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Journal of Microbiological Methods 84 (2011) 194–201

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Typing of *Campylobacter jejuni* and *Campylobacter coli* isolated from live broilers and retail broiler meat by *flaA*-RFLP, MLST, PFGE and REP-PCR

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ARTICLE INFO

Article history:
Received 21 September 2010
Received in revised form 16 November 2010
Accepted 19 November 2010
Available online 2 December 2010

Keywords:
flaA-RFLP
PFGE
MLST
REP-PCR
Campylobacter jejuni
Campylobacter coli

ABSTRACT

We analyzed 100 *Campylobacter* spp. isolates (*C. jejuni* and *C. coli*) from Grenada, Puerto Rico and Alabama, which were collected from live broilers or retail broiler meat. We analyzed these isolates with four molecular typing methods: restriction fragment length polymorphism of the *flaA* gene (*flaA*-RFLP), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and automated repetitive extragenic palindromic polymerase chain reaction (REP-PCR) using the DiversiLab system. All methods performed similarly for the typing of *C. jejuni* and *C. coli*. The DNA extraction method appears to influence the results obtained with REP-PCR. This method was better for the typing of *C. jejuni* than *C. coli*, however both REP-PCR and *flaA*-RFLP generated types that were indistinguishable between *C. jejuni* and *C. coli* and appeared to be random, without any relationship to species, location, or source of isolates. PFGE and MLST generated typing results that had a better correlation with the geographic location of the isolates and showed higher concordance with the Wallace coefficient. The adjusted Rand coefficient did not show higher concordance among the methods, although the PFGE/MLST combination exhibited the highest concordance. PFGE and MLST revealed a better discriminatory power for *C. coli* isolates than REP-PCR or *flaA*-RFLP. The use of readily available online tools to calculate the confidence interval of the Simpson's index of diversity and the adjusted Rand and Wallace coefficients helped estimate the discriminatory power of typing methods. Further studies using different *C. jejuni* and *C. coli* strains may expand our understanding of the benefits and limitations of each of these typing methods for epidemiological studies of *Campylobacter* spp.

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1. Introduction

Advances in DNA techniques applied to typing bacterial foodborne pathogens have improved our understanding of the epidemiology of these pathogens. The successful implementation of a typing method in microbiology laboratories depends on the discriminatory power, which measures the ability of a method to assign a specific molecular fingerprint or operational taxonomic unit to an isolate, the typeability, which is the proportion of a population of distinct strains that can be assigned a type by a method (Hunter, 1990), and the reproducibility of the method, which is the proportion of strains typed the same way on repeat testing, preferably after a period of a few months, by a typing method (Hunter, 1990; Riley, 2004; Maslow et al., 1993a). However, other additional, important considerations in modern molecular microbiology laboratories are the epidemiologic concordance (i.e., the agreement between the grouping of strains by a given method and the available epidemiological information about those strains (van Belkum et al., 2007) and the possibility of automation for high throughput analysis.

Molecular typing techniques are used to characterize the intra-species variability of an organism. These techniques can also be used to track specific types (strains with similar or identical fingerprinting patterns) in epidemiological studies. In the case of *Campylobacter jejuni* and *C. coli*, two species that share similar niches and result in similar disease presentations in humans, few studies have accessed the concordance of results obtained with different techniques on the same set of isolates. Furthermore, we are not aware of any study comparing four typing methods in a set of *C. jejuni* and *C. coli*.

Campylobacter jejuni and *C. coli* are important foodborne pathogenic bacteria worldwide, representing the most reported bacterial foodborne diseases since 2005 in the EU (Anonymous, 2005) and the second most important bacterial foodborne disease in the USA (Anonymous, 2008). The techniques most commonly used for typing *Campylobacter* isolates are based on the amplification of small DNA segments of the bacterial chromosome (repetitive polymerase chain reaction technique, REP-PCR), the amplification and restriction of specific genes (fragment length polymorphism, *flaA*-RFLP), the restriction and migration of large chromosomal segments (pulsed-

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doi:10.1016/j.mimet.2010.11.016

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field gel electrophoresis, PFGE), or the sequencing of housekeeping gene fragments (multilocus sequence typing, MLST) (Dingle et al., 2001; Gibson et al., 1997; Nachamkin et al., 1993; Stern et al., 1984; Yan et al., 1991).

