INTRODUCTION

In the United States, pathogens recovered from food have been found to cause 48 million cases of human illness every year (CDC, 2011a). Pathogens are defined as agents that cause damage to a host (Buzby et al., 1996; Casadevall and Pirofski, 2002). Bean et al. (1990) found that of the 4 types of pathogens (viruses, bacteria, fungi, and parasites), bacteria are attributed to over 90% of confirmed foodborne infections and deaths reported to the Center for Disease Control and Prevention. The 5 most prevalent foodborne bacteria [Campylobacter spp., Salmonella (nontyphoidal), Escherichia coli O157:H7, Escherichia coli non-O157 STEC, and Listeria monocytogenes] are estimated to cost the United States $6.9 billion every year in physician visits, medical supplies, hospital services, medications, and poorly performed or missed work (ERS-USDA, 2000; CDC, 2008).

Of the 5 most prevalent bacteria, Campylobacter spp. are the most frequent cause of bacterial gastroenteritis. This condition is known as campylobacteriosis (Newell and Fearnley, 2003). Campylobacteriosis is estimated to affect over 2.4 million people annually. Of every 1,000 cases, one out of every 1,000 may also lead to a rare autoimmune disorder of the peripheral nervous system, known as Guillain-Barré syndrome (CDC, 2008). Infected individuals experience a rapid decline in muscle strength in the limbs and respiratory system (Nachamkin et al., 1998; Keener et al., 2004). In the United States, the mean cost per patient with Guillain-Barré syndrome is estimated around $318,966, totaling to $1.7 billion annually (Frenzen, 2008).

Some of the identified routes of transmission by which humans can acquire Campylobacter include consumption of unpasteurized milk (Yang et al., 2003; Schildt et al., 2006; Heuvelink et al., 2009), raw or undercooked meat and poultry (Mehle et al., 1981; Stanley et al., 1998; Little et al., 2008), contaminated water and vegetables (Knill et al., 1982; Kumar et al., 2001; Evans et al., 2003), or by contact with feces from infected humans and domestic pets (Bruce, 1981; Luechtefeld and Lou Wang, 1982). Poultry is considered to be the main reservoir for campylobacteriosis in humans. Epidemiological studies have proven that 50 to 70% of human illness due to Campylobacter is credited to the consumption of contaminated poultry and poultry products (Harris et al., 1986; Allos, 2001; Keener et al., 2004). In 2006, Americans on average consumed 27.7 kg of chicken, an increase from 2000 when chicken

ABSTRACT

Human campylobacteriosis, an infection caused by the bacterium Campylobacter, is a major issue in the United States food system, especially for poultry products. According to the Center for Disease Control, campylobacteriosis is estimated to affect over 2.4 million people annually. Campylobacter jejuni and Campylobacter coli are 2 species responsible for the majority of campylobacteriosis infections. Phenotypic and genotypic typing methods are often used to discriminate between bacteria at the species and subspecies level and are often used to identify pathogenic organisms, such as C. jejuni and C. coli. This review describes the design as well as advantages and disadvantages for 3 current phenotypic techniques (biotyping, serotyping, and multilocus enzyme electrophoresis) and 6 genotypic techniques (multilocus sequence typing, PCR, pulse-field gel electrophoresis, ribotyping, flagellin typing, and amplified fragment length polymorphisms) for typing pathogenic Campylobacter spp.

Key words: Campylobacter, genotyping, phenotype, poultry, food safety

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Phenotypic and genotypic methods for typing Campylobacter jejuni and Campylobacter coli in poultry

Review

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consumption was 24.6 kg (US Poultry and Egg, 2011). With the increase in consumption of chicken products and the high number of cases of campylobacteriosis every year, it is imperative that steps be taken to identify the source of the bacterium.

Most cases of campylobacteriosis in humans are sporadic, making it hard to trace the sources and routes of transmission (Altekruse et al., 1999; Wassenaar and Newell, 2000). Although infection can be sporadic, typing systems help identify strains isolated from human cases and allow for the comparison of those strains at the species and subspecies levels that are found in poultry and on poultry products. Identification of these isolates provides scientists with the ability to study the pathogenesis of infections, detect and investigate outbreaks, and assist with surveillance and prevention of campylobacteriosis in humans (Nielsen et al., 2000). This review describes the design as well as advantages and disadvantages of 9 current phenotypic and genotypic typing systems for Campylobacter spp. (Table 1).

