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# Identification of virulence genes and antimicrobial resistance in *Campylobacter* spp. from sheep from the state of Pernambuco in Brazil

**Identificação de genes de virulência e resistência antimicrobiana em *Campylobacter* spp. de ovinos do estado de Pernambuco no Brasil**

**Identificación de genes de virulencia y resistencia antimicrobiana en *Campylobacter* spp. de ovejas del estado de Pernambuco en Brasil**

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**Abstract**
The objective of this study was to genetically identify virulence and antimicrobial resistance in DNA from *Campylobacter* spp. from sheep in the state of Pernambuco, Brazil. The presence of virulence genes was investigated from the polymerase chain reaction. The genetic profile of antimicrobial resistance in samples of sheep origin was investigated by sequencing of the 23S rDNA region to identify A2074G and A2075G mutations and *gyrA* gene fragments to identify C257T and A256G mutations. Forty samples of *Campylobacter* spp. Of these, 11 were from *Campylobacter jejuni*, 12 from *Campylobacter fetus* subsp. *fetus* and 17 *Campylobacter coli* from sheep herds. In virulence analysis, 37 samples (92.50%) were positive for the *cdtA* gene, 30 (75.00%) for *cdtB* and 28 (70.00%) for *cdtC*. In the *cadF* gene research, 38 (95.00%) samples were positive. For the *racR*, *dnaJ* and *ciaB* genes, 32 (80.00%), 19 (47.50%) and 8 (20.00%) positivity were respectively. Only one sample presented the *pldA* gene and none presented *wlaN* and *virB11*. In genotypic analysis of antimicrobial resistance, all samples had the C257T mutation in the *gyrA* gene, but the A256G mutation was absent. Mutations in 23S rDNA, A2074G and A2075G were also not identified. From the results obtained, we can observe the presence of most virulence genes researched, with high resistance to fluoroquinolones. Thus, studied samples of *Campylobacter* spp. demonstrated the potential to cause infection and stay in the hosts.
**Keywords**: Campylobacteriosis; Pathogenicity; Susceptibility; Virulence.

**Resumo**
Objetivou-se com este estudo realizar a identificação genética da virulência e resistência antimicrobiana em DNA de isolados de *Campylobacter* spp. procedentes de ovinos do estado de Pernambuco, Brasil. A presença de genes de virulência foi investigada a partir da Reação em Cadeia da Polimerase. O perfil genético de resistência aos antimicrobianos nas amostras de origem ovina foi pesquisado por sequenciamento da região de 23S rDNA, para identificação das mutações A2074G e A2075G e de fragmentos do gene *gyrA*, para identificação das mutações C257T

e A256G. Foram analisadas 40 amostras de DNA de *Campylobacter* spp, destas, 11 eram de *Campylobacter jejuni*, 12 *Campylobacter fetus* subsp. *fetus* e 17 *Campylobacter coli* procedentes de rebanhos ovinos. Na análise de virulência, 37 amostras (92,50%) foram positivas para o gene *cdtA*, 30 (75,00%) para *cdtB* e 28 (70,00%) para *cdtC*. Na pesquisa do gene *cadF*, 38 (95,00%) amostras foram positivas. Para os genes *racR*, *dnaJ* e *ciaB* houve positividade de 32 (80,00%), 19 (47,50%) e 8 (20,00%), respectivamente. Apenas uma amostra apresentou o gene *pldA* e nenhuma apresentou *wlaN* e *virB11*. Na análise genotípica de resistência antimicrobiana, todas as amostras apresentaram a mutação C257T no gene *gyrA*, mas a mutação A256G estava ausente. As mutações em 23S rDNA, A2074G e A2075G também não foram identificadas. A partir dos resultados obtidos, observa-se a presença da maior parte dos genes de virulência pesquisados, com alta capacidade de resistência às fluoroquinolonas. Assim, amostras estudadas de *Campylobacter* spp. demonstraram o potencial de causar infecção e se manter nos hospedeiros.
**Palavras-chave:** Campilobacteriose; Patogenicidade; Susceptibilidade; Virulência.

