

ORIGINAL ARTICLE

DNA identification and characterization of *Campylobacter jejuni* and *Campylobacter coli* isolated from caecal samples of chickens in GrenadaR.S. Miller¹, W.G. Miller², M. Behringer¹, H. Hariharan³, V. Matthew³ and O.A. Oyarzabal¹

1 Department of Poultry Science, Auburn University, AL, USA

2 Produce Safety and Microbiology Research Unit, Agricultural Research Service, US Department of Agriculture, Albany, CA, USA

3 Department of Paraclinical Studies, School of Veterinary Medicine, St. George's University, West Indies, Grenada

Keywords*Campylobacter*, chicken, *flaA*-RFLP, Grenada, MLST, PFGE.**Correspondence**Omar A. Oyarzabal, Department of Poultry Science, Auburn University, 260 Lem Morrison Dr., AL 36849, USA.
E-mail: oyarzoa@auburn.edu

2009/0039: received 8 January 2009, revised 11 June 2009 and accepted 23 July 2009

doi:10.1111/j.1365-2672.2009.04507.x

Abstract**Aims:** To speciate *Campylobacter* strains from the caeca of chickens in Grenada using PCR and to evaluate DNA-based typing methods for the characterization of these isolates.**Methods and Results:** Isolates were speciated with two multiplex PCR assays and were typed with *flaA*-RFLP, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Results confirmed that *Campylobacter coli* strains were more predominant than *Campylobacter jejuni* strains. From 56 isolates, 18 were misidentified using biochemical tests. PFGE typing gave the highest discriminatory power among the methods used (Simpson's index of diversity, $D = 0.9061$). However, the combination of *flaA*-RFLP, PFGE and MLST results gave the highest discrimination for subtyping of these isolates ($D = 0.9857$). A band position tolerance of 4% in BioNUMERICS was the most appropriate for the analysis of this database. MLST profiles were generally concordant with PFGE and/or *flaA*-RFLP types. Several isolates exhibited new MLST sequence types (STs), and 43 of the 49 *Camp. coli* strains belonged to the ST-828 clonal complex.**Conclusions:** *Campylobacter coli* was the most prevalent species isolated from broilers and layers in Grenada, and a combination of restriction and sequence methods was most appropriate for the typing of *Camp. coli* isolates. *Campylobacter coli* STs clustered with described poultry-associated *Camp. coli* STs by phylogenetic analysis.**Significance and Impact of the Study:** Further studies to understand the predominance of *Camp. coli* within *Campylobacter* spp. from chickens in Grenada may help elucidate the epidemiology of these pathogens in chickens.**Introduction**

The identification of *Campylobacter jejuni* and *Campylobacter coli* by biochemical testing is hindered by the inertness of these species and by the fact that they differ only by their ability to hydrolyse hippurate (Razi *et al.* 1981; Patton *et al.* 1991; Debruyne *et al.* 2008a,b). The limitations of biochemical tests have prompted the use of complementary molecular techniques to accurately

differentiate between these two *Campylobacter* species. These techniques rely on amplification and/or sequencing of specific genes or gene segments.

In addition to the species identification, molecular techniques are used for determination of genetic relatedness of the *Campylobacter* isolates. The most common techniques used for subtyping of *Campylobacter* strains include amplification and restriction techniques, such as restriction fragment length polymorphism of the *flaA*

gene (*flaA*-RFLP), restriction and migration techniques, such as pulsed-field gel electrophoresis (PFGE), and sequencing techniques, such as multilocus sequence typing (MLST) (On *et al.* 2008).

Grenada is an island nation in the south-eastern Caribbean Sea. In 2003, Grenada produced approximately 14% of the 7.2 million kg of chicken meat consumed on the island (David 2004) and imported the remaining 86%. An average of 25.4 kg per capita of chicken meat is consumed annually in Grenada (Anon. 2008). Information concerning human campylobacteriosis in Grenada is not readily available. Statistics have revealed that gastroenteritis and diarrhoea are among the leading causes of infant mortality between 1992 and 1996. In these years, all causes of gastroenteritis increased by 60% in children <5 years of age and by 73% in children over 5 years (Anon. 1998). As a result of the lack of information regarding the prevalence of *Campylobacter* in poultry meat in Grenada, we decided to analyse *Campylobacter* strains collected from broilers and layer chickens in Grenada.

The objectives of this study were to verify the identity of *Campylobacter* isolates using multiplex polymerase chain reaction assays (mPCR) and to determine the genetic relatedness of the isolates with *flaA*-RFLP, PFGE and MLST. Previously, these isolates were identified to the species level, using culture media and biochemical tests (Hariharan *et al.* 2009). The discrepancies between the results from biochemical tests and the results from mPCR assays, the degree of discrimination of *flaA*-RFLP, PFGE and MLST and the combination of these techniques are discussed.

Materials and methods

Collection and biochemical identification of isolates

Fifty-six *Campylobacter* isolates collected during processing from the caeca of broiler and layers were submitted to the Department of Poultry Science, Auburn University for DNA identification and characterization. These samples were collected from five farms and included 43 strains isolated from broilers and 13 strains isolated from layers. These samples were collected at each farm during processing (processing is performed manually in Grenada) on eight different days during the period of August 2006 to January 2007. The caeca were placed in sterile plastic bags and transported to the laboratory on ice for processing the same day.

