EVALUATION OF SUBTYPING METHODS FOR THE CHARACTERIZATION OF *CAMPYLOBACTER* STRAINS FROM DIFFERENT GEOGRAPHICAL AREAS

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EVALUATION OF SUBTYPING METHODS FOR THE CHARACTERIZATION OF *CAMPYLOBACTER* STRAINS FROM DIFFERENT GEOGRAPHICAL AREAS

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A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
December 19, 2008

EVALUATION OF SUBTYPING METHODS FOR THE CHARACTERIZATION OF *CAMPYLOBACTER* STRAINS FROM DIFFERENT GEOGRAPHICAL AREAS

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Robert Scott Miller graduated from the College of Science and Mathematics at Auburn University with a Bachelor of Science degree in Microbiology in August 2003. After degree completion he worked as a Research Assistant II from October 2003 until the present date in the Poultry Science Department's Microbiology Laboratory at Auburn University. Robert married Jennifer Jones in September 1995. They have two children, Robert Sean Miller and Elizabeth Nicole Miller.

THESIS ABSTRACT

EVALUATION OF SUBTYPING METHODS FOR THE CHARACTERIZATION OF *CAMPYLOBACTER* STRAINS FROM DIFFERENT GEOGRAPHICAL AREAS

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Master's of Science, December 19, 2008
(B.S., Auburn University, 2003)

112 Typed Pages

Directed by Omar A. Oyarzabal

Bacterial foodborne diseases are a common cause of lost productivity for industrially developed nations. In developing nations, these illnesses can be deadly for the young, elderly, and immunocompromised. The ability to accurately characterize the bacteria responsible for foodborne disease is a very important tool for researchers and public health providers.

Reported data shows that *Campylobacter* is one of the most common causes of acute bacterial enteritis globally. The purpose of this study was to evaluate molecular typing techniques used to characterize *Campylobacter* strains. To this end, 30 *C. jejuni* strains from Puerto Rico, 56 *C. jejuni* and *C. coli* strains from Grenada, and 55 *C. jejuni* and *C. coli* strains from the southeastern US were evaluated with pulse-field gel

electrophoresis (PFGE), multilocus sequence typing (MLST), and flagellin A restriction fragment length polymorphism (*fla*A-RFLP). *Fla*A-RFLP was the less expensive method and required less labor than PFGE, but the discriminatory power was low and *C. jejuni* and *C. coli* strains grouped together after the analysis. PFGE proved to be the most discriminatory, but most labor intensive, method. PFGE's limitation as a characterization technique is the failure to restrict some *Campylobacter* genomes due to DNA methylation or lack of restriction site. MLST, while lacking PFGE's discriminatory power, can characterize all strains and may provide the best tool for characterization of *Campylobacter* in temporal, epidemiological studies.

This research suggests that a combination of two of the three techniques, depending on the purpose of the characterization, is the best approach when employing molecular typing methods to characterize *C. jejuni* and *C. coli*.

ACKNOWLEDGMENTS

My special thanks to Dr. Omar A. Oyarzabal for providing the opportunity and much needed encouragement to earn my master's degree. I also want to thank my committee members, Dr. Sandra J. Ewald and Dr. Jong Wook Hong for their time and support. Thanks to Fernando Rebollo-Carratto for providing broiler fecal samples from Peurto Rico, Harry Hariharan for *Campylobacter* strains from Grenada, Dr. Lee Zhang of the Auburn University Genetics Analysis Laboratory, and Nick Hermersmann of Lucigen Corporation for providing DNA sequencing.

I would be remiss if I did not thank the following students that worked alongside me in the laboratory for their assistance: Sarah Pierce, Leslie Speegle, Ami Sumners, Megan Behringer, and Anna Stanford. Your help was invaluable.

There is no way to express my thanks to my wife, Jennifer, and our children, Sean and Elizabeth, for their support and love. This graduate program has been a labor of love for all of us. Thank you for your understanding.

I dedicate this thesis and my degree to my parents, Robert Stanford and Kathy Diane Miller. You built the foundation that led to this accomplishment the first time you told me that I could accomplish anything I was willing to work toward. You have always made the most of every blessing you received. Thank you for passing that on to Tim and me. But perhaps most importantly thanks for loving me through everything, even when I would not listen to your advice.

Style manual or journal used Journal of Food Protection.

Computer software used Microsoft Office 2007, Bionumerics version 4.50 (Applied Maths).

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I. LITERATURE REVIEW

Foodborne Diseases

Foodborne diseases are illnesses caused by infectious or toxic substances that enter the body through ingestion of food or beverage. Over 250 foodborne diseases are known, many of which are caused by microbial pathogens, such as bacteria, viruses, fungi, protozoa, and multicellular parasites. The Centers for Disease Control and Prevention (CDC) estimates 76 million cases of foodborne disease every year in the US, leading to 325,000 hospitalizations and 5,000 deaths (Anonymous. 2005). The estimated cases of foodborne illness in the US caused by known pathogens are listed in Table 1. Worldwide estimates of foodborne diseases are difficult to determine, but the World Health Organization (WHO) estimates that 1.8 million deaths worldwide are attributed to foodborne diseases every year (Anonymous. 2007).

Between 1993 and 1997, the CDC reported 2,751 outbreaks of foodborne diseases (Lynch 2006), with an increase in the number of outbreaks to 6,647 between 1998 and 2002 (Olsen 2002). Bacteria were the most common cause of foodborne outbreaks in both studies, with an incidence of 55% in the period 1998-2002 and 75% in the period 1993-1997 (Lynch 2006; Olsen 2002). It is estimated that foodborne disease outbreaks linked to five bacterial pathogens, *Campylobacter, Salmonella, Escherichia coli* O157:H7, *E. coli* non-O157:H7STEC, and *Listeria,* lead to approximately $6.9 billion in

medical costs, lost productivity, and premature death every year in the US alone (Buzby 2001).

The *Campylobacter* Genus

In 1866, Theodore Escherich described nonculturable spiral bacteria found in stool specimens and large intestinal mucosa of neonates and kittens suffering from diarrhea. The failure of the bacteria to grow on solid media after microscopic detection and typical morphology associated with gastroenteritis lead many researchers to believe that the bacteria Escherich described in 1866 was indeed *Campylobacter* spp. (Kist 1986). McFadyean and Stockmen, veterinary surgeons in Great Britain, reported a bacterium that resembled a vibrio in aborted ovine fetuses. The scientific name, *Vibrio fetus*, would not appear for another decade. Over the years, more species and subspecies of *Vibrio* were described, with isolation from human blood occurring in 1938, 1947, and 1958 (Butzler 2004). In 1963, the *Vibrio* fetus and *Vibrio* jejuni were transferred to the newly created *Campylobacter* genus (On 2001). The *Campylobacter* genus is currently comprised of 16 species and 6 subspecies (http://www.the-icsp.org/taxa/Campylobacterlist.htm). Table 2 shows the current taxonomic listing for the *Campylobacter* genus.

Epidemiology of *Campylobacter*

Campylobacter spp. has been isolated from various wild and domesticated animals. Specifically, *C. jejuni and C. coli* have been shown to cause disease in cats, dogs, ferrets, mink, primates, hamsters, guinea pigs, rats, sheep, pigs, and cattle. While *C. jejuni* rarely

causes disease in birds, it appears that birds have a higher carriage rate of *C. jejuni* than other animals (Young and Mansfield 2005). Due to this domestic poultry are the most important *C. jejuni* reservoir for humans with chicken being the primary source for human infection (Allos 2001).

Research to reduce *Campylobacter* colonization in chicken has been stymied by the lack of an obvious route of transmission to negative flocks. A study by Callicott et al. in 2006 is one of several that suggest that vertical transmission is not responsible for infection of broiler flocks. The horizontal transmission from environmental sources has been suspected for sometime but are not very well understood.

Using molecular techniques to determine strain relation can be difficult due to *C.jejuni*'s weak clonality and inter- and intraspecies horizontal genetic exchange (Suerbaum 2001, Dingle 2001).

Also contributing to the difficulties of understanding *Campylobacter* epidemiology is the lack of a single molecular subtyping method capable of providing comprehensive characterization with a proper degree of discrimination for all *C. jejuni* and *C. coli* strains while targeting appropriate genetic variation for a given study (i.e. relationship of recent outbreak strain versus longer term evolutionary studies).

It is essential to understand a few terms common to epidemiology and molecular subtyping before proceeding into a discussion of molecular subtyping methods. An **isolate** is a population of microbial cells in pure culture derived from a single colony on an isolation plate and identified to the species level (Riley 2004, text).

A **strain** is an isolate or group of isolates exhibiting phenotypic and/or genotypic traits belonging to the same lineage, distinct from those of other isolates of the same species

(Riley 2004, text). A **clone** is an isolate or group of isolates descending from a common precursor strain by nonsexual reproduction exhibiting phenotypic or genotypic traits characterized by a strain-typing method to belong to the same group (Riley 2004, text).

Methods for molecular subtyping of bacteria

Molecular subtyping is the characterization of proteins or nucleic acids to aid in epidemiological investigations (Swaminathan 2001). Researchers use molecular subtyping to determine the relatedness of bacterial strains. Understanding strain relatedness aids in taxonomic classification of organisms into related groups, phylogenetic analysis or evolutionary development of an organism, and epidemiological studies of the distribution and determinants of disease (Riley 2004).

Currently practiced molecular subtyping techniques can be organized into four different categories: hybridization and amplification, amplification and restriction, restriction and migration, and sequencing. Polymerase chain reaction (PCR) is an example of hybridization and amplification. Flagellin A restricted fragment length polymorphism (*fla*A-RFLP) utilizes amplification and restriction. Pulsed field gel electrophoresis (PFGE) relies on restriction and migration while multilocus sequence typing (MLST) is based on sequencing.

To understand how molecular subtyping techniques are evaluated some important terms must be defined. **Typeability** is the proportion of strains that is unambiguously typed by the subtyping method. **Reproducibility** is the percent of strains classified as the same subtype upon repeated testing. **Discriminatory power** is the ability of a technique to generate discrete units of information from different isolates at the sub-species level

(Riley 2004). The **discriminatory power** (D_i) of a method is based on its ability to differentiate between unrelated strains and is determined by the number of types assigned by the method and the relative frequency of those types (Hunter 1988).

Since the early 1980s, many techniques for molecular subtyping have been proposed and used with differing levels of success. This thesis will present the results of the evaluation of three subtyping techniques, *fla*A-RFLP, PFGE, and MLST applied to *Campylobacter jejuni* and *C. coli* strains from different geographical areas.

Flagellin A restriction fragment length polymorphism (*fla*A-RFLP)

*Fla*A-RFLP is a typing system based on both sequence variation and restriction. *Fla*A-RFLP was one of the first subtyping techniques used on *Campylobacter* spp. (Nachamkin et al., 1993). *Fla*A-RFLP involves amplification with a polymerase chain reaction assay (PCR) and subsequent digestion of the flagellin A subunit gene with *Dde*I. After standard electrophoresis separation, the bands are stained and visualized with ultraviolet light.

*Fla*A-RFLP has several advantages, such as low cost, simple PCR protocols for amplification and use of traditional electrophoresis for the capturing of the results. One of the disadvantages of this technique is the low discriminatory power, which is usually less than PFGE (Neilsen 2000). Another disadvantage relates to the inconsistencies in the mobility of the DNA fragments in the gel, a limitation of any technique based on the separation of the fragments using gel electrophoresis (Meinersmann 1997).

