Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Molecular typing, serotyping and cytotoxicity testing of *Campylobacter jejuni* strains isolated from commercial broilers in Puerto Rico

O.A. Oyarzabal[1], S. Backert[2], L.L. Williams[3], A.J. Lastovica[4], R.S. Miller[1], S.J. Pierce[1], S.L. Vieira[5] and F. Rebollo-Carrato[6]

1 Department of Poultry Science, Auburn University, Auburn, Alabama, USA
2 Department of Medical Microbiology, Otto von Guericke University, Magdeburg, Germany
3 Department of Food and Animal Sciences, Alabama A&M University, Normal, Alabama, USA
4 Department of Biotechnology, Faculty of Natural Sciences, University of the Western Cape, Bellville, South Africa
5 Department of Animal Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
6 University of Turabo, Estación Universidad, Gurabo, Puerto Rico

Keywords
broiler, *Campylobacter,* chicken, cytotoxicity assays, MLST, PFGE, serotyping.

Correspondence
Omar A. Oyarzabal, 260 Lem Morrison Dr., Department of Poultry Science, Auburn University, AL 36849, USA.
E-mail: oyarzoa@auburn.edu

2007/1629: received 8 October 2007, revised 13 February 2008 and accepted 13 February 2008

doi:10.1111/j.1365-2672.2008.03809.x

Abstract

Aims: Thirty *Campylobacter jejuni* strains isolated from fecal samples ($n = 94$; 32%) from 13 positive farms ($n = 17$; 76%) from commercial broiler chickens in Puerto Rico were analysed by molecular methods.

Methods and Results: Isolates were identified with multiplex polymerase chain reaction assays, tested for their antimicrobial susceptibility and characterized with pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), serotyping and bacterial cytotoxicity in mammalian cells. Isolates exhibited high resistance to vancomycin (minimum inhibitory concentration, MIC of >256 μg ml^{-1}) and trimethoprim (MIC of >32 μg ml^{-1}); few were resistant to clindamycin (MIC_{90} 4 μg ml^{-1}), erythromycin (MIC_{90} 8 μg ml^{-1}) and tetracycline (MIC_{90} 8 μg ml^{-1}); but none was resistant to azithromycin (MIC_{90} 4 μg ml^{-1}), ciprofloxacin (MIC_{90} 1 μg ml^{-1}) or gentamycin (MIC_{90} 4 μg ml^{-1}). Most strains restricted with *Sma*I, but a combination of *Sma*I–*Kpn*I digestion was more discriminatory. MLST analysis yielded four sequence types (ST), and ST-2624 was the predominant one. Phylogenetic analysis revealed a high degree of recombination for *glnA* and *pgm* genes. The predominant serotypes were O:3 and O:5. Most strains had lowest cytotoxicity potential with Caco-2 cells, medium cytotoxicity with INT-407 and Hep-2 cells and high cytotoxicity with CHO cells.

Conclusion: A low degree of antimicrobial resistance, 13 PFGE profiles, 4 ST and a large variability in cytotoxicity assays were found for these strains.

Significance and Impact of the Study: This is the first characterization of *C. jejuni* strains isolated from broilers in Puerto Rico. The genetic diversity of these strains suggests that several techniques are needed for strain characterization.

Introduction

Little information is available about human campylobacteriosis in Puerto Rico. After *Salmonella*, *Campylobacter jejuni* has been reported as the second most common foodborne bacterial pathogen isolated from humans, with an incidence of 3·5%, from a reference laboratory in San Juan (Lopez-Ortiz and Solivan 1999). However, there is no information on the incidence and characteristics of *Campylobacter* spp. in commercial broilers raised in the island. The poultry industry is the second largest agricultural industry in Puerto Rico, with broiler meat comprising

more than 69% of the total meat consumed, with an average of 80 lb of broiler meat consumed per capita for the year 2005. Yet, from the total consumption of broiler meat only 34% is produced locally while the rest is imported as frozen products from the United States (Anon 2005a).

In *C. jejuni*, epidemic clones responsible for human disease are characterized by unique combinations of virulence genes or their alleles (Maynard Smith *et al.* 1993; Musser 1996), and some clones have been associated with domestic animals raised for human consumption, primarily broiler chickens. Therefore, it is assumed that contaminated raw poultry products and consuming undercooked poultry are major risk factors for human campylobacteriosis (Butzler and Oosterom 1991; Tauxe 1997; Nadeau *et al.* 2002). However, drawing conclusions about the pathogenicity potential of *C. jejuni* strains from different sources has always been a challenge. The weak clonality of *C. jejuni* brings several limitations when accurate estimations are needed for source tracking efforts aimed at determining the attribution of infections to food sources. Besides the differences in the interpretation of the results from the same techniques applied to different *C. jejuni* isolates, different molecular approaches may answer specific questions but may not be complementary to each other to help identifying sources and improve the prediction of the appearance of epidemic clones in foods. Few studies have addressed the complementation of methods for epidemiological studies. The use of genomotyping may represent a holistic approach to identifying potentially pathogenic clones. Yet, these methods still do not provide a complete overview on the variability in the genomic profiles among *C. jejuni* strains from different areas (Kärenlampi *et al.* 2007). In addition, sequencing methods such as multilocus sequence typing (MLST) are more expensive than molecular typing methods based on restriction profiles, such as pulsed-field gel electrophoresis (PFGE). With this in view, we collected samples from commercial live broilers in Puerto Rico to isolate *Campylobacter* strains and analyse them with molecular techniques to understand their genomic composition and determine the virulence potential. We collected *Campylobacter* strains from faecal material taken from commercial broiler chickens. These strains were analysed using different molecular techniques to determine possible traits that would predict their risk for humans. For this purpose, the isolates were: (i) identified to the species level using culture media and multiplex polymerase chain reaction (mPCR) assays; (ii) tested for their antimicrobial susceptibility, and analysed using PFGE, MLST and serotyping. In addition, their cytotoxicity potential in mammalian cell lines was investigated.

Materials and methods

Sample collection

Ninety-four faecal samples from 17 farms (34 houses or flocks; two houses per farm) were tested for the presence of *Campylobacter* spp. The isolates were identified to the species level using two mPCR assays developed for the identification of *C. jejuni* and *Campylobacter coli*. Isolates were also tested with a hippurate test (Remel, Lenexa, KS, USA).

The faecal samples were collected from 17 commercial broiler farms in Puerto Rico between March and May 2005 covering different areas in the center–south region of the island. Sampled farms contained chicken houses, some with double-deck houses, with five to seven samples collected from each farm. Each sample consisted of *c.* 2–3 g of freshly deposited faecal material collected from three to four different areas of the houses with sterile tongue depressors. The samples were placed in tubes containing Cary–Blair semi-solid medium supplemented with 5% lysed horse blood and shipped in refrigeration (*c.* 8–10°C) for analysis to the Department of Poultry Science, Auburn University, USA.

Campylobacter isolation

The faecal samples were analysed for *Campylobacter* spp. by direct plating on modified Campy–Cefex (mCC; Oyarzabal *et al.* 2005) and on modified charcoal cefoperazone deoxycholate agar (mCCDA; Hutchinson and Bolton 1984) within 4–6 days of collection. All samples were also enriched in Preston broth (Oxoid Inc., New York) (42°C for 24 h) under microaerobic conditions (10% CO_2, 5% O_2 and 85% N_2; Airgas, Radnor, PA, USA) generated using jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD, USA). Enriched samples were swabbed (*c.* 0·1 ml) onto mCC and mCCDA plates. All plates were then incubated under microaerobic conditions (42°C for 48 h) and screened for typical *Campylobacter* colonies. The colonies were considered presumptive positive if they showed the typical morphology and motility under phase-contrast microscopy (Optiphot-2; Nikon, Tokyo, Japan). All presumptive isolates were collected and individually 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

Stock cultures were transferred to mCC plates that were incubated under microaerobic conditions (42°C for 24 h)

to prepare fresh bacterial DNA. DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA, USA). The DNA samples were tested with two mPCR assays developed for the identification of *C. jejuni* and *C. coli*. These PCR assays were performed in 25-μl aliquots as described elsewhere (Oyarzabal *et al.* 2005, 2007a). 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) and DNA bands were stained with ethidium bromide, visualized using a ultraviolet (UV) transilluminator (Gel-Doc System; Syngene, Frederick, MD, USA) followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene). All strains were also tested with a commercial hippurate test (Hardy Diagnostics, Santa Maria, CA, USA).