The identification of these isolates using biochemical tests have been described elsewhere (Hariharan *et al.* 2009). Briefly, identification of the *Campylobacter* strains was performed as follows: freshly grown cultures were subjected to catalase, oxidase (BBL, Becton, Dickinson

and Co., Sparks, MD, USA), hippurate (Remel, Lenexa, KS, USA) and latex agglutination tests (JCL, Panbio, Columbia, MD, USA). All isolates were also tested for their susceptibility to nalidixic acid (30 µg disc) and cephalothin (30 µg disc) on Mueller–Hinton agar supplemented with 5% sheep blood. All isolates were positive with the latex agglutination test. Based on biochemical tests, hippurate-positive isolates were identified as *Camp. jejuni*, while nalidixic acid-susceptible, hippurate-negative isolates were identified as *Camp. coli*, and nalidixic acid-resistant, hippurate-negative isolates were identified as *Campylobacter lari* (Hariharan *et al.* 2009).

Isolates were stored at –85°C in 2% sterile, skim milk in cryovials. From these stock cultures, strains were grown on blood agar plates (24 h at 42°C under micro-aerobic conditions) and the growth was used to inoculate tubes containing a mix of Cary–Blair and Brucella broth (1 : 3) supplemented with 5% lysed horse blood (20 ml final volume). These tubes were shipped under refrigeration (*c.* 8°C) to the Microbiology Laboratory at Auburn University, USA, for further studies. At Auburn University, isolates were recovered by plating the samples on modified Campy-Cefex (mCC, Oyarzabal *et al.* 2005). Samples were also enriched in Bolton broth (Oxoid, New York, NY, USA) for 24 h at 42°C under microaerobic conditions (10% CO₂, 5% O₂, and 85% N₂; Airgas, Radnor, PA, USA) provided with an evacuation-replacement system (MACSmics Jar Gassing System; Microbiology International, Frederick, MD) and anaerobic jars. Enriched samples were swabbed (*c.* 0.1 ml) onto mCC plates. All plates were then incubated at 42°C under microaerobic conditions for 48 h and screened for typical *Campylobacter* colonies. Colonies were presumed positive if they showed typical morphology and motility under phase contrast microscopy (Optiphot-2; Nikon, Tokyo, Japan). Presumptive isolates were identified using API Campy tests (bioMerieux, Hazelwood, MO, USA), and DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA, USA). Isolates were then stored at –80°C in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 30% glycerol (v/v) and 5% lysed horse blood for further identification and characterization.

PCR identification of *Campylobacter* isolates

DNA of *Campylobacter* isolates was tested using two multiplex PCR assays that were performed in 25 µl aliquots, as described elsewhere (Oyarzabal *et al.* 2005, 2007). These mPCRs selectively detect *Camp. coli* based on amplifications of the *aspA* (Linton *et al.* 1997) and *ceuE* (Gonzalez *et al.* 1997) genes, and *Camp. jejuni* based on the amplification of the *hipO* gene (Persson and Olsen 2005) and an

undefined gene (Winters and Slavik 1995). The isolates biochemically classified as *Camp. lari* were tested with a PCR assay that uses primers that attach to hypervariable regions of the 16S rDNA and that has been shown to unambiguously identify *Camp. lari* (Oyarzabal *et al.* 1997). A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA, USA) was used for amplification. Amplicons were detected in 1.5% UltraPure™ Agarose-1000 (Invitrogen Corporation, Carlsbad, CA, USA). DNA identification of PCR bands and restricted DNA fragments of *flaA*-RFLP and PFGE were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System; Syngene, Frederick, MD, USA), and a digital picture was recorded using GeneSnap (Syngene).

flaA-RFLP characterization

Extracted DNA of *Campylobacter* isolates was tested for *flaA*-RFLP analysis, using 25 µl PCR reactions as described elsewhere (Nachamkin *et al.* 1993; Harrington *et al.* 2003). A PTC-100 Programmable Thermal Controller was used for amplification and the restriction enzyme was *DdeI* (New England Biolabs, Ipswich, MA, USA). DNA was restricted at 37°C for 2 h. After restriction, DNA bands were detected by electrophoresis in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland).

PFGE characterization

We used a standard PFGE protocol (Ribot *et al.* 2001) with a few modifications described elsewhere (Oyarzabal *et al.* 2008). When collecting the cells to make the plugs, an OD_{600 nm} of 1.0 was used instead of 0.57–0.82. *Salmonella choleraesuis* ssp. *choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) restricted with *XbaI* was used as the DNA size marker. *Campylobacter* DNA was digested with *SmaI* and *XmaI*. The strains that did not show restriction with these enzymes were also tested with *XbaI* and *BamHI*. Restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper; Bio-Rad Laboratories) in 1% SeaKem® Gold Agarose gels that were stained subsequently with ethidium bromide. Visualization was carried out with a UV transilluminator (Gel-Doc System), and a digital picture was recorded using GeneSnap.

