739, Oxoid) incubated for 48 hrs under the above mentioned conditions. Colonies were transferred to *Brucella* broth (BD) tubes and incubated overnight at 41.5°C. Cell culture was diluted to match a McFarland Turbidity Standard 0.5, and 110 µL of the cell suspension were applied to one circle on Whatman FTA classic cards (GE Healthcare, USA). The samples were then covered and allowed to dry at room temperature. Cards were identified and stored at room temperature until further use (3 months after collection). FTA cards were shipped for analysis at to the John Kirkpatrick Skeeles Poultry Health Laboratory, Department of Poultry Science, University of Arkansas (Fayetteville, AR, USA).

DNA isolation

Whatman FTA® cards were processed following manufacturer instructions and using an adapted protocol described by Pulido-Landinez et al. (2012). Briefly, 3 FTA disks measuring 2.0-mm was punched out of each filter paper sample by using a 2.0-mm Harris Micro Punch (Whatman Inc.) and transferred in to sterile 1.5 mL Eppendorf tubes before adding 200 µL of FTA purification reagent. To avoid cross contamination between samples, the collecting device was cleaned by cutting three disks from a non-inoculated FTA card. Tubes were vortexed for 5 s (Vortex Maxi Mix Plus) and incubated at 25°C for 5 min. The purification reagent was discarded and replaced with fresh reagent to repeat this step one more time. Disks were then washed twice with 200 µL of TE buffer under the similar conditions indicated for the purification reagent and discarding the buffer in between washes. TE buffer was completely removed and the

filter membrane is added directly to the PCR tube containing the usual enzymes and reagents. Is important that all the liquid has been removed before performing analysis. It is recommended that analysis be conducted within 3 h of the disk washing. If this is not possible, the punch can be stored at 4°C or -20°C in the dark for up to 1 wk.

PCR analysis of the QRDR of gyr**A *and sequencing screening PCR conditions***

A 717-bp fragment of the QRDR of *gyr*A with a new set of primers designed for this study was amplified using forward primer cjejuniAF2 (5′-GCCTCACGTTTAAGCTCT-′3) and reverse primer cjejuniAR3 (5′-TGGGTGCTGTTATAGGTCGT-′3), obtained from *C. jejuni* genome. Polymerase chain reaction amplification was performed in a 50 μL total reaction volume; which included 5 μL of 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.5 μL (5U/μL) of *Taq* thermostable DNA polymerase (Invitrogen), 2 μL of $MgCl_2$ (25 mM), 1μL of dNTPs (dATP, dCTP, dGTP and dTTP, each at 2.5 mM), 5 μL of extracted template DNA and 0.8 μL of each primer. Sterile Milli-Q water was added q.s.p 50 μL. PCR parameters were carried out in a Thermal Cycler (Biometra) under the following cycling conditions: initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1.5 min with a final extension at 72°C for 10 min. A 10 μL sample of each PCR product was ran through a 1% agarose gel (Invitrogen) to confirm the specificity and size of the product. Before genetic sequencing, the PCR product was purified using QIAquick PCR purification kit (QIAGEN, Inc., Valencia, CA, USA) and PCR purification spin protocol (Qiagen), designed for the isolation of DNA fragments from PCR reactions.

Genetic sequencing

Mutations present in each gene were confirmed by DNA sequencing. Then, seven samples were chosen for the sequencing. An ABI 3130xe (AME Bioscience; Toroed, Norway) analyzer was used for automated DNA sequencing by the DNA Technologies Laboratory at the University of Arkansas. Sequencing was performed in both forward and reverse sequences, using samples containing 20 ng of PCR product and 3.4 pmol of the appropriate primer. Electropherograms for both forward and reverse complement DNA sequences were evaluated using the Sequence Scanner Software v1.0 (Applied Biosystems, USA).

RESULTS AND DISCUSSION

The results of antimicrobial susceptibility testing and the resistance rate to ciprofloxacin were calculated. The MICs results as well as MIC_{50} and MIC_{90}, are shown in Table 1. All 50 isolates showed 100% resistance to ciprofloxacin.

Table 1。 *In vitro* results of minimal inhibitory concentrations determinations of ciprofloxacin.

		MIC (µg/mL)			
Microorganism (Number of strains)	**Antimicrobial agent**	Range	MIC_{50}	MIC_{90}	R*%
***Campylobacter jejuni* (50)**	Ciprofloxacin	4-32	16	32	100

Breakpoint values, MIC values, and resistance percentage for 50 *C. jejuni* strains.
MIC_{50}= (n χ 0.5); MIC_{90}= (n χ 0.9); R*= resistance rate.

