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Evolution of *Campylobacter jejuni* of poultry origin in Brazil

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ABSTRACT

Campylobacter jejuni is the most common pathogen associated with foodborne diseases. Persistent presence of this pathogen contaminating the environment in slaughterhouses and chicken products have been reported worldwide. Although many efforts have been employed for reducing *C. jejuni* contamination, few studies have been conducted to understand the dynamics of *C. jejuni* in slaughterhouses over time. In this study, we evaluated the virulence, antibiotic resistance and genetic diversity profiles of 99 *C. jejuni* isolated from chilled chicken carcasses collected in Brazilian slaughterhouses during two distinct periods (2011–2012 and 2015–2016). The virulence profile was evaluated for the presence of *flaA*, *ciaB*, *cadF*, *pldA* and *cdtABC* genes. Antibiotic resistance was evaluated for amoxicillin-clavulanic acid, gentamicin, erythromycin and tetracycline. Genetic diversity was assessed using RAPD-PCR. The prevalence of *C. jejuni* was significantly reduced in 2015–2016 as well the number of antibiotic (and multidrug) resistant isolates, except for tetracycline. However, isolates from 2015 to 2016 showed higher prevalence of multiple virulence genes and genetic diversity profile compared to isolates from 2011 to 2012. During the studied period, stricter regulations to control pathogens in poultry farms and slaughterhouses were implemented in Brazil, which may have contributed to the profile variation observed due to changes of selective pressures on bacterial populations.

1. Introduction

Foodborne diseases are a growing public health concern worldwide. Infections caused by *Campylobacter jejuni* have been a major global problem, leading the ranking of foodborne diseases in Europe (European Union, EFSA and European Centre for Disease Prevention and Control, 2012). Although *C. jejuni* infections may be self-limiting for most patients, in rare cases the illness evolve to severe symptoms or post-infectious complications such as Guillain-Barré, Miller-Fisher syndromes or even death. It is estimated that 37.600 deaths per year globally are caused by *Campylobacter* infections (WHO, 2015).

Risk factors associated with campylobacteriosis in humans are handling, preparation and consumption of raw, undercook or untreated poultry meat (Silva et al., 2011). Chicken has been identified as a reservoir of *Campylobacter* and related to 50–80% of human cases (EFSA, 2010). About 20–30% of campylobacteriosis cases in the European Union may be attributed to poultry meat consumption (EFSA, 2010). The pathogen has been recovered from chicken carcasses, poultry meat parts and equipments in processing plants worldwide (Hansson et al., 2005; Peyrat et al., 2008; García-Sánchez et al., 2017). Chicken carcass contamination during visceral rupture and inappropriate cleaning and disinfection of equipments have been identified as the major problems for *Campylobacter* contamination in the slaughterhouse (Hansson et al., 2005; Peyrat et al., 2008). In addition to the presence of *C. jejuni* in slaughterhouses, which poses a serious risk of infection for workers and consumers, the issue is aggravated by biological aspects of the bacterium. Although observed as a microaerobic and fastidious organism during *in vitro* cultivation, *C. jejuni* shows mechanisms allowing its survival in harsh environmental conditions, such as in acid and oxidative stress (Gomes et al., 2018) and in modified atmosphere packaging (Meredith et al., 2014), suggesting that the organism may be able to adapt to changes and persist in unfavorable environments. Some of the molecular mechanisms involved in *C. jejuni* adaptation are related to the selection of virulence and antibiotic resistance genes and genetic variability.

The increasing number of reports regarding *C. jejuni* antibiotic resistance and virulence are moving forward the efforts to understand the role and prevalence of several key factors associated with such

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mechanisms. A set of virulence genes has been established as fundamental players for *C. jejuni* survival in the environment and within-host. For instance, gene *ciaB* (*Campylobacter* invasion protein B) is involved in protein secretion and is essential for cell invasion and colonization (Cróinín and Steffen, 2012; Eucker and Konkel, 2012), cluster *cdtABC* (cytolethal distending toxins A, B and C) encodes cytotoxins that disrupt the mucosal barrier resulting in host cell death (Pickett and Whitehouse, 1999; Dasti et al., 2010; Ripabelli et al., 2010; Martinez et al., 2006), *cadF* (*Campylobacter* adhesin to fibronecting F) encodes a protein involved in internal binding to the gut epithelial cells (Ziprin et al., 1999), *flaA* (flagellin A) is responsible for cellular motility (Poly et al., 2007), and *pldA* (outer membrane phospholipase A) is involved in adhesion and colonization (Pickett and Whitehouse, 1999). The presence of multiple virulence genes could enhance *C. jejuni* capacity to cause damage or disease in humans. Therefore, monitoring *C. jejuni* prevalence and population typing in poultry farms, slaughterhouses and retail stores is important to evaluate the sources of infection and changes in bacterial populations over time to come up with new strategies for reducing the risks of food contamination.