Pulsed-field gel electrophoresis (PFGE)

PFGE typing relies on macrorestriction of the entire bacterial genome by infrequent cutting endonucleases. In the case of *C. jejuni*, the genome size is ~1.7 Mb and several enzymes can produce discernable bands by this technique. In the US, *Sma*I is the endonuclease most commonly used to restrict *Campylobacter* strains (Ribot, 2001), and is the endonuclease preferred by CDC's Pulsenet PFGE protocol for *Campylobacter jejuni* (Anonymous 2008). CampyNet, the European counterpart of Pulsenet, initially proposed the use of *Kpn*I as the primary endonuclease for PFGE digestion (Michaud 2001), but now it recommends the restriction with *Sma*I (Gilpin 2006).

Until 1984, researchers were unable to differentiate fragments of DNA larger than 40 kilobases by standard electrophoresis methods (Schwartz and Cantor, 1984). By applying voltage gradients at various angles and for various lengths of time, Schwartz and Cantor proved that large fragments of DNA can move through an agarose gel made at higher concentrations than traditional gel electrophoresis. Two years later, the countour-clamped homogeneous electric field (CHEF) apparatus was designed by Chu et al. (1986). This machine allows for 120-degree-angle reorientation of the DNA bands between 24 electrodes organized as a hexagon as they travel through the gel.

PFGE has since been used to type many pathogenic and nonpathogenic microorganisms, and is considered the "gold standard" by epidemiologists for bacterial subtyping. Two advantages of typing with PFGE are a high level of discrimination and reproducibility among inter and intra-laboratory patterns when following the standardized protocols. Some of the disadvantages are the cost of the equipment, the time required to

obtain the results, the high training needed to run the technique, and the fact that some strains do not restrict with current enzymes used in standard PFGE protocols.

Multilocus sequence typing (MLST)

Developed by Maiden et. al (1997), MLST is a variation on the multilocus enzyme electrophoresis (MLEE) technique developed in the 1980s (Maiden 1998). MLST operates under the same philosophy as MLEE by indexing neutral genetic variation from multiple chromosome locations (alleles) while exploiting nucleotide sequencing to identify this variation (Dingle 2001). The advantage of MLST over MLEE is that it determines variation in internal fragments of allele sequences rather than determining allele variation by enzyme mobilities through electrophoresis. MLEE, like other traditional and molecular typing schemes, is not portable because the indexed variation is difficult to compare among laboratories. MLST takes advantage of the electronic portability of sequence data, and allows for exchange, over the Internet, of molecular typing data for global epidemiology between laboratories (Maiden 1998).

For *Campylobacter* typing, MLST involves the sequencing of internal fragments of seven housekeeping genes. The sequences are compared with previously identified sequences, or alleles, at that locus and are assigned allele numbers at each of the seven loci. The seven allele numbers define the strain's allelic profile. Each allelic profile is assigned a sequence type (ST) which is used to describe the strain (Aanensen 2005). Sequence types are then grouped in clonal complexes based on their sequence similarities (Miller 2005).

Housekeeping loci evolve slowly because they are under stabilizing selection for conservation of metabolic function. Therefore, by indexing nucleotide sequence

variations, MLST provides highly discriminated data, and also enables the population structure of an organism to be established. Consequently, these data are not sensitive to the frequent chromosome rearrangements seen in *C. jejuni*, which adversely affects typing techniques such as PFGE (Dingle 2002).

MLST offers several advantages over other subtyping methods. Minimal experimental variation in results facilitates interlaboratory comparisons and the establishment of a standard nomenclature for DNA sequence data. Unlike gel images, sequence data provide information on the precise differences between strains (Sails 2003). MLST also requires much less time to perform than PFGE, the current gold standard for epidemiological studies, but perhaps the greatest benefit of MLST is its ability to type all strains.

Because MLST employs sequence variation in housekeeping genes, it is best suited for long term epidemiology studies, since it identifies variation building slowly within a population (Dingle 2001). Therefore MLST, when used alone, has less discriminatory power than PFGE for short term epidemiological studies. According to Sails et al. (2003), MLST's D_I=0.859 is lower than PFGE's D_I=0.930.

Analysis of molecular subtyping data

There are two major approaches for the classification of strains, cladisitic and phenetic. The **cladistic** approach determines evoluntionary relationships of strains based on shared derived characteristics in extant taxa. The **phenetic** approach groups strains based on similarities or differences without drawing evolutionary connections. Mathematical

procedures can then be used to evaluate characteristics that are numerically encoded (Riley 2004b). The phenetic approached will be used in this thesis.

Both phenetic and cladistic approaches order each taxon in terms of its character states which represent a range of particular characters of a specific taxon. The basic unit of the phenetic approach is the OTU or **operational taxonomic unit**. An OTU is defined as a strain or group of strains specified by a particular set of character states. OTUs similarities or distances can be determined by applying mathematical models (Riley 2004b).

Cluster analysis or the analysis of relations between many strains, rather than pairs of strains, allows for the grouping of strains by a heirarchic structure into phenograms. In MLST, cluster analysis is achieved through nearest-neighbor clustering (also called neighbor-joining clustering). Nearest neighbor cluster analysis constructs phenograms using a similarity matrix derived from sequential pairwise comparisons of the two most similar OTUs or clusters of OTUs (Riley 2004b).

Electrophoretic fingerprint patterns, like those derived from PFGE and RFLP, are most commonly clustered using unweighted pair-group method using arithmetic averages (UPGMA), although the neighbor-joining clustering can also be used. UPGMA creates phenogams by calculating the average distance between one cluster of OTUs to a comparison cluster, where each OTU is given equal weight (Riley 2004b). Throughout this thesis, UPGMA analysis will be used to evaluate PFGE and *Fla*-A-RFLP data, while neighbor-joining will be used for the analysis of MLST data.

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Table 1 *Campylobacter* taxa (2007)

Campylobacter jejuni	*Campylobacter concisus*
C. jejuni subsp. *jejuni*	*Campylobacter upsaliensis*
C. jejuni subsp. *doylei*	*Campylobacter curvus*
C. jejuni subsp. fetus	*Campylobacter rectus*
Campylobacter coli	*Campylobacter helveticus*
Campylobacter lari	*Campylobacter lanienae*
Campylobacter fetus	*Campylobacter mucosalis*
C. fetus subsp. *fetus*	*Campylobacter showae*
C. fetus subsp. *venerealis*	*Campylobacter hominis*
Campylobacter hyointestinalis	*Campylobacter gracilis*
C. hyointestinalis subsp. l*awsonii*	*Campylobacter sputorum*
C. hyointestinalis subsp. h*yointestinalis*	*C. sputorum* subsp. *bubulus*
	C. sputorum subsp. *sputorum*

II. MOLECULAR TYPING OF *CAMPYLOBACTER JEJUNI* STRAINS ISOLATED FROM COMMERCIAL BROILERS IN PUERTO RICO

Introduction

Little information is available about human campylobacteriosis in Puerto Rico. After *Salmonella*, *C. jejuni* has been reported as the second most common foodborne bacterial pathogen isolated from humans 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 lbs 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 US (Anonymous 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 the consumption of undercooked poultry are major risk factors for human campylobacteriosis (Butzler and Oosterom, 1991; Nadeau et

al., 2002; Tauxe 1997). 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 identify 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, DNA microarrays are more expensive and more difficult to perform than molecular typing methods based on restriction profiles, such as pulsed-field gel electrophoresis (PFGE), and even sequencing methods such as multilocus sequence typing (MLST).

With these challenges in mind, we decided to collect samples from commercial live broilers in Puerto Rico to isolate *Campylobacter* strains and analyze them with DNA-based techniques to understand their genomic relatedness and potential implication in human campylobacteriosis. *Campylobacter* strains were collected from fecal material taken from commercial broiler chickens and analyzed using different molecular techniques to determine possible traits that would predict their risk for humans. For this purpose, isolates were 1) identified to the species level using culture media and multiplex

polymerase chain reaction (mPCR) assays and 2) characterized with PFGE, MLST, and flagellin A restriction fragment length polymorphism (*fla*A-RFLP).

Materials and Methods

Sample collection

Fecal samples were collected by Fernando Rebollo-Carratto, a student of the University of Turabo at Gurabo, Puerto Rico 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. Five to seven samples were collected from each farm. Each sample consisted of ~2-3 g of freshly deposited fecal material collected from four different areas of the houses with sterile tongue depressors. Samples were placed in tubes containing Cary-Blair semi-solid medium supplemented with 5% lysed horse blood and shipped in refrigeration (~8-10^{o}C) for analysis to the Department of Poultry Science, Auburn University, USA.

Campylobacter isolation

Fecal samples were analyzed 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 four to six days of collection. Samples were also enriched in Preston broth (Oxoid, Ltd.) for 24 h at 42^{o}C under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) generated using anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International,Frederick, MD). Enriched samples were swabbed (~0.1 ml)

onto mCC and mCCDA plates. All plates were then incubated at 42^{o}C under microaerobic conditions for 48 h and screened for typical *Campylobacter* colonies. 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^{o}C in tryptic soy broth (Difco, Detroit, MI) supplemented with 30% glycerol (vol/vol) 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 at 42^{o}C for 24 h to prepare fresh bacterial DNA. DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). DNA samples were tested with two multiplex PCR assays that 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) was used for amplification. Amplicons were detected in 1.5% UltraPure™ Agarose-1000 (Invitrogen Corporation, Carlsbad, CA) and DNA bands were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD) followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD). All strains were also tested with a commercial hippurate test (Hardy Diagnostics, Santa Maria, CA).

PFGE analysis

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* ss. *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. 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 sec and final 35.38 sec. Restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper, Bio-Rad, Hercules, CA) 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, Frederick, MD). PFGE profiles were used to perform a pair comparison and cluster analysis using the Dice correlation coefficient, and a dendogram was created using the unweighted pair group method using mathematical average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used to analyze the different PFGE patterns (De Boer et al., 2000).

MLST, 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). PCR amplification was performed in 25-µl reactions using Platinum PCR Supermix (Invitrogen Corporation). Amplicons were detected on 1.5% ethidium bromide agarose gels and purified with a MiniElute PCR Purification Kit (Qiagen Inc., Valencia, CA). The concentration of amplified DNA was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two pre-made PCR mixes, OmniMix® HS (Cephied, Sunnyvale, CA) and Platinum PCR Supermix (Invitrogen Corporation) were used in a second PCR amplification using the suggested sequencing primers. Products of the second amplification were sequenced at the Auburn University Genetic Analysis Laboratory. Sequences were 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 Neighbor-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) (Haubold and Hudson 2000), for the standardized index of association.

*fla*A-RFLP

Flagellin A RFLP was carried out in 25 μl aliquots as described elsewhere (Harrington et al 2003). A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA) was used for amplification. Amplicons were detected in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland). DNA bands were stained with ethidium bromide and visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD). An image was recorded (tiff format) using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

Results

Strain identification

Thirty *C. jejuni* isolates were collected from 94 broiler samples. From 17 farms that were sampled, 13 farms gave *Campylobacter* growth, and all isolates were confirmed as *C. jejuni* with two multiplex PCR assays and with a hippurate test.