**Resumen**
El objetivo de este estudio fue realizar la identificación genética de virulencia y resistencia antimicrobiana en ADN de *Campylobacter* spp. de ovejas en el estado de Pernambuco, Brasil. La presencia de genes de virulencia se investigó mediante la reacción en cadena de la polimerasa. El perfil genético de la resistencia antimicrobiana en muestras de origen ovino se investigó mediante la secuenciación de la región 23S rDNA para identificar las mutaciones A2074G y A2075G y los fragmentos del gen gyrA para identificar las mutaciones C257T y A256G. Se analizaron 40 muestras de ADN de *Campylobacter* spp, de las cuales 11 eran de *Campylobacter jejuni*, 12 de *Campylobacter fetus* subsp. *fetus* y 17 *Campylobacter coli* de rebaños de ovejas. En el análisis de virulencia, 37 muestras (92,50%) resultaron positivas para el gen *cdtA*, 30 (75,00%) para *cdtB* y 28 (70,00%) para *cdtC*. En la investigación del gen *cadF*, 38 (95,00%) muestras resultaron positivas. Para los genes *racR*, *dnaJ* y *ciaB*, 32 (80,00%), 19 (47,50%) y 8 (20,00%) fueron positivos, respectivamente. Solo una muestra tenía el gen *pldA* y ninguna tenía *wlaN* y *virB11*. En el análisis genotípico de resistencia antimicrobiana, todas las muestras tenían la mutación C257T en el gen *gyrA*, pero la mutación A256G estaba ausente. Tampoco se identificaron mutaciones en 23S rDNA, A2074G y A2075G. De los resultados obtenidos se observa la presencia de la mayoría de los genes de virulencia investigados, con una alta capacidad de resistencia a las fluoroquinolonas. Así, las muestras estudiadas de *Campylobacter* spp. demostró el potencial de causar infección y permanecer en los huéspedes.
**Palabras clave:** Campilobacteriosis; Patogenicidad; Susceptibilidad; Virulencia.

## 1. Introduction

*Campylobacter* spp. It is a pathogen found in many hosts, infecting everything from animals to humans (Oliver et al., 2009). *Campylobacter* thermophilic species are considered to be the main causes of gastroenteritis in humans in developed and developing countries. These species can also cause neurological problems, such as Guillain-Barré syndrome (Rajendran et al., 2012; Rawat et al., 2018). Risk factors associated with campylobacteriosis in humans include: meat consumption (Fredrigo et al., 2016) and raw milk (Del Collo et al., 2017), contaminated with animal feces that are eliminating the agent.

A genetic study conducted in England found that 4.3% of cases of *Campylobacter jejuni* infections in humans occurred from acquisition by sheep sources (Wilson et al., 2008). In sheep herds, campylobacteriosis causes various reproductive problems, such as miscarriages, stillbirth, placentitis, births of weak lambs, and death of the female due to septicemia (Sahin et al., 2008; Hamali et al., 2014).

Efforts have been made to understand the pathogenicity mechanisms of *Campylobacter* spp. Several virulence genes are considered fundamental for the survival of the bacteria in the environment and in the host (Kienesberger et al., 2014). Among these genes, the main ones are: *cadF*, which encodes a protein responsible for bacterial adhesion to host cells (Ziprin et al., 2001; Graham et al., 2008); *ciaB*, *pldA* and *virB11* are essential for cell invasion and colonization (Grant et al., 1997; Bacon et al., 2000; Rivera-Amill et al., 2001); *racR* and *dnaJ*, which are involved in the resilience of *Campylobacter* spp. at different temperatures (Bras et al., 1999); *wlaN* is associated with the development of Guillain-Barré syndrome (Linton et al., 2000); and the *cdtA*, *cdtB* and *cdtC* genes encoding cytotoxins (Lara-Tejero & Galan, 2001).