Epsilon test (Etest®)

Antimicrobial susceptibility tests were performed with Etest antibiotic strips (AB Biodisk North America, Inc., Piscataway, NJ, USA) as suggested by the manufacturer. Mueller–Hinton agar plates were prepared using S-8000 plate maker and poured using a APS 320 automated petri dish filler (Microbiology International, Frederick, MD, USA). A higher than the recommended inocula, corresponding to *c.* 1·0 at $OD_{600\ nm}$, was used to seed the Mueller–Hinton agar plates. The isolates were tested for their sensitivity to azithromycin, ciprofloxacin, clindamycin, erythromycin, gentamycin, tetracycline, trimethoprim and vancomycin.

Sequencing of the *tet*(O) gene and plasmid analysis

The amplification of a 559-bp fragment of the *tet*(O) gene was done using primers DMT 1 and DMT 2 (Gibreel *et al.* 2004). A premade mix (OmniMix® HS; Cepheid, Sunnyvale, CA, USA) was used for the PCR reactions and the amplification conditions were 95°C for 4 min, 30 cycles of 95°C for 1 min (denaturation), 61°C for 1 min (hybridization) and 72°C for 1 min (extension), and a final extension of 72°C for 4 min. A 2015-bp segment of the *tet*(O) gene was amplified using primers *tet*(O)-F 109–1108 (5′-TTG TTT TGG GGC TAT TGG AG-3′), *tet*(O)-R 109–1108 (5′-TCA CCG TTT GTC GGA ACA TA-3′), *tet*(O)-F 750–1500 (5′-GAG AAA TAT ATG TCA GGG AAA CCG T-3′), *tet*(O)-R 750–1500 (5′-CGG TCT TTC CAT ATA TAT AAC AGT AGG C-3′), *tet*(O)-F 1089–2193 (5′-TAT GTT CCG ACA AAC GGT GA-3′) and *tet*(O)-R 1089–2193 (5′-CCC AAT TCC CAT TCA TCA TAA-3′). The amplified products were sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems). The sequence of the 1997-bp segment of the *tet*(O) gene from one strain was submitted to the European Bioinformatics Institute (EBI) and was assigned the accession numbers (AM884250). The strains were tested for the presence of plasmids using unrestricted agarose plugs in PFGE gels and with the QIAprep® Spin Mini Prep kit and QIAfilter™ Plasmid Midi kit (Qiagen Inc., Sunyvale, CA, USA) for plasmid extraction. Positive *Escherichia coli* strains (AU4 AU19) containing known plasmids were used as positive controls in gel electrophoresis.

PFGE analysis

Thirty isolates were tested with a standard PFGE protocol (Ribot *et al.* 2001) with the modifications suggested by the Centers for Disease Control and Preventions (http://www.cdc.gov/pulsenet/protocols.htm) was used. A digestion of *Salmonella choleraesuis* ssp. *choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) was used as the DNA size marker. This marker was included in three lanes per each PFGE gel, the two lanes at the end and one in the middle. *Campylobacter* DNA was digested with *Kpn*I, *Sal*I or *Sma*I, as well as a combination of *Sma*I/*Kpn*I. The strains that were not digestible with *Sma*I were also tested with the following enzymes: *Apa*I, *Bam*HI, *Bgl*II, *Hind*III, *Ksp*I (*Sac*II), *Not*I, *Nru*I, *Sac*I, *Spe*I and *Xba*I. The switch times for the *Sma*I/*Kpn*I combination were initial 6·76 s and final 35·38 s. Restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper; Bio-Rad) in 1% agarose gel stained with ethidium bromide, visualized with a UV transilluminator (Gel-Doc System; Bio-Rad), and a picture recorded using GeneSnap (Syngene). PFGE profiles were used to perform a pair comparison and cluster analysis using the Dice correlation coefficient, and a dendogram was created by the Unweighted Pair Group method using Mathematical Average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX, USA). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used to analyse the different PFGE patterns (De Boer *et al.* 2000).

MLST and sequence analysis and phylogenetic calculations

MLST was performed following the guidelines available at the *C. jejuni* MLST website (http://pubmlst.org/campylobacter/) and guidelines from previous publications (Dingle *et al.* 2001). The PCR amplification was performed in 25-μl reactions using a premade mix (Invitrogen Corporation). Amplicons were detected on 1·5% ethidium bromide agarose gels and purified with a MiniElute PCR

Purification Kit (Qiagen Inc.). The concentration of amplified DNA was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two premade PCR mixes, OmniMix® HS (Cephied, Sunnyvale, CA, USA) and Platinum PCR Supermix (Invitrogen Corporation) were used for PCR amplification for sequencing using the suggested internal primers. The amplified products were sequenced at the Auburn University Genetic Analysis Laboratory. The sequences were then aligned, assembled and edited using MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html), ClustalW (http://www.ebi.ac.uk/clustalw/index.html) or BioEdit (Hall 1999) version 7.0.5.3 (http://www.Mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees calculations for specific genes based on their sequences were conducted using Neighbour-Joining analysis of MEGA (Molecular Evolutionary Genetics Analysis) version 4 (http://www.megasoftware.net). Split decomposition analysis to test for recombination was done using SplitsTree analysis (Bandelt and Dress 1992; Huson 1998) with free software (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=splitstree&referer=pubmlst.org). Linkage disequilibrium analysis was calculated with Start (http://pubmlst.org/software/analysis/start/) for the index of association and with Lian version 3.5 (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst), as published by Haubold and Hudson (2000), for the standardized index of association.

Serotyping

Serotyping was done by passive haemagglutination of the soluble thermostable antigens of *C. jejuni* (Penner and Hennessey 1980). Antigenic extracts of *Campylobacter* strains were prepared by boiling and centrifugation (Lastovica *et al.* 1986). Type-specific antisera were titrated by a passive haemagglutination test against antigen-sensitized sheep erythrocytes, as described by Penner and Hennessey (1980). Sensitized sheep erythrocytes were tested by passive haemagglutination against 60 reference antisera and those found to be non-ypable were retested against seven other provisional antisera prepared against infrequently occurring serotypes. The serotype of the isolate was designated according to the antisera in which agglutination was observed.

Cell culture and cytotoxicity assays

Twenty *C. jejuni* strains, representing all 12 PFGE profiles obtained with the combination *Sma*I/*Kpn*I, were analysed for their cytotoxicity potential on human colon (Caco-2, HTB-7), human larynx epithelial (Hep-2, CCL-23), Chinese hamster ovarian (CHO, CCL-61) and human intestine epithelial (INT-407) cells. Cytotoxicity assays were performed by measuring the supernatant lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis (Hu *et al.* 2006). Cell lines were obtained from the American Type Culture Collection and maintained in 25-cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing 10% foetal bovine serum (Atlanta Biologicals, Norcross, GA, USA). The virulence potential of *C. jejuni* strains was determined using an *in vitro* cytotoxicity assay (Maldonado *et al.* 2005). Briefly, confluent host cell monolayers were trypsinized and grown in 24-well plates (37°C; 7% CO_2 and humidity for up to 72 h). *Campylobacter jejuni* was added at a multiplicity of infection (MOI) of 100. After 6 h of incubation at 37°C in the presence of 7% CO_2, tissue culture cell supernatants were centrifuged (300 ***g***, 5 min), and the release of endogenous LDH from the cytosol of damaged, mammalian cells was measured by dispensing a 0·1-ml aliquot of cell supernatant combined with 0·1 ml of LDH substrate into each well of a 96-well microtitre plate. Per cent cytotoxicity values were determined based on the amount of LDH released from 9-min readings for both the positive and negative controls in each experiment, as follows:

$$\text{percent cytotoxicity} = (A_{\text{exp}} - A_{\text{PBS}})/(A_{\text{Triton-X}} - A_{\text{PBS}}) \times 100$$

where A_{exp} is the absorbance of the sample, A_{PBS} is the absorbance of phosphate-buffered saline and $A_{\text{Triton-X}}$ is the absorbance of Triton X-100 at 1% concentration. At least three experiments were run for each strain and each cell line. In addition, per cent cytotoxicity values among different cell lines were analysed using the GLM (General Linear Model) procedure of SAS (SAS Institute Incorporated, Cary, NC, USA), and means were analysed for differences with the Duncan's multiple range test, with significance set at $P \leq 0{\cdot}05$.

