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MINIREVIEW

Genotyping of *Campylobacter* spp.

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INTRODUCTION

The genus *Campylobacter* comprises a group of closely related gram-negative bacteria which primarily colonize the gastrointestinal tracts of a wide variety of host species. Some of these bacteria are commensals, but many, particularly *Campylobacter jejuni*, and its close relative *Campylobacter coli*, are enteric pathogens of humans and domestic animals. *C. jejuni* and *C. coli* are common causes of human acute bacterial enteritis worldwide (65). Surveys have suggested that in developed regions, such as Europe and the United States, the incidence of disease associated with *Campylobacter* infections is as high as 1% of the population per annum. In Great Britain the incidence of infection continues to rise, and there were 58,000 reported cases of campylobacteriosis in 1998; this far exceeds the number of reported cases of salmonellosis (5). A detailed understanding of the epidemiology of human infections is essential for the control of this disease. It is widely assumed that campylobacteriosis is primarily a food-borne disease. Case control studies (89) have suggested that a major source of human infection is the handling and consumption of contaminated poultry meat. This suggestion is consistent with studies showing that gastrointestinal tracts of birds are commonly colonized by campylobacters. However, other meat-producing animals, including pigs, cattle, and sheep, are also frequently colonized by *Campylobacter* spp. The relative contributions of these and other potential sources, such as domestic pets, wild birds, wild animals, and contaminated water, to human infection are currently not known. Targeted control of food-borne bacterial pathogens generally depends on identification of sources and routes of transmission. However, because *Campylobacter* spp. are ubiquitous in the environment, cases are sporadic for the most part, and outbreaks are rare, source tracing has proved to be difficult. This difficulty has been compounded by a lack of suitable and readily available methods for identifying or typing, and thereby tracing, individual *Campylobacter* strains that cause human infections. Such tools would also be useful for studying the epidemiology of *Campylobacter* spp. in food-producing animals in the farm environment.

The diversity within *C. jejuni* and *C. coli* has been well established and is detectable at both the phenotypic and genotypic levels. To date, the most widely used phenotypic procedure has been serotyping. There are two generally accepted, well-evaluated serotyping schemes. The Penner scheme is based on heat-stable (HS) antigens using a passive hemagglutination technique (77). The Lior scheme is based on using heat-labile (HL) antigens (50) and a bacterial agglutination method. The major disadvantages of both of these techniques are the high number of untypeable strains and the time-consuming and technically demanding requirements of the techniques. Production and quality control of antiserum reagents for serotyping schemes are costly; consequently, these reagents are not widely available. A recently developed scheme (23) based on HS antigens in which modified antibody production and antigen detection techniques are used may be an improvement for routine use, but this scheme does not solve the problem of restricted reagent availability or the problem of the high level of nontypeability. Because of such problems, the value of serotyping techniques for national and global epidemiological studies has been restricted. Thus, there is a well-recognized need for alternative subtyping schemes. Recently, molecular subtyping methods have been developed. The major advantage of genotyping techniques is that potentially they could be universally available. Some of these techniques, like ribotyping, pulsed-field gel electrophoresis (PFGE), and flagellin typing (*fla* typing), are already in use in a number of laboratories (62). In this review we describe the technologies currently available for genotypic subtyping of *Campylobacter* spp., discuss the advantages and problems of each technique, and indicate the current value of each method.

FLAGELLIN TYPING (*FLA* TYPING)

The flagellin gene locus of *C. jejuni* contains two flagellin genes (*flaA* and *flaB*), which are arranged in tandem and are separated by approximately 170 nucleotides. Because both highly conserved and variable regions are present (56), this locus is suitable for restriction fragment length polymorphism (RFLP) analysis of a PCR product. The conserved regions in this locus are also partially conserved in species other than *C. jejuni*. Thus, the primers used to develop a typing scheme for *C. jejuni* may be used to generate similar schemes for related pathogens. Indeed, *fla* typing has proved to be valuable for the majority of *C. coli* strains and some strains of *Campylobacter lari*, *Campylobacter helveticus* (71), and *C. jejuni* subsp. *doylei* (47a).