PFGE analysis

The macrorestriction 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 six to 14. Ten strains did not restrict with *Kpn*I, while two strains did not restrict with *Sal*I, *Sma*I, or *Sma*I/*Kpn*I. Besides the suggested pulse time for *Kpn*I (Anon. 2007a), 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 due to presence of endogenous proteinases, formaldehyde-treated plugs were digested with different restriction enzymes. The two strains that did not restrict with *Sma*I 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 either. However these strains were restricted with *Bam*HI, *Nru*I, *Sal*I and *Sac*I. It is not clear whether lack of restriction with *Sma*I is due to the lack of *Sma*I restrictions sites or the presence of methylated DNA.

MLST analysis

The use of both OmniMix® HS and Platinum PCR Supermix for PCR amplification gave clean amplicons for sequencing data for MLST analysis. Table 1 shows the allelic profiles and clonal complexes for the *C. jejuni* strains. No new allelic sequences were found for any of the seven house-keeping genes. However, the predominant allelic profile was new and assigned as 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 4 alleles for *pgm* (10, 11, 86 and 89) and *glnA* (4, 17, 21 and 30). A calculation of phylogenetic trees for *pgm* and *glnA* based on their sequences and using the Neighbor-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 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 diversity 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 (standard deviation), and showed linkage disequilibrium for this population of *C. jejuni* (Table 2). 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 (Figure 1A & 1B). When compared to PFGE, MLST was less discriminatory for strain characterization, and strains that grouped under ST-2624 had nine different PFGE profiles (Figure 2).

*fla*A-RFLP analysis

Thirteen unique restriction profiles were obtained with *Dde*I enzyme with the number of DNA bands varying from 4 to 8 (Figure 3). The two largest groupings contained 5 and 6 strains respectively.

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, and *fla*A-RFLP. From the total consumption of broiler meat, only 34% is produced locally, while the rest is imported as frozen products. Therefore, although the

island may be geographically separated from the continental US, the introduction of new *C. jejuni* strains may be possible through the importation of retail poultry products.

The year average temperature in Puerto Rico is between 70 to 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 lower than the 57% reported for commercial broilers in the southeast US (Potturi-Venkata et al., 2007a), and may be accounted by the shipping of the samples in refrigeration for several days, which may have resulted in the reduction in the number of *Campylobacter* cells in the fecal samples. Flocks were sampled two weeks before processing, when *Campylobacter* is usually present at high numbers (Potturi-Venkata et al., 2007a,b). The methodology used was optimized with combinations of plates and enrichment broth for the isolation of *Campylobacter* spp. from fecal material collected from live broilers (Potturi-Venkata et al., 2007b).

Studies that compared the macrorestriction 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* (Kärenlampi et al., 2003; Michaud et al., 2001). 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. Although the number of restriction profiles was similar between *Kpn*I and *Sma*I, only 66% of the isolates were restricted with *Kpn*I versus more than 90% that were restricted with *Sma*I, or *Sal*I. *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 un-resolved 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 a macrodigestion with *Sma*I followed by the digestion with *Kpn*I resulted in a larger number of bands and profiles (Table 3) and may be more discriminatory to track the sources of *Campylobacter* isolates in poultry production. 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 notorious in the area below 250 kbp (Figure 2), an element that made the PFGE profiles more challenging to analyze using the visual scoring system suggested by Tenover and co-workers (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). The fact that all strains showed restriction with frequently cutting enzymes suggests that those strains actually lack some restriction sites or that some sites are methylated. In large eukaryotic genomes ($> 10^8$ base pairs), this lack of restriction, especially the CpG nucleotide deficiency, has been usually associated to 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). It appears that cytosine methylation in bacteria is not generally associated with CpG dinucleotide methylation, especially in *C. jejuni*, which is CpG deficient (Wang et al., 2004). The methylation related problems in *Campylobacter* may be associated with ApC sites.

PFGE macrorestriction with *Sma*I has been reported to be more discriminatory 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 *fla*A-RFLP 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 analyzing *C. jejuni* with PFGE.

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 England (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).

Within the individual genes, *glnA* and *pgm* showed the highest diversity 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 analyze 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 versus 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 analyzed from *C. jejuni* associated to cattle in the UK. 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 to 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, 1999; Maynard Smith et al., 1993), and the standardized I_A (sI_A) that corrects for the number of loci analyzed (Haubold and Hudson, 2000). The sI_A value of 0.73 and a calculated variance that was significantly higher than the expected variance ($P \leq 0.0127$) suggested a high degree of recombination among the MLST genes within this population of *C. jejuni* isolates (Table 2). It is important to remember that a hypothetical sI_A value of 0 represents alleles that are in linkage equilibrium due to free recombination.

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 (Musser1996). However, the analyses of linkage disequilibrium on comparison of gene trees have revealed that few bacteria are indeed clonal (Feil et al., 2001; Maynard Smith et al., 1993; Spratt and Maiden, 1999; Supply et al., 2003). For MLST from *C. jejuni*, a high frequency of inter- and intra-species recombination, calculated to be more than 50 times the frequency of mutation, has been shown to alter the sequence profiles of the housekeeping genes (Schouls et al., 2003). This high recombination frequency plus the mutation rate 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; Potturi-Venkata et al., 2007a,b; Schouls et al., 2003) and retail broiler products (Dickins et al., 2002; Oyarzabal et al., 2007a). Therefore, MLST profiling, although useful to determine temporal phylogenetic comparison, may be less discriminatory 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, versus 10 different PFGE profiles suggest that macrorestriction profiling is still a powerful system for the tracking of *C. jejuni* strains.

*Fla*A-RFLP presented 13 restriction profiles. Even though *fla*A-RFLP has a higher number of profiles, 13, than PFGE, 12, PFGE's discriminatory power in this study is higher than *fla*A-RFLP, D=0.9233 and D=0.8718 respectively. This drop in discrimination is not surprising, since *fla*A-RFLP targets only one gene for typing rather

than the entire genome. But when debating time and expense, *fla*A-RFLP could be substituted in the place of PFGE for *C. jejuni* strains.

The molecular characterization of bacterial pathogens is essential to understand the short-term and long-term epidemiological implications of a given foodborne pathogen (Spratt et al., 1999). The long-term goal of an active surveillance program is to predict, with high degree of certainty, the rise and spread of a lineage with high pathogenicity potential for humans, or epidemic clone (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 different results and the difficulty in the interpretation of these results become more a limitation in studies on pathogens with high capacity for horizontal gene transfer (Oyarzabal et al., 2007b), where even the typability of the isolates 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 limitations 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 studies of larger populations of strains will result in more complex information to analyze.

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Figure Captions

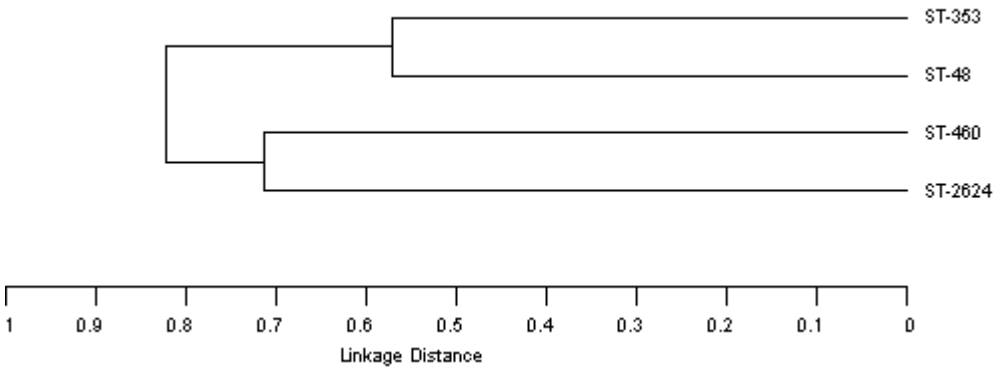
Figure 1A Lineage assignment using the UPGMA cluster analysis calculated with START version 1.0.8. ST-2624 and ST-460 were closely related and more distant from ST-48 and ST-353. **B**: Decomposition analysis on allelic profile data using SplitsTree (Anonymous 2007d; Hudson 1998) resulted in a network-like shape, a shape that suggests recombination.

Figure 2 PFGE macrorestriction profiles (*Sma*I/*Kpn*I) of *C. jejuni* isolates belonging to ST-2624. Pair comparison and cluster analysis performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used for the determination of the different PFGE patterns.

Figure 3 *fla*A-RFLP restriction profiles of all Puerto Rico strains

Figure 1

A


ST-353
ST-48
ST-460
ST-2624
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Linkage Distance

B

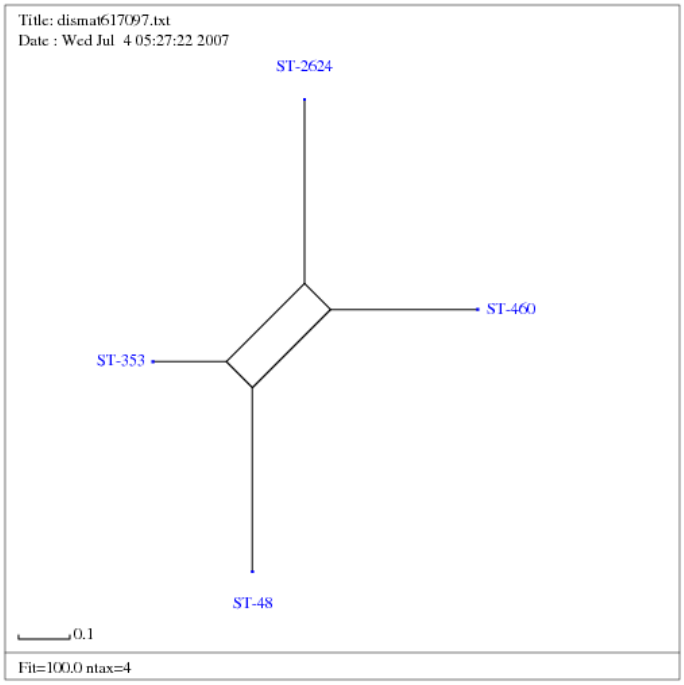

Title: dismat617097.txt
Date : Wed Jul 4 05:27:22 2007
ST-2624
ST-460
ST-353
ST-48
0.1
Fit=100.0 ntax=4

Figure 2

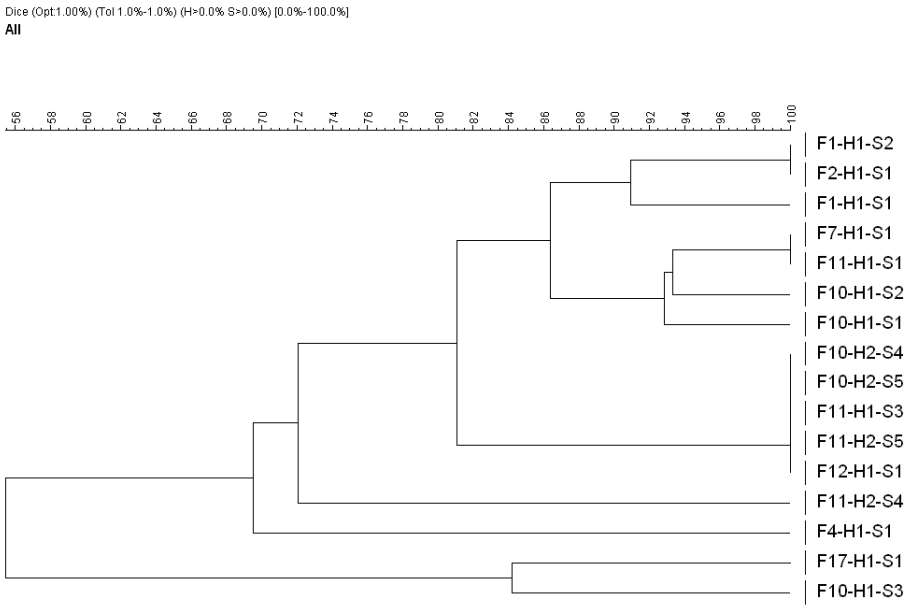

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
All
56
58
60
62
64
66
68
70
72
74
76
78
80
82
84
86
88
90
92
94
96
98
100
F1-H1-S2
F2-H1-S1
F1-H1-S1
F7-H1-S1
F11-H1-S1
F10-H1-S2
F10-H1-S1
F10-H2-S4
F10-H2-S5
F11-H1-S3
F11-H2-S5
F12-H1-S1
F11-H2-S4
F4-H1-S1
F17-H1-S1
F10-H1-S3