MLST characterization

MLST of all strains was performed using the *Camp. coli* primer sets described elsewhere (Miller *et al.* 2005). Each MLST amplification mixture contained: 50 ng genomic DNA, 1× MasterAmp PCR buffer (Epicentre, Madison, WI, USA), 1× MasterAmp PCR enhancer (Epicentre),

2.5 mmol l⁻¹ MgCl₂, 250 µmol l⁻¹ (each) dNTPs, 50 pmol each primer and 1 U *Taq* polymerase (New England Biolabs, Beverly, MA, USA). MLST amplifications were performed on a Tetrad thermocycler (Bio-Rad Laboratories) with the following settings: 94°C, 30 s; 53°C, 30 s; 72°C, 2 min (30 cycles). Amplicons were purified on a BioRobot 8000 workstation (Qiagen, Valencia, CA, USA). Cycle sequencing reactions were performed on a Tetrad thermocycler, using the ABI PRISM BigDye terminator cycle sequencing kit (version 3.1; Applied Biosystems) and standard protocols. Cycle sequencing extension products were purified using BigDye XTerminator (Applied Biosystems). DNA sequencing was performed on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems), using POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software. Alleles and sequence types (STs) were identified using the PERL program MLSTPARSER (Miller *et al.* 2005). Novel alleles and STs were submitted to the PUBMLST *Camp. jejuni/Camp. coli* database (<http://pubmlst.org/campylobacter/>).

Analysis of PFGE and *flaA*-RFLP results

TIF images of the restriction profiles for *flaA*-RFLP and PFGE were incorporated into BioNUMERICS ver. 4.50 (Applied Maths, Austin, TX, USA) for analysis. Pair-comparisons and cluster analyses were made using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. The optimization and position tolerance for band analysis were set at 4%, and a cut-off of 90% was used for the determination of the different restriction patterns for *flaA*-RFLP and PFGE. The Simpson's index of diversity (Hunter and Gaston 1988) was used to determine the discriminating power of each restriction technique.

Results

Farm information

The age at slaughter was different, with broilers being processed at 6 weeks while layers were processed at 72 weeks of age. The number of *Camp. coli* isolates was higher than the number of *Camp. jejuni* isolates in samples from all farms, and *Camp. jejuni* represented no more than 33% of the isolates collected from samples from a given farm (Table 1, farm D).

Identification of *Campylobacter* strains

From 60 cultures shipped to Auburn University, four were not recovered after enrichment. Therefore, 56

Table 1 Number and ID of the isolates collected from five farms in Grenada

Farm	Species	ID	No. isolates
A	Broilers	<i>Campylobacter coli</i>	18*
		<i>Campylobacter jejuni</i>	2
	Layers	<i>Camp. coli</i>	7
		<i>Camp. jejuni</i>	0
B	Broilers	<i>Camp. coli</i>	7
		<i>Camp. jejuni</i>	0
D	Broilers	<i>Camp. coli</i>	4
		<i>Camp. jejuni</i>	1
	Layers	<i>Camp. coli</i>	0
		<i>Camp. jejuni</i>	1
E	Broilers	<i>Camp. coli</i>	9†
		<i>Camp. jejuni</i>	2
K	Layers	<i>Camp. coli</i>	4†
		<i>Camp. jejuni</i>	1

*Two isolates did not grow (not included in this count).

†One isolate did not grow (not included in this count).

isolates were tested at Auburn University. The attempts to speciate these 56 isolates using the API test resulted in several ambiguous results. However, using two mPCR assays (Oyarzabal *et al.* 2005, 2007), 49 isolates were identified as *Camp. coli*, and seven were identified as *Camp. jejuni*. Isolates identified as *Camp. lari* ($n = 3$) by biochemical tests (Hariharan *et al.* 2009) were identified as *Camp. coli* by the mPCR assays and failed to generate amplicons in a PCR assay that uses primers specific for *Camp. lari* (Oyarzabal *et al.* 1997). In addition, 13 isolates identified as *Camp. jejuni* and two identified as *Camp. coli* by biochemical tests were identified as *Camp. coli* and *Camp. jejuni* respectively, with the mPCR assays.

Results from the mPCR assay showed that *Camp. coli* was the most prevalent species and that *Camp. jejuni* accounted for no more than 15% of the isolates. The higher prevalence of *Camp. coli* was strikingly similar in isolates collected from layers or from commercial broiler chickens (Table 2).

Two of these isolates (G185 and G187) gave mixed results (both *Camp. jejuni* and *Camp. coli* bands) during PCR analysis. These strains were re-isolated using a 0.65- μ m filter (Millipore Corp., Billerica, MA, USA), and one isolated colony from each sample was analysed again by

Table 2 Origin of the isolates and distribution by species

Origin	Species	Number	Percentage
Broilers	<i>Campylobacter jejuni</i>	5	12
	<i>Campylobacter coli</i>	38	88
Layers	<i>Camp. jejuni</i>	2	15
	<i>Camp. coli</i>	11	85
Total	<i>Camp. jejuni</i>	7	12.5
	<i>Camp. coli</i>	49	87.5

mPCR, *flaA*-RFLP and PFGE. G185 was identified as *Camp. coli* and gave a restriction profile with PFGE, while G187 was identified as *Camp. jejuni* but did not restrict with PFGE using *SmaI*, *XmaI*, *XbaI* or *BamHI*. In addition, isolate G194 was a mix of two *Camp. coli* strains. This mixed isolate was identified by ambiguous MLST results during sequencing and was confirmed by an unusually large number of PFGE bands. Again, filtration helped us isolate a pure *Camp. coli* strain from this mix.