Results

Strain identification and antibiotic resistance profiling

Thirty *C. jejuni* isolates (32%) were collected from 94 broiler samples. From 17 farms that were sampled, 13 farms (76%) gave *Campylobacter* growth, and from a total of 34 flocks (houses) sampled, 19 (56%) were *Campylobacter*-positive. All isolates were confirmed as *C. jejuni* with two mPCR assays and with a hippurate test. Six samples were detected as positive by direct plating and 24 samples were detected positive after enrichment. Using Etest® analyses, all isolates exhibited high resistance to vancomycin (minimum inhibitory concentration, MIC of >256 μg ml^{-1}) and trimethoprim (MIC of

>32 μg ml^{-1}), but the resistance to other antimicrobials was low. No strain was resistant to ciprofloxacin, one strain was resistant to clindamycin, three were resistant to erythromycin (MIC of >32 μg ml^{-1}), and one was resistant to tetracycline (Table 1). Twenty-two strains showed tetracycline MIC values between 1 and 8 μg ml^{-1}, and nine of these strains exhibited a 559-bp fragment amplification of the *tet*(O) gene with a PCR assay (Gibreel *et al.* 2004). Through a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/), the sequence of a 1998-bp segment of the *tet*(O) gene from the resistant strain was found to be 100% similar to known sequences (M18896.2, CP000549.1, AY714214.1, AY 701528.1 and AY394561.1). PFGE analysis of unrestricted plugs did not reveal any migrating band that could be interpreted as being of plasmid origin which was further confirmed by direct plasmid preparation. From the 22 isolates that showed a tetracycline MIC value between 1 and 8 μg ml^{-1}, no strain showed the presence of any plasmid. Therefore, the *tet*(O) gene was considered to be chromosomically encoded in these strains.

PFGE results

Thirty *C. jejuni* isolates were analysed by PFGE. The restriction profiles obtained varied among the enzymes tested. *Sma*I restriction resulted in nine profiles, *Kpn*I restriction resulted in eight PFGE profiles, *Sal*I restriction gave seven profiles and the combination of *Sma*I–*Kpn*I digestion yielded 13 different restriction profiles, with a number of DNA bands that varied from 6 to 14. Ten strains did not restrict with *Kpn*I, while two strains did not restrict with *Sal*I, *Sma*I or *SmaI/Kpn*I. Besides the suggested pulse time for *Kpn*I (Anon. 2007), we also tried other switch times, some described elsewhere (De Boer *et al.* 2000; Michaud *et al.* 2001), but the bands in the PFGE gels were usually less defined for *Kpn*I and *Sal*I than for *Sma*I. To prove whether the lack of *Sma*I restriction was because of presence of endogenous proteinases, formaldehyde-treated plugs were digested with different restriction enzymes. The two strains did not restrict with *Apa*I, *Bgl*II, *Hind*III, *Ksp*I (*Sac*II), *Kpn*I, *Not*I, *Sma*I, *Spe*I or *Xba*I, but were restricted with *Bam*HI, *Nru*I, *Sal*I and *Sac*I, suggesting that the lack of bands in the *Sma*I digest is most probably the result of the lack of *Sma*I restrictions sites.

The incorporation of thiourea during the running of the PFGE gels was reported to improve the discrimination of DNA bands (Corkill *et al.* 2000), although the addition of more than 200 μmol l^{-1} of thiourea did not improve resolution (data not shown).

MLST results

The use of both OmniMix® HS and Platinum PCR Supermix for PCR amplification gave clean amplicons for sequencing data for MLST analysis. Table 2 shows the allelic profiles and clonal complexes from the *C. jejuni* strains. No new allelic sequences were found for any of the house-keeping genes. However, the predominant allelic profile was new and assigned as sequence type (ST)-2624, and deposited on the MLST web site (http://pubmlst.org/campylobacter/). Among individual alleles, *glnA* and *pgm* were the genes with considerable variability in allelic profiles, with four alleles for *pgm* (10, 11, 86 and 89) and for *glnA* (4, 17, 21 and 30). A calculation of phylogenetic trees for *pgm* and *glnA* based on their sequences and using the Neighbour-Joining feature analysis of MEGA version 4 (Tamura *et al.* 2007) showed a high variability within the four alleles of each gene (data not shown). The calculation of the linkage disequilibrium using the algorithms

Table 1 Frequency distribution of minimum inhibitory concentration (MIC; μg ml^{-1}) of *Campylobacter jejuni* isolates determined by Etest®*

		Number of occurrences of MIC						
Category	Antimicrobial	<0·03	0·06–0·12	0·25–0·50	1–8	>8	MIC_{50}	MIC_{90}
I	Ciprofloxacin†	–	10	15	5‡	–	0·25	1
	Azithromycin	1	13	7	9	–	0·25	4
II	Clindamycin	–	5	16	8	1	0·5	4
	Gentamycin	–	3	2	25	–	2	4
	Erythromycin§	–	–	8	16	6¶	2	8
III	Tetracycline#	1	–	6	22	1	1	8

*Breakpoints suggested by the Clinical and Laboratory Standards Institute (2006).
†Suggested break point: >4 mg l^{-1}.
‡The highest MIC value was 3 μg ml^{-1}.
§Suggested break point: >32 mg l^{-1}.
¶Three isolates with MIC of 24 μg ml^{-1}.
#Suggested break point: >16 mg l^{-1}.

Table 2 Allelic profiles and clonal complexes from *Campylobacter jejuni* strains

ST allelic profile	Clonal complex	Strains	Per cent of dataset
2624*	NA†	16	53·3
429	48	9	30·0
353	353	4	13·3
460	460	1	3·3

*New sequence type (ST) profile.
†No clonal complex assigned yet.

from DnaSP version 4.10.9 resulted in values of 0·77 (Kelly 1997) and 0·74 (Rozas *et al.* 2001) for *pgm* and 0·42 (Kelly 1997) and 0·53 (Rozas *et al.* 2001) for *glnA*. Three alleles were found for *aspA* (7, 14 and 24) and *tkt* (1, 3 and 59), and only two for *gltA* (2 and 5), *glyA* (2 and 68) and *uncA* (5 and 6). Their calculated genetic diversities at individual loci (LIAN) were: *aspA* = 0·5398; *glnA* = 0·6172; *gltA* = 0·5032; *glyA* = 0·5118; *pgm* = 0·6172; *tkt* = 0·4710 and *uncA* = 0·4258. Overall, the LIAN analysis resulted in a mean genetic diversity (*H*) of 0·5266 ± 0·027 (SD), and showed linkage disequilibrium for this population of *C. jejuni* (Table 3). The phylogenetic tree of the ST profiles was calculated with the UPGMA and showed that the new profile ST-2624 was more closely related to ST-460 than the other two ST profiles. The decomposition analysis was performed with SplitsTree and resulted in a network-like shape suggesting a high degree of recombination within genes (Fig. 1). When compared with PFGE, MLST was less discriminating for strain characterization, and strains that grouped under ST-2624 had seven different PFGE profiles (Fig. 2).