At least seven *fla* typing procedures have been developed (2, 6, 7, 9, 44, 60, 64) (Fig. 1), and there is considerable variation in the PCR-RFLP procedures; the DNA preparation techniques (49, 60), primer design (59) (Fig. 1), annealing temperatures (7, 71), restriction enzymes used (2, 7, 9, 60, 71), and genotype nomenclature all vary. Most of the primer sets that have been described were specifically designed for amplification of *flaA* sequences. The total lengths of the restriction fragments observed suggest that only one *fla* gene is amplified in most cases. However, weak bands that may arise from the second *fla* gene are sometimes visible (68). In some methods three primers are used for combined or separate amplification of *flaA* and *flaB* (6, 59). Due to the strong conservation be-

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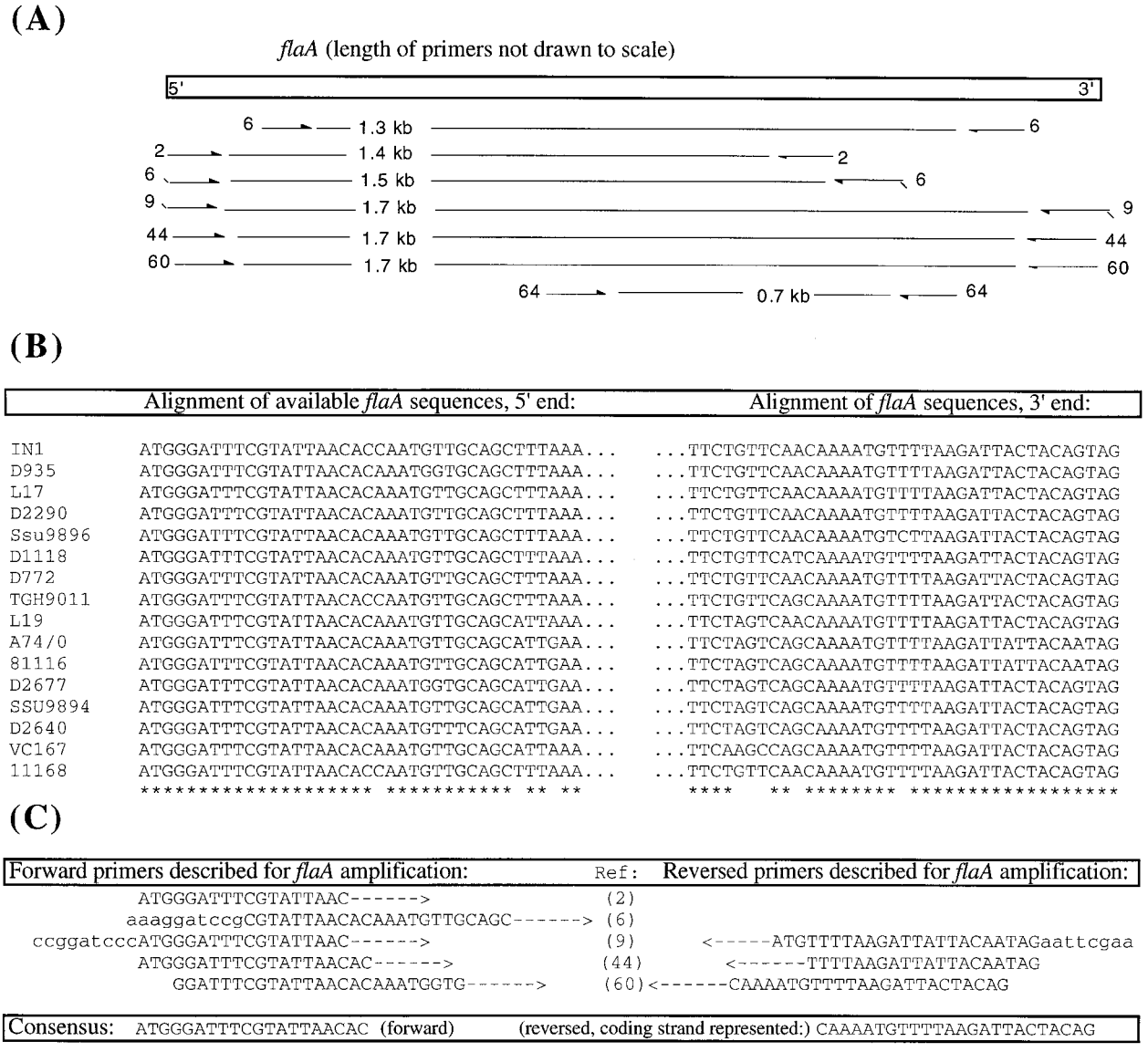

FIG. 1. PCR-RFLP analysis of the flagellin gene(s) (*fla* typing) of *C. jejuni*. (A) Schematic representation of the PCR primers used for genotyping *flaA*. The numbers of the primers (represented by arrows) correspond to the reference numbers (Ref:) in panel C. The lengths of the amplified fragments are indicated. (B) Alignment of the first and last 37 nucleotides of all available *flaA* sequences (alignment courtesy of R. Meinersmann). (C) Previously described primers for *flaA* amplification compared with a recommended consensus sequence derived from all previously published *flaA* sequences. Note that the reverse primer is represented as the coding strand. The sequence of the reverse primer which should be synthesized is 5′-CTGTAGTAATCTTAAAACATTTTG-3′.

tween *flaA* and *flaB* within a strain, addition of a *flaB*-specific primer contributes to differentiation in less than 10% of the isolates that have been examined (2, 59, 61a). A nested primer set has also been developed, but this set appears to unnecessarily complicate the method without enhancing its specificity or sensitivity (12). Some of the primers that have been designed partially overlap (Fig. 1A). Most forward primers match the start of the *flaA* gene, with only minor variations. The reverse primer used is less consistent. The variation in primers has major effects on the PCR/RFLP profiles obtained, and optimization and standardization are urgently needed.