Figure 3

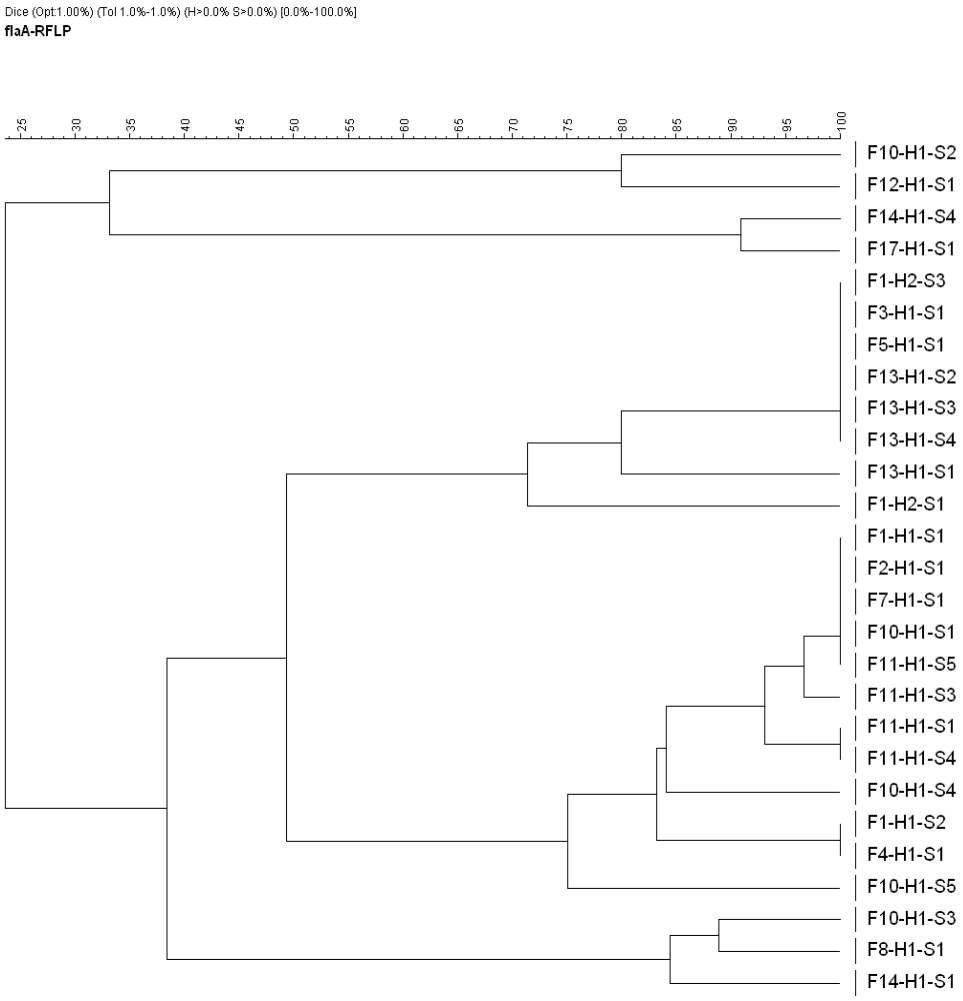

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
flaA-RFLP
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
F10-H1-S2
F12-H1-S1
F14-H1-S4
F17-H1-S1
F1-H2-S3
F3-H1-S1
F5-H1-S1
F13-H1-S2
F13-H1-S3
F13-H1-S4
F13-H1-S1
F1-H2-S1
F1-H1-S1
F2-H1-S1
F7-H1-S1
F10-H1-S1
F11-H1-S5
F11-H1-S3
F11-H1-S1
F11-H1-S4
F10-H1-S4
F1-H1-S2
F4-H1-S1
F10-H1-S5
F10-H1-S3
F8-H1-S1
F14-H1-S1

Table 1 Allellic profiles and clonal complexes from *C. jejuni* strains isolated in this study.

ST Allelic Profile	Clonal Complex	Strains	% of Dataset
2624 [a]	NA [b]	16	53.3
429	48	9	30.0
353	353	4	13.3
460	460	1	3.3

[a]New ST profile.

[b]No clonal complex assigned yet.

Table 2 Index of association calculated with START and the standardized index of association calculated with LIAN showing that the tested population of C. jejuni is in linkage disequilibrium.

		Testing Null Hypotheis (H_0: $V_D = V_e$) [a]		
Summary statistics (Means)			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 [b]	0.73	I_A [c]	4.35	

[a] V_D: Calculated or observed variance; V_e: Expected variance for linkage equilibrium.

[b] sI_A: Standardized index of association

[c] I_A: Index of association.

significantly different ($P < 0.05$) by Duncan's Multiple Range Test (SAS version 9.1).

Table 3 PFGE macrorestriction profiles, ST allelic profiles of *C. 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/ KpnI	ST Allelic Profiles
F4-H1-S1	1	4	1	S-3	NR [a]	Sa-3	SK-4	2624
F5-H1-S1	1	5	1	S-1	NR	Sa-4	SK-3	48
F10-H1-S1	2	10	1	S-2	K-2	Sa-2	SK-5	2624
F10-H1-S2	2	10	1	S-2	K-4	Sa-2	SK-5	2624
F10-H1-S3	2	10	1	S-5	K-5	Sa-2	SK-8	2624
F10-H2-S4	2	10	2	S-6	K-6	Sa-2	SK-7	2624
F10-H2-S5	2	10	2	S-6	K-6	Sa-2	SK-7	2624
F11-H1-S1	3	11	1	S-6	K-7	Sa-2	SK-5	2624
F11-H1-S3	3	11	1	S-6	K-7	Sa-2	SK-7	2624
F11-H2-S4	3	11	2	S-6	K-7	Sa-2	SK-9	2624
F11-H2-S5	3	11	2	S-6	K-7	Sa-2	SK-7	2624
F12-H1-S1	3	12	1	S-7	K-7	Sa-2	SK-7	2624

[a] Not restricted.

III. DNA CHARACTERIZATION OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ISOLATES FROM CHICKENS IN GRENADA

Introduction

Grenada is an island nation in the southeastern Caribbean Sea. In 2003 Grenada produced approximately 14% of the 7.2 million kg of chicken meat consumed on the island (David 2004), importing the remaining 86%. An average of 54 lbs per capita of chicken meat is consumed annually in Grenada (Anonymous, 2008).

Information concerning human campylobacteriosis in Grenada is not readily available, although statistics reveal that gastroenteritis and diarrhea are among the leading causes of infant mortality. Between 1992 and 1996, gastroenteritis increased by 60% in children less than five years of age and by 73.5% in children over five years (Anonymous, 1998). Due to the lack of information regarding *Campylobacter* infection along with Grenada's high importation of chicken meat, we decided to analyze *Campylobacter* strains collected from broilers and layer chickens in Grenada with multiplex polymerase chain reaction assays (mPCR) for determination of their identity and with two fingerprinting techniques, restriction fragment length polymorphism of the flagellin A (*fla*A-RFLP) and pulsed-field gel electrophoresis (PFGE), to determine their genetic relatedness. Previously, these isolates were identified to the species level using culture media and biochemical tests (Hariharan et al., 2008). The discrepancies between the results from biochemical tests and the results from mPCR assays, and the degree of

discrimination of *fla*A-RFLP, PFGE and the combination of both techniques are presented.

Materials and Methods

Sample collection and biochemical identification of isolates

The collection of ceca, the isolation of the *Campylobacter* strains and the identification using biochemical tests have been described elsewhere (Hariharan et al., 2008). Briefly, freshly grown cultures were subjected to catalase, oxidase (BBL, Becton, Dickinson and Co., Sparks, MD, USA), and hippurate tests (Remel, Lennexa, KS, USA), and latex agglutination for *C. jejuni/coli/lari* (JCL, Panbio, Columbia, MD, USA). All isolates were also tested for their susceptibility to nalidixic acid (30 mcg disk) and cephalothin (30 mcg disk) on Mueller-Hinton agar with 5% sheep blood. JCL positive isolates were considered to belong to *C. jejuni/coli/lari* group. Hippurate positive isolates were identified as *C. jejuni*, while nalidixic acid susceptible hippurate negative isolates as *C. coli*, and nalidixic acid resistant, hippurate negative isolates as *C. lari*.

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^{o}C under microaerobic conditions) and the growth was used to inoculate a mix of Cary-Blair and Brucella broth (1:3) supplemented with 5% lysed horse blood for a final volume of 20 ml. These tubes were shipped under refrigeration (~6-8^{o}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, Ltd.) for 24 h at 42°C under microaerobic conditions(10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) generated using anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD). Enriched samples were swabbed (~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 considered presumptive 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) and DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). Isolates were then stored at-80°C in tryptic soy broth (Difco, Detroit, MI) supplemented with 30% glycerol (vol/vol) and 5% lysed horse blood for further identification and characterization.

PCR identification of *Campylobacter* isolates

DNA samples were tested with two multiplex PCR assays that were performed in 25 μl aliquots as described elsewhere (Oyarzabal et al., 2005, 2007). These mPCRs selectively detect *C. coli* based on amplifications of the *asp*A (Linton et al. 1997) and *ceuE* (Gonzalez et al. 1997) genes, and *C. jejuni* based on the amplification of the *hip*O gene (Persson and Olsen 2005) and an undefined gene (Winters and Slavik 1995). A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA) was used for amplification. Amplicons were detected in 1.5% UltraPure™ Agarose-1000 (Invitrogen Corporation, Carlsbad, CA) and DNA bands were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD)

followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

*fla*A-RFLP analysis

Isolates were recovered from frozen stocks on mCC. *flaA*-RFLP analysis was performed in 25 μl PCR aliquots as described elsewhere (Harrington et al 2003; Nachamkin et al., 1993). A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA) was used for amplification. Amplicons were detected in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland) and DNA bands were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD) followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

PFGE analysis

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) and described elsewhere (Oyarzabal et al., 2008) was used. The only modification to described protocols was the preparation of an OD value of 1.0 when collecting the cells. A digestion of *Salmonella choleraesuis* ss. *Choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) was used as the DNA size marker. *Campylobacter* DNA was digested with *Sma*I and *Xma*I. Restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper, Bio-Rad, Hercules, CA) in 1% SeaKem® Gold Agarose (Lonza, Basel,

Switzerland) gel stained with ethidium bromide, visualized with a UV transilluminator (Gel-Doc System, Bio-Rad), and a picture recorded using GeneSnap (Syngene, Frederick, MD).