**BACKGROUND**

Typing systems are based on the idea that clonally related isolates share characteristics that can be tested to differentiate them from unrelated isolates. The 2 common ways of classifying typing systems include phenotypic methods that detect characteristics expressed by the bacteria and genotypic methods that involve analysis of genetic elements based on the bacteria's DNA and RNA (Arbeit, 1995). The methods used in each typing system should follow several essential performance criteria: typeability, reproducibility, stability, sensitivity, discriminatory power, and ease of interpretation (Nielsen et al., 2000). The 2 most important of these criteria are discriminatory power, the ability to differentiate among unrelated isolates, and typeability, the ability of the method to provide unambiguous results for typeable isolates (Burucoa et al., 1999). Most phenotypic and all genotypic typing methods allow for the differentiation of bacterial isolates to the species and subspecies level.

Being able to differentiate between bacterial isolates improves the ability to detect and track food-borne pathogens through all steps of the food system. Each method, whether phenotypic or genotypic, has its own advantages and disadvantages based on the sampling setting, time constraints, and level of conformation required. Use of these typing methods provides more opportunities to understand the population genetics and epidemiology as well as apply more rapid, precise, and efficient food-borne pathogen surveillance and prevention practices (Wiedmann, 2002).

**PHENOTYPIC METHODS**

The classic techniques for differentiating isolates phenotypically are based on the presence or absence of biological or metabolic activities expressed by the organism (Arbeit, 1995). The most popularly used phenotypic methods to differentiate Campylobacter isolates include biotyping, serotyping, and multilocus enzyme electrophoresis. Although most of these methods lack discriminatory power, they are still used and are efficient in characterizing bacterial food-borne pathogens (Wiedmann, 2002).

**Biotyping**

Biotyping is the identification of bacterial isolates through the expression of metabolic activities. These metabolic activities can include colonial morphology, environmental tolerances, and biochemical reactions. The methods used to detect these activities are easy to perform and are relatively inexpensive, making them an ideal method to quickly identify bacterial isolates for further testing.

**Table 1. Advantages and disadvantages of phenotypic and genotypic methods for typing Campylobacter spp. in poultry**

<table>
<thead>
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<th>Typing method</th>
<th>Advantage</th>
<th>Disadvantage</th>
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<tr>
<td>Phenotypic</td>
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<td>Discriminatory power and reproducibility</td>
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<tr>
<td>Biotyping</td>
<td>Reproducibility and typeability (^1)</td>
<td>Discriminatory power, cost, tedious procedures,</td>
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<td>Serotyping</td>
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<td>and time</td>
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<td>Multilocus enzyme electrophoresis</td>
<td>Discriminatory power</td>
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<td>Genotypic</td>
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<td>Multilocus sequence typing</td>
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<td>PCR</td>
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<td>Accuracy of results due to intra- and intergenomic recombination of genes</td>
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<td>Amplified fragment length polymorphism</td>
<td>Discriminatory power, reproducibility, and typeability</td>
<td>Complex analysis and pure culture required to prevent misinterpretation of results due to foreign DNA</td>
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\(^1\)Typeability = the proportion of a population of distinct strains that can be assigned a type marker by that method (Hunter, 1990).
Identification of Campylobacter spp. begins with culturing of the bacteria on some type of medium to observe their colonial morphology. In 1977, Skirrow was the first to develop a blood-agar supplemented with the antibiotics vancomycin, polymyxin B, and trimethoprim, giving the medium selectivity for the growth of Campylobacter jejuni and Campylobacter coli (Skirrow, 1977). Modifications and variations of Skirrow’s original selective medium have taken place as knowledge of the metabolic requirements of Campylobacter and resistance to certain antibiotics have increased. These modified agars use different combinations of antibiotics to discourage proliferation of any non-Campylobacter species or contaminants; however, the selectivity of these media may inhibit the growth of some of the less common species of Campylobacter (Corry et al., 1999).