A consensus primer set for *flaA* amplification has been developed (Fig. 1C) based on (i) the available *flaA* sequences (Fig. 1B), (ii) maximal detection of polymorphisms, (iii) the presence of a G or C at the final position to increase the stability of annealing, and (iv) the overlapping region of previously described forward primers which have proven value for a large number of strains. Based on these criteria, the consensus primer set recommended for *flaA* amplification comprises forward primer 5′-ATGGGATTTCGTATTAACAC-3′ and reverse primer 5′-CTGTAGTAATCTTAAAACATTTTG-3′ (note that in Fig. 1 the coding strand of the reverse primer is shown; however, the complementary strand should be synthesized).

The restriction enzymes that have been used to generate the PCR product fragments also differ significantly. The enzymes *Alu*I (7), *Dde*I (9, 60), *Hin*fI (71), *Eco*RI, and *Pst*I (2) are all currently used in various combinations. It appears that digestion with *Hin*fI alone is not sufficiently discriminatory (68), whereas *Alu*I produces bands that are too small to be practical

(92a). *Dde*I appears to provide the best discrimination, at least for veterinary isolates (6). The level of discrimination can be enhanced by combining *Dde*I with *Hin*fI patterns (61a).

Although *fla* typing has proven to be a useful, reliable, and relatively simple subtyping technique, the variations in the procedure that have been described do not allow results obtained in different laboratories to be compared directly. Because *fla* typing profiles can be readily stored in electronic databases (61), many isolates can be easily compared and global studies are feasible. However, before such comparisons are possible, international standardization of at least the primers and restriction enzymes used is essential.

PFGE

Digestion of bacterial chromosomes by restriction enzymes that cleave the DNA infrequently has proved to be a useful typing technique for many bacteria (21, 90). Although the various DNA fragments obtained are generally very large (20 to 200 kb), they can be separated on the basis of size by using special electrophoretic conditions. Variations in the presence of relevant restriction sites result in genotypic profiles (also called macrorestriction profiles) (73, 84). In order to avoid shearing (which would introduce random breaks), the chromosomal DNA is protected by immobilizing the bacterial suspension in agarose before the cells are lysed. All subsequent enzymatic steps are carried out by means of passive diffusion into the agarose blocks. Any plasmids which occur in *Campylobacter* spp. may potentially be detected by PFGE together with the chromosomal DNA. The blocks containing purified and digested DNA are directly loaded onto agarose gels, which are electrophoresed by using conditions under which the orientation of the electrical field is changed in a pulsed manner (21). PFGE was initially used for *C. jejuni* (98), and the technique was later adapted for *C. coli*, *Campylobacter hyointestinalis*, *Campylobacter fetus*, and *Campylobacter upsaliensis* (8, 26, 81).

There have been inconsistent reports of campylobacters that cannot be typed by PFGE. This problem may be due to DNase production and may be overcome by formaldehyde treatment (29, 73).

The conditions used for PFGE in different studies vary. Differences in electrophoretic conditions can even lead to apparent differences in the profiles obtained for the same DNA preparation. The restriction enzymes used to digest the chromosomal DNA are also different in different studies. Satisfactory results have been obtained with *Sma*I, *Sal*I, *Kpn*I, *Apa*I, and *Bss*HII. *Xho*I appears to be useful for *C. upsalenensis* (8). Using more than one enzyme significantly increases the discriminatory power of the technique (29, 37, 54). Because of the considerable variations in the restriction enzymes and electrophoretic conditions used, comparisons of the PFGE profiles obtained by workers in different laboratories have proved to be difficult so far.