PFGE is regarded as the reference typing method for *Campylobacter* spp. because the entire genome is analyzed to create restriction profiles (Maslow et al., 1993b). The drawback of PFGE is the time and labor necessary to perform this technique properly, and the fact that restriction-based methods are more subjective than sequence-based methods. *flaA*-RFLP amplifies and restricts the *flaA* gene to create a fingerprint profile. MLST utilizes nucleotide polymorphisms in relatively-conserved housekeeping genes, and is a powerful technique for population studies (Maiden et al., 1998). Yet, MLST requires high quality sequences and is the most expensive of all these techniques. A more recent addition to the techniques used for bacterial typing is the automated system to perform REP-PCR. This technique assigns molecular fingerprints based on the amplification of repetitive sequences specific to the organism and commonly found in non-coding regions of bacterial genomes (Hulton et al., 1991; Stern et al., 1984; Versalovic et al., 1991). The automated version of this technique (DiversiLab, bioMérieux, Hazelwood, MO) has been designed to standardize the collection and referencing of bacterial profiles (Healy et al., 2005a). This technique has been applied to the typing of several pathogens, including *Neisseria meningitidis* (Healy et al., 2005a), clinical *Candida* isolates (Wise et al., 2007), Dermatophytes (Pounder et al., 2005), *Fusarium* species (Healy et al., 2005b), fungi of the *Phaeoacremonium* and *Phaeomoniella* species associated with esca syndrome in grapevines (Alves et al., 2004), and for the genotyping and strain identification of Archaea (Cleland et al., 2008). However, it has not been extensively tested with *Campylobacter* isolates, nor has it been tested in studies comparing the performance of this technique and other typing methods on the same isolates.

In this study, we present the results from the evaluation of *flaA*-RFLP, MLST, PFGE and REP-PCR for the typing of *C. jejuni* and *C. coli* isolates collected from live broilers or retail broiler meat. The impact of different DNA extraction kits in the reproducibility of REP-PCR was evaluated, and single techniques and combinations of techniques were assessed for their discriminatory power using the Simpson's index of diversity (Hunter and Gaston, 1988). We also calculated concordance of the methods using the adjusted Rand and Wallace coefficients and the approximate 95% confidence interval for these indices with an online bootstrap method.

2. Materials and methods

2.1. Sample collection

One hundred *Campylobacter* spp. isolates (49 *C. jejuni* and 51 *C. coli*) were recovered from stock cultures stored at −80 °C in cryovials containing 80% Brucella broth, 15% glycerol, and 5% lysed horse blood. These isolates had been collected from live broiler chickens from Grenada (45 isolates) and Puerto Rico (29 isolates), and from retail broiler meat in Alabama (26 isolates). Table 1 shows the origin, the year of collection, and the references describing the method of collection for the isolates collected from live broilers (Miller et al., 2010; Oyarzabal et al., 2008). The isolates from retail broiler meat were collected using standard procedures in our laboratory (Oyarzabal et al., 2007), which include the enrichment of boneless meat samples in Bolton broth and the plating on modified Campy-Cefex (mCC) plates (Oyarzabal et al., 2005). Isolates were plated from frozen stock though 0.6 μm cellulose filters onto mCC plates (Speegle et al., 2009). Plates were incubated at 42 °C for 24 h under microaerobic conditions (10% CO_2, 5% O_2 and 85% N_2; Airgas, Radnor, PA) using the evacuation replacement system MACSmics Jar Gassing System (Microbiology International, Frederick, MD) in anaerobic jars.

Table 1
Origin, year of collection and distribution by species of the isolates used in our studies.

Species	Source	Origin	Number of strains	Year collected	Reference
C. coli	Cecal material from broilers and layers	Grenada	39	2006–2007	Miller et al., 2009
	Retail broiler meat	Alabama	12	2005–2007	This study
C. jejuni	Cecal material from broilers and layers	Grenada	6	2006–2007	Miller et al., 2009
	Fecal material of live broilers	Puerto Rico	29	2005	Oyarzabal et al., 2008
	Retail broiler meat	Alabama	14	2005–2007	This study

2.2. DNA extraction and identification of isolates

DNA was extracted using MoBio UltraClean™ Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA). To evaluate the impact of different DNA isolation protocols, the DNA from 10 isolates was also extracted with Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA), DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA) and the alkaline lysis method (Debruyne et al., 2008). All samples were speciated using a multiplex PCR assay described elsewhere (Linton et al., 1997; Oyarzabal et al., 2007; Persson and Olsen, 2005).

2.3. flaA-restriction fragment length polymorphism (flaA-RFLP) analysis

Extracted DNA was amplified for *flaA*-RFLP analysis as described elsewhere (Nachamkin et al., 1993; Harrington et al., 2003). The *flaA* amplicon was restricted with *DdeI*, and the DNA segments were separated on a 2.5% agarose gel (SeaKem Gold agarose; Lonza, Allendale, NJ) in 1% TBE at 80 V for 1.5 h. DNA fragments were stained with ethidium bromide, visualized using an ultraviolet transilluminator (Gel-Doc system, Syngene, Frederick, MD), and recorded for molecular analysis using GeneSnap (Syngene, Frederick, MD).

2.4. Pulsed-field gel electrophoresis (PFGE)

Campylobacter DNA was prepared for PFGE according to standard protocols (Ribot et al., 2001; Oyarzabal et al., 2008). To prepare the plugs, cells were collected at an OD_{600nm} of 1.3 instead of 0.57–0.82 as suggested by previous protocols (Ribot et al., 2001). DNA was digested with *Sma*I and separated using a CHEF DR II system (Bio-Rad Laboratories, Hercules, CA) on 1% agarose gels (SeaKem Gold agarose; Lonza). *Salmonella enterica* subsp. *enterica* serovar Braenderup strain H9812 (ATCC BAA-664) restricted with *Xba*I was used as the DNA size marker. The gels were stained and visualized as described above.