flaA-RFLP and PFGE results

One isolate did not yield any *flaA*-RFLP profile using the described protocol. The remaining 55 isolates (*Camp. jejuni* and *Camp. coli*) gave 14 *flaA*-RFLP types. Several strains did not show any restriction by PFGE when plugs were made with an OD_{600 nm} of 0.57–0.82 (Ribot *et al.* 2001), but these strains did exhibit restriction when plugs for PFGE were re-made using an OD_{600 nm} of 1.0. At the end, one *Camp. jejuni* isolate and five *Camp. coli* isolates did not show any restriction by PFGE using *SmaI* or *XmaI*. The rest of the isolates gave 19 PFGE types. Five types accounted for 64% of the strains (34 strains), while the other 14 accounted for 21 strains (Fig. 1). Two *Camp. coli* strains (G102 and G151) that did not restrict with *SmaI* or *XmaI* showed a restriction profile with *BamHI*.

MLST results

The allelic profiles from the MLST analysis are presented in Table 3. Several isolates exhibited new STs, which were deposited in the *Camp. jejuni/Camp. coli* PUBMLST database. Additionally, the new allele *tkt365* was identified in all *Camp. coli* ST-3841 isolates. Among the 49 *Camp. coli* isolates typed in this study, remarkably few alleles were identified, and only eight STs were identified: for two loci, *atpA* (*uncA*) and *glyA*, only one allele was present: *atpA17* and *glyA82*, respectively; the *tkf* locus had the largest number of alleles with four identified. Although many of the *Camp. coli* alleles identified here are considered 'common' (i.e. nonhost-associated), chicken- or poultry-associated alleles were also identified within the *Camp. coli* profiles, consistent with their isolation from chickens. These alleles include *gltA65*, *pgm113* and *tkf47*. No alleles associated previously with cattle or swine were identified in this study.

Forty-three of the 49 *Camp. coli* strains were members of the ST-828 clonal complex; the remaining strains (ST-1581) were not associated with any clonal complex. Within the ST-828 clonal complex, ST-1630 included the largest number of strains and also the most numbers of PFGE and *flaA*-RFLP profiles (Table 4).