The first modification of Skirrow’s medium was by Blaser et al. (1979); cephalothin and amphotericin B were added to create Campy-BAP. Researchers compared Skirrow’s and Blaser’s media against a newly developed Preston agar (Bolton and Robertson, 1982). This agar contained rifampicin and cycloheximide to replace cephalothin and amphotericin B. The results indicated that Preston agar had the best isolation rates for the majority of Campylobacter specimens tested (Bolton and Robertson, 1982; Bolton et al., 1983). Bolton et al. (1984) designed charcoal cefoperazone deoxycholate, a blood-free agar that could be used to isolate Campylobacter strains if animal blood is not available or of poor quality. Stern et al. (1992) developed Campy-Cefex agar using the antibiotics cefoperazone and cycloheximide. The authors compared Campy-Cefex agar to Campy-BAP and cefoperazone deoxycholate, showing that Campy-Cefex agar was as productive and selective as the other 2 media. Campy-Cefex agar has been and is still the Campylobacter medium of choice by the US Department of Agriculture—Agricultural Research Service for isolating Campylobacter spp. from poultry (National Advisory Committee on Microbiological Criteria for Food, 2007). In 2001, Campy-Line agar and Campy-Line blood agar were developed using triphenyltetrazolium chloride, a supplement that allowed for easy detection of Campylobacter (Line, 2001). Depending on the type of medium used, Campylobacter colonies may have different appearances. Most colonies are round or irregular in shape, flat, and translucent gray-white or creamy gray in color (Hansson et al., 2004). Isolating Campylobacter spp. using medium can be easy and inexpensive; however, choosing a medium that complies with the type of sample taken is vital and can affect the frequency of Campylobacter recovery (Barros-Velázquez et al., 1999). Age and storage conditions of the medium are also important when looking for high isolation rates for Campylobacter spp. If the medium is stored improperly or becomes old, the selective components will be reduced and, therefore, diminish productivity (Stern et al., 1992).

The thermotolerant nature of C. jejuni and C. coli also discourages the growth of other bacteria on media. Most bacteria, including nonthermophilic Campylobacter spp., do not grow well at 42°C (Barros-Velázquez et al., 1999; Keener et al., 2004). Campylobacter jejuni and C. coli are also strictly microaerophilic, requiring a lower concentration of oxygen (3–15%) and a higher carbon dioxide concentration (3–5%) than is present in the atmosphere (Holt et al., 2000).

After growth on a medium in a microaerophilic environment, a suspect colony can then undergo a series of biochemical reactions to further type the strain. Gram staining, oxidase tests, and catalase tests are the easiest techniques to quickly identify Campylobacter cells. Gram staining uses dyes to differentiate between cells based on their cell wall structure, more specifically the thickness of the peptidoglycan layer. Gram-positive bacteria have a thick peptidoglycan layer and appear bluish-purple, whereas gram-negative bacteria, such as Campylobacter, have a thin peptidoglycan layer and appear red or pink. Campylobacter cells are spirally curved rods, ranging from 0.2 to 0.5 μm wide and 0.5 to 5 μm long, and when 2 cells form short chains, they can appear S-shaped or gull-wing-shaped (Holt et al., 2000). Most Campylobacter spp. are catalase- and oxidase-positive. These bacteria produce catalase to reduce the toxic product hydrogen peroxide (H2O2) created in their environment. Campylobacter are oxidase-positive because they contain cytochrome C, a compound that helps transfer electrons to the cytochrome oxidase complex. These bacteria also reduce fumarate to succinate and are negative in methyl red, acetoin, and indole reactions (Barros-Velázquez et al., 1999; Wassenaar and Newell, 2006). Methyl red can be used to test for acid production by fermentation and yielding a red color at or below pH 4.4. The Voges-Proskauer test involves the production of acetoin made by the degradation of glucose. Campylobacter spp. have a nonfermenting metabolism and do not produce acid or neutral end products, which consequently results in negative reactions. The indole test indicates the presence of the enzyme tryptophanase, which hydrolizes tryptophan to indole. Campylobacter do not hydrolyze indole, the reaction is negative (Sun, 2007). Campylobacter spp. also reduce nitrates but not nitrites (Barros-Velázquez et al., 1999).