Table 3 Calculation of the index and the standardized index of association*

Summary statistics (means)		Testing null hypotheis (H_0: $V_D = V_e$)* Monte Carlo (1000 resamplings)		Parametric
V_D†	9·27	V_D	0·0127	0·0136
V_e†	1·71	*L*	1·9141	1·906
sI_A‡	0·73	I_A§	4·35	

*Calculated with START (index of association) and LIAN (standardized index of association); the tested population of *Campylobacter jejuni* is in linkage disequilibrium.
†V_D, calculated or observed variance; V_e, expected variance for linkage equilibrium.
‡sI_A, standardized index of association.
§I_A, index of association.

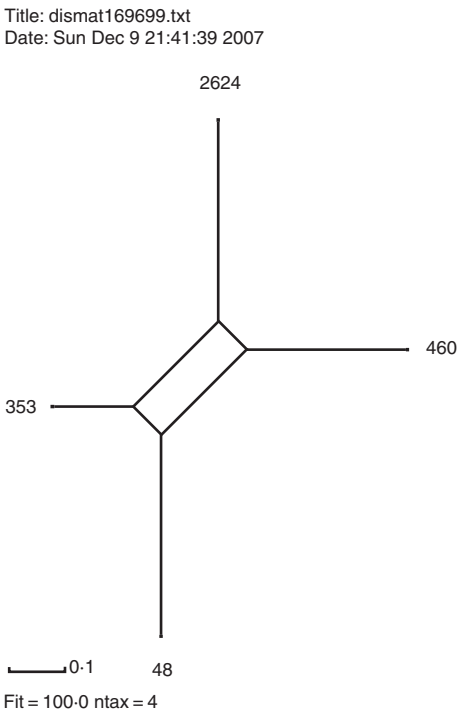

Figure 1 Decomposition analysis on allelic profile data using SplitsTree (Hudson 1998; Anon. 2007d). The network-like shape of the results is suggestive of recombination.

Serotyping results

From the 30 *C. jejuni* isolates analysed with the serotyping scheme, the predominant serotypes were O:3 and O:5. Two strains had a weak reaction with O:21 and 18 strains from the O:3 and O:5 serotypes had a minor component which serotyped as O:21. Ten strains were nontypable by the panel of antisera used.

Cell cultures and cytotoxicity assays

Twenty *C. jejuni* strains representing all 12 PFGE profiles obtained by the combination of *Sma*I/*Kpn*I were characterized for their cytotoxicity potential (measured as LDH release) on selected tissue cell lines (Caco-2, CHO, Hep-2 and INT-407). Each strain was compared with a positive control (Triton X-100), a low control (PBS) and *C. jejuni* ATCC 29428. Cell cytotoxicity was grouped based on the amount of LDH released from each cell line, with categories divided as low (1–20%), medium (21–49%) and high (>50%). Table 4 shows the results grouped in categories for all the cell lines. Only one strain was grouped into the low category for each cell line. When tested with Caco-2 cells, most strains grouped as medium, while most strains grouped as high when tested with CHO cells. Testing with INT-407 and Hep-2 resulted in a similar distribution

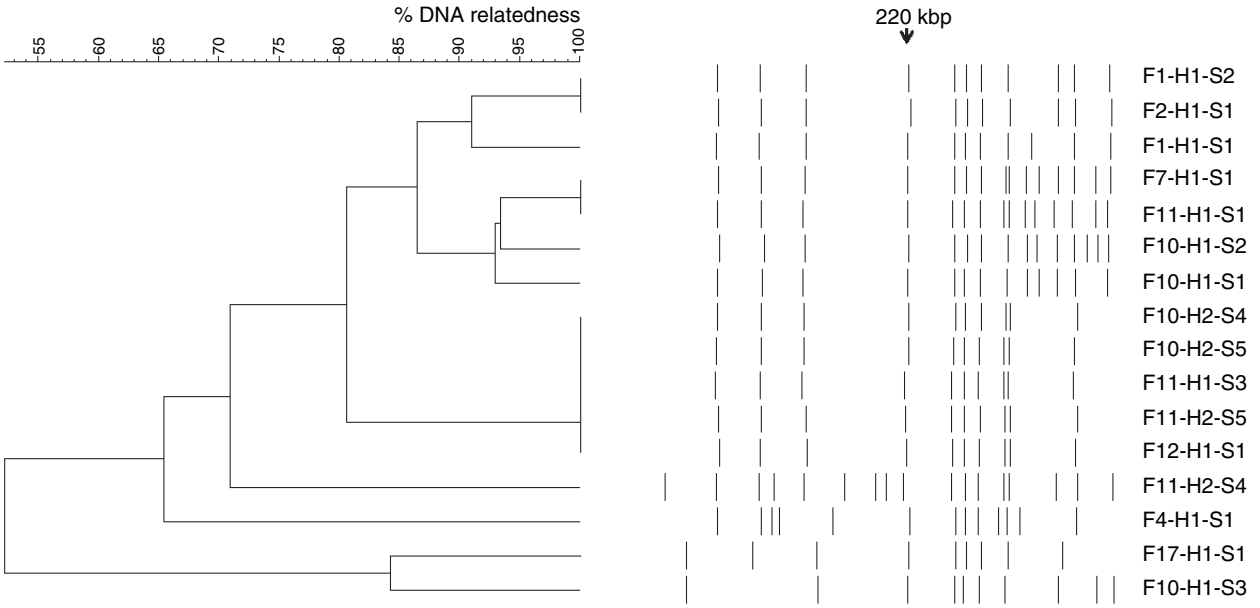

Figure 2 Pulsed-field gel electrophoresis macrorestriction profiles (*Sma*I/*Kpn*I) of *Campylobacter jejuni* isolates belonging to sequence type (ST)-2624. Pair comparison and cluster analysis performed with the Dice correlation coefficient and the Unweighted Pair Group method using Mathematical Average clustering algorithm of BioNumerics version 4.50 (Applied Maths).

Table 4 Cytotoxicity assays of 20 *Campylobacter jejuni* strains*

	LDH (%)†			
Strain	Caco-2	INT-407	Hep-2	CHO
Controls				
Low (D_0F)	0	0	0	0
High (1% Triton-X)	100	100	100	100
ATCC 29428	38	72	94	78
Categories‡				
High	1	9	10	19
Medium	18	10	9	0
Low	1	1	1	1
Mean ± SEM§	35 ± 1·5*	50 ± 2·6†	53 ± 2·8†	70 ± 2·6‡

*Caco-2, human colon adenocarcinoma cells; INT-407, human intestine epithelial cells; Hep-2, human larynx enterocyte-like cells; CHO, Chinese hamster ovarian cells.

†LDH, lactate dehydrogenase release.

‡Categories: high, ≥50%; intermediate, 21–49%; low, 1–20%.

§SEM, standard error of the mean. Means with different superscripts within this row are significantly different ($P < 0·05$) by Duncan's multiple range test (SAS version 9.1).

of strains between the medium and high categories. The mean percentage values of LDS release from INT-407 and Hep-2 cells were similar ($P \geq 0·05$) for all strains. Some strains showed a high variability of results among cell culture assays (Fig. 3a), while others exhibited a closer agreement among the results from different assays (Fig. 3b). All four tissue cultures were effective in determining the cytotoxicity potential of *C. jejuni* strains isolated from poultry, although a large variation in the percentage of LDH release was found for the same isolates tested with different cell lines.