Interpretation of results

TIF images of the restriction profiles for *fla*A-RFLP and PFGE were incorporated into BioNumerics version 4.50 (Applied Maths, Austin, TX) and pair-comparisons and cluster analysis was 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 1%, and a cutoff of 90% was used for the determination of the different restriction patterns. The Simpson's index of diversity (Hunter and Gaston, 1988) was used to determine the discriminating power of each restriction technique.

Results

Strain identification

From 60 cultures shipped to Auburn University, four were not able to be recovered after enrichment. The attempts to speciate the isolates using the API test resulted in several ambiguous results. Therefore, the test was not used for the determination of the species. By using two mPCR assays, 49 isolates were identified as *C. coli* and seven were identified as *C. jejuni*. Isolates identified as *C. lari* by biochemical tests resulted in *C. coli* by the mPCR assays and failed to generate amplicons in a PCR assay that uses primers specific for *C. lari* (Oyarzabal et al., 1997). Table 1 shows the number of isolates for

which discrepancies between biochemical test results and DNA results were found. Two isolates (G185 and G187) gave mixed results (*C. jejuni* and *C. coli* bands) in PCR results suggesting that they were mixed cultures of these two *Campylobacter* spp. These strains were re-isolated using a 0.65 µm filter (Millipore Corp., Billerica, MA) and one isolated colony from each sample was analyzed again for PCR, *fla*A-RFLP and PFGE. G185 was identified as *C. coli* and gave a restriction profile with PFGE, while G187 was isolated as *C. jejuni* but did not restrict with PFGE using *Sma*I or *Xma*I (Figure 1).

*fla*A-RFLP and PFGE results

One isolate did not yield a *fla*A-RFLP profile using the described protocol with restriction by *Dde*I. The remaining 55 isolates (*C. jejuni* and *C. coli*) gave 31 *fla*A-RFLP profiles, with a Simpson's index of diversity of 0.958. However, six clusters contained 47% of the strains.

Of all isolates, five *C. coli* and one *C. jejuni* isolates did not show any restriction profile with the PFGE protocol using *Sma*I or *Xma*I. With PFGE, only 26 profiles were obtained and therefore this technique showed a lower discriminatory power (D = 0.9437). A combination of the *fla*A-RFLP and PFGE profiles results in the highest discrimination for these isolates (D = 0.9906). Some isolates that clustered together by PFGE analysis showed different *fla*A-RFLP patterns (Figure 2).

Discussion

The biochemical differentiation of *C. jejuni* and *C. coli* is based on one test, hippurate hydrolysis. *C. jejuni* strains carry the *hip*O gene which encodes the hippuricase enzyme.

This enzyme catalyzes the hydrolysis of hippuric acid to benzoic acid and glycine (Hani and Chan, 1995; Harvey, 1980). Although all *C. jejuni* test positive for the presence of this gene using molecular assays, the limitation of the biochemical test is that some *C. jejuni* strains do not express the enzyme for this reaction and appear to be hippurate-negative with biochemical tests (Totten et al., 1987).

Similar limitations have been found for the biochemical identification of *C. lari*, in which some *C. jejuni* strains may be resistant to nalidixic acid and can be misidentified as *C. lari* (Oyarzabal et al., 1997). Since the incorporation of molecular techniques for routine identification of *Campylobacter* isolates collected from chickens, approximately 10 to 15 years ago, the report of *C. lari* has dropped considerable. In the US, no confirmed report of *C. lari* has been documented in broiler chickens in the last eight years. Even the presence of *C. lari* in samples collected from commercial turkeys amounts to very few strains from hundreds of *Campylobacter* isolates from this avian species (Smith et al., 2004).

In our studies, the agreement between the two mPCR was 100%; therefore, all isolates that were found to be *C. coli* with one mPCR were also *C. coli* with the other mPCR. One of these mPCR assays uses a set of primers (COL1 and COL2) that amplifies a 894-bp fragment of the *ceu*E gene, a gene that encodes a lipoprotein component of enterochelin. These primers have been found to be specific for *C. coli* in several studies (Debruyne et al., 2008; Gonzalez et al., 1997; Nayak et al., 2005; Oyarzabal et al., 2007). The other mPCR also uses a specific set of primers (CC18F and CC519R) that reacts only with the aspartokinase gene of *C. coli* (Debruyne et al., 2008; Linton et al., 1997; Oyarzabal et al., 2005). Therefore, *C. coli* makes up the majority of the *Campylobacter*

strains from chickens in Grenada, and this *Campylobacter* species appears to be more prevalent than *C. jejuni*.

Some isolates with similar PFGE profiles had different *fla*A-RFLP restriction patterns. Therefore, a combination of fingerprinting methods appears to be necessary for higher discrimination of isolates. But the argument for the continuous differentiation between *C. jejuni* and *C. coli* is becoming more difficult to justify under the new findings that suggest a strong convergence between these two species (Sheppard et al., 2008). It appears that the speciation of *C. jejuni* and *C. coli* should be revisited, especially considering that these two bacteria produce the same disease, are in the same niche and are transmitted by the same routes. Although in most cases *C. jejuni* and *C. coli* isolates from broiler meat tend to cluster independently when analyzed with PFGE, some isolates do become difficult to interpret because they may be confirmed as *C. jejuni* by molecular methods but cluster together with *C. coli* (Figure 2). These findings will continue to challenge our definition of species boundary between two closely related bacteria. This important discussion should be addressed before implementing any particular policy for the presence of *Campylobacter* spp. in food samples. In the USA, the current public health policy for other foodborne pathogens (e.g. *Salmonella*) is not based on species or serotypes. Only for those cases where one species is pathogenic and others are not (e.g. *Listeria* spp.) does speciation become necessary.

Characterization of *Campylobacter* isolates with *fla*A-RFLP has several advantages, such as low cost, simplicity and a good level of standardization among laboratories (Djordjevic et al., 2007). However, *fla*A-RFLP usually yield less discriminatory power for *C. jejuni* isolates than PFGE based on *Sma*I (Han et al., 2007).

Surprisingly, we obtained more restriction profiles with *fla*A-RFLP than with PFGE. These results may be explained in part because most of the isolates were *C. coli*.

To summarize, our results emphasize the need to complement the identification of isolates by biochemical tests with molecular methods if speciation is necessary. The choice of a fingerprinting profile technique sometimes relates to the techniques already in use in some laboratories. However, a thorough investigation should include at least two different techniques because in most cases those techniques will complement each other and yield more discriminatory results.

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Figure Captions

Figure 1. PFGE macrorestriction (A) and *fla*A-RFLP restriction (B) of strains G185 (lines 3, 4, 8, 9) and G187 (lines 1, 2, 5, 6). Mix cultures (lines 1, 3, 5, 9) show more bands than pure cultures. Isolate G187 did not restrict with PFGE but showed a restriction pattern with *fla*A-RFLP. After obtaining a single culture, strain G185 was *C. coli* and G 187 was *C. jejuni*. Ma: DNA size marker for PFGE made from the digestion of *Salmonella choleraesuis* ss. *Choleraesuis* serotype Braenderup H9812 (ATCC BAA-664); Mb: 100-bp marker (Promega, Madison WI).

Figure 2. *fla*A-RFLP restriction profiles of *C. coli* and *C. 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 version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used for the determination of the different PFGE patterns.

Figure 1

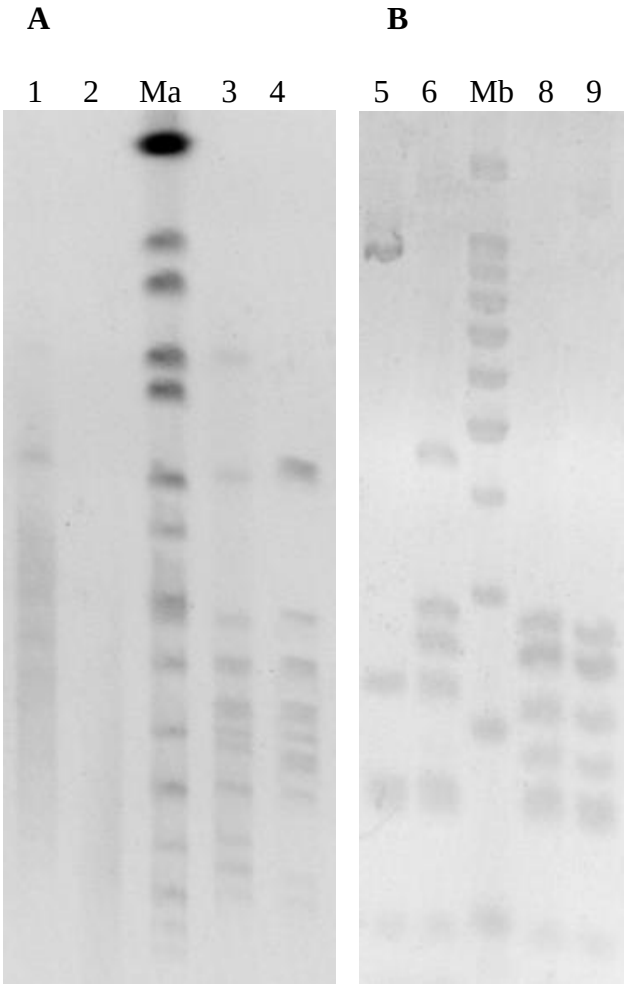

Figure 2


Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
flaA-RFLP
flaA-RFLP
PFGE
40 45 50 55 60 65 70 75 80 85 90 95 100
coli G109 broiler
jejuni G112 broiler
coli G102 broiler
coli G127 layer
coli G123 broiler
coli G113B broiler
coli G126 broiler
coli G106 broiler
coli G113 broiler
coli G140 layer
coli G202-2 broiler
coli G206 broiler
jejuni G142 layer
coli G138A layer
coli G172 broiler
coli G103 broiler
coli G128 layer
jejuni G83 layer
coli G175 broiler
coli G118 broiler
coli G191 broiler
jejuni G188 broiler
coli G117 broiler
coli G125 broiler
coli G105 broiler
coli G130 broiler
coli G194 broiler
coli G185 broiler
coli G158 layer
coli G190 broiler
coli G186 broiler
coli G193 broiler
coli G196 broiler
coli G204 broiler
coli G202 broiler
coli G192 broiler
coli G201 broiler
coli G203 broiler
coli G207 broiler
jejuni G122 broiler
coli G151 layer
coli G104 broiler
coli G146 layer
coli G195 broiler
coli G107 broiler
coli G143 layer
coli G144 layer
coli G108 broiler
coli G95 layer
coli G110 broiler
coli G138B layer
jejuni G170 broiler
coli G115
coli G163 broiler

Table 1. Identification of 17 Campylobacter isolates with discrepancies between biochemical tests and multiplex polymerase chain reaction (mPCR) assays.

Number of Strains	Biochemical ID	mPCR ID	Presence of Hippuricase Gene	Hippurate Activity[1]
13	*C. jejuni*	*C. coli*	-	-
3	*C. lari*	*C. coli*	-	-
1	*C. coli*	*C. jejuni*	+	+

[1] Hippurate activity was determined using a commercial hippurate test (Hardy Diagnostics, Santa Maria, CA).

Table 2. Evaluation of the discriminatory power of different typing techniques applied to *Campylobacter* spp. isolates from Grenada.