The presence of multiple virulence genes may increase the ability to cause damage or disease in humans and animals. Therefore, monitoring its frequency and typing is important to assess sources of infection and changes in bacterial populations over time (Melo et al., 2019). In addition, the indiscriminate use of antimicrobials in the treatment and prevention of livestock

diseases has led to the spread of non-drug-susceptible *Campylobacter* strains, resulting in major concerns for world health authorities (Iovine, 2013; Zhang et al., 2016).

In Brazil, in relation to other pathogens, studies on the pathogenicity of *Campylobacter* spp. Although these studies are still scarce and in view of this limited information and the economic impact that campylobacteriosis can cause to herds and public health, this study aimed to identify virulence and antimicrobial resistance genes in *Campylobacter* spp. from sheep in the state of Pernambuco, Brazil.

## 2. Materials and Methods

### Samples

Forty DNA samples from *Campylobacter* spp. Eleven were *Campylobacter jejuni*, twelve *Campylobacter fetus* subsp. *fetus* (Lúcio et al., 2018). and seventeen *Campylobacter coli*. These DNA samples belong to the bank of the Laboratory of Infectious Diseases of the Universidade Federal Rural de Pernambuco and were obtained from fecal samples of sheep from herds of the state of Pernambuco, Brazil.

### Identification of virulence genes

Ten genes responsible for the expression of *Campylobacter* spp. by Polymerase Chain Reaction (PCR). The *primers* and conditions used in virulence gene detection reactions are described in Table 1.

**Table 1.** *Primers* used for virulence gene amplification.

| Gene | ***Primer* Sequence (5´-3´)** | base pairs | Reference |
|---|---|---|---|
| *cdtA* | AACGACAAATGTAAGCACTC<br>TATTTATGCAAGTCGTGCGA | 487 | Asakura et al. (2008) |
| *cdtB* | GGCTTTGCAAAACCAGAAG<br>CAAGAGTTCCTCTTAAACTC | 553 | Asakura et al. (2008) |
| *cdtC* | AAGCATAAGTTTTGCAAACG<br>GTTTGGATTTTCAAATGTTCC | 397 | Asakura et al. (2008) |
| *ciaB* | TCATGCGGTGGCATTAGAATGGG<br>AGGTCTAACTTCATCAACCCTTTGCCA | 658 | Konkel et al. (1999) |
| *dnaJ* | AGGCTTTGGCTCATCACGTCG<br>GGTCGCTTCACCGCGTATGG | 574 | Konkel et al. (1999) |
| *racR* | TGGGGCTTCAAATCGGTGCTGA<br>GCGACCGATGATAACATCAAGGCT | 326 | Hamidian et al. (2011) |
| *pldA* | AAGCTTATGCGTTTTT<br>TATAAGGCTTTCTCCA | 913 | Datta et al. (2003) |
| *cadF* | TTGAAGGTAATTTAGATATG<br>CTAATACCTAAAGTTGAAAC | 489 | Konkel et al. (1999) |
| *wlaN* | TTAAGAGCAAGATATGAAGGTG<br>CCATTTGAATTGATATTTTTG | 750 | Linton et al. (2000) |
| *virB11* | GAACAGGAAGTGGAAAAACTAGC<br>TTCCGCATTGGGCTATATG | 708 | Bacon et al. (2000) |

Source: Elaborated by the authors.

Positive reaction controls for the *cdtA*, *cdtB*, *cdtC* and *pldA* genes were provided by the Instituto Oswaldo Cruz (CCAMP) and the negative control was ultrapure water. Amplified products were identified by Blue Green-stained (LGCbio®) agarose gel electrophoresis (1.5%), visualized under UV light and documented by an image capture (Loccus biotecnologia, L. PIX, Cotia, Brazil).

**Genetic analysis of antimicrobial resistance**

Genotypic analysis of antimicrobial resistance was performed by 23S rDNA sequencing to identify A2074G and A2075G mutations and *gyrA* gene fragments to identify C257T and A256G mutations (Korczak et al., 2009; Vacher et al., 2013).