Discussion

We pursued this study to determine the prevalent *Campylobacter* species that colonizes commercial broiler flocks in Puerto Rico and to characterize these isolates using PFGE, MLST, antimicrobial susceptibility, heat-stable serotyping and the invasiveness in mammalian cell cultures. The year average temperature in Puerto Rico is between 70° and 90°F, and the temperature recorded outside the farms was between 75° and 80°F. Only few samples were positive by direct plating, but 32% of the samples were positive after enrichment in Preston broth. This number of positive flocks is similar to the 57% reported for commercial broilers in the southeast United States (Stern *et al.* 2001; Potturi-Venkata *et al.* 2007a). Therefore, the shipping of the samples under refrigeration for several days may have accounted for a reduction in the number of campylobacters in the sample, but did not result in a detectable reduction in the prevalence of positive flocks (56%) or farms (76%). The flocks were sampled two weeks before processing, when *Campylobacter* is usually present in high numbers (Potturi-Venkata *et al.* 2007a; b). The methodology used was optimized with combinations of plates and enrichment broth for the isolation of

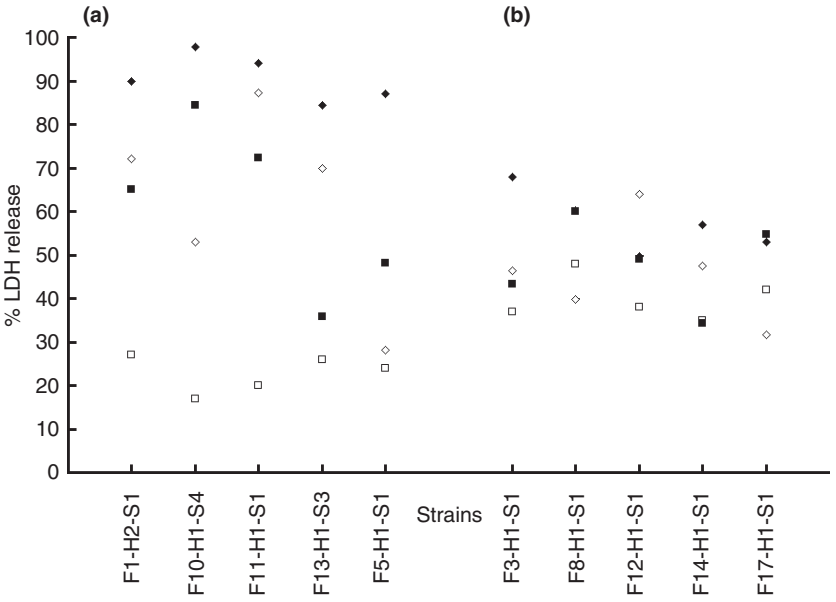

Figure 3 Variability of results among strains tested with different cell line assays (Caco-2, (□) INT-407 (■), Hep-2 (◇) and CHO) (◆). Strains with low agreement of results (a) *vs* those with higher agreement of results (b).

Campylobacter spp. from faecal material collected from live broilers (Potturi-Venkata *et al.* 2007b).

The general incidence of resistance among the antimicrobials tested with the disc diffusion method was low. Multiresistance was not found among these isolates and may be related to the low usage of antimicrobials for disease treatment in animal production in Puerto Rico. The disc diffusion method appears to be reliable for the determination of MIC for erythromycin, ciprofloxacin and tetracycline for *C. jejuni* and *C. coli* (Gaudreau and Gilbert 1997). Although gentamicin and erythromycin resistance is usually low in *C. jejuni*, erythromycin resistance in *C. jejuni* from chicken appears to be increasing in Canada (Lévesque *et al.* 2007). Erythromycin is still an important antimicrobial for treatment of campylobacteriosis in humans. The increase in the inoculum used to seed the plates for the disc diffusion method resulted in more consistent and reliable results recorded at 24 h of incubation, and similar suggestions on the preparation of the inocula and MIC interpretation have been reported to improve the per cent agreement between the disc diffusion method and the agar dilution method for erythromycin (Luangtongkum *et al.* 2007).

None of the isolates exhibited resistance to ciprofloxacin. Our results differed from the antimicrobial resistance reported for *C. jejuni* in Trinidad and Tobago, where 87% of the *C. jejuni* broiler strains were found to be resistant to sulfa/trimethoprim and ciprofloxacin, three antimicrobials that are apparently used routinely by the poultry industry in that country (Rodrigo *et al.* 2007). In France, the resistance to ciprofloxacin by *C. jejuni* from broilers decreased from 31% in 2002 to 9% in 2004, a decrease that has been suggested to be the benefits of policies limiting the use of antimicrobials in food animals (Gallay *et al.* 2007).

The incidence of tetracycline resistance was lower than the incidence recorded for organic or conventionally grown poultry in the United States (Luangtongkum *et al.* 2006), or in Canada, where more than 60% of the *C. jejuni* isolates from chickens are resistant to tetracycline and 8–40% are resistant to ciprofloxacin (Anon 2005b; Kos *et al.* 2006). The sequencing of *c.* 90% of the chromosomally encoded gene that confers tetracycline resistance confirmed that this gene is highly conserved in *C. jejuni* strains isolated from humans and domestic animals (Gibreel *et al.* 2004).

Studies that compared the restriction patterns of *Sma*I and *Kpn*I of human isolates of *C. jejuni* have concluded that *Kpn*I is the enzyme of choice for molecular epidemiology studies of *C. jejuni* (Michaud *et al.* 2001; Kärenlampi *et al.* 2003). However, we found that the use of *Sma*I appears to be better than *Kpn*I for PFGE analysis of *C. jejuni* from live broilers. *Sma*I always restricts more *C. jejuni* strains than *Kpn*I (Michaud *et al.* 2001), and it is the primary restriction enzyme for the protocols developed by the CDC, CampyNet (http://campynet.vetinst.dk/PFGE.html) and studies in Denmark (Nielsen *et al.* 2006). *Sal*I normally generates partial digest despite the experimental conditions (Ho and Monaco 1995), a feature that we noticed by the presence of unresolved fragments in the compression zone of the gels for all the strains. Therefore, this enzyme should be limited in its use for restriction profiling for fingerprinting purposes. The combination of restriction with *Sma*I followed by restriction with *Kpn*I resulted in a larger number of bands and resulted in more PFGE profiles (Table 5). In addition, the

Table 5 Pulsed-field gel electrophoresis (PFGE) macrorestriction profiles, sequence type (ST) allelic profiles and serotyping results of *Campylobacter jejuni* strains isolated from five different farms

	Isolation (day/place)			PFGE profiles					
Isolate ID	Day	Farm	House	*Sma*I	*Kpn*I	*Sal*I	*Sma*I/*Kpn*I	ST allelic profiles	Serotyping
F4-H1-S1	1	4	1	S-3	NR*	Sa-3	SK-4	2624	O:5
F5-H1-S1	1	5	1	S-1	NR	Sa-4	SK-3	48	O:5
F10-H1-S1	2	10	1	S-2	K-2	Sa-2	SK-5	2624	O:21
F10-H1-S2	2	10	1	S-2	K-4	Sa-2	SK-5	2624	O:21
F10-H1-S3	2	10	1	S-5	K-5	Sa-2	SK-8	2624	O:5
F10-H2-S4	2	10	2	S-6	K-6	Sa-2	SK-7	2624	O:3
F10-H2-S5	2	10	2	S-6	K-6	Sa-2	SK-7	2624	O:3
F11-H1-S1	3	11	1	S-6	K-7	Sa-2	SK-5	2624	ND†
F11-H1-S3	3	11	1	S-6	K-7	Sa-2	SK-7	2624	O:3
F11-H2-S4	3	11	2	S-6	K-7	Sa-2	SK-9	2624	O:3
F11-H2-S5	3	11	2	S-6	K-7	Sa-2	SK-7	2624	ND
F12-H1-S1	3	12	1	S-7	K-7	Sa-2	SK-7	2624	ND

*NR, Not restricted.
†ND, Serotyping not done.

combination of molecular methods demonstrated that more than one PFGE profile may be present in one farm, in different houses (F10-H1-S3 and F10-H2-S4) or in the same house or flock (F11-H2-S4 and F11-H2-S5) (Table 5). It is important to restrict the plugs first with *Sma*I and then with *Kpn*I to guarantee that a larger number of strains will be restricted. The increase in the number of fragments with the *Sma*I/*Kpn*I combination was more obvious in the area below 250 kbp (Fig. 2); an element that made the PFGE profiles more challenging to analyse using the visual scoring system suggested by Tenover *et al.* (1995). Therefore, the use of fingerprinting analysis software, such as BioNumerics, appears to be indispensable for a reliable analysis of the results obtained with this combination.