RIBOTYPING

Multiple copies of the rRNA gene loci (coding for 5S, 16S, and 23S rRNA) are present at different positions on the campylobacter chromosome. Because of the strong conservation of regions in the rRNA genes and the presence of highly variable (noncoding) flanking regions, these genes are suitable targets for subtyping purposes. The technique most commonly used for genotyping based on ribosomal genes is agarose gel electrophoresis of digested genomic DNA followed by Southern blot hybridization with a probe specific for rRNA genes. This technique (ribotyping) results in a high level of typeability. However, since most *Campylobacter* spp. contain only three ribosomal gene copies, the discriminatory power of this method is limited. For example, ribotyping cannot distinguish between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* or differentiate strains within these subspecies (13). In general, ribotyping seems to be most useful for determining the species of *Campylobacter* isolates that are difficult to analyze phenotypically (43). Nevertheless, the technique has been used successfully for subtyping, at least to some degree, *C. jejuni*, *C. coli*, *C. upsaliensis* (31, 83), *C. lari* (70), *C. helveticus*, and aerotolerant *Campylobacter* spp. (43). Most investigators have used a probe that was derived by PCR from *Campylobacter* DNA and is specific for 16S rRNA. Some workers have used a single probe which contains both 16S and 23S cDNA derived from *Escherichia coli* (20, 74, 80), which is more discriminatory than a specific probe for 16S rRNA alone (74). Single 23S rRNA-specific probes do not appear to have enough discriminatory power to be useful for subtyping (39). Early ribotyping results failed to clearly differentiate *C. coli* from *C. jejuni* strains (19, 35). However, differentiation can be improved by using two restriction enzymes and a 16S rRNA-specific probe (22).

The enzymes used in ribotyping studies for chromosomal digestion also vary considerably; *Pst*I, *Hae*III, *Hin*dIII, and *Pvu*II have been used alone, in pairs, or even in combinations consisting of three enzymes (19, 28, 35, 40, 70, 72, 74, 80). The differences in the restriction enzymes and probes used generally hamper direct comparisons of the ribotype profiles obtained in different laboratories.

The relatively low discriminatory power of ribotyping and the elaborate nature of the technique make it a relatively unsuitable method for routine genotyping. However, there are two improvements which can add value to the technique. First, rehybridization of the Southern blots with probes for other hypervariable regions of the chromosome can be used to increase the level of discrimination. This approach has been used with a polymorphic lambda clone insert (40, 41). Using this probe alone resulted in discriminatory power that was greater than the discriminatory power obtained with the ribosomal probe, probably because the former probe contained a longer insert. Although the nature of the lambda clone insert is not known, the genomic sequence of a *C. jejuni* strain that became available recently should enable rapid identification of the polymorphic sequences detected. Other probes that have been used with the ribosomal genes include fragments obtained from the genes encoding ATPase, ribosomal protein S12, and elongation factor EF-Tu (31, 97). The second improvement in ribotyping involves automation (riboprinting), which overcomes the labor-intensiveness and enhances the reproducibility of the method. Recently, riboprinting has been used to subtype *C. jejuni* and *C. coli* strains obtained from a range of sources (90a). However, the high cost of riboprinting (both for equipment and consumables) and the low throughput will severely limit its use even though the automated devices can also be used for many other bacteria.

RAPD ANALYSIS

Arbitrary developed primers can be used to amplify random DNA products under low-stringency PCR conditions. Typically, randomly designed 10-mer primers are used under conditions that allow some mismatches to increase the number of primed sites. PCR products are produced when primer sites are situated within the amplification distance (less than 5 kb) and with the correct opposite orientation. The lengths of these

products and the efficiency of annealing and thus amplification vary with the sites primed. Consequently, band patterns consist of both weak and strong amplicons. This complicates interpretation of the results considerably.

Random amplified polymorphic DNA (RAPD) methods have been developed for *Campylobacter* spp. by using various primers and reaction conditions (20, 25, 47, 55, 63). These methods should theoretically give high typeability, but in practice up to 14% of the strains examined may be untypeable due to DNase activity (20, 34, 36). The choice of the primers used is crucial to the outcome of RAPD analysis. The primers are often selected arbitrarily (69). In one comparison of three primers (10-mers), using two primers resulted in complex band patterns and multiple differences between strains; however, one primer amplified only one major PCR product of different size between strains (55). Interestingly, the G+C contents of primers cannot be used to predict their usefulness in RAPD analysis (47).

The major disadvantage of RAPD analysis is poor reproducibility, which effectively outweighs the advantages of rapidity and cost-effectiveness. Minor differences in band patterns can be observed with both duplicate samples (36) and outbreak isolates. These differences can lead to subjective interpretation of the data (20). Such differences occur even when purified DNA is used as the template. The reasons for the variations are not known, but inconsistencies in thermal cyclers, template purity, and procedures have been implicated as possible causes (16, 53, 76). Using computational analysis of band patterns may improve the consistency of the technique and comparisons of data, but whether weaker bands should be ignored tends to be arbitrarily decided and computer-aided representations may simulate higher reproducibility than can be obtained in reality (34). A slightly different approach for amplifying random genomic DNA fragments involves using primers specific for enterobacterial repetitive intergenic consensus (ERIC) sequences. Such primers can be used under high-stringency conditions to match the target sequences. However, using lower annealing temperatures increases the number of bands that can be detected. This approach has been used to determine the genotypic subtypes of *C. jejuni* and *C. upsaliensis* (30), since these species do not contain repetitive sequences which can be used for high-fidelity ERIC amplification. Combinations of an ERIC primer and a randomly chosen primer have also been used (1, 17, 30, 38). However, the reproducibility of the results obtained with the combined RAPD-ERIC amplification technique is still low.