Samples were purified after amplification and bidirectionally sequenced using the *BigDyeTerminator v3.1 CycleSequencing* Kit (AppliedBiosystems, USA) according to the manufacturer's instructions. Sequencing was performed by capillary electrophoretic separation in an *ABI 3500 GeneticAnalizer* sequencer (AppliedBiosystems). Data were collected using *Data Collection software* (AppliedBiosystems) and passed a quality inspection through *Sequencing Analysis Software* (AppliedBiosystems). The sequences were edited using Chromas 2.6.6 and analyzed in ClustalX 2.1.

The bibliographic research carried out was narrative (Cordeiro et al., 2007; Monteiro et al.; 2017) with research of articles in national and international journals from 1990 to 2021.

## 3. Results

The virulence genes *cdtA*, *cdtB* and *cdtC* showed high frequency among isolates, especially *cdtA* in all *Campylobacter* species (100% in *C. jejuni*; 91.7% in *C. fetus* subsp. *fetus* and 88.2% in *C. coli*). The simultaneous presence of *cdtA*, *cdtB* and *cdtC* genes was detected in all *C. jejuni* samples, in three *C. fetus* subsp. *fetus* samples and 8 *C. coli*. The *racR* and *dnaJ* genes were also very frequent. *CiaB* had reasonable frequency in all species.

Although *cadF* had a high frequency in *C. jejuni* and *C. coli* (100.0% and 94.1%, respectively), their presence was not as expressive in *C. fetus subsp. fetus* (8.3%). The *pldA* gene was detected only in a *C. jejuni* isolate. None of the isolates had *virB11* and *wlaN* genes. In general, *C. jejuni* was the species with the highest virulence potential, while *C. coli* had the lowest potential. The detailed frequency of virulence genes can be seen in Tables 2 and 3.

**Table 2.** Virulence factors present in *Campylobacter* spp. in sheep from Pernambuco State, Brazil.

| | ***cdtA*** | ***cdtB*** | ***cdtC*** | ***racR*** | ***dnaJ*** | ***ciaB*** | ***cadF*** | ***pldA*** |
|---|---|---|---|---|---|---|---|---|
| *C. jejuni* | 11/11 (100%) | 11/11 (100%) | 11/11 (100%) | 11/11 (100%) | 09/11 (81.8%) | 3/11 (27.2%) | 11/11 (100%) | 01/11 (9.1%) |
| *C. fetus* subsp. *fetus* | 11/12 (91.7%) | 5/12 (41.7%) | 9/12 (75%) | 9/12 (75%) | 7/12 (58.3%) | 2/12 (16.7%) | 1/12 (8.3%) | 0/12 |
| *C. coli* | 15/17 (88.2%) | 14/17 (82.3%) | 8/17 (47.0%) | 12/17 (70.6%) | 3/17 (17.6%) | 3/17 (17.6%) | 16/17 (94.1%) | 0/17 |

Source: Elaborated by the authors.

**Table 3.** General frequency of virulence genes in *Campylobacter* spp. according to its main role.

| **Role** | **Frequency** |
|---|---|
| Adhesion (*cadF*) | 28/40 (70,0%) |
| Colonization (*racR*) | 32/40 (80,0%) |
| Colonization (*dnaJ*) | 19/40 (47,5%) |
| Invasion (*ciaB*) | 8/40 (20,0%) |
| Invasion (*pldA*) | 1/40 (2,5%) |
| Invasion (*virB11*) | - |
| Toxin production (*wlaN*) | - |
| Toxin production (*cdtA*) | 37/40 (92,5%) |
| Toxin production (*cdtB*) | 30/40 (75,5%) |
| Toxin production (*cdtC*) | 28/40 (70,0%) |

Source: Elaborated by the authors.

In the antimicrobial resistance genotypic analysis, all isolates had the C257T mutation in the *gyrA* gene, but the A256G mutation was absent. The 23S rDNA, A2074G and A2075G mutations were also not identified in the isolates.

