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Molecular typing of *Campylobacter jejuni* strains: comparison among four different techniques

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Abstract

This study compared the ability of pulsed-field gel electrophoresis (PFGE), *flaA* small variable region (SVR) sequencing, analysis of the clustered regularly interspaced short palindromic repeats locus by high resolution melting analysis (CRISPR-HRMA), and multilocus sequence typing (MLST) for typing 111 *Campylobacter jejuni* strains isolated from diverse sources during 20 years in Brazil. For this, we used previous results obtained by PFGE and *flaA*-SVR sequencing from our research group and performed CRISPR-HRMA and MLST typing for the first time. Furthermore, the discrimination index (DI) of each method was accessed. The DI for PFGE, *flaA*-SVR sequencing, CRISPR-HRMA, and MLST was 0.980, 0.932, 0.868, and 0.931, respectively. By PFGE and *flaA*-SVR sequencing, some strains from clinical and non-clinical sources and from humans and animals presented ≥ 80% similarity. Similarly, some strains from different origins presented the same ST and CRISPR-HRMA types. In conclusion, despite the different DI values, all assays provided the same epidemiological information suggesting that a potential transmission may have occurred between *C. jejuni* from clinical and non-clinical sources and from animals and humans in Brazil. Furthermore it was demonstrated the suitability of PFGE that should be used preferably together with MLST and/or *flaA*-SVR sequencing for typing *C. jejuni* strains.

Keywords *Campylobacter jejuni* · Pulsed field gel electrophoresis · *flaA*-SVR sequencing · CRISPR-HRMA · MLST · Molecular epidemiology

Introduction

Campylobacter jejuni has been the most common causative agent of food-borne gastroenteritis in some countries [1–4]. According to the US Centers for Disease Control and Prevention (CDC), in the USA [3], *Campylobacter* spp. have been the most common bacterial pathogens that cause diarrhoea in humans and affect over two million people annually.

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The same trend has been observed in some European countries, which reported more than 246,000 confirmed cases in 2016 of human campylobacteriosis [4]. Based on this high incidence of *Campylobacter* as an important cause of disease in humans, epidemiological studies using typing methods are required in order to trace the source and the route of transmission of this important pathogen [5].

Various molecular methodologies such as pulsed-field gel electrophoresis (PFGE), sequencing of the short variable region (SVR) of the *flaA* gene, analysis of the clustered regularly interspaced short palindromic repeats locus by high resolution melting analysis (CRISPR-HRMA), and multilocus sequence typing (MLST), among others have been used successfully worldwide for studying the genotypic diversity and the epidemiology of *C. jejuni* strains [6–15].

PFGE was developed by Ribot et al. [8] and since then has been commonly used to study the molecular epidemiology of *C. jejuni*, being generally accepted as one of the most discriminatory genotyping method available for typing *Campylobacter* spp. [5, 16, 17]. Meinersmann and colleagues [6] described for the first time the sequencing of the SVR of the *flaA* gene for genotyping *Campylobacter* spp. strains with good discriminatory power. This technique provided a SVR allele that allows the comparison among other strains isolated worldwide deposited in a database available online (http://pubmlst.org/campylobacter/).

The suitability of HRMA in discriminating CRISPR genotypes in *C. jejuni* strains was demonstrated by Price et al. [10]. HRMA has been used to characterize hypervariable regions, such as CRISPR locus, that are a class of short sequence repeats that have been found in some bacterial genomes and are composed of direct repeats interspersed with non-repetitive spacer sequences [18, 19]. HRMA consists of a one-step, closed-tube post-PCR assay that detects nucleotide sequence variation within a specific locus via melting curve analysis of amplicons [20].

The MLST method was developed for *C. jejuni* by Dingle and colleagues [7] based on the sequencing of seven housekeeping genes. The major advantage of this technique over the other genotyping methods is its high reproducibility and the database available online (http://pubmlst.org/campylobacter/) making it possible to access the sequence type (ST) of the strains and to monitor the global trends of *C. jejuni* strains isolated worldwide [5, 7, 17].