Typing Method	Number of Types	Strains per Type	Simpson's Index
*fla*A-RFLP	31	2,1,2,1,1,1,1,1,1,1,1,2,5,3,1,1,2,1,1,1,1,1,1,3,1,3,1,9,1,1,3	0.958
PFGE	26	1,1,1,1,1,4,1,1,2,5,5,9,1,1,1,1,1,3,1,1,3,1,1,1,1,1	0.9437
*fla*A-RFLP/PFGE	48	2,1,1,1,1,1,1,1,1,1,1,1,1,2,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,2,1,1,1,1,1,1,2,5,2,1,1,1,1,1,1	0.9906

IV. FINGERPRINT PROFILING OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ISOLATED FROM RETAIL BROILER MEAT IN ALABAMA

Introduction

The southeastern region of the US produces more broiler meat than the rest of the country combined (Anonymous 2005). According to the U. S. Department of Agriculture (USDA), 17 states account for 94% of all US broiler production (Anonymous 2008a), and nine of those states are found in the southeast (Anonymous 2005). With US broiler meat consumption increasing to 86 lbs. per person in 2006 (Anonymous 2008a) and the CDC estimating 2.4 million infections in 2006 (Anonymous 2008b), *Campylobacter* in broiler meat is rapidly becoming an issue of concern from the public health point of view.

There is little information on the revalence of different *Campylobacter* spp. in broiler meat in the Southeast USA. In addition, we wanted to evaluate different DNA-based techniques for characterization of isolates to then compare the resutls with those obtained from the use of the same techniuqes in strains collected in other geographical areas (Puerto rico and Grenada). The goal of this study was to determine reaction the species of campylobacters present in broiler meat and to characterize them molecularly via pulsed-field gel electrophoresis (PFGE), multilocus sequencing typing (MLST), and flagellin A restriction fragment length polymorphism (*fla*A-RFLP). We purchased retail broiler meat from local supermarkets in Auburn, Alabama, during the summer months of

2005, 2006, and 2007. The purchased meat was processed at two commercial broiler facilities referred hitherto as Plant A and Plant B.

Materials and Methods

Sample collection

A total of 126 broiler retail samples processed at two different broiler processing plants were purchased from different retail stores in Auburn, Alabama, between June and August over a period of three years, 2005-2007. Samples were collected once a week and consisted of breast tenders, boneless breasts and thighs (including boneless thighs). Samples were stored at ~4°C until processing within 5 h of purchase.

Campylobacter isolation

Twenty five grams of sample were aseptically removed from each package and placed in individual sterile Whirl-Pak© bags (Nasco, Fort Atkinson, WI). Bolton broth (100 mL) supplemented with 5% lysed horse blood (School of Veterinary Medcine, Auburn University) was added to the samples. The mix was stomached for one minute in a Laboratory Blender Stomacher 400 (Seward, London, UK). Samples with the enrichment broth were incubated under microaerobiosis conditions (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) generated using anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International,Frederick, MD) at 42°C for 48 h. The enriched samples were then swabbed onto modified Campy-Cefex (mCC) plates and were incubated under microaerobiosis at 42°C for 48h.

Identification by PCR

Presumptive identification was done by selecting colonies and observing them under phase contrast microscopy to determine cell morphology and motility. Presumptive colonies were stored at -80^{o}C for further analysis.

Stock cultures were transferred to mCC plates which were incubated under microaerobic conditions at 42^{o}C for 24 h to prepare fresh bacterial DNA. DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA) and tested with two multiplex PCR assays that were performed in 25 μl aliquots as described elsewhere (Oyarzabal et al., 2005, 2007). A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA) was used for amplification. Amplicons were detected in 1.5% UltraPure™ Agarose-1000 (Invitrogen Corporation, Carlsbad, CA) and DNA bands were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD), followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

PFGE analysis

A standard PFGE protocol (Ribot et al., 2001) with the modifications suggested by the Centers for Disease Control and Prevention (http://www.cdc.gov/pulsenet/protocols. htm) was used. A digestion of *Salmonella choleraesuis* ss. *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 *Sma*I. Restricted DNA fragments were separated with a contour-clamped homogeneous electric field (CHEF Mapper, Bio-Rad, Hercules,

CA) in 1% SeaKem® Gold Agarose (Lonza, Basel, Switzerland) gel stained with ethidium bromide, visualized with a UV transilluminator (Gel-Doc System, Bio-Rad), and a picture recorded using GeneSnap (Syngene, Frederick, MD). PFGE profiles were used to perform a pair comparison and cluster analysis using the Dice correlation coefficient, and a dendogram was created using the unweighted pair group method using mathematical average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used to analyze the different PFGE patterns (De Boer et al., 2000).

MLST, 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). PCR amplification was performed in 25-µl reactions using two pre-made PCR mixes, OmniMix® HS (Cephied, Sunnyvale, CA) and Platinum PCR Supermix (Invitrogen Corporation). Amplicons were detected on 1.5% ethidium bromide agarose gels, and the concentration of amplified DNA was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Sequencing of the amplified products using the suggested internal primers was performed at the Auburn University Genetic Analysis Laboratory and Lucigen Corporation. Sequences were aligned, assembled and edited using MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html).

*fla*A-RFLP

Flagellin A RFLP was carried out in 25 μl aliquots as described elsewhere (Harrington et al 2003)**.** A PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories, Hercules, CA) was used for amplification. Amplicons were detected in 2.5% SeaKem® Gold Agarose (Lonza, Basel, Switzerland) and DNA bands were stained with ethidium bromide, visualized using a UV transilluminator (Gel-Doc System, Syngene, Frederick, MD) followed by recording using a computer program (GeneSnap) for molecular analysis (Syngene, Frederick, MD).

Statistical analysis

A chi square calculation was done with the percentages positive from each processing plant (A and B).

Results and Discussion

Strain identification

Out of the 126 samples, 56 samples had presumptive *Campylobacter* isolates, which were identified by enrichment, selective plates, and phase contrast microscopy (Table 1). Of the 56 presumptive *Campylobacter* isolates, the multiplex PCR protocols identified twenty four strains as *C. jejuni* and thirty one as *C. coli*. One strain could not be stored and was lost.

A higher percentage of *C. coli* strains (56% versus 44% *C. jejuni*), were isolated from retail samples compared to the ration usually found from broiler carcass rinses, in which the ratio is approximately 97% *C. jejuni* and the rest *C. coli* (Oyarzabal et al.

2005). Others reports have also shown a much higher percentage of *C. jejuni* to *C. coli* for processed carcasses in the US (Fitch 2005; Son et al 2007). Yet, the ratio is different when we analyze retails samples. This change in the ratio between the two *Campylobacter* spp. appears to be true also in some European countries. For instance, Madden et, al. (1998) found *C.jejuni* and *C. coli* percentages of 56% and 44%, respectively, in retail chicken in Northern Ireland. We still do not understand all the epidemiological events related to these different ratios found in the same products at different stages over the production chain (processed versus retail). However there appears to be a real change in the ratio because they have been shown using the same techniques for identification and speciation of isolates (Oyarzabal et al. 2005, 2007).

Of the three kinds of broiler meat tested from both plant A and plant B studied (breast tenders, boneless breasts and thighs), tenders had the lowest occurrence of *Campylobacter* contamination, with only 27% of total tenders testing positive for *Campylobacter* (Table 1). Tenders are cut from the *Pectoralis minor* located inside the chicken breast and underneath the *Pectoralis major*, which makes up the breast meat. Therefore, tenders are less likely to become contaminated with *Campylobacter* from intestinal contents during processing.

Forty three percent of boneless breasts were *Campylobacter* positive followed by 79% of thighs testing positive for *Campylobacter* (Table 1). Since both of these cuts have exterior surfaces that are exposed during evisceration, they have more opportunities to come in contact with *Campylobacter* contaminated material (feces) or contaminated carcasses.

Boneless breasts from plant A had a higher percentage of *Campylobacter* positive

samples than boneless breasts from plant B (18% versus 9%, respectively). A chi square analysis showed that this difference was not significant, x2=3.000 (Table 1).

Thighs from plant B had a higher percentage of Campylobacter positive samples than thighs from plant A (18% versus 9%, respectively). A chi square analysis showed that this difference was not significant, x2=1.667 (Table 1).

PFGE analysis

Thirty six macrorestriction profiles were obtained from the 55 strains that were tested using *Sma*I restriction and a similarity cutoff of 90%. Each profile comprised eight to 11 electrophoretic bands. All 55 strains showed electrophoretic bands when digested with *Sma*I. The discriminatory power of PFGE in this study was 0.9805.

No more than 5 strains belonged to one unique profile. Therefore, no dominant strain of *C. jejuni* or *C. coli* was present in commercially processed retail broiler meat produced in these two plants during the sampled time.

Over time the number of PFGE profiles was variable when considering total strains from both plants. The highest number of PFGE profiles (19) was found in 2007. One strain, 1084, which was identified twice by mPCRs as *C. coli*, showed a PFGE profile that falls into the *C. jejuni* grouping (Figure 1). This strain did not show any amplification with the PCR targeting the *fla*A gene and therefore no RFLP profile could be obtained for this strain. To corroborate that strain 1084 is indeed *C. coli*, we will amplify and sequence its 16S rDNA and performed ClustalW analysis for identification. We will also amplify the *fla*A gene of this strain to find out the actual primer set that could be used when strains do not amplify the *fla*A gene.

We have previously seen that PFGE profiling is not completely discriminatory to group *C. jejuni* and *C. coli* in different PFGE clusters. An analysis of a large database for MLST sequences has recently shown that the patterns of genetic exchange between *C. jejuni* and *C. coli* can be interpreted as "converging". Thus, the factors that historically prevented the gene flow between these two species are apparently disrupted and these two species are merging or "despeciating". The consequence of this genetic merge is that any DNA-based method for fingerprinting may not be able to cluster these species in different groupings anymore (Sheppard et al., 2008).

MLST analysis

Twelve strains were analyzed by MLST with the rest of the strains scheduled for completion of analysis before the publication of this data. Table 2 shows the allelic profiles and clonal complexes found for the twelve strains. Two of the ST allelic profiles are new profiles that have not been assigned a ST value yet, although the actual allele profile for each gene has been previously described in the MLST database. The two new profiles are in the process of being submitted to the MLST web site for identification.

Four strains, the largest cluster, belong to ST allelic profile 353 (Table 2). There was one strain in ST allelic profiles 354, 924, 939, 1210, 1212 and 3510. According to epidemiological data, ST allelic profiles 353, 354, 1212, 1210, and 3510 have been linked to human campylobacteriosis in Europe, Australia, and North America. ST allelic profiles 924 and 939 have been found in chickens from the UK, Canada, and the US (Anonymous 2008c).

*fla*A-RFLP analysis

In this study one strain (1084) failed to digest with *Dde*I, yielding 34 profiles based on a 90% similarity cutoff. The discriminatory power of *flaA*-RFLP (0.9783) was very similar to PFGE (0.9805). Unlike the powerful discrimination found for *C. coli* by RFLP in the strains from Grenada (Chapter III), *fla*A-RFLP was less discriminatory than PFGE for retails isolates of *C. coli*.

Because *fla*A-RFLP is a typing process based only on the flagellin A gene, it is no surprise that *C. jejuni* and *C. coli* discrimination is not always possible considering that *C. jejuni* and *C. coli* share a common biological niche and have a high horizontal gene transfer. The fact that *fla*A-RFLP grouped *C. jejuni* and *C. coli* strains together (Figure 2) emphasize the fact that continuous DNA exchange between these two species makes it difficult to separate fingerprinting profiles. These two species shared the same niche (the only *Campylobacter* strains transmitted by commercial broiler products), use the same transmission routes and cause similar disease in humans. Therefore, a question is raised on the practicality of speciating *Campylobacter* isolates from broiler products.