## 4. Discussion

This is the first study in Brazil on virulence factors and genetic potential of antimicrobial resistance in *Campylobacter* spp. from sheep. Studies in other countries have shown the high frequency of virulence genes in *Campylobacter* spp. (Khoshbakht et al., 2014; Giannatale et al., 2014; Siddiqui et al., 2015), a result that was also found for most of the genes tested. The performance of virulence factors has been recognized as the main pathogenic activity of bacteria (Ketley, 1997). The performance of these factors has been clarified, occurring in isolation or in association, causing damage to host cells and triggering the appearance of clinical signs (Wysok & Wojtacka, 2018).

All *C. jejuni* isolates of the present study demonstrated the presence of *cdt* genes suggesting the active toxin production capacity. Distensive cytoletal toxin (CDT) production is regulated by the *cdtA*, *cdtB* and *cdtC* genes (Fouts et al., 2007). The simultaneous presence of the three genes is necessary for CDT toxicity (Lara-Tejero et al., 2001; Lindmark et al., 2009). The cdtA and cdtC subunits bind to the receptor on the surface of the host cell and cdtB is actively transported to the nucleus, where it breaks the double strand of DNA and blocks the cell cycle in the G2 / M phase, leading to cellular apoptosis (Whitehouse et al., 1998).

In Brazil, thirteen samples of *Campylobacter* spp. of chicken carcasses from free markets and hypermarkets, and of these, four (30.7%) presented the three *cdt* genes (Carvalho et al., 2010). *Campylobacter* was later isolated in samples of carcasses, feces and mesenteric lymph nodes from pigs slaughtered in refrigerators. Of 31 positive samples, *cdt* genes were detected in 28 (64.5%) of them (Silva et al., 2012).

The *racR* gene, present in 80% of the tested samples, composes the RacR-RacS system, which measures adaptive responses related to heat stress, representing significant importance in bacterial resistance and colonization. This system may be necessary during the diffusion of intestinal bacteria into the environment and vice versa (Bras et al., 1999). Another *Campylobacter* spp. thermotolerance protein is dnaJ, it has been identified that mutant colonies in their coding region have the delayed ability to form colonies when exposed to the highest temperature. The *dnaJ* mutant was still unable to colonize newborn Leghorn chickens, proving the in vivo role of heat shock proteins (Konkel et al., 1998).

In humans, *Campylobacter's* cellular invasion capability has been demonstrated as an important step in the pathogenic mechanism (Rivera-Amill et al., 2001). However, this bacterial invasion ability is related to the strain involved (Van Vliet, Ketley 2001). Cultivation of *C. jejuni* associated with INT-407 cells leads to the production of a group of invasive proteins, especially ciaB. When *C. jejuni* has a mutation in the *ciaB* gene, although adherence to intestinal mucosa cells exists, the bacterium is not internalized and does not secrete any of the other invasive proteins (Tay et al., 1996).

In the present study, only one *C. jejuni* isolate presented the *pldA* gene. This gene was discovered in an operon that encodes the *Campylobacter coli* enterocelin transport system. Suggesting its participation in the expression of a *Campylobacter* outer membrane phospholipase A, which is associated with hemolytic activity. Since hemolysins are closely related to the causative potential of the disease, the *pldA* gene has since been considered important in the virulence of *Campylobacter* spp. (Grant et al., 1997).

Knowing the significance of *ciaB* and *pldA* genes in colonization of intestinal cells, four mutant strains were used for vaccination in chicks to reduce *Campylobacter* colonization. It was hypothesized that any of these *C. jejuni* mutant strains could transiently colonize the chick caecum, leading to an immune response that would protect them from subsequent challenge. However, inoculation of these strains did not induce biologically significant resistance against subsequent challenge with the parental strain (Ziprin et al., 2001).

*Campylobacter* adhesion in epithelial cells is mediated by multiple adhesins, CadF being considered the main one, highly conserved in *C. jejuni* and *C. coli*, it is a 37kDa membrane protein that binds to fibronectin (Konkel et al., 1997). This result corroborates that found in the present study, since the *cadF* gene was present in all *C. jejuni* isolates, and in high frequency in *C. coli*. However, only one isolate of *C. fetus* subsp. *fetus* presented the *cadF* gene, meaning that in this species other proteins such as PEB1 and JpldA may have significance in cell adhesion.