The frequency of *Campylobacter* is increasing worldwide and trends in antimicrobial resistance have shown a clear association between use of antimicrobial in the veterinary industry and resistant infections in humans, becoming a problem major public health (Alfredson and Korolik 2007; Iovine, 2013). The findings of this study showed a high percentage (100%) of *Campylobacter jejuni* isolates resistant to ciprofloxacin. Although there are few studies in Brazil, a high degree of resistance to ciprofloxacin have been reported in other Brazilian studies (95%-100%) (de Moura et al., 2013; Hungaro et al., 2015).

The results of this research suggest that antimicrobial resistance in *Campylobacter* isolated from broiler carcass is widespread and may be increasing. Since poultry is considered to be one of the most important reservoirs of human infections, this persistent resistance is likely to have important public health consequences. Amplification of DNA directly from storage cards commonly used for preserving samples is particularly effective for future PCR assays. The quantity of DNA extracted from 3 pieces of 2 mm punched disks added to 50 µL PCR reaction volume is sufficient

for amplification. The use of the filter paper disks increases the number of PCR reactions that we can obtain from an individual sample. Amplification of *Campylobacter* genes were successful and demonstrated strong bands for a large amplicon for all of the 50 samples preserved in FTA cards (**Figure 1**). FTA cards protect nucleic acids from nucleases, oxidation, UV damage and microbial and fungal attack. This DNA cards stored at room temperature was worked as effectively as conventional frozen DNA storage (Whatman, 2006). A point mutation in the QRDR of the *gyr*A gene at codon 86 (ACA to ATA), substituting isoleucine for threonine is the most common mechanism of fluoroquinolone in *C. jejuni* and has been shown in many studies (Bachoual et al., 2001; Hakanen et al., 2002; Lucey et al., 2002; Piddock et al., 2003). In the present study, the detection of a mutation regarding ciprofloxacin-resistant isolates revealed that all seven samples analyzed had a mutation in the *gyr*A gene that was detected by sequencing. With the sequencing test we can conclude that DNA extracted from the cards is optimal material for mutational screening preserving DNA integrity for molecular analyses.

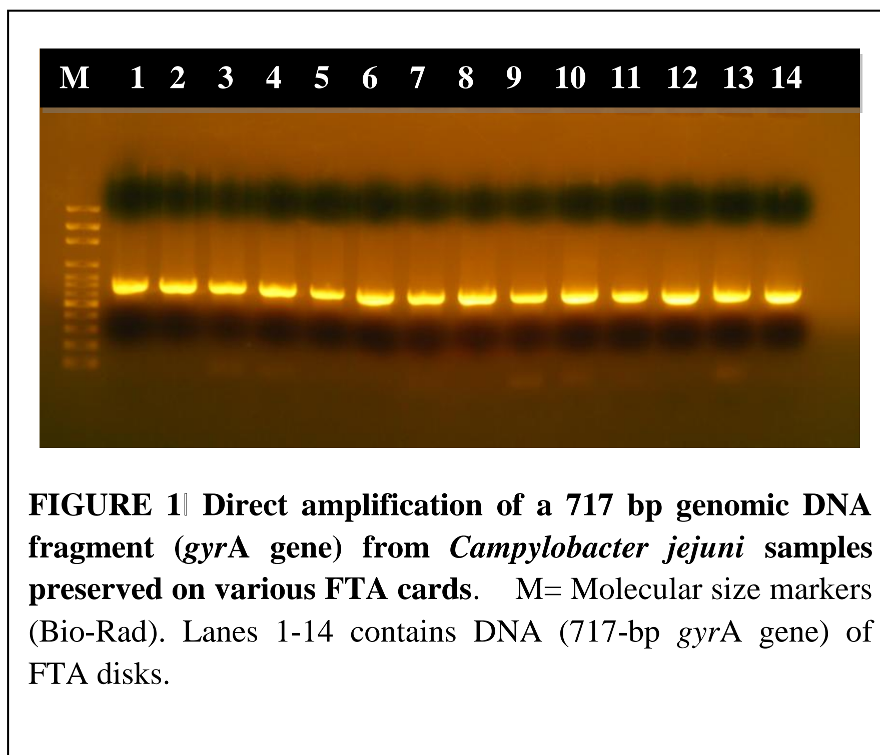

FIGURE 1 Direct amplification of a 717 bp genomic DNA fragment (*gyr*A gene) from *Campylobacter jejuni* samples preserved on various FTA cards. M= Molecular size markers (Bio-Rad). Lanes 1-14 contains DNA (717-bp *gyr*A gene) of FTA disks.