Comparisons of numbers of profiles produced over time and plant by plant were gave ambiguous results. *flaA*-RFLP and PFGE outperformed each other two of the three years, but both yielded 19 profiles for the 2007 strains (Table 3). When analyzing the strains originating from only one plant, *fla*-A RFLP produced 18 profiles to PFGE's 14 for plant B (Table 4).

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Figure Captions

Figure 1 PFGE dendogram of *C. jejuni* and *C. coli* from plant A and B (2005-2007). Cluster analysis was performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNumerics version 4.50. The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used for the determination of the different PFGE patterns.

Figure 2 *fla*A-RFLP dendogram of *C. jejuni* and *C. coli* from plants A and B (2005-2007). Cluster analysis was performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNumerics version 4.50. The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used for the determination of the different *fla*A-RFLP patterns.

Figure 1

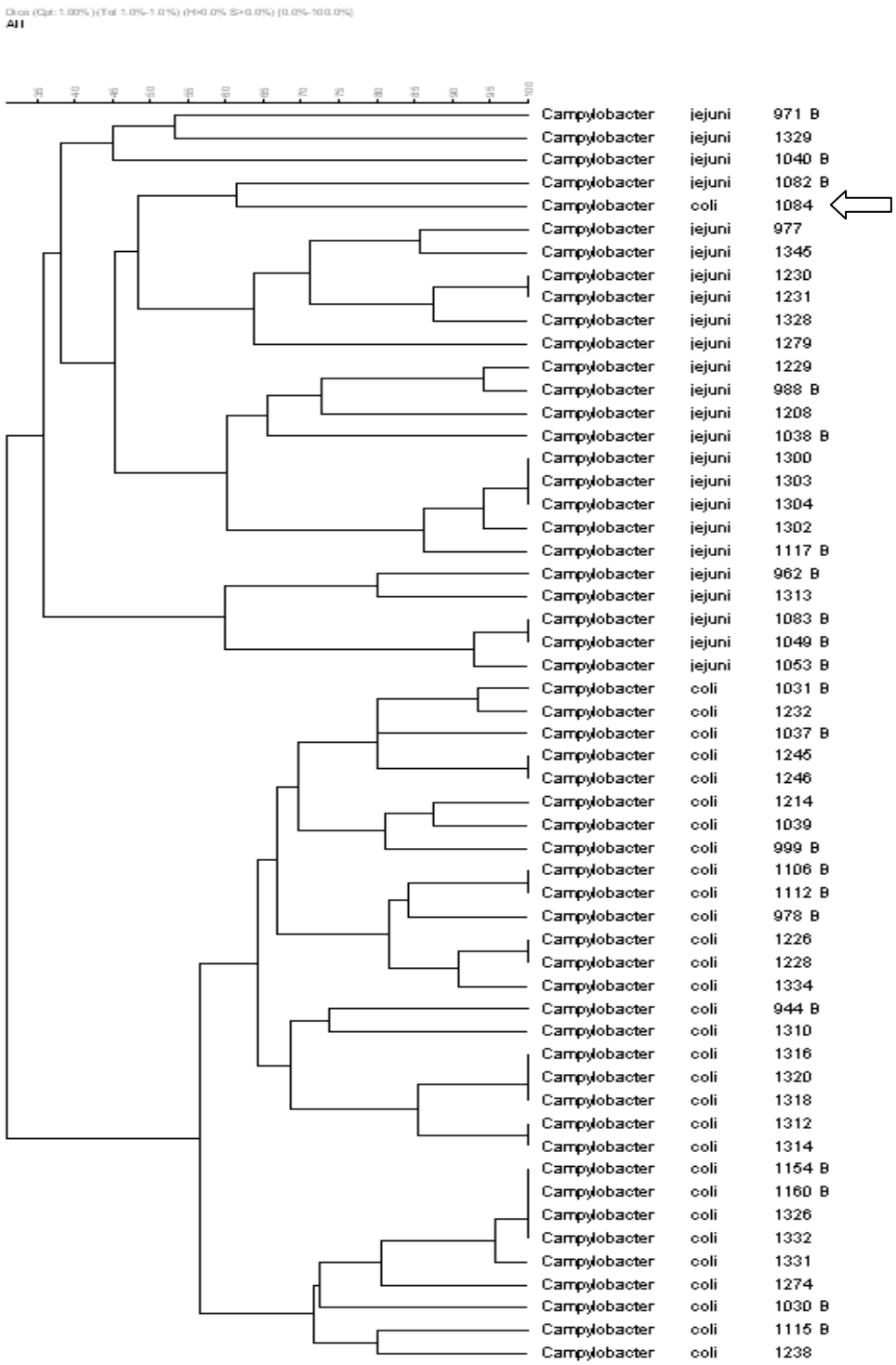
All
Campylobacter jejuni 971 B
Campylobacter jejuni 1329
Campylobacter jejuni 1040 B
Campylobacter jejuni 1082 B
Campylobacter coli 1084
Campylobacter jejuni 977
Campylobacter jejuni 1345
Campylobacter jejuni 1230
Campylobacter jejuni 1231
Campylobacter jejuni 1328
Campylobacter jejuni 1279
Campylobacter jejuni 1229
Campylobacter jejuni 988 B
Campylobacter jejuni 1208
Campylobacter jejuni 1038 B
Campylobacter jejuni 1300
Campylobacter jejuni 1303
Campylobacter jejuni 1304
Campylobacter jejuni 1302
Campylobacter jejuni 1117 B
Campylobacter jejuni 962 B
Campylobacter jejuni 1313
Campylobacter jejuni 1083 B
Campylobacter jejuni 1049 B
Campylobacter jejuni 1053 B
Campylobacter coli 1031 B
Campylobacter coli 1232
Campylobacter coli 1037 B
Campylobacter coli 1245
Campylobacter coli 1246
Campylobacter coli 1214
Campylobacter coli 1039
Campylobacter coli 999 B
Campylobacter coli 1106 B
Campylobacter coli 1112 B
Campylobacter coli 978 B
Campylobacter coli 1226
Campylobacter coli 1228
Campylobacter coli 1334
Campylobacter coli 944 B
Campylobacter coli 1310
Campylobacter coli 1316
Campylobacter coli 1320
Campylobacter coli 1318
Campylobacter coli 1312
Campylobacter coli 1314
Campylobacter coli 1154 B
Campylobacter coli 1160 B
Campylobacter coli 1326
Campylobacter coli 1332
Campylobacter coli 1331
Campylobacter coli 1274
Campylobacter coli 1030 B
Campylobacter coli 1115 B
Campylobacter coli 1238

Figure 2

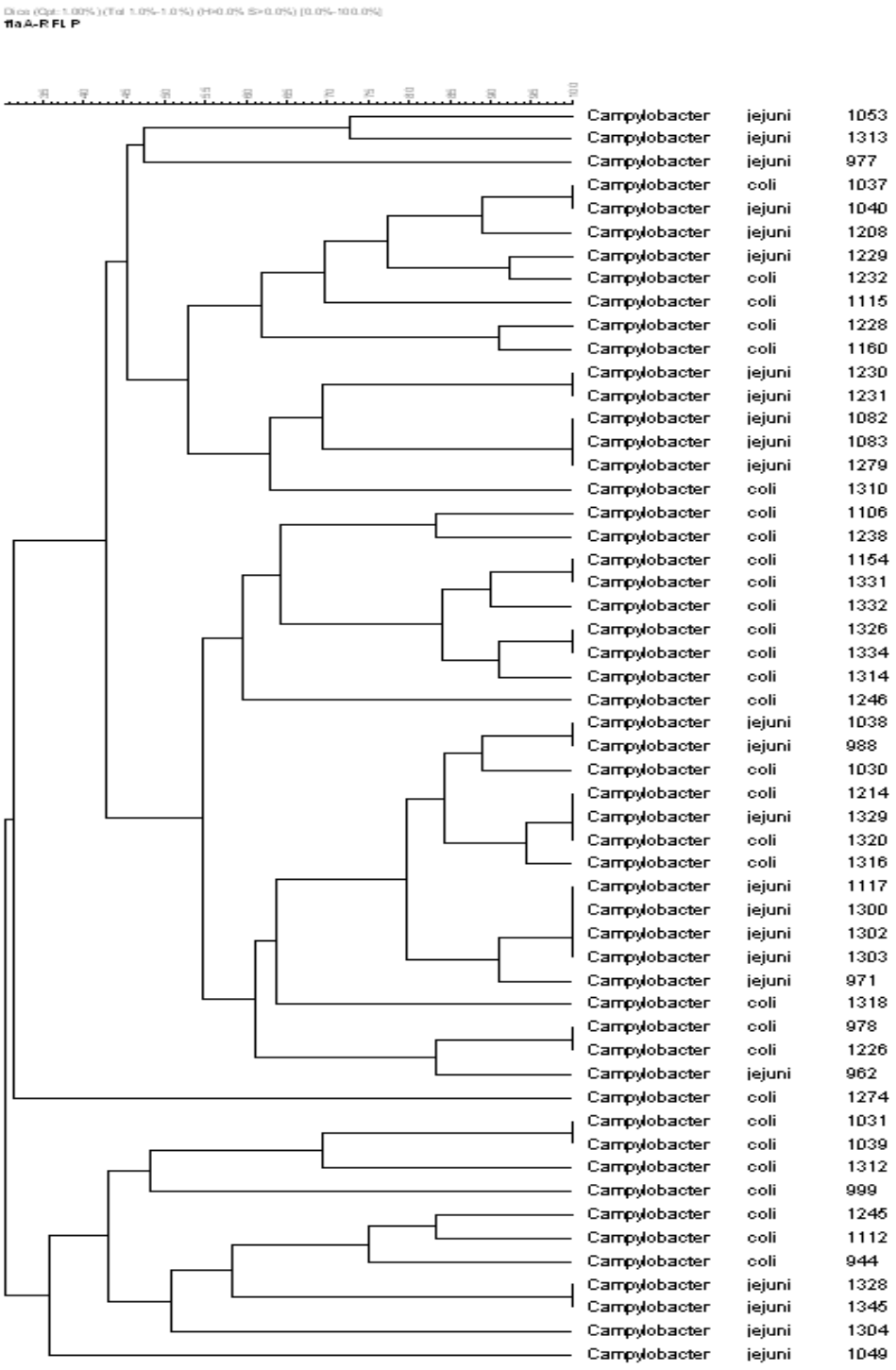
flaA-RFLP
Campylobacter jejuni 1053
Campylobacter jejuni 1313
Campylobacter jejuni 977
Campylobacter coli 1037
Campylobacter jejuni 1040
Campylobacter jejuni 1208
Campylobacter jejuni 1229
Campylobacter coli 1232
Campylobacter coli 1115
Campylobacter coli 1228
Campylobacter coli 1160
Campylobacter jejuni 1230
Campylobacter jejuni 1231
Campylobacter jejuni 1082
Campylobacter jejuni 1083
Campylobacter jejuni 1279
Campylobacter coli 1310
Campylobacter coli 1106
Campylobacter coli 1238
Campylobacter coli 1154
Campylobacter coli 1331
Campylobacter coli 1332
Campylobacter coli 1326
Campylobacter coli 1334
Campylobacter coli 1314
Campylobacter coli 1246
Campylobacter jejuni 1038
Campylobacter jejuni 988
Campylobacter coli 1030
Campylobacter coli 1214
Campylobacter jejuni 1329
Campylobacter coli 1320
Campylobacter coli 1316
Campylobacter jejuni 1117
Campylobacter jejuni 1300
Campylobacter jejuni 1302
Campylobacter jejuni 1303
Campylobacter jejuni 971
Campylobacter coli 1318
Campylobacter coli 978
Campylobacter coli 1226
Campylobacter jejuni 962
Campylobacter coli 1274
Campylobacter coli 1031
Campylobacter coli 1039
Campylobacter coli 1312
Campylobacter coli 999
Campylobacter coli 1245
Campylobacter coli 1112
Campylobacter coli 944
Campylobacter jejuni 1328
Campylobacter jejuni 1345
Campylobacter jejuni 1304
Campylobacter jejuni 1049

Table 1 Positive samples from plants A and B

Product	No. of samples	Negative	Positive %		
			Total	Plant A	Plant B
Tenders	45	31	14 (27)	8	6
Boneless breast	62	35	27 (43)	18	9
Thighs (including boneless thighs)	19	4	15 (79)	5	10
Total	126	70	56 (50)	31 (24)	25 (26)

Table 2 Allellic profiles and clonal complexes from *C. jejuni* and *C. coli* strains isolated in this study.