Research from sheep and cattle feces samples in Iran has confirmed the presence of the *cadF* gene in all samples tested (40/40). The authors suggested the potential capacity of *C. jejuni and C. coli* of sheep and cattle origin to cause infection in humans (Khoshbakht et al., 2014). Other studies have also shown the significant presence of *cadF* in *Campylobacter* spp. from various animal species (Bang et al., 2003).

The type IV secretion system is associated with the cell membrane and may be for *Campylobacter* a tool in gene transfer and virulence factor secretion (Bacon et al., 2000; Kienesberger et al., 2014). The *virB11* gene, a component of the type IV secretion system, has been found at low frequencies (Bang et al., 2003; Wieczorek, Osek, 2008) or even absent in *Campylobacter* spp. (Ghorbanalizadgan et al., 2014), as also identified in this study.

The genetic marker *wlaN* was related to the expression of mimetic gangliosides involved in Guillain-Barré Syndrome, an autoimmune human disease that causes acute paralytic neuropathy (Linton et al., 2000). According to the so-called "molecular mimicry" hypothesis, antibodies generated against *Campylobacter* LOS cross-react with gangliosides found in nervous tissue (Yuki, 1997). Given this relevance, the *wlaN* gene was researched, but was not detected in any sample, suggesting no relationship with the Guillain-Barré Syndrome in these strains analyzed.

Results regarding antimicrobial resistance are in line with previous studies elsewhere (Keller & Perreten, 2006; Luangtongkum et al., 2009). The most frequently found C257T mutation in the *gyrA* gene was present in all isolates tested. This single mutation in the gene confers a high resistance to antimicrobials: nalidixic acid and fluoroquinolones (Gootz & Martin, 1991; Wieczorek, Osek, 2013).

Associated with multidrug efflux pumps (CmeABC), point V mutations in the 23S rRNA gene at positions 2074 or 2075 confer resistance to macrolides (Iovine, 2013; Wieczorek, Osek 2013). These mutations were not found in the isolates, thus demonstrating that genotypic resistance to macrolides does not occur in the analyzed samples, unlike that observed with fluoroquinolones.

A study conducted in Switzerland with 329 strains of *C. jejuni* and *C. coli* identified 35% of *C. coli* strains with quinolone-only mutations, 15% with macrolide-only mutations, and 6% with mutations that conferred resistance to both antimicrobial classes. While the strains of *C. jejuni* did not show macrolide resistance mutations, although they showed 31% of mutations responsible for quinolone resistance (Korczak et al., 2009).

In the USA, 320 strains of *C. jejuni* and 115 strains of *C. coli* obtained from feedlot cattle were analyzed. The results indicated that fluoroquinolone resistance reached 35.4% in *C. jejuni* and 74.4% in *C. coli*. Although all fluoroquinolone-resistant *C. coli* isolates harbored a single *gyrA* mutation, *C. jejuni* isolates had other mutations in the gene (Tang et al., 2017).

The resistance of *Campylobacter* spp. macrolides and fluoroquinolones, the antimicrobials of choice for fighting infection, have significantly increased in humans and animals worldwide (Kaakoush et al., 2015). This reality, regarding the availability of therapies for infections, is of concern, as this infection with antibiotic resistant strains has been associated with longer disease duration, increased risk of invasive disease, death and increased hospital expense costs (Helms et al., 2005). Since most humans are infected with *Campylobacter* from animals, increased resistance in these sources directly impacts the human population (Adak et al., 2005).

## 5. Conclusions

From the results obtained with this study, first performed with sheep samples in Brazil, we can observe the presence of most virulence genes researched, with high resistance to fluoroquinolones. Thus, the isolates studied showed the potential to cause infection and remain in the host tissues, which may cause economic losses for sheep and also warn the public health risk.

More studies are needed for the characterization of *Campylobacter*, in order to have a better understanding for the implementation of measures to control the propagation of these strains.

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