2.5. Analysis of flaA-RFLP and PFGE results

Digital images of the restriction profiles for *flaA*-RFLP and PFGE were converted into TIFF format and uploaded into BioNumerics ver. 5 (Applied Maths, Austin, TX, USA) for analysis. Pair comparisons and cluster analyses were made using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. The optimization tolerance was set at 2% and the position tolerance for band analysis was set at 4%. A cut-off of 90% was used to separate different restriction patterns for *flaA*-RFLP and PFGE.

2.6. Multilocus sequence typing (MLST)

MLST was performed as described elsewhere (Miller et al., 2010). In the case of *Campylobacter*, MLST amplifies a segment of seven housekeeping genes: *aspA* (aspartate ammonia-lyase, allele = 477 bp), *atpA* (or *uncA*, ATP synthase F1 sector, alpha subunit, allele = 489 bp), *glnA*

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(glutamine synthetase, allele = 477 bp), *gltA* (citrate synthase, allele = 402 bp), *glyA* (serine hydroxymethyltransferase, allele = 507 bp), *pgm* (phosphoglucomutase, allele = 498 bp) and *tkt* (transketolase, allele = 459 bp) for a total composite sequence length (all 7 loci) of 3309 bp.

MLST of all strains was performed using primer sets described elsewhere (Miller et al., 2005). Each MLST amplification mixture contained: 50 ng genomic DNA, 1× MasterAmp PCR buffer (Epicentre, Madison, WI), 1× MasterAmp PCR enhancer (Epicentre), 2.5 mM $MgCl_2$, 250 μM (each) dNTPs, 50 pmol each primer, and 1 U *Taq* polymerase (New England Biolabs, Beverly, MA). MLST amplifications were performed on a Tetrad thermocycler (Bio-Rad Laboratories) and cycle sequencing extension products were purified using BigDye XTerminator (Applied Biosystems). DNA sequencing was performed on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Alleles and sequence types (STs) were identified using the Perl program MLSTparser3 (Miller et al., 2009). Novel alleles and STs were submitted to the PubMLST *C. jejuni*/*C. coli* databases (http://pubmlst.org/campylobacter/).

2.7. Repetitive polymerase chain reaction (REP-PCR)

REP-PCR was performed on DNA extracted with MoBio UltraClean™ Microbial DNA isolation kit (MoBio Laboratories). Isolates were tested using an automated commercial system (DiversiLab, bioMérieux), which includes a *Campylobacter* kit, micro-fluidic chips, and the Caliper LabChip Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA). PCR amplifications were performed in a PTC-100 Programmable Thermal Controller (Bio-Rad Laboratories) and the amplified products were loaded into micro-fluidic chips prepped with specific DNA reagents and supplies. The micro-fluidic chips were then loaded into the Caliper LabChip Agilent 2100 Bioanalyzer, and fingerprints were analyzed using the 2100 Expert Software and information transferred to the DiversiLab Web Interface v. 3.3.40 using the DiversiLab Upload Client. Cluster analyses were performed using the Pearson correlation coefficient, and a dendrogram was created by web interface software. This software uses a cutoff of 95% to analyze different REP-PCR profiles.

2.8. Evaluation of individual and combined typing methods

Using the Online Tool for Quantitative Assessment of Classification Agreement (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool), we calculated the discriminatory power of each technique by determining the Simpson's index of diversity (SID) for each method and each species (*C. jejuni* or *C. coli*) and for the complete set of strains (Hunter and Gaston, 1988). The SID describes a method's ability to assign a different type to two unrelated strains sampled randomly and taken from the population of a given species (van Belkum et al., 2007). The formula used to calculate the SID is:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j$$

where a_j is the number of strains in the population which are indistinguishable from the jth strain, and N is the number of strains in the population (Hunter, 1990; Hunter and Fraser, 1989).

The concordance of the methods was determined by calculating the adjusted Rand and Wallace coefficients. With the bootstrap methods (1000 iterations), we calculated the approximate 95% confidence (CI) interval for SID, adjusted Rand and Wallace coefficients, and the statistical differences ($P<0.05$) for typing methods based on the categorization of data by the adjusted Rand and Wallace coefficients (Carrico et al., 2006; Pinto et al., 2008). The adjusted Rand coefficient is used to evaluate the extent of agreement between two typing methods. This coefficient incorporates the intercluster distances and is a powerful method to evaluate cases of entity pairs grouped in the same cluster by one method but separated by another (Pinto et al., 2008). The Wallace coefficient is an estimate of how much new information is obtained from a typing method in comparison with another one. This coefficient calculates the probability that entities grouped in the same cluster under a method are also in the same cluster under another method, and vice versa. Therefore, a high Wallace's coefficient value indicates that partitions defined by one method could have been predicted by another method (Carrico et al., 2006).