Despite the high incidence of *Campylobacter* as a cause of human gastroenteritis in many countries, this pathogen has been underdiagnosed and underreported in Brazil. There is a paucity of studies that molecularly characterized *C. jejuni* strains isolated in this country [14, 21–24].

Thus, the aim of this study was to compare the suitability of PFGE, *flaA*-SVR sequencing, HRMA of the CRISPR locus, and MLST to type *C. jejuni* strains isolated from diverse sources in Brazil. For this, we used previous results of our research group [14] obtained by PFGE and *flaA*-SVR sequencing and performed CRISPR-HRMA and MLST typing for the first time.

Materials and methods

Bacterial strains

A total of 111 *C. jejuni* strains isolated from human diarrheal faeces (41 strains), human blood (02 strains), monkey faeces (19 strains), chicken faeces (14 strains), chicken meat (33 strains), and sewage (02 strains) from some cities in the States of Minas Gerais, São Paulo, Rio de Janeiro, and Rio Grande do Sul located in the Southeast and South regions of Brazil between 1996 and 2016 were typed. Specifically, the strains isolated from monkeys were isolated from ones of the species *Saimiri, rhesus, and cynomolgus* all from captive monkeys. These strains were selected from the collections of the *Campylobacter* Reference Laboratories of the Oswaldo Cruz Institute of Rio de Janeiro (Fiocruz-RJ) and of the Adolfo Lutz Institute of Ribeirao Preto (IAL-RP) in Brazil. They were systematically chosen to represent isolates from sporadic cases from different clinical and non-clinical samples of the two collections of the reference laboratories mentioned above.

Molecular typing methods

The ability of PFGE and *flaA*-SVR sequencing to type *C. jejuni* strains isolated in Brazil was done based on the results reported in a previous study of our research group [14]. Furthermore, the use of HRMA of the CRISPR locus and MLST for typing *C. jejuni* strains is described for the first time in the present study.

PFGE typing

Agarose blocks were prepared with each 111 *C. jejuni* strains studied using the CDC PulseNet protocol for *C. jejuni* [8]. The plugs were digested with 40 U of SmaI (Life Technologies, Carlsbad, CA, USA) at 25 °C for 2 h. Macrorestriction fragments were resolved by counter-clamped homogeneous electric field electrophoresis in a CHEF-DR III apparatus (Bio-Rad Laboratories) as described in details by Gomes et al. [25]. The pulse times were ramped from 6.8 to 35.4 s over 19 h, as described by Ribot et al. [8]. A *Salmonella* serotype Braenderup H9812 strain digested with 40 U of *Xba*I at 37 °C for 2 h was used as a reference for the molecular mass standard. The gels were stained with ethidium bromide (0.5 μg/mL) for 30 min and destained in distilled water for 80 min. The restriction fragments were viewed under UV light. The relatedness among the PFGE profiles was analysed using the

software package BioNumerics 7.0 (Applied Maths, Keistraat, Belgium). Only bands that represented fragments between 20.5 and 1135 Kb in size were included in the analysis. A dendrogram of genotypic similarity was constructed by the unweighted pair group method with arithmetic mean (UPGMA) method using the Dice similarity coefficient and a position tolerance of 1.5%.

flaA-SVR sequencing

All amplifications of the *flaA* gene were performed according Frazão et al. [14] using the primer pair described by Wassenaar and Newell [16]. Briefly, amplicons of 1713 bp were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.5 μg/mL) and observed under UV light. Thereafter, the amplicons were purified for sequencing with a PureLink Quick PCR Purification kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. For performing the sequencing of the SVR of the *flaA* gene, the purified amplicons were resubmitted to amplification using the primer pair described by Meinersmann et al. [6]. Automated DNA sequencing was performed with an ABI 3500xL sequencer (Life Technologies, Carlsbad, CA, USA). The dendrogram was generated with the software package BioNumerics 7.0 (Applied Maths, Keistraat, Belgium), using the UPGMA method with Jukes and Cantor distance correction model. Bootstrap values (1000 samples) were used to estimate the robustness of the phylogenetic analysis. The *flaA*-SVR nucleotide allele was obtained on the database found at http://pubmlst.org/campylobacter/flaA.