OTHER GENETIC TYPING METHODS

Although several other genetic methods have been developed to identify campylobacters to the species level, most of them are not suitable for subtyping analysis within species. Recently, the following three novel genetic subtyping methods with promising potential have been described: AFLP analysis, multiplex PCR-RFLP analysis, and gene sequencing.

AFLP analysis. AFLP is not an abbreviation, although it may be read as amplified fragment length polymorphism. This technique was originally developed for genetic analysis of plants and has been adapted for subtyping a number of bacteria (42, 48, 91). This method is based on complete digestion of chromosomal DNA with two restriction enzymes, one with a 4-bp recognition site and the other with a 6-bp recognition site. PCR amplification of the digestion products is based on the restriction sites and is designed so that only those fragments flanked by both restriction sites are amplified. The primers used for amplification are radioactively or fluorescently labelled, and the resultant labelled PCR products are analyzed on denaturing polyacrylamide gels. This allows separation at the nucleotide level of fragments that are typically 50 to 500 nucleotides long. The number of bands generated (80 to 100 bands is optimal) can be reduced by incorporating into the PCR primers one or more specific nucleotides adjacent to the restriction site. Thus, only those fragments containing the specific nucleotide adjacent to the restriction site are detected and analyzed. The method can be adapted to any (bacterial) species. However, the restriction enzymes and adjacent specific nucleotides used must be optimized for each species. AFLP analysis has recently been used to subtype *C. jejuni* (14, 46). The major advantage of this technique is that a random portion of the whole genome is sampled. The disadvantage is that the technique is complex (it is comparable to PFGE) and requires a major capital investment (an automated DNA sequencer and appropriate software). Nevertheless, it seems likely that this technique will be used more widely in the near future.

Multiplex PCR-RFLP analysis. Polymorphic genes other than *fla* can be used for PCR-RFLP analysis. Moreover, several polymorphic genes can be combined and analyzed by using a multiplex PCR in order to obtain increased discriminatory power. Such a multiplex PCR was developed for *C. jejuni* (79) by using the polymorphic genes *gyrA* and *pflA*. This method gives a level of discrimination similar to that of PFGE, and the level of discrimination can be enhanced by including *fla* as a third gene target. One possible disadvantage of using *gyrA* is that because of its involvement in induction of antibiotic resistance, selection pressures may cause some genotypes to be overrepresented. Other obvious gene candidates for PCR-RFLP analysis include *flgE*, the gene coding for the flagellar hook protein, which is different in different strains (52), and the genes coding for the major outer membrane proteins, which are clearly polymorphic. Multilocus enzyme electrophoresis (MEE) is often considered a genotypic method, even though it determines differences in phenotypic expression of enzymes in different strains. Almost certainly, genetic diversity is one of the mechanisms that underlie the variations detected in MEE patterns. Therefore, the ongoing analysis of MEE in population genetics studies (55a) may lead to identification of new hypervariable genetic loci which can be used as targets for PCR-RFLP analysis. Finally, the HS antigen-dependent serotyping scheme (77) has revealed that there is significant diversity in lipopolysaccharide phenotypes. The genes that encode lipopolysaccharide production have recently been identified (24). These genes may also be suitable candidates for alternative PCR-RFLP typing schemes.

Some genes can be eliminated as candidates. The genes that encode the cytolethal distending toxin (*cdt* genes), a potential virulence factor of *C. jejuni* and *C. coli* (78), are too strongly conserved to differentiate strains (92a). Likewise, the fact that the hippuricase gene (*hipO*) is strongly conserved eliminates this locus as a genotyping target (82).

Nucleotide sequencing. Direct nucleotide sequencing (with or without PCR amplification) is becoming increasingly automated and consequently is a reasonable alternative method for genotyping bacterial isolates. The advantage of sequence analysis is that it is highly reproducible and the results are easy to interpret. However, the complex data and superb discriminatory power make interpretation highly dependent on computerized comparison programs and the parameters set by the software packages used. Sequence analysis has been applied to the flagellin locus in several studies (33, 56).