3. Results and discussion

In this manuscript we present the results from typing a set of *C. jejuni* and *C. coli* strains with *flaA*-RFLP, MLST, PFGE, and REP-PCR. Isolates of *C. jejuni* and *C. coli* from different sources and geographical areas were evaluated with these typing methods. We chose a set of *C. jejuni* and *C. coli* strains that belong to three different regions and that were either isolated from live broilers or retail broiler meat. These typing methods were evaluated for their discriminatory power, typeability, reproducibility and their epidemiological concordance. Their clustering concordance was assessed using the adjusted Rand and Wallace coefficients, which evaluate pairwise agreement of data partitioned by different methods using a 2 by 2 mismatch matrix.

3.1. flaA-RFLP results

flaA-RFLP exhibited a typeability of 95% and resulted in 19 distinct types. Seven types overlapped between species. Seven of the distinct types contained only one isolate, while four types contained each more than 10 isolates. One type (K) was represented by 18% of the isolates (Table 2). *flaA*-RFLP has been used in several studies for the typing of *Campylobacter* spp. and has even been evaluated for its reproducibility in an inter-laboratory study (Harrington et al., 2003). This technique is inexpensive and very easy to reproduce, and although similar types (strains resulting in similar band patterns) are found between *C. jejuni* and *C. coli*, this technique can be easily incorporated in laboratories that may need to type a relatively small number of isolates. Most laboratories that have incorporated molecular methods can do *flaA*-RFLP on *Campylobacter* isolates. Even the interpretation of the results can be done using the pairwise binary band matching (Tenover et al., 1995), which is still a simple but powerful method for epidemiological studies involving a relatively small number of isolates (Vauterin and Vauterin, 2006). However, the recombination within and between the two flagellin loci creates instability in the polymorphism of these genes and has been found to be a limitation for the use of this technique for the long-term study of *Campylobacter* strains (Harrington et al., 1997). It appears that the variability in the *Dde*I restriction sites in the *flaA* gene is more limited for *C. coli* than for *C. jejuni* (Harrington et al., 1997). In this study, two *C. jejuni* strains and three *C. coli* strains did not show any amplification or bands with the *flaA*-RFLP typing method. Therefore, this method had the lowest typeability of all the methods tested.

3.2. MLST results

MLST resulted in 26 distinct profiles. Thirteen sequence types (ST) were represented by only one isolate, while three types (353, 1630 and 2624) contained more than 10 isolates each. Within *C. jejuni* isolates, sequence type 353 was present in the samples from the three geographical areas included in the study. Only one new MLST sequence type (4376) for a *C. jejuni* strain was found. This profile was deposited in the *C. jejuni*/*C. coli* PubMLST database.

MLST is an important technique for population studies of *Campylobacter* spp. (Maiden and Dingle, 2008). Because the *C. jejuni* and *C. coli* MLST methods utilize seven housekeeping genes common to all isolates from both species and primer pairs that efficiently

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Table 2
Number of types obtained with REP-PCR (lower case letters), PFGE (Roman numerals), *flaA*-RFLP (upper case letters), MLST (ST numbers) and all combined methods (Arabic numerals).

Species	Origin	Region	*flaA*-RFLP	MLST (ST#)	PFGE	REP-PCR	PFGE + MLST[a]
C. coli	Live broilers	Grenada	A, B, D, I, J, K, L, O, Q[b]	825, 894, 1173, 1581, 1630, 3839, 3840, 3841	V, XVIII, XIX, XX, XXI, XXII, XXIII, XXVI, XXVII, XXVIII, XXIX, XXXI	h, i, l, m, n, q, r, t, v, w	8, 23, 24, 25, 26, 27, 30, 31, 36, 37, 38, 39, 40, 43, 44, 45, 46
	Retail broiler meat	Alabama	C, D, E, F, J, K	829, 1082, 1119	XVI, XVII, XXI, XXIV, XXV, XXVI	g, m, r, u	21, 22, 28, 29, 32, 33, 34, 35
C. jejuni	Live broilers	Grenada	D, I, L, M, Q	51, 353, 354, 824	II, IV, IX, VI, XXX[c]	f, h, k, q, v	5, 7, 9, 12, 42, 48
		Puerto Rico	F, G, H, J, K, L, N, S[b]	48, 353, 460, 2624	I, II, VII, X, XI, XIV[d]	a, b, c, d, e, g, j, k, l, o, q, x, z, ad	2, 4, 10, 14, 15, 10, 19, 49
	Retail broiler meat	Alabama	F, K, P, R	51, 353, 354, 924, 939, 1210, 1212, 3510, 3694, 4376	I, III, VIII, IX, XII, XIII, XV, XXX, XXXII	c, k, l, p, q, s, aa, ab, ac	1, 3, 6, 7, 13, 17, 18, 20, 41, 47

[a] Strains within the same PFGE type (e.g., type I) were separated by MLST to create a numerical numbering system.
[b] Two strains did not show any type.
[c] Three strains did not show any type.
[d] Four strains did not show any type.

amplify genomic DNA from either species, MLST sequence data can be easily obtained for any *C. jejuni* or *C. coli* strain (Miller et al., 2005). One base pair (bp) difference in any of the seven alleles results in a different MLST sequence type. Thus, if two isolates are different at any base along the 3309 bp composite sequence length for all seven alleles, then they have different sequence types by MLST. The MLST typeability in this study was 100%.