HRMA of the CRISPR locus

This analysis was performed using the protocol described by Souza and Falcão [26]. Briefly, the reaction was performed in 20 μl reaction mixture that contained 10 μl of MeltDoctor HRM Master Mix (Life Technologies, Carlsbad, CA, USA), 1.2 μl of each primer, 4 μl of genomic DNA at 5 ng/μL, and 3.6 μl of DNase and RNase-free distilled water. The primers used were described by Price et al. [10]. The PCR conditions were 1 cycle of 95 °C for 10 min and 45 cycles consisting of 95 °C for 15 s and 58 °C for 1 min. Real-time PCR cycling was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the HRMA step was performed immediately after PCR cycling. The amplicons were heated to 95 °C for 10 s and then cooled to 60 °C for 1 min. The melting curves were generated by increasing the temperature from 60 to 95 °C in 1.6 °C/s increments, and fluorescence was detected every 0.1 s. The normalized melting curve data was analysed with HRM 2.0.1 software program (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The software grouped together amplicons that had similar normalized melting curves so that the differences among sets of amplicons could be easily identified.

MLST typing

For MLST we used the whole genome sequencing of the 106 *C. jejuni* strains carried out on a NextSeq Illumina instrument (Illumina, San Diego, CA, USA). The Illumina reads were assembled using CLC Genomics Workbench version 10.0.1 (CLC bio, Aarhus, Denmark) [27]. Five strains (Cj 14, Cj 21, Cj 28, CCAMP 698, and CCAMP 1065) were excluded of the MLST typing because the draft genome sequences obtained for these strains were not of good quality. Sequence types (STs) were assigned using the *Campylobacter* database at http://pubmlst.org/campylobacter/. New alleles and STs were submitted to the database. All the *C. jejuni* strains studied were registered, and their data are available in the database cited above. The draft genome sequences of all 106 *C. jejuni* strains were submitted to GenBank, and the accession numbers are presented in Frazão et al [14].

Discrimination index (DI)

The discriminatory index of the four techniques used in this study was assessed by Simpson's diversity index as described by Hunter and Gaston [28].

Results

PFGE typing and *flaA*-SVR sequencing

By PFGE, it was obtained 64 different PFGE types, and the 111 *C. jejuni* strains were grouped into three main clusters designated PFGE-A, PFGE-B, and PFGE-C. The similarity of the three clusters was above 47.7%. Specifically, the cluster PFGE-A comprised 48 (43.2%) strains exhibiting a similarity of more than 50.4% isolated from humans, monkey and chicken faeces, chicken meat, and sewage in São Paulo, Minas Gerais, and Rio de Janeiro States between 1996 and 2016. Cluster PFGE-B was composed of 25 (22.5%) strains that exhibited a similarity above 52.3% isolated from humans, chicken faeces, and chicken meat between 1996 and 2015 in São Paulo, Minas Gerais, Rio de Janeiro, and Rio Grande do Sul. Cluster PFGE-C comprised 38 (34.2%) strains with a similarity above 59.9% isolated from humans, monkey and chicken faeces, chicken meat, and sewage in Minas Gerais, São Paulo, and Rio de Janeiro States between 1996 and 2011. These three main clusters were subdivided into 20 subclusters composed of some strains with ≥ 80% of similarity [14].

The dendrogram of the *flaA*-SVR sequencing revealed 35 SVR-types and grouped the 111 *C. jejuni* strains studied into

two clusters named as SVR-A and SVR-B, with a similarity of more than 83.4%. Cluster SVR-A comprised 13 (11.7%) strains with ≥ 89.2% of similarity isolated from humans, monkey and chicken faeces, and sewage between 2004 and 2016 in São Paulo, Minas Gerais, and Rio de Janeiro States. Cluster SVR-B was composed of 98 (82.3%) strains exhibiting a similarity of more than 91.2%, isolated from humans, monkey and chicken faeces, chicken meat, and sewage between 1996 and 2015 in São Paulo, Minas Gerais, Rio de Janeiro, and Rio Grande do Sul States. The most frequently *flaA*-SVR alleles detected were gt57, gt45, gt49, and gt21 with 19, 13, 13, and 09 strains, respectively, isolated from different sources, years, and states of Brazil [14].