COMPARISON OF GENOTYPIC AND PHENOTYPIC SUBTYPING METHODS

Genotyping methods may be compared on the basis of a range of criteria, including sensitivity, availability, reproducibility, rapidity, ease of use, and cost. One of the most important characteristics is discriminatory power. Given the recent development and restricted use of many techniques, comparisons are sometimes difficult and the data may be incomplete (Table 1).

A major problem is the lack of a standard set of strains which can be used for comparative evaluations. Selection of such a set is particularly important as the members of some strain groups have closer phenotypic and genotypic relationships than the members of other groups have. For example, serotype HS19 strains are very homogeneous genetically, whereas the strains belonging to serotypes such as HS2 and the HS4 complex are very heterogeneous; moreover, different serotypes may be genetically related despite differences in HS antigen expression (51). Consequently, the results of subtyping obtained with a homogeneous group of strains are less discriminatory than the results of serotyping obtained with a heterogeneous group of strains. Increasingly, there is a need for a standard set of strains which can be used to evaluate new subtyping techniques. A European group, CAMPYNET, has recently been established to collect and catalogue such a standard set of 100 *C. jejuni* and *C. coli* strains. This strain set will be available internationally (10).

Although definitive studies have yet to be done, it is becoming clear that at least with heterogeneous groups of strains, PFGE and *fla* typing have greater discriminatory power than RAPD analysis or ribotyping and that ribotyping is the least discriminative technique. Comparing genotypic methods with the currently widely used phenotypic typing methods is crucial, particularly so that comparisons can be made with retrospective data. Serotyping has tended to be the "gold standard." Because of the putative role of flagellin in the Lior serotyping scheme, several attempts have been made to correlate flagellin genotype with Lior serotype. Although direct correlations can be demonstrated for some serotypes (2), the correlations are generally weak (9, 59–61, 71). This finding supports the controversy surrounding the role of flagellin as the dominant HL antigen in this serotyping scheme (4). PFGE typing of members of several serogroups identified by the Lior typing scheme has revealed little, if any, correlation (87, 88). Similarly, identical ribopatterns can be obtained for members of multiple HL antigen serotypes (40).

Most genotypic methods have been compared to HS antigen serotyping; however, the numbers and diversities of the serotypes tested have differed considerably. *C. jejuni* serotype HS1, HS2, and HS4 isolates exhibited considerable genetic diversity within the serogroups as determined by both PFGE (28, 73) and *fla* typing (9, 68) and, to a lesser extent, by ribotyping and RAPD analysis (20, 28, 40, 73). A comparison of RAPD fingerprinting and HS antigen serotyping results suggested that sometimes there can be correlations between the data obtained with these two typing schemes (55); however, such correlations are unpredictable (1, 36).

In general, strains that belong to the same serotype are not always similar genetically. Most *C. jejuni-C. coli* serotypes comprise heterogeneous genotypes, and strains belonging to different serotypes can be genetically related. However, lineages of clones can be recognized in certain HS antigen serotypes (27). There may also be genetic similarity between serotypes (e.g., serotypes HS9 and HS38), as demonstrated by using PFGE and *fla* typing (51). Interestingly, weak serological cross-reactivity is not indicative of genetic linkage, since HS63, which cross-reacts with the HS9-HS38 group, is not genetically related to HS9 or HS38.

Nontypeability is a significant problem with serotyping and most likely is a consequence of the incompleteness of the serum set used for typing. Most genotyping methods have considerably higher typeability than serotyping, and most serologically nontypeable isolates can be grouped with known serotypes by PFGE (27) and ribotyping (22), suggesting that a lack of antigen detection is a major factor in nontypeability and that the strains are otherwise not atypical.

Generally, genotypic methods have greater discriminatory power than phenotypic methods, such as biotyping or phage typing (28, 68, 72, 74, 85). However, combining a genotypic method with serotyping usually results in greater discriminatory power and more complete strain identification than genotyping alone. Similarly, combining two independent genetic methods can also improve discrimination.

GENETIC INSTABILITY AND THE CONSEQUENCES FOR GENOTYPING

For successful application of any typing technique, inherent stability of the subtype marker is essential. The stability of the genotypes of *Campylobacter* strains has been tested in vitro. In general, subculturing, freezing, and storage of strains have no effect on the genotype (9). This observation is in contrast to the results obtained with some of the phenotypic methods, such as serotyping, which can require multiple subcultures (sometimes up to 10 passages) to optimally detect antigen expression (22a, 49a) or are subject to variation in expression (57). However, long-term subculturing of *C. coli* porcine isolates can result in significant changes in the PFGE band patterns (66). Moreover, studies of outbreak-related isolates have suggested that differences in flagellin genotypes can also occur (33). These observations indicate that the potential for genetic instability is present in campylobacters. This potential appears to reflect the following four possible mechanisms: (i) recombinations within the duplicate duplicated flagellin genes, (ii) uptake of extracellular DNA by natural transformation, (iii) programmed DNA recombination, and (iv) random recombination on a genomic scale (96). Preliminary evidence suggests that at least some of these mechanisms can be detected by genotypic methods (Table 1).