Brazil is the second largest chicken producer (13 056 000 tonnes in 2017) in the world and the leading exporting country (4 320 000 tonnes) of chicken products (ABPA, 2018). The Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) has applied regulations for pathogen control in poultry farms and processing plants. In addition, the Brazilian industry is under the European Union regulations for exported products. However, despite the important position of the Brazilian chicken industry for the global trade market, cases of campylobacteriosis are underreported and molecular studies of *C. jejuni* diversity and virulence are still scarce in the country. Therefore, we assessed the prevalence of *C. jejuni* in chilled chicken carcasses processed by the largest Brazilian exporting company during two distinct periods 3 years apart (2011–2012 and 2015–2016). Isolates were evaluated for their antimicrobial resistance, virulence and genetic diversity profiles. We found significant changes in *C. jejuni* profiles between the two periods, showing reduction of pathogen prevalence but enhanced virulence pattern.

2. Material and methods

2.1. Study area and sample collection

A total of 1070 chicken carcasses were collected in poultry slaughterhouses (which were also meat processing plants), belonging to the largest Brazilian poultry meat company that supplies meat products to the national and the international markets. Chicken carcasses were sampled in two distinct periods, from August 2011 to February 2012 (420 carcasses) and from September 2015 to February 2016 (650 carcasses). All chilled broiler chicken carcasses were randomly collected after packaging.

2.2. Isolation of Campylobacter spp.

Campylobacter spp. isolation followed the standard procedure (ISO 10272-1:2006), with modifications. Packs were asseptically opened and each chicken carcass was transferred to a sterile stomacher bag. Chicken carcasses were rinsed with 400 mL of 0.1% sterile peptone water (Difco, New Jersey, United States). During rinsing, carcasses were gently rubbed in particular body sites, such as the neck, wings and thighs. Thirty millilitres of the rinsate was added to 30 mL of Bolton broth in double concentration (CM0983, Oxoid, Hampshire, UK) supplemented with an antibiotic mix (SR0183E, Oxoid) and with 5% horse blood (Laborclin, Paraná, Brazil). Bolton broth tubes were incubated in a microaerobic atmosphere (5–15% O_2 and 10% CO_2) using a Microaerobac (Probac do Brasil, Sao Paulo, Brazil) at 37 °C for 44 h ± 4 h. After Bolton broth incubation, a membrane filtration method was used for plating the samples in *Campylobacter* Blood Free Selective Agar (Modified CCDA-Preston) (CM0739, Oxoid) plates suplemented with antibiotic (SR0155E, Oxoid). Briefly, a 0.65 µm pore size cellulose membrane filter (Millipore, Massachusetts, United States) was placed on top of the medium and 300 µL of each enrichment in Bolton broth was added to the plate. After approximately 15 min, the membrane was dry and it was removed from the agar plate. Modified CCDA-Preston plates were incubated at 37 °C for 44 h ± 4 h in a microaerobic atmosphere as described above.

2.3. Molecular identification of Campylobacter jejuni

Two typical *Campylobacter* spp. colonies were randomly selected from each modified CDDA-Preston plate for further analysis. Selected colonies were suspended in Bolton broth (Oxoid) and grown overnight. Total DNA was extracted using a commercial kit (Wizard Genomic DNA Purification kit, Promega, Madison, Wisconsin, United States) according to manufacturer's instructions. Identification of *C. jejuni* was performed using a previously described multiplex PCR protocol (Harmon et al., 1997), which distinguishes *C. jejuni* from *C. coli*. Briefly, PCR reactions were prepared using GoTaq Green Master Mix kit (Promega), 2 µL of template DNA, 20 pmol of primer set C1 and C4 (Invitrogen, California, United States) and 40 pmol of primer set pg3 and pg50 (Invitrogen) (Table 1). Gel electrophoresis and staining were also performed as previously described (Harmon et al., 1997). DNA of *C. jejuni* ATCC 33291, *C. jejuni* NCTC 11351 and *C. jejuni* IAL 2383 were used as positive controls and a blank tube (no DNA template, but water) was used as a negative control.

2.4. Molecular identification of virulence genes

A set of seven well-known virulence genes of *C. jejuni* were screened using PCR (Table 2). PCR reactions were performed using GoTaq Green Master Mix kit (Promega) as instructed by manufacturer. Amplification protocols were conducted as originally described (Table 2). Positive and negative controls were the same as described in section 2.3.