HRMA of the CRISPR locus

The results of the HRMA of the CRISPR locus of the 111 *C. jejuni* strains studied are presented in the Table 1. Twenty two different melting profiles were obtained, and each one was named as variant. Variant 3 was the most prevalent and comprised 36 strains isolated from humans (2), monkey faeces (1), chicken faeces (3), chicken meat (29), and sewage (1). The second most prevalent was variant 5 with 16 strains isolated from humans (9), monkey faeces (1), and chicken faeces (6). Variant 4 was the third most prevalent and comprised eight strains isolated from humans (2), monkey faeces (5), and sewage (1). The following nine variants were represented by only one strain of this study: V13, V15, V16, V17, V18, V19, V20, V21, and V22 (Table 1).

MLST

By MLST 41 different STs were observed among the 106 *C. jejuni* strains typed by this methodology (Table 2). Fourteen STs (8741, 8743, 8744, 8745, 8746, 8747, 8748, 8749, 8751, 8752, 9081, 9082, 9083, and 9084) were described for the first time in the *Campylobacter* spp. MLST database. The ST353 was the most prevalent in this present study with 22 strains isolated from humans (8), monkey faeces (2), and chicken meat (12). The second most prevalent ST was the ST8741 with one strain isolated from human and 11strains isolated from chicken meat. Thirty-one STs do not belonged to any known clonal complex (CC), and CC ST-353 and CC ST-403 were the most prevalent ones, comprising 38 and 11 strains, respectively (Table 2).

Discrimination index (DI)

The DI for PFGE, *flaA*-SVR sequencing, HRMA of the CRISPR locus, and MLST was 0.980, 0.932, 0.868, and 0.931, respectively.

Discussion

C. jejuni is the most common causative agent of food-borne gastroenteritis in many countries [1–4]. However, despite the high incidence of *Campylobacter* as a cause of human gastroenteritis in some countries, this pathogen has been underdiagnosed and underreported in Brazil, and there is a paucity of studies that molecularly characterized *C. jejuni* strains in this country [14, 21–24].

This study compared four methodologies in order to access the capability of these techniques to type and to differentiate 111 *C. jejuni* strains isolated from different sources in Brazil. For this, we used previous results of PFGE and *flaA*-SVR sequencing obtained by our research group [14] and also presented here CRISPR-HRMA and MLST results for the first time.

PFGE technique grouped the 111 *C. jejuni* strains studied in three main clusters, with a genotypic diversity above 47.7% and revealed 64 PFGE types which indicates the high diversity among the majority of the strains studied. However, these three clusters were divided in 20 subclusters that contained 99 strains with a genetic similarity ≥ 80%. The analysis of these subclusters showed a high similarity of some *C. jejuni* strains studied suggesting potential for transmission between clinical and non-clinical sources and between animals and humans in Brazil [14].

By *flaA*-SVR sequencing, the 111 *C. jejuni* strains studied were grouped in two clusters designated SVR-A and SVR-B comprising strains from clinical and non-clinical sources exhibiting a similarity ≥ 83.4%. Additionally, this technique showed that the most frequently detected *flaA*-SVR alleles were allele gt57, gt45, and gt49 with 19, 13, and 13 strains, respectively. Some strains isolated from different sources, places of isolation, and years in Brazil were indistinguishable sharing the same *flaA*-SVR allele [14]. In some countries, the alleles most frequently observed were different from the alleles obtained in the present work. For instance, in the USA, the most prevalent alleles were the alleles gt3 and gt6 of the *C. jejuni* isolated from humans [9]. According to Wassenaar and colleagues [29] in Norway, Basque Country, and Iceland, the alleles gt36, gt32, and gt34 were the most prevalent in *C. jejuni* strains isolated from poultry and humans. A study performed in Portugal showed that gt34 was the most frequently detected among *C. jejuni* strains isolated from humans, food, and animals [30].