Other biochemical tests that Campylobacter spp. can undergo include hippurate hydrolysis, rapid H2S production, nalidixic acid resistance, and DNA hydrolysis. Ninety-five percent of C. jejuni strains are hippurate-negative and do not produce H2S, whereas all C. coli strains are hippurate-negative and produce H2S (Totten, 1987; Barros-Velázquez et al., 1999; Holt et al., 2000). Skirrow and Benjamin (1980) were the first to propose biotyping schemes for C. jejuni, C. coli, and C. laridis using the hippurate hydrolysis test, the rapid H2S test in iron-containing medium, and a test for resistance to nalidixic acid. Hébert et al. (1982) quickly followed with their own biotyping scheme. Instead of using the rapid H2S test and resistance to nalidixic acid, they looked at DNA hydrolysis and growth on charcoal yeast extract agar. Lior (1984) used a combi-
nation of the 2 schemes, biotyping by hippurate hydrolysis, rapid H$_2$S production, and DNA hydrolysis, along with serotyping to characterize thermophilic isolates from human and nonhuman sources. He found that the discriminatory power was greatly increased when using the serotyping scheme along with the biotyping scheme. All of the tests described by Skirrow and Benjamin, Herbert, and Lior are still popular and commonly used when biotyping *Campylobacter* spp.

All of the tests described above can be used alone or in combination to isolate and identify *Campylobacter* spp. Although it is easy and cost effective to obtain commercial kits and the equipment to run these biochemical reactions, the kits may yield poor reproducibility and discriminatory power due to variation of gene expression caused by environmental factors and lack of biochemical diversity between isolates. The efficiency of these tests can be increased by using another phenotypic method, serotyping, along with biotyping.

### Serotyping Using Heat-Stable and Heat-Labile Antigens

Serologic typing, or serotyping, is based on the knowledge that microorganisms have differing cellular surface structures (Arbeit, 1995). In serotyping, antibodies and antisera are used to detect surface antigens present on bacteria, thereby distinguishing strains by the differences in their surface structures (Wiedmann, 2002). *Campylobacter* spp. have several structures found on their cell surface, including lipopolysaccharides, capsular polysaccharides, membrane proteins, and extracellular organelles (Linton et al., 2001); most of these structures also have a role in host-bacterium interactions.

Berg et al. (1971) were the first to separate *Campylobacter* into 3 serotypes based on heat-stable antigens. Penner and Hennessy (1980) demonstrated in their preliminary experiments that antigens (heat-stable at 100°C) were capable of agglutinating in antisera and could produce specific antibodies in rabbits; this preliminary work ultimately lead to the Penner scheme of serotyping. This particular scheme differentiates strains on the basis of heat-stable antigens found on the cell surface (Moran and Penner, 1999). Lior et al. (1982) followed with their own serotyping scheme based on an easy-to-perform slide agglutination technique, which uses live bacteria and specific absorbed antisera for the detection of heat-labile antigens. This system was developed according to classical methods used to identify flagellar antigens of gram-negative species, including *Salmonella* spp. and *E. coli* (Moran and Penner, 1999). Soon after, Patton et al. (1985) compared the Penner and Lior schemes, finding both methods of serotyping to be typeable and reproducible, suggesting, however, that they be used together for the best differentiating results. Nielsen et al. (2000) and McKay et al. (2001) found that using the Penner scheme alone yielded low discriminatory power, but when supplemented with other typing methods, like fla-denaturing gradient gel electrophoresis, riboprinting, or pulsed-field gel electrophoresis (PFGE), the serotyping scheme could be definitive in identifying *Campylobacter* isolates. The major disadvantages of both of these schemes are that they are time-consuming, have tedious requirements, require costly reagents, and there are also a high number of untypeable strains.