One of the benefits of MLST is the unambiguous speciation of *C. coli* and *C. jejuni*, which cannot be performed unambiguously by 16S rDNA sequencing. Two strains of the same species have a relatively small number of bp differences across the seven loci, and in many instances contain allele sequences in common at one or more loci. However, the average nucleotide variation between strains of *C. jejuni* and *C. coli* is approximately 10% and strains of these two species rarely share common alleles. If they do, it is generally attributed to horizontal gene transfer. Because *C. coli* and *C. jejuni* can be typed with the same primer pairs and their sequences assembled into one alignment, the difference between *C. coli* and *C. jejuni* alleles is visually obvious. The unequivocal speciation of *C. coli* and *C. jejuni* in this study by MLST was not observed with other typing methods, namely REP-PCR and *flaA*-RFLP. Furthermore, another benefit of MLST is that assignment of DNA sequences to MLST alleles and sequence types is not prone to the variation and interpretation of restriction band profiling and band migration, and MLST sequence data can be readily compared between laboratories (Maiden and Dingle, 2008).

3.3. PFGE results

PFGE showed a typeability of 96% and produced 32 distinct types, with none of the types overlapping between species. Sixteen unique types were generated for *C. jejuni* and 16 unique types were also generated for *C. coli*. Of the total number of profiles, 13 types contained only one strain while two types (XII and XXX) contained more than 10 isolates each (Table 2). Some types were unique to specific species and geographic areas, such as types X, XI, XIV which were unique to *C. jejuni* from Puerto Rico. Other types shared different geographic areas within the species, such as types I and II for *C. jejuni* and XXVI for *C. coli* (Table 2).

PFGE creates a unique profile based on the restriction of the whole chromosomal DNA (Peters, 2009). This technique usually yields Simpson's diversity index values above 0.9 for *Campylobacter* isolates. It is a technique with a high discriminatory power, but the typeability is never 100% (96% in this study) and although faster protocols have been developed, the quality control for gel analysis is very important, especially for the computerised analyses of the results (van Belkum et al., 2007). Although PFGE protocols have not been further developed for automation and some strains do not show restriction profiles with this technique due to the presence of DNAses or an apparent methylation of segments of the genome that may prevent restriction enzymes from cutting the DNA (Gibson et al., 1994; Oyarzabal et al., 2008), we have successfully used PFGE to follow the contamination of boiler carcasses during the processing of broiler chickens (Potturi-Venkata et al., 2007). PFGE is also a powerful, relatively inexpensive technique for short term epidemiological analysis or for a large number of strains (Baqar et al., 2010; Ribot et al., 2001).

The lower Simpson's diversity index for PFGE profiles for *C. jejuni* when compared to *C. coli* was due to the fact that four *C. jejuni* strains did not show restriction with this method (Table 3). Although MLST showed more types for *C. jejuni*, the Simpson's diversity index for MLST was higher for *C. coli* because two STs (2624 and 353) predominated among the *C. jejuni* isolates.

3.4. Effect of DNA extraction on REP-PCR results

Different REP-PCR profiles were found for the same isolates independently of which DNA extraction methods was used. For instance, two isolates (10-1, G 187) showed a similar profile using DNA extracted with either the MoBio or Prepman kit, and a different profile for the extraction with Qiagen (Fig. 1A). In some cases, several isolates grouped together regardless of the DNA extraction method employed (Fig. 1B). DNA extraction using the alkaline lysis method consistently failed to generate patterns that could be interpreted by the automated system, and the REP-PCR analysis of DNA extracted with this method failed to show any appropriate profile in the densitometric curves (Fig. 2).

3.5. REP-PCR results

Automated REP-PCR using DNA extracted with the MoBio kit showed a typeability of 100% and produced 29 distinct profiles, with five of the profiles identified in both species. Twelve types contained only one isolate, while three types (h, k and m) included more than 10 isolates each (Table 2).

When analyzing the distribution among the profiles, it appears that REP-PCR generated profiles that showed a random distribution. For instance, profile k, the REP-PCR profile that contained the largest number of *C. jejuni* strains, does not draw any type of correlation between the sorting of strains by the other methods, nor does it seem to distinguish samples by location or source. REP-PCR also showed results which varied according to the DNA extraction method. In our studies, the DNA extraction method used appeared to limit REP-PCR reproducibility. Therefore, a strict adherence to a DNA isolating method is important to avoid reproducibility limitations. With REP-PCR the typeability was 100% and the method is almost completely automated.

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Table 3
Number of types by method and species, and Simpson's index of diversity (SID) and confidence interval (CI) at 95% by method and species, or combined for both species by each method.