Taken together, the results obtained by PFGE and *flaA*-SVR sequencing showed that some strains isolated from different sources, years, and places of isolation presented a high genotypic similarity among each other, suggesting that a potential transmission may have occurred between *C. jejuni* from clinical and non-clinical sources and from humans and animals over the course of 20 years in different states of the Southeast and South regions in Brazil [14].

Table 1 Distribution of the 22 CRISPR-HRMA variants (V) among the 111 *Campylobacter jejuni* strains studied

V	Strains	State	Source	Period	Total
V1	Cj14 e Cj30	SP	Human	2003 to 2009	2
V2	CCAMP 488, Cj03, Cj16, Cj31, CCAMP 489, CCAMP 1478	SP, RJ	Human	1996 to 2010	6
V3	Cj02, Cj12, CCAMP 476, CCAMP 478, CCAMP 479, CCAMP 685, CCAMP 764, CCAMP 1013, CCAMP 1014, CCAMP 1015, CCAMP 1016, CCAMP 1018, CCAMP 1019, CCAMP 1020, CCAMP 1021, CCAMP 1023, CCAMP 1024, CCAMP 1025, CCAMP 1032, CCAMP 1047, CCAMP 1050, CCAMP 1051, CCAMP 1052, CCAMP 1053, CCAMP 1054, CCAMP 1055, CCAMP 1056, CCAMP 1057, CCAMP 1058, CCAMP 1059, CCAMP 1060, CCAMP 1491, CCAMP 1518, CCAMP 1520, CCAMP 1521, CCAMP 1523	SP, MG, RJ, RS	Human, Animal, Food, The environment	1996 to 2015	36
V4	CCAMP 497, CCAMP 687, CCAMP 689, CCAMP 696, CCAMP 828, CCAMP 830, CCAMP 845, CCAMP 980	RJ	Human, animal, the environment	1997 to 1999	8
V5	Cj01, Cj04, Cj11, Cj15, Cj17, Cj22, Cj28, Cj29, CCAMP 470, CCAMP 471, CCAMP 472, CCAMP 480, CCAMP 481, CCAMP 699, CCAMP 770, CCAMP 1574	SP, MG, RJ	Human, animal	1996 to 2016	16
V6	Cj06, Cj07, CCAMP 789	SP,RJ	Human, animal	1997 to 2003	3
V7	Cj18, Cj23, Cj24, CCAMP 621, CCAMP 698	SP, RJ	Human, animal	1996 to 2006	5
V8	Cj20, Cj25, Cj26, Cj27, CCAMP 473, CCAMP 493	SP, MG, RJ	Human, animal	1998 to 2011	6
V9	Cj33, CCAMP 588	SP, RJ	Human	2001, 2012	2
V10	CCAMP 81, CCAMP 162, CCAMP 163, CCAMP 672, CCAMP 674, CCAMP 675	RJ	Animal	2003 to 2006	6
V11	Cj13, CCAMP 487, CCAMP 506, CCAMP 1065, CCAMP 1493	SP, RJ	Human, animal, food	1996 to 2015	5
V12	CCAMP 159, CCAMP 700, CCAMP 991	RJ	Human, animal	1999 to 2004	3
V13	CCAMP 501	RJ	Human	1999	1
V14	CCAMP 594, CCAMP 1039, CCAMP 1048, CCAMP 1266	MG, RJ	Human, animal, food	2001 to 2009	4
V15	Cj21	SP, RJ	Human, animal	2006, 2015	1
V16	CCAMP 1061	MG	Food	2009	1
V17	CCAMP 1080	RJ	Animal	2009	1
V18	CCAMP 1519	RS	Food	2015	1
V19	CCAMP 1140	RJ	Animal	2009	1
V20	CCAMP 1466	RJ	Animal	2009	1
V21	CCAMP 1497	RJ	Human	2014	1
V22	CCAMP 1538	RS	Animal	2015	1