2.5. Antibiotic susceptibility testing

Campylobacter resistance to antibiotics was assessed determining the minimal inhibitory concentration (MIC) values against four antibiotics routinely used to treat infections in humans and in veterinary medicine. Samples were suspended in 0.9% NaCl to obtain 5.10^5 UFC/mL (0.5 McFarland Standard). Twenty microliters of the bacterial suspension were transferred into 180 µL cation-adjusted (20–25 mg Ca_2+/L, 10–12.5 mg Mg_2+/L) Muller Hinton broth (Oxoid) with 5% lysed sheep blood (Laborclin), according to ISO 20776-1 (EUCAST, 2018). The suspension was mixed and transferred to wells, which contained different concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 $mg.L^{-1}$) of a selected antibiotic (amoxicillin–clavulanic acid, erythromycin, gentamicin and tetracycline). The mutiwell plate was sealed and incubated at 41 ± 1 °C for 40–48 h in microaerobic conditions as described above. Breakpoints for *C. jejuni* were considered for erythromycin ($> 4\ mg.L^{-1}$) and tetracycline ($> 2\ mg.L^{-1}$), whereas breakpoints for *Enterobacteriales* were considered for amoxicillin–clavulanic acid ($> 8\ mg.L^{-1}$) and gentamicin ($> 4\ mg.L^{-1}$), according to the EUCAST (2018) guidelines. In addition, a 10 µL of each

Table 1
Primers used in the identification of *Campylobacter jejuni*.

Primer Name	Sequence 5′-3′	Molecular Weight (bp)	Reference
pg 3	GAACTTGAACCGATTTG	460 (*C. jejuni* and *C. coli*) 160 (*C. jejuni*)	Harmon et al. (1997)
pg 50	ATGGGATTTCGTATTAAC		
C1	CAAATAAAGTTAGAGGTAGAATGT		
C4	GGATAAGCACTAGCTAGCTGAT		

Table 2
Primers for identification of virulence genes in *Campylobacter jejuni*.

Genes	Primers	Sequence 5′-3′	Molecular Weight (pb)	Volume/DNA/Annealing	Reference
flaA	flaA-F	ATGGGATTTCGTATTAACAC	1728	50uL/20ng/45 °C/1min	Hanel et al. (2004)
	flaA-R	CTGTAGTAATCTTAAAACATTTTG			
pldA	pldA-361	AAGAGTGAGGCGAAATTCCA	385	50uL/20ng/45 °C/1 min	Zheng et al. (2006)
	pldA-726	GCAAGATGGCAGGATTATCA			
cadF	cadFI-F2B	TTGAAGGTAATTTAGATATG	400		
	cadFI-R1B	CTAATACCTAAAGTTGAAAC			
ciaB	ciaBI-652	TGCGAGATTTTTCGAGAATG	527		
	ciaBI-1159	TGCCCGCCTTAGAACTTACA			
cdtA	cdtA-F	CTATTACTCCTATTACCCCACC	420	25uL/80ng/57 °C/1 min	Martinez et al. (2006)
	cdtA-R	AATTTGAACCGCTGTATTGCTC			
cdtB	cdtB-F	AGGAACTTTACCAAGAACAGCC	531		
	cdtB-R	GGTGGAGTATAGGTTTGTTGTC			
cdtC	cdtC-F	ACTCCTACTGGAGATTTGAAAG	339		
	cdtC-R	CACAGCTGAAGTTGTTGTTGGC			

diluted inoculum was plated in a modified CCDA-Preston agar (Oxoid) to check the bacterial growth of the respective dilution well. *C. jejuni* IAL 2383 and *C. jejuni* NCTC 11351 were used as positive controls, whereas a blank sample was used as a negative control.

2.6. Determination of genetic diversity

Random Amplified Polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity among isolates. Two 10-mer primers, HLWL74 (5′-ACGTATCTGC-3′) (Mazurier et al., 1992) and 1290 (5′-GTGGATGCGA-3′) (Akopyanz et al., 1992), were used for PCR amplification in a Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Harmburg, Germany). PCR mixture (20 µL total volume) was prepared with 10 ng of template DNA; 20 mM of Tris-HCL; 50 mM of KCl; 2 mM of $MgCl_2$; 200 µM of each triphosphate deoxynucleotide (dNTP); 30 picomols of primer (Invitrogen) and 1 U of Taq DNA polymerase (Invitrogen). PCR amplification and electrophoresis were performed as previously described (Akopyanz et al., 1992; Mazurier et al., 1992). DNA fingerprint gel images were analysed using GelCompare II software (Comparative Analysis of Electrophoresis Patterns) version 1.5 (Applied Maths, Korthrijk, Belgium). A similarity matrix was obtained by comparing pairs of strains using the Dice similarity coefficient considering 1% of tolerance for each primer. A dendrogram was built using UPGMA (unweighted pair group method with arithmetic mean) based on the average of experiments for the two primers.

2.7. Statistical analysis

The two independent datasets (2011–2012 and 2015–2016) were compared by the binomial test of proportions using GraphPad Prism 5.0 (GraphPad Software, California, United States). Results were considered statistically significant when $P < 0.05$.