The FTA Classic card has been tested as a potentially efficient, safe way and economic method for DNA and RNA storage and for collecting fresh cells (Dobbs et al., 2002; Natarajan et al., 2000; Mbogori et al., 2006; Pulido-Landinez et al., 2012; Jignal et al., 2014). Once a biological sample is applied to an FTA Card, chemicals rapidly lyse cells, inactivate proteins and immobilize and stabilize the DNA. Nucleic acid are physically entrapped, immobilized and stabilized for storage at room temperature (Whatman, 2006). The DNA is protected from damage and degradation making high quality samples for future analysis.

In conclusion, assessment of antimicrobial resistance and tracks changes in microbial populations permits the early detection of resistant strains of public health importance. Surveillance findings are required to inform clinical therapy decisions and to assess the impact of resistance control interventions. The characteristics of the profiles suggest that the DNA has maintained its integrity after long term storage at ambient temperature and is a suitable template for PCR and sequencing from *Campylobacter* samples. The application of this technology has the potential in numerous methodologies, especially when working in remote areas and in developing countries where access to laboratory facilities and equipment is limiting.

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CONSIDERAÇÕES FINAIS

O objetivo deste estudo foi analisar a ocorrência, padrões de resistência antimicrobiana e sua relação fenotípica e genotípica, bem como a caracterização de marcadores de virulência em isolados de *Campylobacter* spp. obtidos a partir de fontes de origem aviar de diferentes pontos na linha do abate de matadouros-frigoríficos do Estado do Rio Grande do Sul. Um total de 141 amostras de origem aviar, incluindo fezes (n=8), água de *chiller* (n=18), carcaças de frango durante o processo do abate (n=26) e carne de frango pronta para o consumo (n=89) foram avaliadas. Assim, mediante o uso da técnica de m-PCR foi determinada a presença de *Campylobacter jejuni* em 140 amostras (99.2%), enquanto que *Campylobacter coli* foi identificado na amostra restante (0,7%).

A análise de marcadores de patogenicidade dos isolados através da técnica de PCR permitiu estabelecer patrões genéticos de *Campylobacter* spp. presentes em produtos de origem avícola na região Sul do Brasil. A compreensão da ecologia de genes patogênicos de espécies bacterianas tem se expandido graças aos avanços no campo da biologia molecular. Adesão e invasão são passos críticos que devem ocorrer antes que a doença se desenvolva, e seu entendimento são peças fundamentais na epidemiologia. Neste contexto, o esforço para reduzir as infecções por *Campylobacter* spp. em humanos está diretamente ligado a uma melhor compreensão dos aspectos biológicos deste microrganismo e, particularmente, dos seus mecanismos de virulência, que contribuem na patogênese da doença.

Os resultados dos testes de resistência antimicrobiana mostraram uma grande diversidade de sorovares e origem dos isolados. A partir dos resultados obtidos neste trabalho, pôde-se verificar que cepas de *C. jejuni* de origem avícola foram altamente resistentes a fluoroquinolonas em comparação com as amostras de origem humana. Atualmente a maior preocupação é o surgimento de cepas patogênicas resistentes como consequência da utilização de antimicrobianos em animais de produção, e o potencial de transferência dessas cepas a partir de produtos alimentares para seres humanos. A combinação de métodos fenotípicos e genotípicos para a caracterização da resistência fornece ótimos resultados. Os métodos baseados em PCR e PCR-RFLP para caracterizar mutações associadas às FQ oferecem alternativas viáveis para o rastreio de

um grande número de isolados provando ser uma opção útil, rápida e econômica para testes diagnósticos de rotina. Os resultados do presente estudo apontam a necessidade de reforçar as estratégias de gestão da qualidade em toda a cadeia de produção avícola.

CONCLUSÕES

- Foi possível identificar a diversidade dos sorovares de *Campylobacter spp.* circulantes em matadouros-frigoríficos da Região Sul do Brasil mediante o isolamento microbiológico e identificação genotípica de 140 cepas de *Campylobacter jejuni* (99.2%) e uma cepa de *Campylobacter coli* (0.7%) mediante a técnica de Multiplex-PCR.