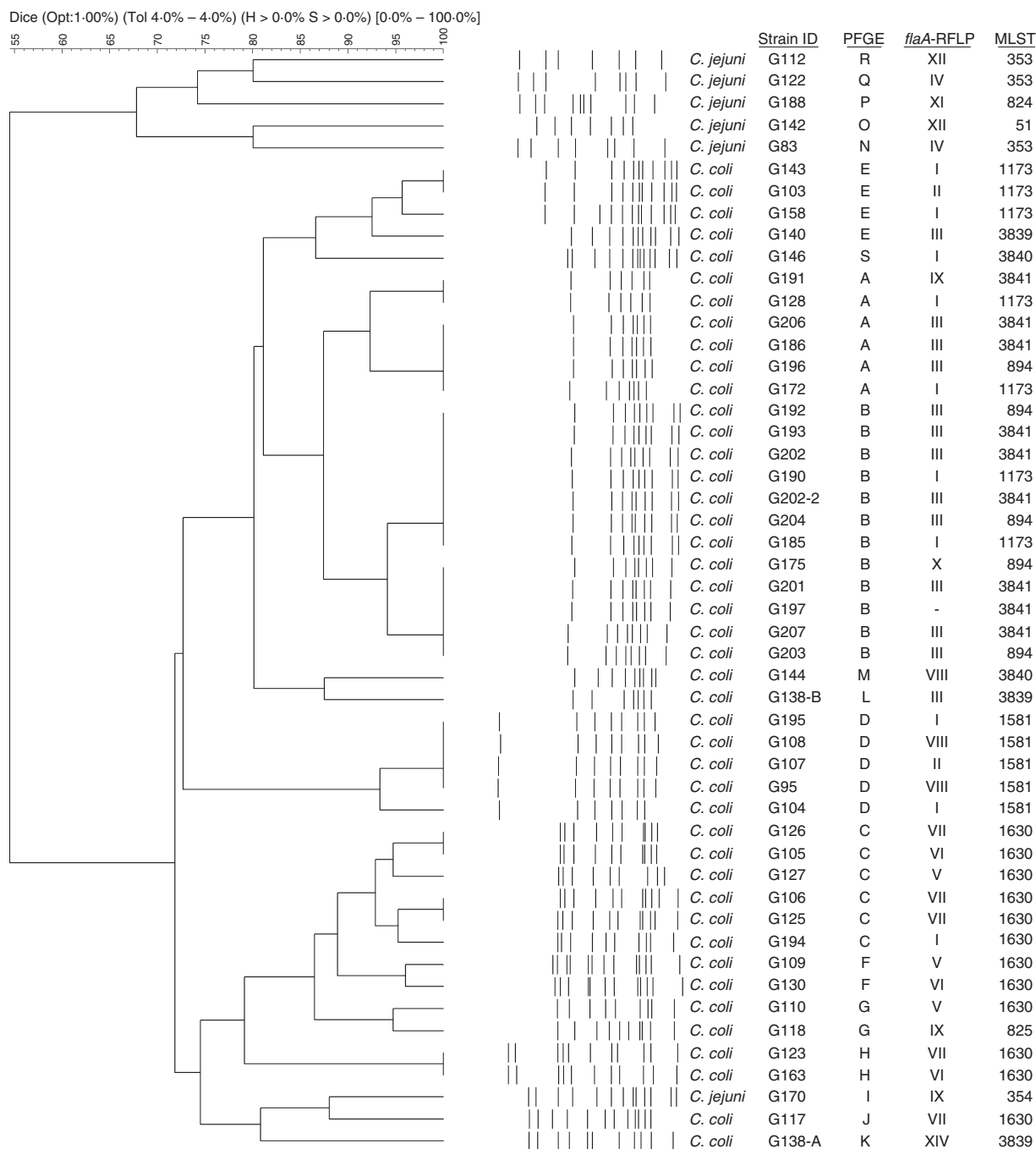


Figure 1 Pulsed-field gel electrophoresis (PFGE) restriction profiles of *Campylobacter coli* and *Campylobacter jejuni* isolates from Grenada. Pair comparison and cluster analysis performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNUMERICS ver. 4.50. The optimization and position tolerance for band analysis were set at 4%, and a cut-off of 90% was used for the determination of PFGE types. The right columns show the PFGE (letters), *flaA*-RFLP (roman numerals) and multilocus sequence typing (numbers) types for each isolate.

Discriminatory power of the three typing methods

Twelve MLST STs were identified, with a Simpson's index of diversity of 0.8636. Most of the major PFGE and *flaA*-

RFLP clusters were concordant generally with ST, and contained strains with the same or phylogenetically similar STs. The combination of *flaA*-RFLP, PFGE and MLST results gave the highest discrimination for these isolates,

Table 3 Allelic profiles and clonal complexes from 56 *Campylobacter coli* and *Campylobacter jejuni* strains isolated from chickens in Grenada

Allelic profiles	Clonal complex	ST	No. of strains	Country	Source	Isolated from	
Known	ST-828	1630	16	Canada	Human stool	Broilers and layers	
		894	5	USA	Turkey	Broilers	
		825	1	UK, Spain	Human stool, environmental water, and pig	Broilers	
	ST-443*	1173	7	USA	Chicken	Broilers and layers	
		51	1	Netherlands, UK, Canada, Curaçao, USA	Human stool, chicken	Layers	
		ST-354	354	2	UK, Australia, Canada, USA, Belgium	Human stool, human blood culture, chicken, chicken offal or meat, calf, sheep	Broilers
		ST-353	353	3	UK, Curaçao, USA and Canada	Human stool, human blood culture, chicken, chicken offal or meat	Broilers and layers
		ST-257	824	1	UK and Australia	Human stool	Broilers
	–†	1581	6	Denmark	Human stool	Broilers and layers	
	New	ST-828	3841	9	Grenada	Chicken	Broilers
3840			2	Grenada	Chicken	Layers	
3839			3	Grenada	Chicken	Layers	

ST, sequence types.

**Camp. jejuni* STs in bold.

†No clonal complex assigned yet.

with a total of 42 different combined types for 56 strains. The combination of results gave the highest Simpson's index of diversity for these strains (Table 5).

Discussion

The biochemical differentiation of *Camp. jejuni* and *Camp. coli* is based on one test, hippurate hydrolysis

Table 4 Pulsed-field gel electrophoresis (PFGE) and *flaA*-RFLP types and number of strains per each multilocus sequence typing (MLST) profile found in this study

Species	MLST	No. of types		No. of strains
		PFGE	<i>flaA</i> -RFLP	
<i>Campylobacter coli</i>	3841	A, B	III, IX*	9
	3840	M, S	I, VIII	2
	3839	E, K, L	III, XIV	3
	1630	C, F, G, H, J, NR†	I, V, VI, VII	16
	1581	D, NR‡	I, II, VIII	6
	1173	A, B, E	I, II	7
	894	A, B	III, X	5
<i>Campylobacter jejuni</i>	825	G	IX	1
	824	P	XI	1
	354	I, NR‡	III, IX	2
	353	N, R, Q	IV, XII	3
	51	O	XIII	1

*One strain failed to restrict with *flaA*-RFLP.

†NR, no restriction. Four strains failed to restrict with PFGE.

‡One strain failed to restrict with PFGE.