V Variant, *MG* Minas Gerais, *SP* São Paulo, *RJ* Rio de Janeiro, *RS* Rio Grande do Sul

According to Price et al. [10], HRMA technique can be used as an alternative to DNA sequencing to analyse the CRISPR locus of the *C. jejuni* strains. Price and colleagues [10] studied 29 *C. jejuni* strains and obtained by CRISPR-HRMA eight different variants or profiles. These findings of Price et al. [10] provided a novel approach for genotyping

Table 2 Distribution of the clonal complexes (CC) and sequence types (ST) of the 106 *C. jejuni* strains typed by MLST

CC	ST	Human $n = 39$	Animal $n = 33$	Food $n = 32$	The environment $n = 2$	Total $n = 106$
21	21	1				5
	50	1				
	1359			2		
	9081	1				
42	469	1				2
	3997	1				
45	45	1				1
48	332	1				11
	475	6	1			
	8744		3			
49	8749		1			1
52	52	2	4			6
206	2086	1				1
353	353	8	2	12		38
	8741	1		11		
	8743		1			
	8752		1			
	9083	1				
	9084			1		
354	354	1	1			6
	1723		1			
	3852	1				
	6257	1				
	8747		1			
403	403	2	4			11
	1775	2	3			
443	51		3			9
	463	1		5		
607	607	1				1
658	1398		1			2
	8745	1				
NA	791	1				1
NA	1962		1			1
NA	2042		1			1
NA	2289	1				1
NA	2304		2			2
NA	6091		1			1
NA	8746				1	1
NA	8748		1		1	2
NA	8751			1		1
NA	9082	1				1

NA not assigned to any CC; New STs are in bold

C. jejuni strains with resolving power similar to PFGE. In the present work, the analyses of the CRISPR locus was done for the first time for *C. jejuni* strains isolated in Brazil. However, despite of the lowest DI value, this methodology provided the same epidemiological information as the other techniques performed in the present study and can be used for complementing the PFGE findings.

The typing of the 106 *C. jejuni* strains by MLST showed 41 different STs of which 14 STs were deposited in the database for the first time. Some strains isolated from different sources, years, and places of isolation belonged to the same ST. For instance, the ST353 comprised strains isolated from humans, animals, and food. Similarly, ST475, ST52, ST354, ST403, and ST1775 were represented by strains isolated from humans and animals, and ST8741 and ST463 comprised strains isolated from humans and food. It is interesting to mention that according to the *Campylobacter* spp. database, the ST8741 is comprised exclusively by *C. jejuni* strains of this present study. However, the ST353 showed to be a more diverse ST-containing strains isolated in some European countries, South America, USA, Canada, and Japan, between 1982 to 2017, from different sources, such as human faeces and blood, chicken meat, chicken faeces, environmental waters, and dog, among others.

In this way, the results of HRMA of the CRISPR locus and MLST described above and presented here for the first time reinforced our previous hypothesis suggesting that a potential transmission may have occurred between *C. jejuni* from clinical and non-clinical sources and from humans and animals isolated during 20 years in Brazil, once a same CRISPR-HRMA variant and a same ST comprised strains isolated from clinical and non-clinical sources and from humans and animals.

According to the DI, in this present study, PFGE was the most discriminatory method for typing the *C. jejuni* strains studied, and the same trend was observed in other works worldwide [9, 11, 31]. Furthermore, both MLST and *flaA*-SVR sequencing presented almost the same discriminatory index, high reproducibility even if performed in different laboratories, and possibility of comparison with strains of other countries deposited in the databases.

In conclusion, PFGE was more efficient than *flaA*-SVR sequencing, HRMA of the CRISPR locus, and MLST for differentiating the *C. jejuni* strains studied. However, all assays provided the same epidemiological information and suggested that a potential transmission may have occurred between *C. jejuni* from clinical and non-clinical sources and from animals and humans in Brazil, confirming the suitability of PFGE that should be used preferably together with MLST and/or *flaA*-SVR sequencing for typing *C. jejuni* strains.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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