	C. coli		*C. jejuni*		Combined spp.	
Method	SID (No. of types)	CI	SID (No. of types)	CI	SID (No. of types)	CI
REP-PCR	0.83 (12)	0.76–0.89	0.91 (22)	0.85–0.96	0.93 (30)	0.908–0.956
PFGE	0.91 (16)	0.87–0.95	0.89 (17)[a]	0.83–0.95	0.95 (32)	0.930–0.968
flaA-RFLP	0.85 (13)[b]	0.80–0.90	0.88 (15)[c]	0.83–0.93	0.90 (20)	0.872–0.925
MLST	0.87 (11)	0.83–0.91	0.83 (14)	0.76–0.89	0.92 (25)	0.906–0.946
PFGE + MLST	0.96 (25)	0.94–0.98	0.91 (24)	0.85–0.96	0.97 (49)	0.953–0.985
All Combined Methods[d]	0.99 (45)	0.99–1.00	0.99 (41)	0.99–1.00	0.99 (86)	0.996–0.999

[a] Four strains did not show any type.
[b] Two strains did not show any type.
[c] Three strains did not show any type.
[d] Simpson's diversity index calculated for the combination of all methods.

Because the annealing temperature during the PCR amplification step is usually lower in this technique, the patterns are evaluated by calculating the correlations among densitometric curves (Rademaker and de Bruin, 1997). Therefore, the analysis is done using the Pearson correlation (curve matching) and not the Dice correlation coefficient (band position), which is used for the analysis of *flaA*-RFLP and PFGE data (Grundmann et al., 1997; Vauterin and Vauterin, 2006). *Campylobacter jejuni* exhibited a Simpson's index of diversity 0.1 higher than *C. coli* and therefore more studies should be done to determine if these values are related to the bacterial species or are a reflection of this particular set of strains. REP-PCR, as with *flaA*-RFLP, generated some types that were indistinguishable between *C. coli* and *C. jejuni*.

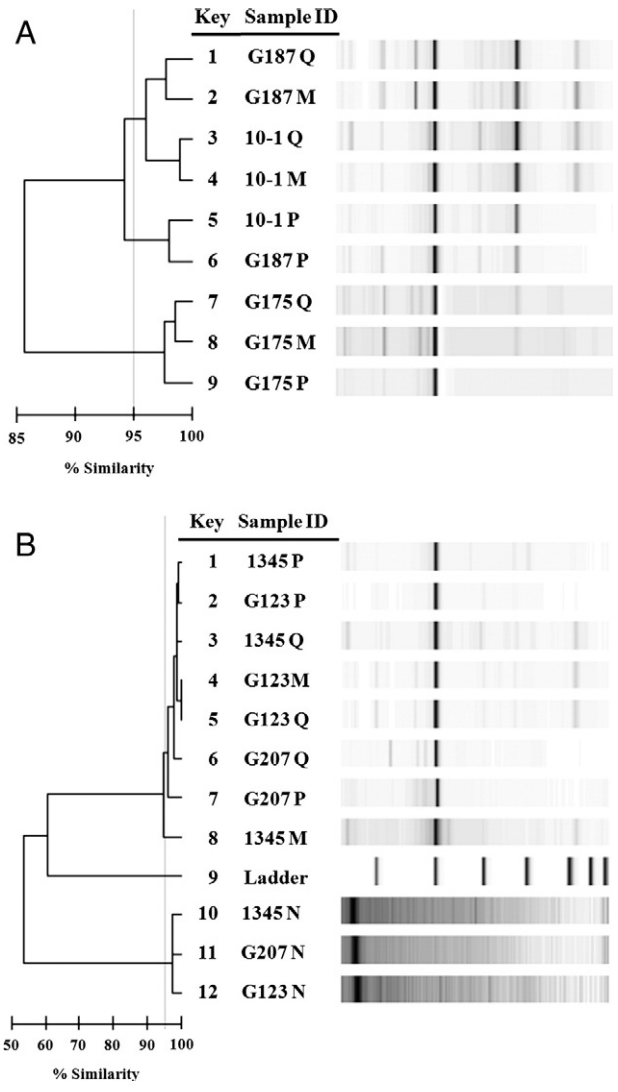

Fig. 1. REP-PCR results of *Campylobacter* isolates from live broilers (G175, G-187 and 10-1; panel A), live broilers (G123, G207 panel B), and retail broiler meat (1345, panel B). DNA was extracted with the MoBio UltraClean™ Microbial DNA Isolation Kit (M, MoBio Laboratories), Prepman Ultra Sample Preparation reagents (P, Applied Biosystems), DNeasy Blood & Tissue kit (Q, Qiagen) or by the alkaline lysis method (N).

3.6. Comparison of the four typing methods

Some REP-PCR and *flaA*-RFLP types were identified in both *C. coli* and *C. jejuni*, while each PFGE and MLST type was restricted to either *C. coli* or *C. jejuni* (Table 2). Table 3 shows the SID and confidence intervals for each method and for each species, and combined results for all strains. The SID for REP-PCR and *flaA*-RFLP was larger for *C. jejuni* than *C. coli*. The opposite was true for SID calculated for MLST and PFGE. When the results from all the methods were combined, the SID increased to 0.99, and there were 86 types and an SID of 0.99 for the calculation based on all isolates. SID assumes values between 0 and 1, with a value closer to 1 meaning that most of the strains are assigned different types. In practice, SID should be at least in the order of 0.90 to 0.95 for a typing system to be considered robust (van Belkum et al., 2007).