(Debruyne *et al.* 2008a). Although all *Camp. jejuni* carry the hippuricase gene, some *Camp. jejuni* strains do not express the enzyme for this reaction and appear to be hippurate negative. Therefore, the use of molecular methods for species identification should not be overlooked when trying to differentiate *Camp. jejuni* from *Camp. coli*. In our studies, the agreement between the two mPCRs was 100%. One of these mPCR assays target the *ceuE* gene and is specific for *Camp. coli* (Gonzalez *et al.* 1997; Nayak *et al.* 2005; Oyarzabal *et al.* 2007; Debruyne *et al.* 2008a), while the other mPCR reacts only with the aspartokinase gene of *Camp. coli* (Linton *et al.* 1997; Oyarzabal *et al.* 2005; Debruyne *et al.* 2008a). It is apparent that *Camp. lari* has not been documented in live chickens or commercial broiler meat, because of the incorporation of molecular techniques for routine identification of *Campylobacter* isolates.

Interestingly, in our results, *Camp. coli* made up the majority of the *Campylobacter* strains from broilers and

Table 5 Simpson's index of diversity for each typing method and a combination of all methods used in this study

Typing method	Simpson's index	No. of types
<i>flaA</i> -RFLP	0.8741	14
MLST	0.8636	12
PFGE	0.9061	19
Combination (all three methods)	0.9857	42

MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis.

layers in these samples from Grenada. No information was available for specific flocks, and therefore we could not link any flock to any given breed. Most reports from the rest of the world show that *Camp. jejuni* is the most predominant *Campylobacter* species in live, commercial broilers, and in retail broiler meat (Genigeorgis *et al.* 1986; Annan-Prah and Janc 1988; Hofshagen 2007; Oyarzabal *et al.* 2007). However, two studies have reported a different ratio between *Camp. coli* and *Camp. jejuni* for some flocks.

El-Shibiny *et al.* (2005) reported a higher prevalence of *Camp. coli* in free-range and organic chickens over 5 weeks of age. These authors identified their isolates using PCR protocols developed originally by Linton *et al.* (1997). Coincidentally, we also used the primers targeting the *aspA* gene of *Camp. coli* from Linton *et al.* (1997) in one of our multiplex PCR assays. El-Shibiny *et al.* (2005) concluded that their results were related to the extended rearing period of the free-range chickens (56 days) and organic chickens (73 days), and to the possible exposure to more environmental sources than commercially raised chickens. In another report, Petersen *et al.* (2001) found one breeder flock, out of two tested, with higher *Camp. coli* prevalence (36%), and suggested that adult hens are more susceptible to colonization by *Camp. coli*. In our study, we did not find any correlation of age and *Camp. coli* presence, and the prevalence of *Camp. coli* in broilers and layer was similar. Because layers were much older birds (72 weeks) than broilers (6 weeks), we think that the most probable explanation for these results is that layers acquire *Camp. coli* from environmental sources. Because several farms reared both broilers and layers, it is also logical to think that layers may serve as the source of contamination for broilers.

Several ST numbers from MLST analysis were found in broilers and layers, and some have been already reported from other sources (Table 3). A previous study had identified putative host-associated *Camp. coli* MLST alleles and STs (Miller *et al.* 2006). Additionally, a phylogenetic analysis, using concatenated allele sequences as described previously (Stoddard *et al.* 2007), indicated that the *Camp. coli* STs identified in this study clustered with other poultry-associated *Camp. coli* STs present within the PUBMLST database. These findings suggest again that exposure to unique environmental sources may account for this large percentage of *Camp. coli* isolates in layers and in broilers. We will continue some studies to identify the source of contamination for layers.

Campylobacter jejuni isolates grouped with *Camp. coli* isolates by *flaA*-RFLP, and one *Camp. jejuni* strain grouped within the *Camp. coli* by PFGE. However, all *Camp. coli* and *Camp. jejuni* isolates were readily distinguishable following MLST, with strains from the two

species forming distinct groups. Additionally, the MLST speciation matched 100% with the mPCR results. It appears that the argument for the continuous differentiation between *Camp. jejuni* and *Camp. coli* is becoming more challenging, especially now that a strong convergence between these two species has been described (Sheppard *et al.* 2008). Although lateral DNA transfer between the two species has been reported (Meinersmann *et al.* 2003; Miller *et al.* 2006), the extent of exchange and the frequency of transfer are not fully known because studies usually focus on a small section of the whole genome.

The PFGE analysis was performed using variable band position tolerance (1–4%) in BioNUMERICS. The use of a less stringent position tolerance (1–2%) resulted in a larger number of types, while a more stringent position tolerance (3–4%) yielded results that were more concordant with a general analysis by visual inspection of macrorestriction profiles using accepted guidelines (Tenover *et al.* 1995). The number of reports about the influence of band tolerance settings in BioNUMERICS, for the analysis of large databases of *Camp. coli* isolates, is limited. Carriço *et al.* (2005) evaluated the PFGE profiles of 1798 isolates of *Streptococcus pneumoniae* with two coefficients, Dice and Jaccard coefficients, and tested different position tolerances for band analysis. These authors found that a 2% position tolerance was most appropriate for their database. Perhaps for the analysis of *Camp. coli* isolates, a more stringent position tolerance may be suitable to account for the genetic variability found by PFGE analysis. However, a larger number of *Camp. coli* isolates should be tested to validate this assumption.