3. Results and discussion

3.1. Prevalence of Campylobacter jejuni was significantly reduced in 2015–2016

Reducing chicken carcass contamination by *Campylobacter* spp. at the slaughterhouse has been identified as a mean of reducing human infection (Rosenquist et al., 2003). In our study, we collected chilled chicken carcasses after packaging in two distinct periods of time from slaughterhouses of the same Brazilian company to evaluate pathogen contamination between years. Only 9.25% (99/1070) of chicken carcasses were found positive to *C. jejuni* considering all samples collected in this study. The prevalence of carcass contamination is highly variable among countries, for instance, ranging from 11.3% in Brazil (Carvalho et al., 2013) to 87.5% in France (Hue et al., 2010). Differences in prevalence may be related to poultry management, meat processing conditions and control programs as well as to methodology differences among studies, which may jeopardize a comprehensive comparative analysis.

Sample collections were carried out pratically over the same months during the two periods (August to February), showing similar sampling

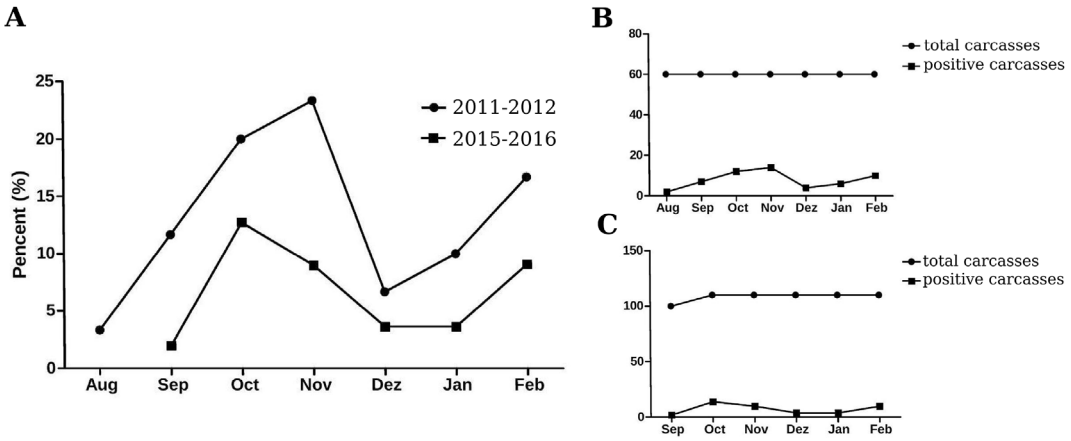

Fig. 1. Sample collection patterns and *C. jejuni*-positivity during the two sampling times. A) Curves show the percentage of *C. jejuni*-positive carcasses in 2011–2012 and 2015–2016. B) Number of carcasses collected during 2011–2012 and number of *C. jejuni*-positive carcasses during the same period. C) Number of carcasses collected during 2015–2016 and number of *C. jejuni*-positive carcasses during the same period.

patterns over time as well as positivity (Fig. 1). Therefore, no sampling bias was introduced in the study. An increasing prevalence is observed for both periods from September onwards, which marks the beginning of spring in South America, and corroborates with previously observed seasonal trends in *Campylobacter* prevalences (Hofshagen and Kruse, 2005; Hansson et al., 2007). However, the sample positivity was remarkably decreased by December in both periods. This month marks the raining season and therefore, poultry farms integrated to the studied Brazilian company treat the litters with 500–900 g/m of calcium oxide (CaO) to reduce humidity. Previous works have shown that alkalinization of poultry litter and manure soil using calcium-based products affects the viability of pathogens, such as *Salmonella* Enteritidis (Bennett et al., 2003) and *Salmonella* Typhimurium (Nyberg et al., 2011). Raised poultry litter pH may have impacted the *C. jejuni* viability.

During the collection time comprised between 2011 and 2012, 13.1% (55/420) of the chilled chicken carcasses were positive for *C. jejuni*. In contrast, only 6.8% (44/650) of chilled chicken carcasses were found positive in the period comprised between 2015 and 2016, showing a significant reduction ($p = 0.0007$) compared to the previous sampling period. The prevalence reduction observed for the second period (2015–2016) may be related to the introduction of protective measures at the farm level and more rigorous control measures at the slaughterhouse due to the National Pathogen Control Program (PNCP) implemented by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) by the Ordinance SDA number 17/2013 (BRASIL, 2013). During the past the decade (2000–2010), an increasing prevalence of typhoid and non-typhoid *Salmonella* spp. in chicken carcasses was observed in Brazil (Voz-Rech et al., 2015; Cardoso et al., 2015). Therefore, in 2013 a Scientific Comission was established for a PNCP aiming to study the prevalence of *Salmonella* spp. in poultry farms and slaughterhouses and to establish official measures for each critical control point (BRASIL, 2013). In 2016, a regulatory document (Instrução Normativa n. 20/2016) officially implemented the control program (BRASIL, 2016). All controls established in the slaughterhouses due the official *Salmonella* control program may have contributed to the decreasing prevalence of *C. jejuni* in chicken carcasses. In addition to that, as a big exporting company, the preparation to the early announced European Union (EU) regulation 2017/1495 (Comission Regulation, 2017) regarding *Campylobacter* presence and countings in chicken carcasses exported to the EU may have accounted for the observed changes.