In general, a method that yields SID values of 0.95 can be considered more or less 'ideal' (van Belkum et al., 2007). Therefore, any typing method that generates SID values above 0.9 is appropriate for typing *C. jejuni* and *C. coli* isolates. However, selection of the method depends on many variables, some of which include the cost and the degrees of difficulty necessary to perform the technique and interpret the results. For instance, it is not difficult, expensive or complex to perform PFGE analysis on a small number of *C. jejuni* isolates. Although large numbers of PFGE profiles can only be interpreted with computer programs, the cost of performing such an analysis is not a significant increase over the cost of analyzing smaller sample sets. However, a more important consideration appears to be the congruence of the results with epidemiological data. For us, it is more important for a method to have a strong epidemiological validation than to have the highest discriminatory power. In other words, the random generation of profiles that do not correlate with epidemiological data may not provide valuable typing data.

The concordance of the methods was low for all combinations, based on the calculation of the adjusted Rand coefficient and 95% CI with 1000 bootstraps (Table 4). The highest concordance was found between MLST and PFGE (adjusted Rand = 0.453, 95% CI = 0.405–0.671). The MLST/PFGE concordance was different ($P<0.05$) than the concordance by the combinations of *flaA*-RFLP/PFGE, REP-PCR/PFGE, REP-PCR/*flaA*-RFLP, MLST/*flaA*-RFLP, and MLST/REP-PCR (Table 4). The concordance based on the Wallace coefficient (Table 5) was also

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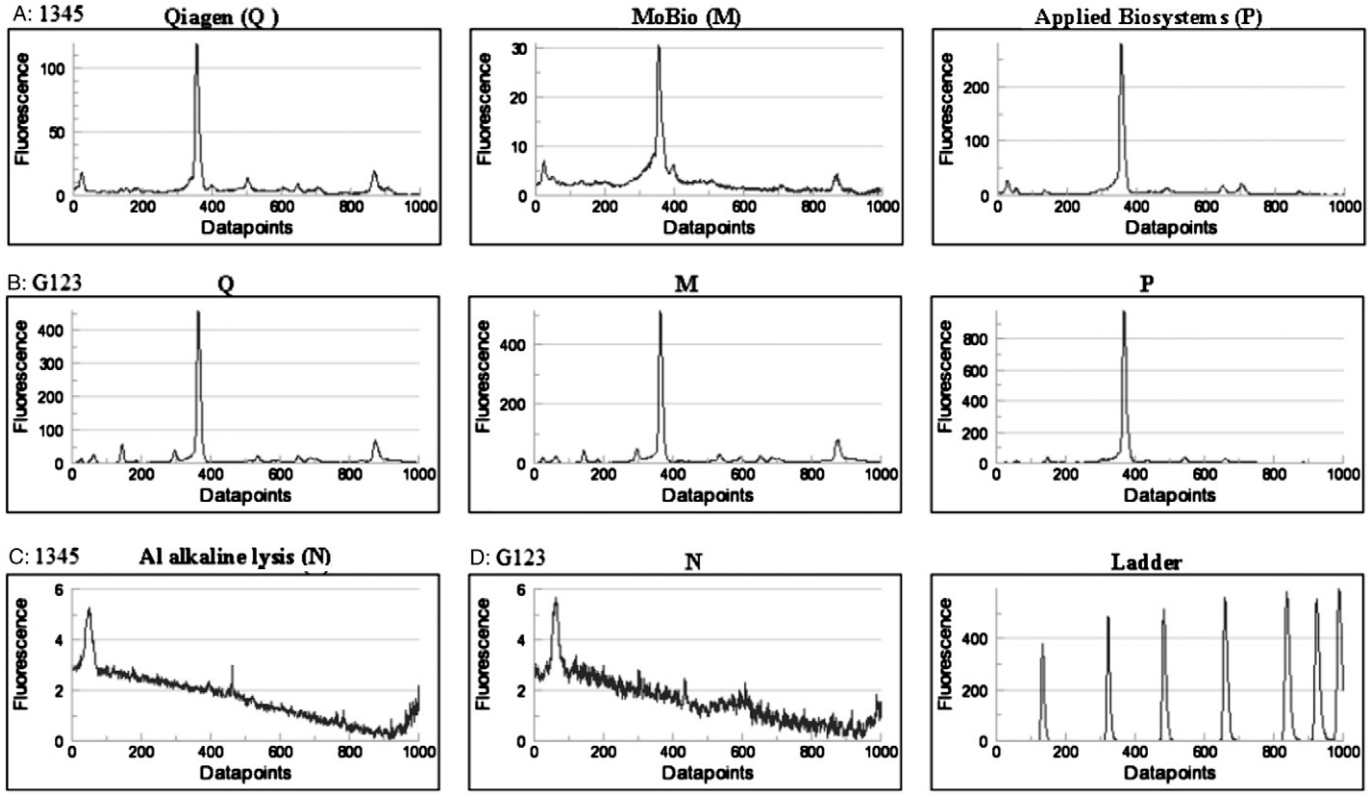