Some isolates with similar PFGE profiles had different *flaA*-RFLP restriction patterns, or vice versa. Therefore, a combination of fingerprinting methods was necessary to achieve a higher discrimination of isolates. The characterization of *Campylobacter* isolates with *flaA*-RFLP has several advantages, such as low cost, simplicity and a good level of standardization among laboratories (Djordjevic *et al.*, 2007). Using a 90% cut-off for the analysis of the results, we obtained more restriction profiles with PFGE than with *flaA*-RFLP. These results agree with other reports suggesting that PFGE using *Sma*I is usually more discriminatory for *Camp. jejuni* and *Camp. coli* isolates than *flaA*-RFLP (Newell *et al.* 2000; Han *et al.* 2007). Yet a combination of PFGE, a restriction profile technique, with MLST, a sequencing technique, provided the most discriminatory combination.

To summarize, our results emphasize that *Camp. coli* was the predominant *Campylobacter* spp. from broilers and layers in these samples from Grenada and that there is a need to complement the identification of isolates by biochemical tests with molecular methods if speciation is

necessary. The choice of technique for fingerprinting sometimes relates to the techniques already in use in some laboratories. However, a thorough investigation should include at least two techniques, a restriction based and a sequenced based, to complement each other and yield more discriminatory results.

Acknowledgements

This publication made use of the *Campylobacter jejuni* Multi Locus Sequence Typing website (<http://pubmlst.org/campylobacter/>) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford (Jolley et al., 2004, *BMC Bioinformatics* 5, 86). The development of this site has been funded by the Wellcome Trust.

References

- Annan-Prah, A. and Janc, M. (1988) The mode of spread of *Campylobacter jejuni/coli* to broiler flocks. *Zentralbl Veterinarmedizin B* 35, 11–18.
- Anon. (1998) Grenada. *Health in the Americas* 3, 285–293. [Online]. <http://www.paho.org/English/HIA1998/Grenada.pdf>. (accessed 5 January 2009).
- Anon. (2008) CARICOM chicken meat and egg consumption 2002, MT. Caribbean Poultry Association. [Online]. http://www.caribbeanpoultry.org/docs/cpi/CARICOM_Poultry_Consumption_2002.pdf. (accessed 5 January 2009).
- Carriço, J.A., Pinto, F.R., Simas, C., Nunes, S., Sousa, N.G., Frazão, N., de Lencastre, H. and Almeida, J.S. (2005) Assessment of band-based similarity coefficients for automatic type and subtype classification of microbial isolates analyzed by pulsed-field gel electrophoresis. *J Clin Microbiol* 43, 5483–5490.
- David, A. (2004) Livestock genetics resources report, incomplete draft. Government of Grenada, Ministry of Agriculture, Lands, Forestry, and Fisheries, Food and Agriculture Organization. [Online]. <ftp://ftp.fao.org/docrep/fao/010/a1250e/annexes/CountryReports/Grenada.pdf>. (accessed 5 January 2009).
- Debruyne, L., Samyn, E., De Brandt, E., Vandenberg, O., Heyndrickx, M. and Vandamme, P. (2008a) Comparative performance of different PCR assays for the identification of *Campylobacter jejuni* and *Campylobacter coli*. *Res Microbiol* 159, 88–93.
- Debruyne, L., Gevers, D. and Vandamme, P. (2008b) Taxonomy of the family *Campylobacteraceae*. In *Campylobacter*, 3rd edn ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 3–17. Washington, DC: American Society for Microbiology Press.
- Djordjevic, S.P., Unicomb, L.E., Adamson, P.J., Mickan, L., Rios, R. and the Australian *Campylobacter* Study Group. (2007) Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing are reliably predicted by restriction fragment length polymorphism analyses of the *flaA* gene. *J Clin Microbiol* 45, 102–108.
- El-Shibiny, A., Connerton, P.L. and Connerton, I.F. (2005) Enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens. *Appl Environ Microbiol* 71, 1259–1266.
- Genigeorgis, C., Hassuneh, M. and Collins, P. (1986) *Campylobacter jejuni* infection on poultry farms and its effect on poultry meat contamination during slaughtering. *J Food Prot* 49, 895–903.
- Gonzalez, I., Grant, K.A., Richardson, P.T., Park, S.F. and Collins, M.D. (1997) Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceuE* gene encoding a putative virulence determinant. *J Clin Microbiol* 35, 759–763.
- Han, K., Jang, S.S., Choo, E., Heu, S. and Ryu, S. (2007) Prevalence, genetic diversity, and antibiotic resistance patterns of *Campylobacter jejuni* from retail raw chickens in Korea. *Int J Food Microbiol* 114, 50–59.
- Hariharan, H., Sharma, S., Chikweto, A., Matthew, V. and Deallie, C. (2009) Antimicrobial drug resistance as determined by the E-test in *Campylobacter jejuni*, *C. coli*, and *C. lari* isolates from the ceca of broiler and layer chickens in Grenada. *Comp Immunol Microbiol Infect Dis* 32, 21–28.
- Harrington, C.S., Moran, L., Ridley, A.M., Newell, D.G. and Madden, R.H. (2003) Inter-laboratory evaluation of three flagellin PCR/RFLP methods for typing *Campylobacter jejuni* and *C. coli*: the CAMPYNET experience. *J Appl Microbiol* 95, 1321–1333.
- Hofshagen, M. (2007) Rapport 1b – 2007: Action plan against *Campylobacter* spp. in Norwegian broilers. [Online]. http://www.vetinst.no/eng/content/download/496/4102/file/Report_2007_01b_Campylobacter_Actionplan_2006.pdf. (accessed 5 January 2009).
- Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 26, 2465–2466.
- Linton, D., Lawson, A.J., Owen, R.J. and Stanley, J. (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* 35, 2568–2572.
- Meinersmann, R.J., Dingle, K.E. and Maiden, M.C. (2003) Genetic exchange among *Campylobacter* species. *Genome Lett* 2, 48–52.
- Miller, W.G., On, S.L., Wang, G., Fontanoz, S., Lastovica, A.J. and Mandrell, R.E. (2005) An extended multilocus sequence typing system (MLST) for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. *J Clin Microbiol* 43, 2315–2329.
- Miller, W.G., Englen, M.D., Kathariou, S., Wesley, I.V., Wang, G., Pittenger-Alley, L., Siletz, R.M., Muraoka, W. et al. (2006) Identification of host-associated alleles by multilocus sequence typing of *Campylobacter coli* strains from food animals. *Microbiology* 152, 245–255.