3.2. Increased pathogenic potential of Campylobacter jejuni isolates from 2015 to 2016

Bacterial virulence is the ability to cause infection and/or colonization in a host, which is conferred by genetic factors determining bacterial phenotypes. Therefore, the pathogenity of *C. jejuni* depends on which virulence factors an isolate carries in its genome. We molecularly screened all 99 *C. jejuni* isolated from chicken carcasses for seven well-known virulence-associated genes (*flaA*, *pldA*, *cadF* and *ciaB, cdtABC*). Among 55 isolates from 2011 to 2012, 46 (83.6%) carried at least one virulence gene. The prevalence was higher amongst the 44 isolates from 2015 to 2016 of which 43 (97.7%) carried at least one virulence gene. The prevalence for all tested genes, but *flaA*, was significantly higher ($p < 0.05$) for isolates from 2015 to 2016 (Table 3). Virulence may be strengthened by the expression of a particular gene. However, the product of a single gene may be not always enough to establish infection or colonization, which could be the result of a coordinated expression of distinct virulence genes. Therefore, we searched for virulence gene profiles amongst *C. jejuni* isolates (Table 4). We found out a total of 12 distinct virulence gene profiles (P1 to P12) for isolates from the two periods. Surprisingly, isolates from 2015 to 2016 showed a less distributed profile range (P1, P5, P8 and P12) compared to 2011–2012 (P1-P4, P6-P7, P9-P12). Most isolates from both periods carried out all tested virulence genes (profile P1), showing higher prevalence ($p = 0.00004$) for 2015–2016 (84.1% of isolates) compared to 2011–2012 (45.5% of isolates). In contrast, profile 12 (absence of all virulence genes) showed lower prevalence ($p < 0.05$) for 2015–2016 (2.3%) compared to 2011–2012 (16.4%). Therefore, despite the reduced prevalence of *C. jejuni* in 2015–2016, it was observed an increased virulence potential of isolates from this sample collection period.

Table 3
Percentage of virulence genes in *Campylobacter jejuni* isolated from chicken carcasses in two different periods in Brazil.

Virulence Genes	2011-2012	2015-2016
	(N = 55) n(%)	N = 44 n(%)
flaA	41 (74.5)[a]	37 (84.1)[a]
pldA	35 (63.6)[a]	43 (97.7)[b]
cadF	37 (67.3)[a]	43 (97.7)[b]
ciaB	37 (67.3)[a]	42 (95.5)[b]
cdtABC	36 (65.5)[a]	43 (97.7)[b]

N - number of isolates; n(%) - number and percentage of isolates that have a virulence gene; Superscript letters [(a, b)] - distinct letters in a row indicate that numbers are significantly different ($p < 0.05$).

Table 4
Virulence profiles of strains of *Campylobacter jejuni* isolated from chicken carcasses in different periods in Brazil.

Virulence Profiles	2011-2012	2015-2016
	N = 55 n(%)	N = 44 n(%)
P1: *flaA, pldA, cadF, ciaB, cdtABC*	25 (45.5)[a]	37 (84.1)[b]
P2: *flaA, pldA, cadF, ciaB*	5 (9.1)	0 (0)
P3: *flaA, cadF, ciaB, cdtABC*	2 (3.6)	0 (0)
P4: *flaA, pldA, ciaB, cdtABC*	1 (1.8)	0 (0)
P5: *pldA, cadF, ciaB, cdtABC*	0 (0)	5 (11.4)
P6: *pldA, cadF, ciaB*	3 (5.5)	0 (0)
P7:*flaA, cadF, cdtABC*	1 (1.8)	0 (0)
P8: *pldA, cadF, cdtABC*	0 (0)	1 (2.3)
P9: *flaA, cdtABC*	7 (12.7)	0 (0)
P10: *cadF, ciaB*	1 (1.8)	0 (0)
P11: *pldA*	1 (1.8)	0 (0)
P12: absence of all genes	9 (16.4)[a]	1 (2.3)[b]

Legend: N - total number of strains; n,% - number and percentage of strains that have the virulence gene; Superscript letters [(a, b)] - distinct letters in a row indicate that numbers are significantly different ($p < 0.05$).