ST Allelic Profile	Clonal Complex	Strains
?[a]	?	1
?[a]	?	1
353	353	4
354	354	1
1212	607	1
1210	353	1
924	607	1
939	353	1
3510	353	1

[a] New ST profile. These alleles have not been submitted to the MLST web site for identification.

Table 3 Number of profiles per technique per year

Year	Number of profiles per technique per year		
	PFGE	*fla*A-RFLP	MLST*
2005	13	10	3
2006	7	9	4
2007	19	19	5

* Only 12 strains were analyzed with MLST.

Table 4 Number of profiles per technique per processing plant

Plant	Technique		
	PFGE	*fla*A-RFLP	MLST*
A	23	21	6
B	14	18	6

* Only 12 strains were analyzed with MLST.

V. SUMMARY - EVALUATION OF FINGERPRINT PROFILING OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* FROM DIFFERENT GEOGRAPHICAL AREAS USING PULSED-FIELD GEL ELECTROPHORESIS

When strains from all three geographic areas were analyzed, 121 strains grouped into 58 profiles. As stated earlier, failure of PFGE enzymes to digest the genomic DNA of some strains is the largest drawback to PFGE as a molecular subtyping technique. Out of 139 strains analyzed in these studies, two *C. jejuni* strains from Puerto Rico, five *C. jejuni* strains from Grenada, and eleven *C. coli* strains from Grenada failed to digest with *Sma*I. As seen in the Puerto Rico data, the problem can be overcome to some degree by using a combination of restriction enzymes rather than a single one.

Only some samples from the same geographic areas clustered at 100% identity, but clusters from the same geographic areas did not group together (Figure 1). One explanation for this is that both Puerto Rico and Grenada import a large percentage of their consumed broiler meat from the US, but both also produce a small amount of broiler meat domestically, allowing for a more diverse *Campylobacter* population on the two islands. More likely the diversity shown by PFGE between the three geographic areas is caused by *Campylobacter's* naturally high frequency of recombination and mutation rate. The *C. jejuni* strains grouped into two groups split by the *C. coli* strains (Figure 1).

The overall conclusion of this research is that one single fingerprinting method is not sufficient to identify relatedness among *C. jejuni* or *C. coli* isolates. The combination

of two restriction profiling methods overcomes the limitations that one method may have (low discrimination or lack of profiling with some strains). The inclusion of a sequence based method (MLST) does complement methods based on electrophoretic profiling and allow for the determination of clonality based on sequences from seven house-keeping genes. Yet, MLST is not discriminatory enough to assess the special relatedness of *Campylobacter* strains. MLST may be used for the temporal analysis of strains but the frequent horizontal rearrangements that these bacteria undergo makes it difficult to assess which clone is the oldest in phylogenetic studies.

There is no "quality control" procedures that have been established for the analysis of electrophoretic profiling (PFGE, RFLP, etc.) of bacterial isolates, including *Campylobacter* isolates. The scientific literature related to the use of these techniques for DNA relatedness emphasize the standardization of the actual eletrophoretic run (Ribot et al., 2001) but not too much attention has been paid to the limitation of DNA extraction. In the case of PFGE, the methylation of adenine in GATC sequences does limit the action of some restriction enzymes and result in some strains being refractory to PFGE analysis (Edmonds et al., 1992). In the case of RFLP, the limitation is due to changes in the sequences where the primers attached and therefore result in the lack of amplification of some strains.

We are analyzing the data by converting the electrohoretic bands numbers so that each band size is represented by a number. The numbers are expressed in kilo base pairs (kbp) for PFGE bands and in base pairs (bp) for RFLP bands. Our preliminary analysis of RFLP data from the Puerto Rico is shown in Table 1. Although most strains are within the 1,700 bp range (amplicon obtained for RFLP), some strains exhibited more bands that

altogether make up for more than 1,700 bp (see strains F1-H1-S1, F4-H1-S1 and F10-H1-S5 in Table 1). We are trying to interpret these data to determine if a partial digestion is found for some strains. We are also analyzing the possibility that two *C. jejuni* strains were collected and tested together, although the PFGE analysis does point to isolated strains being originally collected (observe the PFGE profiles of strains F1-H1-S1, F4-H1-S1 and F10-H1-S5 in Figure 2, Chapter 2). We hope our efforts will result in a series of guidelines to prepare samples for successful RFLP and PFGE analysis.

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Table 1 Band sizes and the sum of all bands for each fingerprinting profile collected with *fla*A-RFLP

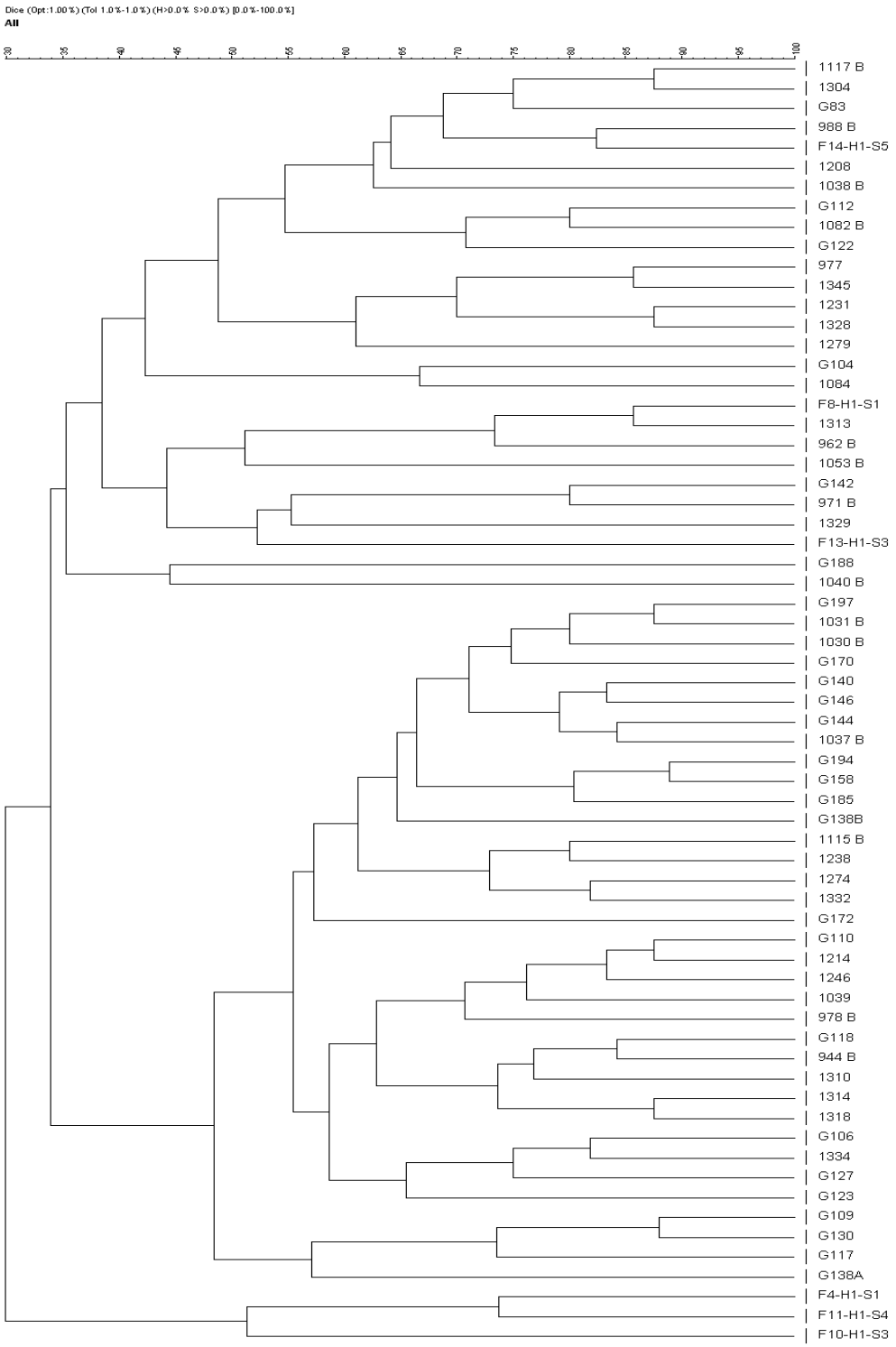
Strain ID	Bands (bp)								Sum (bp)
F11-H1-S3	548	348	285	229	161	93			1664
F11-H1-S1	559	346	290	228	162	99			1683
F11-H1-S4	550	355	295	233	160	97			1692
F10-H1-S4	530	339	279	224	162				1534
F1-H1-S1	977	628	542	347	285	224	158	97	***3258***
F4-H1-S1	970	630	544	343	278	227	160	96	***3249***
F10-H1-S5	1053	764	551	344	285	230	170	98	***3495***
F10-H1-S3	742	259	223	166					1391

.

Figure Captions

Figure 1 PFGE macrorestriction profiles (SmaI) of *C. jejuni* and *C. coli* isolates from Puerto Rico, Grenada, and southeastern US. Pair comparison and cluster analysis performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%, and a cutoff of 90% was used for the determination of the different PFGE patterns.

Figure 1


Dice (Opt:1.00 %) (Tol 1.0 %-1.0 %) (H>0.0 % S>0.0 %) [0.0 %-100.0 %]
All
100
1117 B
1304
G83
988 B
F14-H1-S5
1208
1038 B
G112
1082 B
G122
977
1345
1231
1328
1279
G104
1084
F8-H1-S1
1313
962 B
1053 B
G142
971 B
1329
F13-H1-S3
G188
1040 B
G197
1031 B
1030 B
G170
G140
G146
G144
1037 B
G194
G158
G185
G138B
1115 B
1238
1274
1332
G172
G110
1214
1246
1039
978 B
G118
944 B
1310
1314
1318
G106
1334
G127
G123
G109
G130
G117
G138A
F4-H1-S1
F11-H1-S4
F10-H1-S3

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