- Foi analisada a presença dos principais genes de virulência nos isolados de *Campylobacter jejuni* mediante a técnica de PCR, que indicaram a deteção dos genes: *fla*A (78.5%), *cad*F (77.8%), *vir*B (22.1%) e *wla*N (10.7%). O operon (*cdt*ABC) estava presente em 74.2%, enquanto o *cdt*A, *cdt*B e *cdt*C, foram detectados em um 85%, 80% e 92.1%, respectivamente. O gene *iam* não foi detectado em nenhum dos isolados avaliados.

- Foram identificadas em 141 amostras de *Campylobacter* spp. mediante ensaios fenotípicos genotípicos e uma alta porcentagem de resistência a β-latâmicos (65%) (91/141). Cinquenta cepas (35.5%) são resistentes a tetraciclinas e 26 (18.5%) tem a presença da bomba de efluxo. Neste contexto, podemos concluir que 36 cepas (25.6%) são resistentes a dois diferentes grupos de antimicrobianos (β-latâmicos e Tetraciclinas).

- Foi determinada mediante a concentração inibitória mínima a frequência na resistência a agentes antimicrobianos em 50 isolados de *Campylobacter jejuni* confirmando uma porcentagem de 98% de sensibilidade a eritromicina. Em contraste, 94% (47/50) dos isolados foram resistentes à ciprofloxacina e quarenta e cinco cepas (90%) resistentes ao ácido nalidíxico.

- Foram identificadas diferenças na resistência a ciprofloxacina em amostras de origem avícola e humana. Os resultados revelaram 100% de resistência para *C. jejuni* e *C. coli* de origem avícola e resistência de 9.1% para *C. jejuni* e 40% para *C. coli* de origem humana.

- Foi utilizada com eficiência a técnica de RFLP-PCR na detecção da resistência a ciprofloxacina tanto para *C. jejuni* quanto para *C. coli*, demonstrando ser uma prova específica, sensível e de alto poder discriminatório e de alta repetitividade. Além disso, foi possível obter resultados em um dia.

- Foram utilizados com sucesso os cartões FTA em técnicas moleculares, acrescentando uma ferramenta importante para futuras pesquisas, garantindo viabilidade e oferecendo um meio seguro para o transporte de DNA.

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APENDICE A:

63rd Western Poultry Disease Conference & XXXIX
Convención de la Asociación Nacional de Especialistas en Ciencias Avícolas, A.C.

Detection of Cytolethal Distending Toxin (CDT) Genes in *Campylobacter jejuni* isolated from Chicken Carcasses.

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Presentation Type: Oral

Summary

One of the major virulence factors related to the pathogenicity of *Campylobacter jejuni* in human and animal infections is the cytolethal distending toxin (CDT), leading to cell cycle arrest in G2/M and subsequent cell death. Its activity is encoded by the *cdt* gene operon, which consists of three adjacent genes (*cdt*A , *cdt*B , *cdt*C). In this work was isolated and identified strains of *Campylobacter jejuni* from refrigerated carcasses of three slaughterhouses in the state of Rio Grande do Sul, Brazil; and detected using PCR the presence of *cdt* genes operon. Thirty strains of *Campylobacter jejuni* were isolated and identified by phenotypic and genotypic methods. The *cdt* complex was detected in 63.33 % of the isolates, showing a high possibility of the introduction of this toxin which is linked to the pathogenesis of inflammatory diarrhea in humans.

Keywords: *Campylobacter jejuni*, Cytolethal Distending Toxin (CDT), chicken carcass, PCR.

Resumen

Uno de los principales factores de virulencia relacionados a la patogenicidad de *Campylobacter jejuni* en infecciones humanas y animales es la toxina distensora citoletal (CDT), conduciendo a arresto del ciclo celular en G2/M y posterior muerte celular. Su actividad está codificada por el complejo de genes *cdt*, que consiste en tres genes adyacentes (*cdt*A, *cdt*B, *cdt*C). El objetivo del presente trabajo fue aislar e identificar cepas de *C. jejuni* a partir de carcasas refrigeradas de tres plantas de sacrificio del estado de Rio Grande del Sur, Brasil; detectar mediante la técnica de PCR la presencia del complejo de genes *cdt*. Treinta cepas de *Campylobacter jejuni* fueron aisladas e identificadas por métodos fenotípicos y genotípicos. La presencia del complejo *cdt* fue detectada en el 63,33% de las cepas aisladas, demostrando la alta posibilidad de la presentación de la toxina que está relacionada con la patogénesis de la diarrea inflamatoria en humanos.

Palabras clave: *Campylobacter jejuni*, Toxina Distensora Citoletal (CDT), pollo en canal, PCR.