Several studies have shown that the flagellin locus can be subject to recombination events. Such events were originally observed by using artificially introduced antibiotic resistance cassettes (3, 93), but recent evidence has indicated that intragenomic recombination events also occur naturally in human strains (33). Moreover, it has been shown that uptake and integration of flagellin gene fragments can occur in the laboratory by natural transformation processes (4, 92, 94). Indirect evidence that DNA exchange can occur over time under natural conditions has been obtained from flagellin sequencing of strains of putative clonal origin (33). Such recombination events could alter the *fla* genotype of a given strain. So far, *fla* genotypes have proven to be stable during storage (9) and are probably stable during short-term outbreaks, such as poultry flock infections. Nevertheless, the possibility that genotypic instability occurs in response to environmental pressures cannot be ignored. If exchanges of flagellin sequences can occur under natural conditions, then using *fla* typing as the sole method of strain identification is unwise. In epidemiological studies, combining *fla* typing with any of the other genotyping or phenotyping methods is probably sufficient to identify changes in a given *fla* type due to DNA exchange. The inherent

TABLE 1. Advantages and disadvantages of various genotyping methods and serotyping for subtyping campylobacters

Method	Discriminatory power[a]	Typeability (%)[b]	Reproducibility[c]	Sensitivity to genetic instability	Time required	Cost	Availability
fla typing	Reasonable	100	Good	Yes	<1 day	Low	Good
PFGE	Good	100	Good	Yes	3–4 days	Average	Limited
Ribotyping	Poor	100	Good	Yes	3–4 days	Average	Complex method
Automated ribotyping	Good	NAV[d]	Good	NAV	8 h	High	Limited
RAPD	Average	~80	Low	Yes	<1 day	Low	Good
AFLP	Good	100	Good	No	2–3 days	Average	Complex method
Sequencing	Very good	100	Good	NA[e]	2–3 days	Average	Limited
Serotyping	Average	~80	Good[f]	NA	<1 day	Low	Limited

[a] The discriminatory power of a method is its ability to differentiate between genetically unrelated strains.
[b] Typeability is 100% if all strains tested are typed by a given method.
[c] The reproducibility of a method is determined by its ability to identify duplicate samples. This is irrespective of external factors, such as genetic instability.
[d] NAV, not available.
[e] NA, not applicable.
[f] Weak serological cross-reactions can complicate interpretation of data. Multiple passages may be required to fully express antigens.

potential for instability in *fla* typing probably makes this method unsuitable for global or long-term time-related epidemiological studies.

Other genotypic methods can be influenced by genomic instability as a result of programmed or random rearrangements of genomic segments. Programmed genomic rearrangements have been described for the surface layer protein locus of *C. fetus* (15) and take place at a high frequency. Because of the length of the DNA fragments involved, such rearrangements are unlikely to be detected by PFGE analysis but could possibly influence the outcome of RAPD analysis. Such recombination events make the *sap* gene locus, coding for the surface layer protein, not a suitable candidate for typing purposes, despite the recent development of such a typing technique (13).

Recent observations suggest that the genome of *C. jejuni* may undergo mosaic rearrangements on a genomic scale. Such events would be detectable by genotypic methods that monitor the whole genome and may underly the variation that has been observed in single colonies of epidemiologically related isolates investigated by PFGE (95). Since PFGE of strains is not generally a routine procedure, genetic instability may occur more frequently than previous reports suggest. Certainly, significant changes in PFGE genotypes have been observed after extensive subculturing (66). Furthermore, minor but consistent changes in PFGE genotypes have been observed in temporally separated isolates obtained from humans with chronic infections (86). Slight differences in RAPD profiles of epidemiologically related poultry isolates have also been interpreted as being the result of genetic instability (1). Finally, even ribotyping may detect genetic instability (32).

So far, most reports of genetic instability have been anecdotal. However, evidence that most genotypic methods can be affected by this phenomenon is accumulating rapidly. Because of the nature of the methods, RAPD and AFLP analyses, which generate multiple small bands, should be least sensitive to changes in the genome. Certainly, the AFLP patterns of isolates of clonal origin and variable PFGE patterns correctly cluster strains (14, 92a). Moreover, AFLP fingerprints were not affected by genetic manipulation of the flagellin locus, indicating that the AFLP technique is generally not sensitive to genetic rearrangements at least in this locus (14). Multiplex PCR-RFLP should also have the potential to be relatively insensitive to genetic instability, if the individual PCR target genes are stable. For this reason, the flagellin gene locus may not be the optimal choice for a locus to include in a multiplex PCR typing scheme.