The lack of improvement by adding formaldehyde in the PFGE protocol strongly suggests that the lack of restriction patterns found for some isolates and specific enzymes is not related to the presence of DNAses in those strains (Gibson *et al.* 1994), but may be related to the lack of restriction sites or restriction site methylation. In large eukaryotic genomes ($>10^8$ base pairs), this lack of restriction, especially the CpG nucleotide deficiency, has been usually associated with DNA methylation, which is almost universal in these organisms (Bestor 1990). DNA methylation involves the addition of a methyl group to DNA bases, primarily to carbon #5 of the cytosine pyrimidine ring. This theory has traditionally been applied to explain the lack of restriction of bacterial genome. However, recent findings question this assertion and suggest that cytosine methylation is not the primary reason for the CpG dinucleotide deficiency in bacterial genomes (Wang *et al.* 2004).

PFGE restriction with *Sma*I has been reported to be more discriminating than MLST for outbreak investigations, although a combination of MLST plus sequencing of the *flaA* short variable region may provide a level of discrimination equivalent to PFGE for outbreak investigations (Sails *et al.* 2003). Our comparison of the PFGE, MLST and serotyping profiles of *C. jejuni* strains isolated in the same geographical area suggests that the PFGE profiles may change quickly within *C. jejuni* strains and may account for the variety of genotypes frequently found when analysing *C. jejuni* with PFGE (Table 5).

It is apparent that a ST profile of *C. jejuni*, or clone, appears to dominate in a geographic area for a variable period. The prevalent ST profile in our report is a new combination of previously described alleles. The other three MLST profiles found have been identified in *C. jejuni* isolated from humans, animals and the environment. ST-460 has been reported by the Centers for Disease Control and Prevention, and ST-48 and ST-353 were described in the United Kingdom (Dingle *et al.* 2001, 2002). ST-48 was the most predominant ST in a study done in New South Wales, Australia (Mickan *et al.* 2007), and has been associated to serotype O:4 of the Penner serotyping system (Penner and Hennessey 1980). ST-353 has been associated with O:3, O:11 and O:37 of the heat-resistant serotyping system (Dingle *et al.* 2002). In our study ST-48 was represented by O:3 and O:5 serotypes, while ST-353 was represented only by serotype O:3. ST-2624 was represented by all the serotypes found in this study, including the minor O:21.

Within the individual genes, *glnA* and *pgm* showed the highest diversity (four alleles) and the analysis of their sequence diversity showed a variability that has been described for other genes in *C. jejuni* (Suerbaum *et al.* 2001). Both the diversity within genes and the analysis of the allelic profile data using SplitsTree and the UPGMA

cluster analysis resulted in graphs with alleles connected to each other by multiple pathways, with a network-like structure suggesting recombination (Suerbaum *et al.* 2001). *pgm* has been used to analyse the recombination of *C. jejuni* from MLST data, and along with *unc* (*atpA*, ATP synthase alpha subunit) they appear to come to *C. jejuni* from other *Campylobacter* spp. (Fearnhead *et al.* 2005). In our study, the diversity of the allelic profiles for *unc* was minimal, with two alleles and only one base difference (C *vs* T) in base 864 of the *atpA* gene (NCBI accession number AL111168). This is different from the variety of allelic profiles, with multiple polymorphic sites, reported by French *et al.* (2005) for *unc* alleles analysed from *C. jejuni* associated to cattle in the United Kingdom. It appears that the allelic variability for each of the seven housekeeping genes for MLST profiles varies according to the origin of the strains and different geographical areas.

We calculated both the index of association (I_A), as described for the analysis of the degree of linkage in multilocus enzyme electrophoresis (Maynard Smith *et al.* 1993; Maynard Smith 1999), and the standardized I_A (sI_A) that corrects for the number of loci analysed (Haubold and Hudson 2000). The sI_A value of 0·73 and a calculated variance that was significantly higher than the expected variance ($P \leq 0{\cdot}0127$) suggested a high degree of recombination among the MLST genes within this population of *C. jejuni* isolates (Table 3).

MLST results have revealed that populations of different bacteria frequently exchange genetic material, and produce interclonal variance in virulence traits. These findings based on molecular population genetic studies have resulted in the tenet that the unit of bacterial pathogenicity is the clone, or cell line (Musser 1996). However, the analyses of linkage disequilibrium on comparison of gene trees have revealed that few bacteria are indeed clonal (Maynard Smith *et al.* 1993; Spratt and Maiden 1999; Feil *et al.* 2001; Supply *et al.* 2003). In *C. jejuni*, a high frequency of inter- and intraspecies recombination may account for the high degree of variation (addition and or deletions) of restriction sites and PFGE profiles found in *C. jejuni* isolated from live broilers chickens (Dingle *et al.* 2001; Schouls *et al.* 2003; Potturi-Venkata *et al.* 2007a,b) and retail broiler products (Dickins *et al.* 2002; Oyarzabal *et al.* 2007a). Therefore, MLST profiling, although useful in determining temporal phylogenetic comparison, may be less discriminating for spatial, short-term epidemiological studies of *C. jejuni* strains from live broilers. The fact that we found only four ST allelic profiles, with one being completely new, *vs* 10 different PFGE profiles suggest that microrestriction profiling is still a powerful system for the tracking of *C. jejuni* strains.

Most of the studies of cytotoxicity on *Campylobacter* spp. make use of tissue culture cell models, with a subjective, qualitative characterization of the degree of morphological cell rounding. We measured instead the supernatant LDH that was released upon cell lysis. Our results measuring LDH from the cytotoxicity assays in cell lines showed a wide range of variation that has been described using different mammalian cell lines on *C. jejuni* isolated from different sources, including broiler chickens (Gilbert and Slavik 2004; Coote *et al.* 2007). The variability of these assays is dependent on the way the assays are performed, may appear to even change with strains over time (Coote *et al.* 2007), and demonstrate that cytotoxin production by *C. jejuni* is quite complex when compared with that of other enteric pathogens (Misawa *et al.* 1995). Our results showed that some strains had a high agreement among the results from the same cell line, while other strains showed no agreement among the results from the same cell line. The number of isolates that grouped in the low cytotoxicity category was much smaller than previously reported (Gilbert and Slavik 2004). Although there is no direct correlation between the pathogenicity of *C. jejuni* and the presence of the toxins (Lam 1993), cytotoxicity assays have been used to infer the potential pathogenicity of *C. jejuni* isolates. For instance, there is an important correlation between invasiveness and colitis in patients from which the isolates were obtained, although some strains attach to cell lines but do not invade the cells (Everest *et al.* 1992). The findings of low toxicity reported with CHO (Nadeau *et al.* 2003; Coote *et al.* 2007) do not correlate with the present results.

Globally, there are differences in *Campylobacter* antigens and on the typability of *C. jejuni* strains with Penner antisera and it is common to encounter strains that are untypable with this method (Eyles *et al.* 2006; Nielsen *et al.* 2006). Some of the strains from this study were not recognized by any antisera, and a weak serotyping found with O:21 makes this serotype less significant, as the titre was too low to be compared with the titres obtained with O:3 and O:5. Serotype O:5 has been found in different geographical areas, and has recently been described in *C. jejuni* isolates from humans and water sources in New Zealand (Eyles *et al.* 2006). *Campylobacter jejuni s*erotypes O:3 and O:5 have been found in the blood cultures and diarrhoetic stools of paediatric patients (Lastovica 1996). Serotype O:21 has been also found in paediatric diarrhoeic patients, in chickens without disease and in terminally ill ostriches with enteritis in South Africa (A.J. Lastovica, unpublished results).