APENDICE B:

Anais do VII Simpósio Brasileiro de Microbiologia Aplicada

III Encontro Latino Americano de Microbiologia Aplicada

IX Encontro Nacional dos Estudantes de Pós-Graduação de Microbiologia Agrícola

XI Fórum dos Coordenadores dos Programas de Pós Graduação em Microbiologia da Área de Ciências Agrárias

Detection of *Campylobacter* Adhesion to Fibronectin (*cad*F) gene in *Campylobacter jejuni* strains isolated from Poultry

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Abstract

Campylobacter jejuni is one of the most common causes of bacterial diarrhea worldwide and is the primary bacterial cause of food-borne illness. Other clinical presentations of *Campylobacter* infection are meningitis, septicaemia, localized extraintestinal infections, reactive arthritis and immune-reactive complications such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS). Adherence to and invasion of epithelial cells are the most important pathogenic mechanism of *Campylobacter* diarrhea. Nowadays, the best characterized *Campylobacter jejuni* adhesin are the *Campylobacter* adhesion to fibronectin (CadF). The product of *cad*F gene is an adhesion and fibronectin-binding protein involved in the process of invasion, influencing microfilament organization in host cells. In this work was isolated and identified ninety strains of *Campylobacter jejuni* from samples of poultry of ten slaughterhouses in the state of Rio Grande do Sul, Brasil; were isolated and identified by phenotypic and genotypic methods. The isolates are detected using polymerase chain reaction. The primary goal of this work was to determine the frequency of *cad*F gene in *Campylobacter jejuni* isolates from poultry. The *cad*F gene cluster was detected in 63.33% (57/90) isolates. The presence of *cad*F gene related to the adherence and invasion in *Campylobacter* and play an important role in the colonization of a host as well as in the development of *Campylobacter jejuni* mediated enteritis. Thus, as *Campylobacter* may be transferred from animals to human, it is important to know whether all *Campylobacter* isolates obtained from different sources are equally virulent.

Keywords: *Campylobacter jejuni*, *cad*F, carcass, PCR.

APENDICE C:

AVISULAT 2014

IV Congresso Sul Brasileiro de Avicultura, Suinocultura, e Laticínios - Feira de Equipamentos, Serviços e Tecnologia.

Detecção do gene *wla*N de *Campylobacter jejuni* em isolados de origem avícola.

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Resumo

Campylobacter jejuni é a principal causa de doença diarréica de origem alimentar bacteriana em todo o mundo. A fonte mais comum de infecções humanas são produtos avícolas. A doença clínica em humanos varia desde infecções assintomáticas até diarréia sanguinolenta inflamatória grave. Campylobacteriose é a antecedente mais frequente da neuropatia periférica pós-infecciosa, associadas às síndromes de Guillain-Barré e Miller Fisher. As cepas de *Campylobacter jejuni* possuem grande variabilidade na estrutura dos Oligo-Polissacarídeos (LOS) com base nas variações do conteúdo genético do lócus da biossíntese. Estes LOS apresentam modificações específicas envolvidas no mimetismo molecular de gangliosídeos caracterizados por um ataque imune mediado sobre os nervos periféricos, particularmente na bainha de mielina e nervos sensoriais motores. Um dos principais fatores de virulência relacionados à patogenicidade de *Campylobacter jejuni* em infecções humanas e animais é a presença do gene *wla*N. Este gene codifica uma β1,3-galactosiltransferase que está implicada no mimetismo e tem correlação na colonização e invasão de células Caco-2 em modelos *in vivo* e *in vitro*. O objetivo do presente trabalho foi isolar e identificar cepas de *Campylobacter jejuni* de 10 matadouros-frigoríficos do estado do Rio Grande do Sul, Brasil; detectar mediante a técnica de Reação em Cadeia da Polimerase (PCR) a presença do gene *wla*N. Foram isoladas e identificadas por métodos fenotípicos e genotípicos 80 amostras de *Campylobacter jejuni*. A presença do gene *wla*N foi detectada no 12,5% das amostras isoladas, demonstrando a alta possibilidade da presença do gene em produtos de origem avícola. A variabilidade destas estruturas externas parece ser um importante fator de virulência permitindo que as bactérias superem fatores externos adversos e consigam causar doença melhorando suas propriedades antigênicas ao mudar a habilidade de infectar seus hospedeiros.

Palavras-chave: *Campylobacter*, *wla*N, Guillain-Barré*,* PCR.

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