Fig. 2. Normalized REP-PCR data from densitometric curves. DNA from isolates collected from live broilers (G123) or retail broiler meat (1345) was extracted with the methods described in Fig. 1. The values in the densitometric curves for the samples whose DNA was extracted using the Applied Biosystems kit (P) were consistently higher than the samples whose DNA was extracted using the MoBio (M) or Quigen (Q) kits. The alkaline lysis method (N) produced very low densitometric curves with unreadable patters.

higher for the combination MLST/PFGE (Wallace = 0.401, 95% CI = 0.351–0.627), although it was the highest for the combination PFGE/MLST (Wallace = 0.614, 95% CI = 0.544–0.832). This highest PFGE/MLST combination was different ($P<0.05$) than the rest of the combinations, based on the Wallace coefficient and 95% CI of 1000 bootstraps (Table 5).

Typing methods produce categorical data that can be grouped in clusters, which in turn can be identified by visual or computerised methods. The categorization of datasets into clusters can group strains that share common traits (van Belkum et al., 2007). Therefore to perform comparative typing, a quantitative approach to reveal the concordance between typing methods should be applied (van Belkum et al., 2007). Although the objective validation of type data has always been limited by the lack of a statistical analysis framework, the calculation of the adjusted Rand and the Wallace coefficients, and their confidence intervals, allow for the quantification of the congruence between the results of two different methods (Pinto et al., 2007, 2008; Wallace, 1983). In our studies, the adjusted Rand gave very low congruence among the different methods. However, the Wallace coefficient for congruence of the result from PFGE and MLST gave a value of 0.6, which is greater than the 0.5 value that provides a CI within the desired 95% coverage (Pinto et al., 2008). The congruence of the results from two or more typing methods varies according to the size of the sample taken, and even from different samples from the same population (Pinto et al., 2008). Therefore, it is expected that our results may differ from a similar analysis using different strains of *C. coli* and *C. jejuni*. However, we believe that the high congruence between PFGE and MLST data may be repeatable in other studies.

Combining PFGE, a restriction and band migration system, with MLST, a sequencing method, provides complementary, powerful and discriminatory results. In this study, the clustering concordance of these techniques is not identical, but complementation of the results leads to a higher level of isolate discrimination. However, as sequencing methods become less expensive and automation of their analyses increase, restriction and band migration system may fall short from providing an accessible and universal database for comparison of results.

4. Conclusions

In summary, the DNA extraction method appears to influence the results obtained with REP-PCR. REP-PCR was better for the typing of *C. jejuni* than *C. coli*. Both REP-PCR and *flaA*-RFLP generated types that were indistinguishable between *C. jejuni* and *C. coli* and appeared to be at random without any relationship to species, location or source of isolates. PFGE and MLST generated typing results that had a better

Table 4
Adjusted Rand coefficient and 95% CI of 1000 bootstraps of the methods tested in this study. The calculation was done using the Online Tool for Quantitative Assessment of Classification Agreement (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool).

	PFGE	*flaA*-RFLP	REP-PCR	MLST
PFGE	1.000			
flaA-RFLP	0.157 (0.176–0.353)	1.000		
REP-PCR	0.072 (0.142–0.278)	0.044 (0.082–0.207)	1.000	
MLST	0.453 (0.405–0.671)	0.214 (0.207–0.378)	0.171 (0.190–0.363)	1.000

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Table 5
Wallace coefficient and 95% CI of 1000 bootstraps of the methods tested in this study. The calculation was done using the Online Tool for Quantitative Assessment of Classification Agreement (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool).

	PFGE	*flaA*-RFLP	REP-PCR	MLST
Wallace coefficient	0.048	0.100	0.069	0.073
PFGE		0.326 (0.339–0.588)	0.153 (0.210–0.420)	0.614 (0.544–0.832)
flaA-RFLP	0.156 (0.157–0.351)		0.103 (0.134–0.254)	0.243 (0.231–0.435)
REP-PCR	0.105 (0.167–0.307)	0.149 (0.191–0.349)		0.236 (0.240–0.477)
MLST	0.401 (0.351–0.627)	0.331 (0.313–0.529)	0.224 (0.232–0.439)	

correlation with the location and the date of isolate collection than the results obtained with REP-PCR and *flaA*-RFLP. PFGE and MLST had also a better discriminatory power for *C. coli* isolates than REP-PCR of *flaA*-RFLP, and also had a better concordance of the results. Therefore, it is important to have clear epidemiological questions in mind before deciding on the typing technique to incorporate. The use of available online tools to calculate the confidence interval of SID, and adjusted Rand and Wallace coefficients will help estimate the discriminatory power of typing methods. Further studies using different *C. jejuni* and *C. coli* may expand our understanding of the benefits and limitations of each of these typing methods for epidemiological studies of *Campylobacter* spp.

Acknowledgments

We thank Robert S. Miller for his technical assistance.

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