APPLICATIONS

The established genotypic methods are beginning to have proven value for a number of applications. Most methods clearly reveal the enormous diversity of *C. jejuni* and *C. coli* genotypes present in all of the host and environmental niches that have been investigated, including cases of human campylobacteriosis, poultry, pigs (97), abattoirs (85), sewage plants (45), and seawater (36). Many other *Campylobacter* species also exhibit genetic diversity; these species include *C. upsaliensis* isolates from dogs and humans, as detected by ribotyping (83), and *C. lari* isolates from oysters, as demonstrated by RAPD analysis (18).

Albeit rare, outbreak infections caused by *Campylobacter* spp. do occur. Genotyping methods can now routinely be used to identify outbreak-related strains. Some laboratories perform preliminary screening by serotyping, but this approach may disguise some clonal relationships that are obvious only when genotyping is used. The genotyping methods whose value has been proven in outbreak investigations include ribotyping (40, 72), PFGE (28, 80, 88), *fla* typing (60), and RAPD analysis (30, 38). Outbreak-related *C. upsaliensis* isolates have also been identified by using PFGE (31).

Genotyping techniques are also becoming more useful for investigating sources of infection and routes of transmission in animals and humans. Such techniques have been particularly useful in epidemiological investigations of poultry infections, which may be considered short-term outbreaks. Although there is diversity between farms, as well as between flocks on a farm, most birds in a flock are infected with a restricted number of genotypes at any one time (6, 11, 47, 54). The limited diversity is useful in tracing or eliminating potential sources of transmission. For example, RAPD and ribotyping analyses have shown that litter can be eliminated as a mode of transmission of *C. jejuni* between broiler flocks (75), while *fla* typing (61a) and riboprinting (41a) of sequential flocks in the same broiler houses have revealed that persistent environmental sources were present.

It can be anticipated that in the future genotyping will allow us to identify lineages of *Campylobacter* strains that are more or less virulent or are restricted to certain hosts. Direct correlations between genotype and virulence have yet to be established. Notably, the ribotypes of *C. jejuni* strains isolated from diarrheic stools or blood of patients could not be distinguished in the study of Jackson et al. (41). Although serotyping has been of limited value in revealing the pathogenic potential of

Campylobacter isolates, certain HS antigen serotypes of *C. jejuni* (mainly O:19 and O:41) are associated with some severe postinfectious neurological diseases, such as Guillain-Barré syndrome (GBS). Recently, genetic techniques have been used to demonstrate that the O:19 group is a distinct genetically homogeneous *C. jejuni* group (63). However, no differences can be detected between isolates from patients with GBS and isolates from patients without GBS (25, 64), suggesting that this manifestation of the disease is primarily host dependent rather than strain specific. A PCR-based genotyping method has now been developed to identify O:19 isolates independent of serotyping (58). It seems likely that in the future additional genotypic methods will be developed to detect and exploit markers of pathogenicity.

A picture of common genetic lineages, clones, and interstrain genetic relationships within *C. jejuni* and *C. coli* strains is beginning to emerge (27, 34, 40, 61, 84). Some PFGE types or groups appear to be host specific (67). Similarly, some *fla* profiles appear to be associated only with humans, while other types are restricted to poultry (45). Studies will eventually indicate which serotypes are most likely to cause disease, and the information obtained can be used in risk assessment models to differentiate clones that are food hazards from avirulent strains present in food-producing animals.

CONCLUSIONS AND FUTURE DIRECTIONS

It is expected that genotyping of *Campylobacter* isolates will be increasingly used in the future. The techniques that are available now are suitable for global epidemiological studies; however, standardization and harmonization of the various procedures (i.e., the restriction enzymes used, the electrophoretic conditions used, the nomenclature, etc.) are urgently needed. In addition, reliable comparisons of large numbers of isolates and exchanges of data between laboratories have now become essential. Thus, international databases of digitized genotypes are required. These issues are now being addressed by international consortia (10).

It is not yet possible to identify the most suitable campylobacter genotyping method that fulfills all of the requirements for molecular epidemiological investigations. Using several techniques in a multilayer strategy may be the best approach. For the greatest discriminatory power, a single- or multiple-locus method should be combined with a chromosomal method. However, most laboratories have to deal with large numbers of strains and have time and resource constraints. For these laboratories a single typing method would be optimal. For short-term limited-outbreak situations, such as infections in broiler flocks, *fla* typing is fast, easy, and cheap and has sufficient discriminatory power. For global epidemiological studies, AFLP analysis is the most promising method for providing reproducible profiles which appear to be insensitive to the genetic instability that complicates other methods.

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