Campylobacter populations are subject to selective and non-selective bottlenecks resulting from, for instance, stress conditions or changes in their niches. Selective bottlenecks may influence a particular genomic loci, preserving more competitive strains (Aidley et al., 2017). The introduction of a stricter pathogen control management, according to the PNCP, may have contributed to a reduction in *C. jejuni* numbers in chicken carcasses as well to a selection of the observed virulence traits. The dominant virulence profile includes adherence (*pldA*), colonization (*pldA* and *cadF*), motility (*flaA*), invasion (*ciaB*), and toxin (*cdtABC*) genes, which are necessary for a rapid adaptation to and survival in the environment and have been found conserved in the genus *Campylobacter* (Fouts et al., 2005; Iraola et al., 2014). These genes play central roles for *C. jejuni* survival in and/or outside the host and their absence would lead to disadvantageous phenotypic behaviours (de Vries et al., 2015; Konkel et al., 1999; Ziprin et al., 1999; Grant et al., 1997; Jain et al., 2008). For instance, *flaA* (Flagellin A) mutants have showed non-motile phenotypes or severely attenuated motility *in vitro* (de Vries et al., 2015); *ciaB* (*Campylobacter* invasive antigen B) mutants have showed a significant reduction in internalization and deficient secretion process *in vitro* (Konkel et al., 1999); *cadF* (*Campylobacter* adhesin to fibronecting) mutants were showed to be incapable to colonize the

chicken cecum (Ziprin et al., 1999); *pdlA* (Outer membrane phospholipase A) mutants have showed reduced hemolytic activity compared to the wild-type strain in *Campylobacter coli* (Grant et al., 1997); and *cdtABC* (Cytolethal distending toxin A, B, C) mutants have showed impared ability to adhere and to invade mouse epithelial cells compared to the wild type *C. jejuni* (Jain et al., 2008). These virulent phenotypes are also associated with enterocolitis. Therefore, long-term assessments of *C. jejuni* virulence markers is essential to identify potential sources for human infection and to aid with fast and proper prevention and control strategies.

3.3. Antibiotic-resistant isolates were less prevalent in 2015–2016

Bacterial antibiotic resistance is a major concern in public health. Over the past years, several studies have reported the development of antibiotic-resistance *C. jejuni* strains (Tang et al., 2017; Szczepanska et al., 2017; Pollett et al., 2012), which can be influenced by drug usage in animal production and human medicine. Antibiotic resistant *C. jejuni* and *C. coli* isolates have been identified in contaminated carcasses, equipments and in particular areas of the slaughterhouses (Torralbo et al., 2015). We tested all 99 *C. jejuni* isolates detected in chicken carcasses against four commonly used antimicrobial agents. A total of 77 (77.8%), 55 (55.6%), 25 (25.3%) and nine (9.1%) isolates were resistant to tetracyclin, amoxicillin-clavulanic acid, erythromycin and gentamicin, respectively. Similar prevalences (up to 70%) for macrolid, tetracycline and amoxicillin-clavulanic acid resistance have been previously described (EFSA, 2015; Nguyen et al., 2016). Interestingly, the number of isolates resistant to amoxicillin-clavulanic acid, erythromycin and gentamicin was significantly reduced ($p < 0.05$) in 2015–2016 (Table 5). We also checked for multidrug resistance (MDR) profile (Table 6). Twenty isolates (20.2%) showed resistance to three or more antimicrobial agents. Among them, a significant small number of isolates from 2015 to 2016 showed MDR profiles ($p = 0.0047$) when compared to isolates from 2011 to 2012. Reduced prevalences for some antibiotics observed in this study might be a result of recent policies established in Brazil and of a greater control of antibiotic usage in poultry farms.

A regulatory document (BRASIL, 2012) banning the use of macrolids in poultry farms was published by MAPA in 2012. The reduced number of erythromycin resistant isolates is likely a result of a decreasing exposure to antibiotics (and therefore, to selective pressures on respective antibiotic resistant genes) after law enforcement. Similarly, the reduced number of MDR isolates is likely a result of multi-sectoral efforts, such as the Brazilian requirements (Instrução Normativa n. 14/2012), the implementation of PNCP (Ordinance SDA number 17/2013; Instrução Normativa n. 20/2016) as previously mentioned, the Food and Drug Administration recommendations (FDA, 2013) and, and due to an increasing demand of antibiotic-free products by some importing countries. Microbial susceptibility to antibiotics has also been recently described for *Campylobacter* isolates from chilled chicken collected between 2015 and 2016 in the United Kingdom (FSA, 2018). In addition, MAPA has recently created a National Program for Antibiotic Resistance Prevention and Control in Animal Agriculture (BRASIL, 2017) and therefore, a decrease in animal antibiotic exposure and pathogen resistance will be expected.