### Multilocus Enzyme Electrophoresis

In multilocus enzyme electrophoresis (MEE), bacterial isolates are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing conditions (Downes, 2001; Wiedmann, 2002). Each intracellular enzyme represents a discrete, independent characteristic of an isolate; these enzymes differ in size, electrical charge, and conformation, resulting in slight alterations in migration rates across a gel. Enzyme activities are determined by locating their position on a gel after adding color-generating substrates to the enzymes (Arbeit, 1995; Downes, 2001). The variation of the electrophoretic mobility of an enzyme is dependent on mutations at the gene locus that causes amino acid substitutions, which alter the charge of the protein. These mobility variants are called electromorphs. The unique profile of an electromorph produced by each strain of a bacterial isolate is called an electromorph type (ET; Arbeit, 1995). The ET number that represents the combination of alleles of the different enzymes being tested is compared with interphylogenetic relationships among isolates (Klena and Konkel, 2005). Multilocus enzyme electrophoresis was first used by Aeschbacher and Piffaretti (1989) to characterize *Campylobacter* spp. to determine the relationships of *C. jejuni* and *C. coli* populations between strains from nonhuman and human sources. Patton et al. (1991), and later Bolton et al. (1996), compared the discriminatory power of MEE to other widely used phenotypic and genotypic methods using epidemic strains of *C. jejuni*. Both groups found MEE to be one of the most discriminatory methods used. Although almost all strains can be typed with MEE, it is not greatly used due to its low reproducibility, difficulty of comparison of results between laboratories, and its ability to generate untypeable alleles (Downes, 2001; Klena and Konkel, 2005).

### Genotypic Methods

When determining the genotype of an organism, locatable regions within a genome are examined, which allows for the differentiation of many subtypes within a species as well as the genomic relatedness among isolates (Downes, 2001). Genotypic methods provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeability, and discrimi-
natory power when compared with phenotypic typing methods (Wassenaar and Newell, 2000; Wiedmann, 2002). Commonly used genotypic typing methods include multilocus sequence typing, PCR, PFGE, ribotyping, flagellin typing, and amplified fragment-length polymorphism (AFLP).

**Multilocus Sequence Typing**

With the advancement in technology and an increase in knowledge of gene sequencing, a genotypic typing method called multilocus sequence typing (MLST) has been developed based on the well-established principles of MEE (Maiden et al., 1998). Multilocus sequence typing differs from MEE in that it assigns alleles directly by DNA sequencing of 7 to 11 housekeeping genes, rather than indirectly through the electrophoretic mobility of their gene products. In MLST, each sequence of a locus is assigned an allele number in order of its discovery. These alleles correspond to the electromorph in MEE. The alleles are combined into an allelic profile and are given a sequence type, which is equivalent to the ET used in MEE. Allelic profiles are then compared, which shows how closely related the isolates are to each other. The more sequence types the isolates have in common, the more they are related, and vice versa (Urwin and Maiden, 2003). The sequence data can then be compared among laboratories using an online database (http://pubmlst.org). Multilocus sequence typing was introduced by Maiden et al. (1998) to overcome the problems of comparing results of typing schemes between laboratories. As a result, they were able to develop a portable, highly discriminatory method for characterizing pathogenic microorganisms. Dingle et al. (2001) developed an MLST method specifically for the characterization of *C. jejuni* strains. Although most *Campylobacter* infections from poultry are caused by *C. jejuni*, it is still important to have the capability to detect all possible infectious *Campylobacter* spp. Therefore, Miller et al. (2005) designed an extended MLST method that characterizes not only *C. jejuni* but also *C. coli*, *C. lari*, and *C. upsaliensis*. The advantages of using MLST include high discriminatory power, reproducibility, ease of interpretation, and transferability of information among laboratories (Dingle et al., 2001). Multilocus sequence typing can also detect mixed cultures, genetic exchange, and recombination between *Campylobacter* spp. (Miller et al., 2005). Although MLST results are easy to reproduce, interpret, and transfer, it is a complex and expensive technique to perform (Lévesque et al., 2008).

**PCR**

Polymerase chain reaction, sometimes known as molecular photocopying, is a typing method that amplifies target segments of DNA (Atlas and Bej, 1994). By using oligonucleotides (primers) that bind to the target segments of DNA at the 5' and 3'-ends, DNA polymerase can be directed to the targeted site for amplification. This process when repeated ultimately leads to an exponential increase in the total amount of DNA. Mullis and Faloona (1987) revolutionized the way scientists approach the study and analysis of genes. Several variations to the original PCR technique have been developed that are useful in detecting *Campylobacter* spp. These techniques include reverse-transcriptase PCR, multiplex PCR, and quantitative real-time (QRT)-PCR.