The molecular characterization of bacterial pathogens is essential to understand the short- and long-term epidemiological implications of a given foodborne pathogen (Spratt and Maiden 1999). The long-term goal of an active surveillance programme is to predict, with high degree of certainty, the rise and spread of an epidemic clone with high pathogenicity potential for humans

(Maynard Smith *et al.* 1993). For weakly clonal organisms molecular methods have yielded different data, and there appears to be a lack of agreement among methods applied to the same group of strains. For instance, different databases have been collected and maintained for *C. jejuni*, such as fingerprinting profiles based on PFGE or sequencing data based on MLST. MLST results in 100% typability of the isolates, but the cost of this method (Dingle *et al.* 2002) and the apparent low number of alleles found in a relatively small population of isolates may limit its application for epidemiological studies. Yet, the prediction of the appearance of epidemic clones in a given geographic area may still be limited even if an active surveillance is in place. The difficulty in interpretation of a wide range of results is a severe limitation in studies on pathogens with high capacity for horizontal gene transfer (Oyarzabal *et al.* 2007b), where even typability results may vary according to the different methods. The small population of *C. jejuni* that we sampled may be a limiting factor of this study, but at the same time exemplifies some of the limitation of each of the methods employed for strain characterization. The diversity found within *C. jejuni* and the lack of agreement among the different methods suggests that studies of larger populations of strains will result in more complex information to analyse. In conclusion, these results suggest that the use of more than two typing methods may be necessary to fully understand the variability of *C. jejuni* strains colonizing commercial broilers in Puerto Rico.

Acknowledgements

A.J. Lastovica gratefully acknowledges the financial support of the South African Medical Research Council. The work of S. Backert is supported by SafeFoodera program and BMBF (NBL3-2, 01ZZ0407). This publication made use of the *C. jejuni* MLST website (http://pubmlst.org/campylobacter/) developed by Keith Jolley and Man-Suen Chan, sited at the University of Oxford, and funded by the Wellcome Trust.

References

Anon. (2005a) *Report on the consumption of broiler meat in Puerto Rico*. Santurce, P.R.: Office of Agricultural Statistics, Department of Agriculture of Puerto Rico.

Anon. (2005b) *Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), Preliminary Results*. Minister of Public Works and Government Services Canada, Ottawa, Ontario K1A 0S5. Cat No.: HP2-4/2005-1E-PDF.

Anon. (2007) PulseNet Protocols. *Campylobacter jejuni*. http://www.cdc.gov/pulsenet/protocols.htm.

Bandelt, H.J. and Dress, A.W. (1992) Split decomposition: a new and useful approach to phylogenetic analysis of distance data. *Mol Phylogenet Evol* **1**, 242–252.

Bestor, T.H. (1990) DNA methylation: evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. *Phil Trans R Soc Lond B* **326**, 179–187.

Butzler, J. and Oosterom, J. (1991) *Campylobacter*: pathogenicity and significance in foods. *Int J Food Microbiol* **12**, 1–8.

Clinical and Laboratory Standards Institute (2006) *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Approved standard M45-A*. Wayne, PA: Clinical and Laboratory Standards Institute.

Coote, J.G., Stewart-Tull, D.E.S., Owen, R.J., Bolton, F.J., Siemer, B.L., Candlish, D., Thompson, D.H., Wardlaw, A.C. *et al.* (2007) Comparison of virulence-associated *in vitro* properties of typed strains of *Campylobacter jejuni* from different sources. *J Med Microbiol* **56**, 722–732.

Corkill, J.E., Graham, R., Hart, C.A. and Stubbs, S. (2000) Fast-track communication pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. *J Clin Microbiol* **38**, 2791–2792.

De Boer, P., Duim, B., Rigter, A., van der Plas, J., Jacobs-Reitsma, W. and Wagenaar, J.A. (2000) Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. *J Clin Microbiol* **38**, 1940–1946.

Dickins, M.A., Franklin, S., Stefanova, R., Shutze, G.E., Eisenach, K.D., Wesley, I.V. and Cave, D. (2002) Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-filed gel electrophoresis. *J Food Protect* **65**, 957–962.

Dingle, K.E., Colles, F.M., Wareing, D.R., Ure, R., Fox, A.J., Bolton, F.E., Bootsma, H.J., Willems, R.J. *et al.* (2001) Multilocus sequence typing system for *Campylobacter jejuni*. *J Clin Microbiol* **39**, 14–23.

Dingle, K.E., Colles, F.M., Ure, R., Wagenaar, J.A., Duim, B., Bolton, F.J., Fox, A.J., Wareing, D.R.A. *et al.* (2002) Molecular characterization of *Campylobacter jejuni* clones: a basis for epidemiologic investigation. *Emerg Infect Dis* **8**, 949–955.

Everest, P.H., Goosens, H., Butzler, J.P., Lloyd, D., Knutton, S., Ketley, J.M. and Williams, P.H. (1992) Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. *J Med Microbiol* **37**, 319–325.

Eyles, R.F., Brooks, H.J.L., Townsend, C.R., Burtenshaw, G.A., Heng, N.C.K., Jack, R.W. and Weinstein, P. (2006) Comparison of *Campylobacter jejuni* PFGE and Penner subtypes in human infections and in water samples from the Taieri River catchment of New Zealand. *J Appl Microbiol* **101**, 18–25.

Fearnhead, P., Smith, N.G.C., Barrigas, M., Fox, A. and French, N. (2005) Analysis of recombination in *Campylobacter jejuni* from MLST population data. *J Mol Evol* **61**, 333–340.

Feil, E.J., Holmes, E.C., Bessen, D.E., Chan, M.-S., Day, N.P., Enright, M.C., Goldstein, R., Hood, D.W. *et al.* (2001) Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proc Natl Acad Sci USA* **98**, 182–187.

French, N.P., Barrigas, M., Brown, P., Ribiero, P., Williams, N.J., Leatherbarrow, H., Birtles, R., Bolton, E. *et al.* (2005) Spatial epidemiology and natural population structure of *Campylobacter jejuni* colonizing a farmland ecosystem. *Environ Microbiol* **7**, 1116–1126.

Gallay, A., Prouzet-Mauléon, V., Kempf, I., Lehours, P., Labadi, L., Camou, C., Denis, M., de Valk, H. *et al.* (2007) *Campylobacter* antimicrobial drug resistance among humans, broiler chickens, and pigs, France. *Emerg Infect Dis* **13**, 259–266.

Gaudreau, C. and Gilbert, H. (1997) Comparison of disc diffusion and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli*. *J Antimicrob Chemother* **39**, 707–712.

Gibreel, A., Tracz, D.M., Nonaka, L., Ngo, T.M., Connell, S.R. and Taylor, D.E. (2004) Incidence of antibiotic resistance in *Campylobacter jejuni* isolated in Alberta, Canada, from 1999 to 2002, with special reference to *tet*(O)-mediated tetracycline resistance. *Antimicrob Agents Chemother* **48**, 3442–3450.

Gibson, J.R., Sutherland, K. and Owen, R.J. (1994) Inhibition of DNAase activity in PFGE analysis of *Campylobacter jejuni*. *Lett Appl Microbiol* **19**, 357–358.

Gilbert, C. and Slavik, M. (2004) Determination of toxicity of *Campylobacter jejuni* isolated from humans and from poultry carcasses acquired at various stages of production. *J Appl Microbiol* **97**, 347–353.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Haubold, B. and Hudson, R.R. (2000) LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage analysis. *Bioinformatics* **16**, 847–848.

Ho, M.F. and Monaco, A.P. (1995) PFGE in physical mapping. In *Pulsed Field Gel Electrophoresis, A Practical Approach* ed. Monaco, A.P. pp. 21–43 New York, NY: Oxford University Press Inc.

Hu, L., Bray, M.D., Osorio, M. and Kopecko, D.J. (2006) *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infect Immun* **74**, 2697–2705.

Hudson, D.H. (1998) SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73.

Hutchinson, D.N. and Bolton, F.J. (1984) An improved blood-free selective medium for isolation of *Campylobacter jejuni* from faecal specimens. *J Clin Pathol* **37**, 956–995.

Kärenlampi, R., Rautelin, H., Hakkinen, M. and Hänninen, M.-L. (2003) Temporal and geographical distribution and overlap of Penner heat-stable serotypes and pulsed-field gel electrophoresis genotypes of *Campylobacter jejuni* isolates collected from humans and chickens in Finland during a seasonal peak. *J Clin Microbiol* **41**, 4870–4872.

Kärenlampi, R., Rautelin, H. and Hänninen, M.-L. (2007) Evaluation of genetic markers and molecular typing methods for prediction of sources of *Campylobacter jejuni* and *C. coli* infections. *Appl Environ Microbiol* **73**, 1683–1685.