The identification of a persistent number of tetracycline-resistant isolates from chicken carcasses during the two periods is alarming because this is a commonly used drug to treat bacterial infections. Several studies have shown that most of *C. jejuni* isolates (up to 100%) carry a *tetO* gene (Mazi et al., 2008; EFSA, 2015; Nguyen et al., 2016; Wozniak-Biel et al., 2017; Reddy and Zishiri, 2017), which codes for a membrane protein involved with tetracycline efflux, and this might be related to the high prevalence and inhibitory concentrations observed in this study. Resistance to tetracycline has been a worrisome problem. For instance, an EFSA study with 28 EU Member States showed extremely high resistance of *Campylobacter* isolates from humans, broilers and

Table 6
Resistance profiles in *Campylobacter jejuni* isolated from poultry meat.

Antimicrobial agents	2011/2012 (N = 55)	2015/2016 (N = 44)
TET-AMO	17	17
TET-ERY	1	1
ERY-AMO	1	–
Total – co-resistant isolates	19 (34.5%)[a]	18 (41.0%)[a]
TET-AMO-ERY	10	2
TET-ERY-GEN	4	1
TET-AMO-GEN	1	–
TET-AMO-GEN-ERY	2	–
Total – MDR isolates	17 (30.1%)[a]	3 (6.8%)[b]
TOTAL	36 (65.5%)[a]	21 (47.7%)[a]

N - total number of isolates in each period; Superscript letter [(a or b)] - distinct letters in a same row indicate that numbers are statistically different using Fisher's exact test. MDR stands for multidrug resistant.

Table 5
Minimum inhibitory concentrations and resistance rate distributions for investigated *Campylobacter jejuni* in the periods 2011/12 (55 strains) and 2015/16 (44 strains).

Antibiotic Concentration (mg/L)	AMO 2011/12	AMO 2015/16	ERY 2011/12	ERY 2015/16	GEN 2011/12	GEN 2015/16	TET 2011/12	TET 2015/16
< 0,125	3	10	7	12	21	12	5	7
0,125	–	–	–	–	–	–	–	–
0,25	–	1	4	1	8	14	1	1
0,5	1	1	6	7	13	15	1	–
1	2	3	4	10	3	–	3	–
2	4	5	8	6	1	1	4	–
4	7	2	5	4	1	1	4	4
8	2	3	6	2	2	–	2	1
16	15	3	–	–	1	–	3	6
32	12	8	4	–	–	–	12	9
64	4	2	1	–	3	–	1	8
128	1	1	–	–	1	–	6	2
256	2	–	–	–	1	1	5	1
> 256	2	5	10	2	–	–	8	5
Total resistant isolates R(%)	36* (65.5)	19* (43.2)	21** (38.2)	4** (9.1)	8* (14.5)	1* (2.3)	41 (74.5)	36 (81.8)

AMO - amoxicillin-clavulanic acid; GEN - gentamycin; ERY - erythromycin; TET - tetracycline; _ (line) - breakpoint according to EUCAST, 2018 and R(%) - resistance rate. Differences were considered significative when * $p < 0.05$, ** $p < 0.005$ using Fisher's exact test.