Reverse transcription PCR, first described by Powell et al. (1987) relies on the application of 2 separate enzymatic activities to isolate and amplify cellular messenger RNA. The first activity results in DNA formation by reverse transcriptase that is complementary to the target RNA sequence. This complementary DNA is also known as cDNA. The second enzymatic reaction amplifies this cDNA through the use of Taq DNA polymerase (Bustin, 2004). This method is extremely sensitive, requires minimal sample sizes, and is able to measure multiple unique target sequences in every sample, giving it an advantage over other PCR techniques (Dieffenbach and Dveksler, 2003). Multiplex PCR was first described by Chamberlain et al. (1988). This technique can amplify 2 or more target sequences by using multiple, unique primer sets resolved within a single PCR reaction (Lee et al., 2009). When amplifying multiple target sequences, it is important to choose target DNA with similar annealing temperatures and lengths to avoid differential yields of amplified products (Atlas and Bej, 1994). Although this method is cost-effective and convenient, optimizing reaction conditions can be tedious and time-consuming.

Quantitative real-time PCR uses fluorescent dyes to determine whether a DNA sequence is present in a sample as well as the number of amplified products in the sample. The first real-time PCR technology was established by Higuchi et al. (1993) and the method combined PCR amplification with the constant monitoring of newly synthesized DNA from each cycle. Quantitative real-time PCR is very fast and allows for inspection of fluorescence due to newly synthesized DNA without introducing errors by stopping the reaction and removing newly synthesized product (Mackay, 2007).

Many studies involving *Campylobacter* spp. have used these variations of PCR to identify and differentiate isolates. Persson and Olsen (2005) developed a robust multiplex PCR method to analyze *C. jejuni* and *C. coli* from pure culture and stool samples. Chuma et al. (2000) also used multiplex PCR to look at the role of sparrows as a source of *Campylobacter* infection in broilers. Hiett et al. (2002) used multiplex PCR along with a set of primers specific for the *Campylobacter* flagellin A gene short variable region to directly detect *Campylobacter* in fluff and eggshell samples. Stintzi (2002) used reverse transcription PCR to measure changes in *C. jejuni* transcription levels as they responded to temperature.
Yang et al. (2003) used QRT-PCR to detect *C. jejuni* in poultry and milk products as well as environmental water samples. Ridley et al. (2008) used QRT-PCR to determine potential environmental sources that could lead to the colonization of a broiler flock by *Campylobacter*. In 2008 and 2010, research was conducted using QRT-PCR to quantify *Campylobacter* from poultry carcass rinses providing valuable data that could be used for risk assessments in the processing plant (Botteldoorn et al., 2008; Josefsen et al., 2010).

The above PCR techniques have truly revolutionized the way that bacterial genes are identified and typed. These techniques are easy to reproduce, highly discriminatory, and available in most laboratories. Although these techniques can be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp.