- Nachamkin, I., Bohachick, K. and Patton, C.M. (1993) Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J Clin Microbiol* **31**, 1531–1536.
- Nayak, R., Stewart, T.M. and Nawaz, M.S. (2005) PCR identification of *Campylobacter coli* and *Campylobacter jejuni* by partial sequencing of virulence genes. *Mol Cell Probes* **19**, 187–193.
- Newell, D.G., Frost, J.A., Duim, B., Wagenaar, J.A., Madden, R.H., van der Plas, J. and On, S.L.W. (2000) New developments in the subtyping of *Campylobacter* species. In *Campylobacter*, 2nd edn ed. Nachamkin, I. and Blaser, M.J. pp. 27–44. Washington, DC: American Society for Microbiology Press.
- On, S.L.W., McCarthy, N., Miller, W.G. and Gilpin, B.J. (2008) Molecular epidemiology of *Campylobacter* spp. In *Campylobacter*, 3rd edn ed. Nachamkin, I., Szymanski, C.M. and Blaser, M.J. pp. 199–211. Washington, DC: American Society for Microbiology Press.
- Oyarzabal, O.A., Wesley, I.V., Barbaree, J.M., Lauerman, L.H. and Conner, D.E. (1997) Specific detection of *Campylobacter lari* by PCR. *J Microbiol Methods* **29**, 97–102.
- Oyarzabal, O.A., Macklin, K.S. and Barbaree, J.M. (2005) Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. *Appl Environ Microbiol* **71**, 3351–3354.
- Oyarzabal, O.A., Backert, S., Nagaraj, M., Miller, R.S., Hussain, S.K. and Oyarzabal, E.A. (2007) Efficacy of supplemented buffered peptone water for the isolation of *Campylobacter jejuni* and *C. coli* from broiler retail products. *J Microbiol Methods* **69**, 129–136.
- Oyarzabal, O.A., Backert, S., Williams, L.L., Lastovica, A.J., Miller, R.S., Pierce, S.J., Vieira, S.L. and Rebollo-Carrato, F. (2008) Molecular typing of *Campylobacter jejuni* strains isolated from commercial broilers in Puerto Rico. *J Appl Microbiol* **105**, 800–812.
- Patton, C.M., Wachsmuth, I.K., Evins, G.M., Kiehlbauch, J.A., Plikaytis, B.D., Troup, N., Tompkins, L. and Lior, H. (1991) Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. *J Clin Microbiol* **29**, 680–688.
- Persson, S. and Olsen, K.E.P. (2005) Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples. *J Med Microbiol* **54**, 1043–1047.
- Petersen, L., Nielsen, E.M. and On, S.L. (2001) Serotype and genotype diversity and hatchery transmission of *Campylobacter jejuni* in commercial poultry flocks. *Vet Microbiol* **821**, 41–154.
- Razi, M.H.H., Park, R.W.A. and Skirrow, M.B. (1981) Two new tests for differentiating between strains of *Campylobacter*. *J Appl Bacteriol* **50**, 55–57.
- Ribot, E.M., Fitzgerald, C., Kubota, K., Swaminathan, B. and Barrett, T.T. (2001) Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J Clin Microbiol* **39**, 1889–1894.
- Sheppard, S.K., McCarthy, N.D., Falush, D. and Maiden, M.C.J. (2008) Convergence of *Campylobacter* species implications of bacterial evolution. *Science* **320**, 237–239.
- Stoddard, R.A., Miller, W.G., Foley, J.E., Lawrence, J., Gulland, F.M.D., Conrad, P.A. and Byrne, B.A. (2007) *Campylobacter insulaenigrae* isolates from Northern elephant seals (*Mirounga angustirostris*) in California. *Appl Environ Microbiol* **73**, 1729–1735.
- Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Winters, D.K. and Slavik, M.F. (1995) Evaluation of a PCR based assay for specific detection of *Campylobacter jejuni* in chicken washes. *Mol Cell Probes* **9**, 307–310.