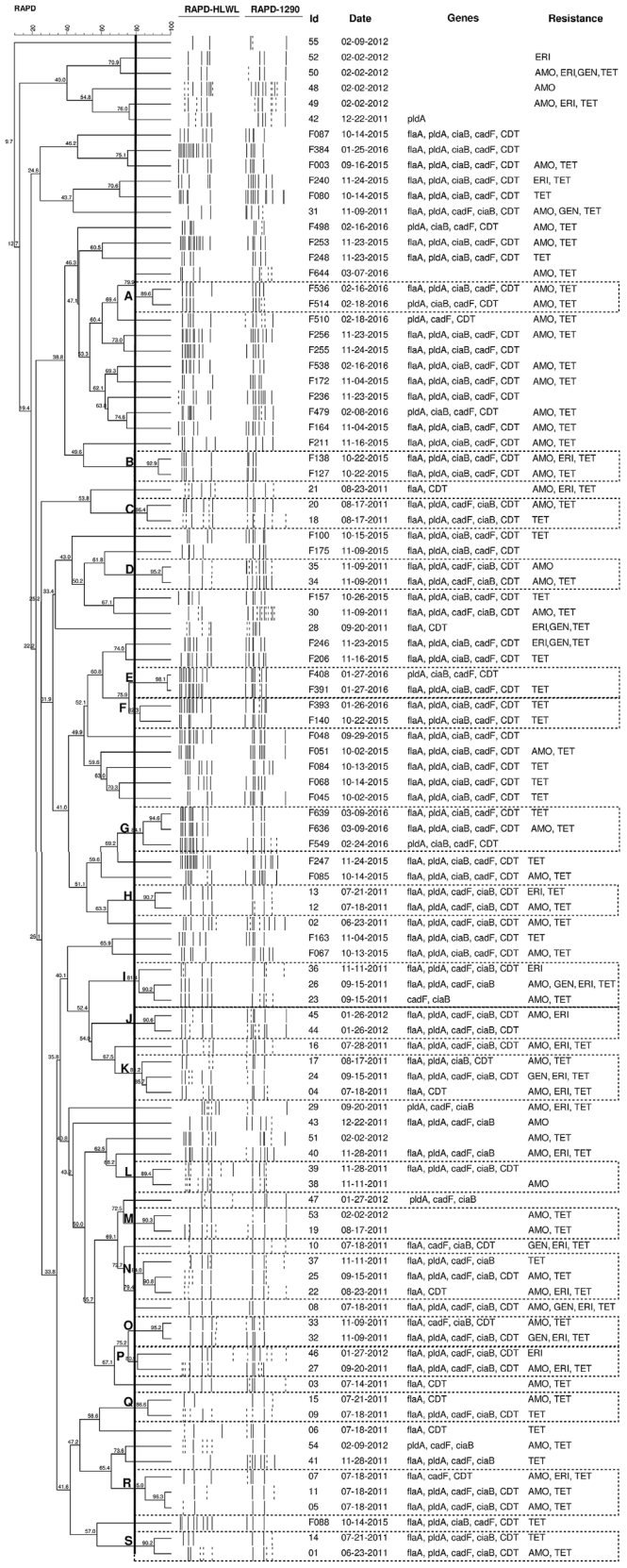

Fig. 2. Dendrogram showing the genetic diversity of 99 *C. jejuni* isolates from 2011 to 2012 and 2015–2016, clustered by UPGMA. Legend: Letters from A to S stand for different clusters. Each cluster grouped isolates showing 80% orhigher similarity to each other.

broiler meat (EFSA, 2018). These findings indicate that specific programs to control tetracycline usage should be implemented worldwide.

3.4. A higher level of genetic diversity was found for isolates from 2015 to 2016

The genetic diversity amongst the 99 *C. jejuni* isolates from 2011 to 2012 and 2015–2016 was assessed using RAPD-PCR. Although RAPD-PCR is not the golden standard for genotyping due to poor reproducibility (Wassenaar and Newell, 2000), it is a cheap, easy and highly discriminatory typing method for investigating genome-wide variability of a large number of isolates (Ono et al., 2003; Acik and Cetinkaya, 2006), particularly for *C. jejuni*, which is a microaerobic and fastidious organism. DNA amplicons were obtained for all isolates using selected primers. Isolates showing a similarity level of 80% or higher were considered genetically similar and grouped in a same cluster. Clustering analysis revealed 19 clusters, named from A to S (Fig. 2). Each cluster grouped isolates from a single sample collection period. Fourteen clusters grouped 32 isolates (63.6% or 32/55) from 2011 to 2012 whereas five clusters were obtained for isolates from 2015 to 2016, grouping only 13 isolates (29.5% or 13/44). Therefore, a higher genetic variability was observed for isolates from 2015 to 2016. *C. jejuni* responses to the environmental changes may be possibly related to this finding.

In poultry farms and in meat processing plants, bacterial populations are periodically under strong pressures due to antibiotic use and sanitary control actions. These pressures may select the fittest strains, such as those bearing virulence genes, and hypermutable strains, which evolve to a number of bacterial variants during a bottleneck event, allowing the organism survival in an altered environment. *C. jejuni* is a highly recombinant bacteria and also capable of phase-variable (PV) switching to generate niche specific genotypes (Bayliss et al., 2012). The PV switching in *C. jejuni* is mainly a result of instability in polyG tracts whose mutations lead to an on-off switch in gene expression, which allows bacteria to overcome variable environment conditions and within-host selection pressures (Bayliss et al., 2012). Recombination events and insertion/deletions in polyG repeats impact genome rearrangements and fragment mobility. Thus, as a result of a rapid bacterial adaptation under a major environmental pressure, sequence alterations may have contributed to the observed genetic diversity. Further studies, however, are necessary to understand the adaptive mechanisms allowing *C. jejuni* isolates to evolve and persist in constraining environments from poultry farms and along the food chain to consumers.

4. Conclusion

This study provides insights regarding bacterial population dynamics in two distinct periods of time, during the transition period until the establishment of stricter Brazilian regulations in poultry farms and slaughterhouses. We showed a significant reduction in *C. jejuni*-positive chilled chicken carcasses between periods. A quantitative risk assessment predicted that a small reduction of 2-log10 in poultry colonization could reduce the human infections by 30-fold (Rosenquist et al., 2003) and therefore, much efforts have been focused in reducing *Campylobacter* loads from poultry production to retail. However, despite the lower prevalence of *C. jejuni* in chicken carcasses, our work demonstrates that selective pressures in the environment may lead to more virulent and genetically diverse strains, which could potentially lead to more severe human infections. Finally, further approaches should be considered when implementing monitoring and control measures for *C. jejuni* for assessing not only the prevalence and countings, but also the evolutionary changes in bacterial populations.

Declarations of interest

None.

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