**PFGE**

After its development by Schwartz and Cantor (1984), PFGE has emerged as one of the best molecular approaches to analyzing bacterial pathogens, including *Campylobacter*. In short, PFGE is a variation of agarose-gel electrophoresis. It begins by embedding bacterial cells in agarose gel, referred to as the plug. The plug is then treated with enzymes and RNases to digest unwanted proteins and RNA, leaving naked, purified chromosomal DNA. After the chromosomal DNA is pure, the plug is cut or broken into segments and treated with restriction enzymes to digest the DNA into segments. While the plug is being treated with restriction enzymes, an agarose gel is poured and allowed to set. Once the gel is set and the DNA is digested by the restriction enzymes, the plug is placed in the wells of the gel and capped in place within the molten agarose. At this point, an electric field is applied across the gel and the current is changed periodically, or pulsed, rather than keeping the current constant. Steele et al. (1998) determined PFGE to be the most discriminatory typing method for *Campylobacter* when compared with fatty acid profile typing, serotyping, and biotyping. In another study, Fitzgerald et al. (2001) determined that PFGE, along with PCR restriction fragment length polymorphism (RFLP), was highly discriminatory in differentiating *Campylobacter* isolates among species present on farms as well as in clinical environments. This method has been proven to be highly discriminatory, and the relative simplicity of the restriction-enzyme profiles facilitates the analysis and comparison of multiple bacterial isolates. Results from studies revealed that reproducibility of PFGE analysis between laboratories can be high if standard protocols are used in each laboratory (Ribot et al., 2001). As a result, PFGE is the method of choice for epidemiologic subtyping of pathogenic bacteria by most federal, state, and local health laboratories in the United States. An outbreak of *E. coli* O157:H7 in 1993 led to the development of PulseNet (http://www.cdc.gov/pulsenet/), a database of PFGE patterns that assist epidemiologists at the Center for Disease Control and Prevention in detection of food-borne disease case clusters as well as separates outbreak-associated cases from other sporadic cases. PulseNet also facilitates rapid communication between the Center for Disease Control and public health departments, providing earlier identification of a common source outbreak and potential reduction in the amount of illnesses related to that outbreak (CDC, 2011b); however, PFGE does have a few limitations. The equipment required to perform this technique is expensive and the procedure can take up to 4 d (Arbeit, 1995). Also, comparison of the PFGE profiles from the same bacterial isolates obtained by workers in different laboratories can differ due to the variations in sources of the restriction enzymes and electrophoresis conditions used (Wassenaar and Newell, 2000).

**Ribotyping**

Ribotyping is a ribosomal (r)RNA approach for the identification of bacterial isolates (Williams et al., 1998). Multiple copies of the rRNA gene loci that code for the 5, 16, and 23S rRNA are located at different positions on the *Campylobacter* chromosome. The strong conservative nature of the rRNA genes and the presence of noncoding flanking regions make these genes suitable for this typing method (Wassenaar and Newell, 2000). This technique begins by isolating genomic DNA from a *Campylobacter* sample. The genomic DNA is digested with restriction enzymes and products are separated by electrophoresis on a gel. Products are transferred to a blot by Southern blotting techniques and labeled probes specific for ribosomal RNA are hybridized to the RNA. The fragments are then analyzed for patterns. Although ribotyping has a high level of typeability for *Campylobacter* spp., its low number of ribosomal genes gives it poor discriminatory power. Another disadvantage is that this method is tedious, time-consuming, and expensive, which makes it unsuitable for routine genotyping (Wassenaar and Newell, 2000). Automated ribotyping (AR) systems have been recently developed to help decrease labor and increase sensitivity in identifying food-borne pathogens. Automated ribotyping combines the molecular steps into a single efficient machine, making the testing method faster and more reliable (Pavlic and Griffiths, 2009). With *Campylobacter* spp., AR systems are used in conjunction with other genotyping methods to amplify the discriminatory power of the typing method. Manfreda et al. (2003) used multiplex PCR along with AR to demonstrate that the method is highly reproducible and efficient enough to use as a library typing method for *Campylobacter* surveillance. O’Reilly et al. (2006) also showed that by using AR in combination with MLST or RFLP, AR is useful in identifying genotypic grouping of *Campylobacter* spp. from clinical isolates in Australia.
**Flagellin Typing Using RFLP**