Kelly, J.K. (1997) A test of neutrality based on interlocus associations. *Genetics* **146**, 1197–1206.

Kos, V.N., Keelan, M. and Taylor, D.E. (2006) Antimicrobial susceptibilities of *Campylobacter jejuni* isolates from poultry from Alberta, Canada. *Antimicrob Agents Chemother* **50**, 778–780.

Lam, K.M. (1993) A cytotoxicity test for the detection of *Campylobacter jejuni* toxin. *Vet Microbiol* **35**, 133–139.

Lastovica, A.J. (1996) Serotype distribution of isolates of *C. jejuni* subsp. *jejuni, C. jejuni* subsp. *doylei, C. coli* and *C. upsaliensis* from pediatric enteritis patients. In *Campylobacter, Helicobacters and Related Organisms* ed. Newell, D.G., Ketley, J.M. and Feldman, R.A. pp. 481–485 New York, NY: Plenum Press.

Lastovica, A.J., Marshall, R.B. and Penner, J.L. (1986) *Campylobacter* serotyping on shipped antigenic extracts. *Lancet* **1**, 320.

Lévesque, S., Frost, E. and Michaud, S. (2007) Comparison of antimicrobial resistance of *Campylobacter jejuni* isolated from humans, chickens, raw milk, and environmental water in Québec. *J Food Protect* **70**, 729–735.

Lopez-Ortiz, W. and Solivan, R.A. (1999) *Campylobacter jejuni* among patients with gastroenteritis: incidence at a reference microbiology laboratory in San Juan, Puerto Rico. *P R Health Sci J* **18**, 273–276.

Luangtongkum, T., Morishita, T.Y., Ison, A.J., Huang, S., McDermott, P.F. and Zhang, Q. (2006) Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of *Campylobacter* spp. in poultry. *Appl Environ Microbiol* **72**, 3600–3607.

Luangtongkum, T., Morishita, T.Y., El-Tayeb, A.B., Ison, A.J. and Zhang, Q. (2007) Comparison of antimicrobial susceptibility testing of *Campylobacter* spp. by the agar dilution and the agar disk diffusion methods. *J Clin Microbiol* **45**, 590–594.

Maldonado, Y., Fiser, J.C., Nakatsu, H.C. and Bhunia, A.K. (2005) Cytotoxicity potential and genotypic characterization of *Escherichia coli* isolates from environmental and food sources. *Appl Environ Microbiol* **71**, 1890–1898.

Maynard Smith, J. (1999) The detection and measurement of recombination from sequence data. *Genetics* **153**, 1021–1027.

Maynard Smith, J., Smith, N.H., O'Rourke, M. and Spratt, B.G. (1993) How clonal are bacteria? *Proc Natl Acad Sci USA* **90**, 4384–4388.

Michaud, S., Menard, S., Gaudreau, C. and Arbeit, R.D. (2001) Comparison of *Sma*I-defined genotypes of *Campylobacter jejuni* examined by *Kpn*I: a population-based study. *J Med Microbiol* **50**, 1075–1081.

Mickan, L., Doyle, R., Valcanis, M., Dingle, K.E., Unicomb, L. and Lanser, J. (2007) Multilocus sequence typing of *Campylobacter jejuni* isolates from New South Wales, Australia. *J Appl Microbiol* **102**, 144–152.

Misawa, N., Ohnishi, T., Itoh, K. and Takahashi, E. (1995) Cytotoxin detection in *Campylobacter jejuni* strains of human and animal origin with three tissue culture systems. *J Med Microbiol* **43**, 354–359.

Musser, J.M. (1996) Molecular population genetic analysis of emerged bacterial pathogens: selected insights. *Emerg Infect Dis* **2**, 1–17.

Nadeau, E., Messier, S. and Quessy, S. (2002) Prevalence and comparison of genetic profiles of *Campylobacter* strains isolated from poultry and sporadic cases of campylobacteriosis in humans. *J Food Prot* **65**, 73–78.

Nadeau, E., Messier, S. and Quessy, S. (2003) Comparison of *Campylobacter* isolates from poultry and humans: association between *in vitro* virulence properties, biotypes and pulse-field gel electrophoresis clusters. *Appl Environ Microbiol* **69**, 6316–6320.

Nielsen, E.M., Fussing, V., Engberg, J., Nielsen, N.L. and Neimann, J. (2006) Most *Campylobacter* subtypes from sporadic infections can be found in retail poultry products and food animals. *Epidemiol Infect* **134**, 758–767.

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. (2007a) 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., Rad, R. and Backert, S. (2007b) Conjugative transfer of a chromosomally-encoded antibiotic resistance from *Helicobacter pylori* into *Campylobacter jejuni*. *J Clin Microbiol* **45**, 402–408.

Penner, J.L. and Hennessey, J.N. (1980) Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. *J Clin Microbiol* **12**, 732–737.

Potturi-Venkata, L.-P., Backert, S., Vieira, S.L. and Oyarzabal, O.A. (2007a) Evaluation of logistic processing to reduce cross-contamination of commercial broiler carcasses with *Campylobacter* spp. *J Food Prot* **70**, 2549–2554.

Potturi-Venkata, L.P., Backert, S., Lastovica, A.J., Vieira, S.L., Norton, R.A., Miller, R.S., Pierce, S.J. and Oyarzabal, O.A. (2007b) Evaluation of different plate media for direct cultivation of *Campylobacter* spp. from live broilers. *Poul Sci* **86**, 1304–1311.

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.

Rodrigo, S., Adesiyun, A., Asgarali, Z. and Swanston, W. (2007) Antimicrobial resistance of *Campylobacter* spp. isolated from broilers in small poultry processing operations in Trinidad. *Food Cont* **18**, 321–325.

Rozas, J., Gullaud, M., Blandin, G. and Aguadé, M. (2001) DNA variation at the rp49 gene region of *Drosophila simulans*: evolutionary inferences from an unusual haplotype structure. *Genetics* **158**, 1147–1155.

Sails, A.D., Swaminathan, B. and Fields, P.I. (2003) Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by *Campylobacter jejuni*. *J Clin Microbiol* **41**, 4733–4739.

Schouls, L.M., Reulen, S., Duim, B., Wagenaar, J.A., Willems, R.J.L., Dingle, K.E., Colles, F.M. and Van Embden, J.D.A. (2003) Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. *J Clin Microbiol* **41**, 15–26.

Spratt, B.G. and Maiden, M.C.J. (1999) Bacterial population genetics, evolution and epidemiology. *Phil Trans R Soc Lond B Biol Sci* **354**, 701–710.

Stern, N.J., Fedorka-Cray, P., Bailey, J.S., Cox, N.A., Craven, S.E., Hiett, K.L., Musgrove, M.T., Ladely, S. *et al.* (2001) Distribution of *Campylobacter* spp. in selected U.S. poultry production and processing operations. *J Food Prot* **64**, 1705–1710.

Suerbaum, S., Lohrengel, M., Sonnevend, A., Ruberg, F. and Kist, M. (2001) Allelic diversity and recombination in *Campylobacter jejuni*. *J Bacteriol* **183**, 2553–2559.

Supply, P., Warren, R.M., Bañuls, A.-L., Lesjean, S., van der Spuy, G.D., Lewis, L.-A., Tibayrenc, M., van Heldenand, P.D. *et al.* (2003) Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. *Mol Microbiol* **47**, 529–538.

Tamura, K., Dudley, J., Nei, M. and Kumar, S.(2007) *MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution*, doi:10.1093/molbev/msm092. [Online.] http://www.megasoftware.net. Accessed 23 September 2007.

Tauxe, R. (1997) Emerging foodborne diseases: an evolving public health challenge. *Emerg Infect Dis* **3**, 425–434.

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.

Wang, Y., Rocha, E.P.C., Leung, F.C.C. and Danchin, A. (2004) Cytosine methylation is not the major factor inducing CpG dinucleotide deficiency in bacterial genomes. *J Mol Evol* **58**, 692–700.