The flagella of *Campylobacter* are complex and their proteins are encoded by a major flagellin gene, flaA, and a minor flagellin gene, flaB. The flaA and flaB genes are approximately 59 kDa in size and are highly homologous (Guerry, 2007). The flaA gene is regulated by the classic flagellin promoter σ28, whereas the flaB gene is regulated by the σ54-dependent promoter (Hendrixson, 2008). Early studies conducted by Morooka et al. (1985), Pavlovskis et al. (1991), Nachamkin et al. (1993), and Wassenaar and Newell (2000) illustrated that motility is necessary for the interaction of *Campylobacter* with the intestinal epithelia cells in humans as well as other animal species. Recent studies have suggested that the level of σ54 genes may play a major regulatory role in flagella expression in *C. jejuni*. These studies have led to the idea that flagella hold virulence factors for these pathogenic bacteria (Guerry, 2007). Because both flaA and flaB genes are different from one another and highly conserved, this flagellin gene locus is suitable for detection by RFLP from PCR products. In flagellin gene typing, the flaA gene is first amplified by PCR. The amplified DNA is then digested with specific restriction endonucleases. Restriction fragment length polymorphism probes hybridize to the digested fragments, and the fragments are viewed by using agarose gel electrophoresis, giving distinguishable patterns. The discriminatory power of flagellin gene typing is determined by the type of restriction enzyme used (Fitzgerald et al., 2001). Like the Center for Disease Control PulseNet database for PFGE patterns, a database called PubMLST is available for flaA isolates (http://pubmlst.org/). The PubMLST database is a publically accessible, easy-to-use database that allows for scientists to submit, search, and share information on flaA gene typing (PubMLST, 2011). Although flagellin gene typing is quick and can have high discriminatory power, it is suggested that this method should not be the sole technique used in epidemiological grouping of isolates. The use of the flaA gene for typing has been recently questioned due to the intra- and intergenic recombination within the flagellin genes (Mellmann et al., 2004). This recombination alters the flagellin sequence by allowing sequences located in the flaA and flaB genes to be transferred back and forth between these 2 genes while leaving the flagellin sequence otherwise genotypically unaltered (Harrington et al., 1997). It has also been reported that exogenous DNA from naturally competent bacteria can be incorporated into the flaA and flaB genes of *Campylobacter* through this recombination process (Wassenaar et al., 1995). The recombination of these genes makes long-term use of this technique unlikely.

**AFLP**

Amplified fragment length polymorphism is a technique capable of genotyping genomic DNA from any organism. The method of genotyping bacteria by AFLP was developed by Vos et al. (1995). This technique requires genomic DNA to be digested with restriction enzymes. The digested DNA is then ligated with double-stranded adaptors and amplified using primers complementary to the adaptors and restriction site sequences. Subsets of the restriction fragments are amplified a second time using selective primers that incorporate a label onto the products. The products are separated using electrophoresis and denaturing polyacrylamide gel before being visualized (Vos et al., 1995). Amplified fragment length polymorphism provides an advantage that other genotypic methods of typing do not. The data can be analyzed to determine the genetic relatedness among bacterial strains as well as identify or type strains, and it is also able to generate typing of any DNA regardless of origin and complexity (Vos et al., 1995). Duim et al. (2000) and Schouls et al. (2003) demonstrated the ability of AFLP analysis to identify 2 *Campylobacter* spp., *C. jejuni* and *C. coli*, from poultry samples. They were also able to show that these 2 species had similar AFLP banding patterns to strains they had identified in humans. Schouls et al. (2003) found, however, that there could be problems with analysis due to complex banding patterns, which are PCR-based and are prone to variation. Another disadvantage of this method is that it requires the organism of interest to be isolated from all other organisms, this is because the DNA from other organisms can disturb the AFLP pattern. Since being developed, AFLP has been established as a broadly applicable genotypic typing method with high discriminatory power, typeability, and reproducibility (Savelkoul et al., 1999).

**Conclusions**

Typing methods play a critical role in the identification, monitoring, and prevention of *Campylobacter* infections. The use of multiple phenotypic and genotypic typing methods can improve species and subspecies discrimination and is appropriate when trying to identify pathogenic organisms like *C. jejuni*, *C. coli*, and *C. laridis* (Wassenaar and Newell, 2000). Serotyping and biotyping methods are standard when it comes to identifying bacterial isolates in a laboratory setting. These phenotypic methods, however, cannot provide as much discriminatory power as genotyping methods. Through ongoing research, PFGE and AFLP have been found to have the greatest discriminatory power when compared with techniques like ribotyping and flagellin typing. Polymerase chain reaction, a genotyping technique itself that is often used in concert with most other genotypic methods, has been found to be an efficient and reliable typing method, providing superior results concerning the discrimination of bacterial isolates. Although it is not yet possible to identify a perfect typing method for pathogenic *Campylobacter* spp., currently available techniques, when used in concert, fulfill the requirements for epidemiological inquiries in laboratory
settings. The development and fine-tuning of an ideal typing method could make routine subtyping of *Campylobacter* spp. feasible.

**REFERENCES**


