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Application of Different Chromosomal DNA Restriction Digest Fingerprints to Specific and Subspecific Identification of *Campylobacter* Isolates

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Eleven strains comprising representatives of different subspecies, biotypes, and serotypes of *Campylobacter jejuni* and reference strains of *C. coli*, *C. fetus* subsp. *fetus*, *C. hyointestinalis*, and *C. sputorum* subsp. *sputorum* were studied to assess the utility of different DNA profiles for measuring fine differences between allied bacteria. Strains were compared by analyses of *Hae*III and *Xho*I digest patterns of chromosomal DNA and Southern blot hybridization patterns of *Xho*I digests obtained with an *Escherichia coli* 16S + 23S rRNA gene probe. Visual comparisons and numerical analyses of the *Hae*III and *Xho*I digest patterns both revealed clear differences between the five *Campylobacter* species and between representatives of *C. jejuni* subspecies and biotypes. Only strains with the same Penner serotype gave identical total digest polymorphisms. The advantages of *Xho*I total digests and Southern blot hybridization patterns were that they were less complex than the *Hae*III patterns and easier to compare visually. However, numerical analysis of *Xho*I data resulted in reduced discrimination. We conclude that DNA fingerprinting using either *Hae*III or *Xho*I fragment polymorphisms has considerable potential as a generally applicable method for identification of *Campylobacter* isolates, especially at the infrasubspecific level.

Electrophoretic fingerprinting based on restriction endonuclease digestion of chromosomal DNA is being used increasingly to identify strains of medically important bacteria, especially for epidemiological purposes, when other, more conventional methods, such as serotyping or phage typing, are unsuitable or unavailable (24). Restriction digest analysis has been applied to various pathogens of the genus *Campylobacter* because of the limitations of currently available biochemical and typing methods for species and strain identification. Species studied by this technique have included *C. jejuni* (4, 15, 26, 30), which is now one of the most frequently identified agents of acute infectious diarrhea in developed countries, *C. coli* (14), *C. laridis* (15), *C. fetus* subsp. *fetus* (7), and *C. pylori* (16, 19). The DNA digest patterns produced by restriction endonucleases such as *Hae*III, *Hind*III, *Bst*EII, and *Xho*I appear to be highly stable (19), and present evidence suggests that they provide a sensitive means of differentiating individual strains. Such patterns are often difficult to compare if they are complex, but the use of numerical methods of analysis has enabled quantitative relationships between *Campylobacter* species to be determined (6).

A second type of DNA fingerprint used in bacterial strain identification is based on the detection of restriction fragment length polymorphisms containing rRNA genes (rDNA) (10). The rRNA cistrons provide valuable genomic markers, as they are highly conserved in bacteria but differ widely between species in both number and location (2, 3, 9–12, 22, 38). The advantage of such patterns is greater clarity than total DNA digests, which allows easier interpretation and avoidance of complications due to the possible presence of plasmid DNA. Recently, an oligonucleotide probe specific for the *Campylobacter* 16S rRNA gene was used to detect differences within and between various thermophilic *Campylobacter* species (32). The patterns indicated the presence of two to four copies of the 16S rRNA gene and clearly demonstrated the potential of rRNA cistron probes as an identification method in epidemiological studies of *Campylobacter* infections.

In the present study, genomic fingerprinting techniques were applied to a selected set of *Campylobacter* reference strains representing different species, subspecies, biotypes, and serotypes. The aim was to determine which method offered the greatest potential for detecting strain differences in terms of discriminatory power and ease of interpretation. Computer-assisted numerical analyses of *Hae*III and *Xho*I digests were used to provide estimates of quantitative similarities between total band patterns. The results were compared with band patterns of Southern blot hybridizations of *Xho*I digests using a broad-spectrum, unfractionated *Escherichia coli* 16S + 23S rRNA probe, which provides a convenient means of detecting rRNA genes even among taxonomically distinct bacteria (5, 10, 37).

MATERIALS AND METHODS

Bacterial strains. The 11 reference *Campylobacter* strains used comprised 7 strains of *C. jejuni* and 1 strain each of *C. coli*, *C. fetus* subsp. *fetus*, *C. hyointestinalis*, and *C. sputorum* subsp. *sputorum*. A small set of strains was used so that they could be run simultaneously on one gel to avoid problems of gel-to-gel reproducibility. The strains were chosen to reflect genomic variation within and between *C. jejuni* and other allied species. Cultures were obtained from the National Collection of Type Cultures (NCTC), and their numbers, subspecies status, biotypes, and serotypes (when known) are listed in Table 1. They were cultivated at 37°C for 1 day on 5% (vol/vol) defibrinated horse blood agar under microaerophilic conditions in an anaerobic jar (catalyst removed) that was evacuated to a pressure of 560 mm of Hg (ca. 74.7 kPa) and filled with 10% CO_2–90% H_2.

DNA preparation, digestion, and separation of fragments.

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TABLE 1. *Campylobacter* strains used in this study

Organism	Strain	Biotype according to:		Serotype (PEN)	Sizes (in kb) of RFLPs[a] in Southern blots of *Xho*I digests
		Skirrow and Benjamin (33)	Lior (17)		
C. jejuni subsp. *jejuni*	NCTC 10983	1	I	02	16.4, 14.5
C. jejuni subsp. *jejuni*	NCTC 11168	1	I	02	16.4, 14.5
C. jejuni subsp. *jejuni*	A174/81	1	I	03	16.4, 14.5
C. jejuni subsp. *jejuni*	NCTC 11351^T	1	I	023	16.4, 14.5
C. jejuni subsp. *jejuni*	NCTC 11392	2	III	06	19.4, 14.5
C. jejuni subsp. *jejuni*	NCTC 12109	2	IV	ND[b]	23.1, 14.5
C. jejuni subsp. *doylei*	NCTC 11950^T		I	ND	19.5
C. coli	NCTC 12110		I	ND	23.1, 22.0, 13.7, 6.2
C. fetus subsp. *fetus*	NCTC 10842^T				23.1, 16.4, 14.5, 11.7
C. hyointestinalis	NCTC 11608^T				23.9, 21.1, 18.8, 8.4
C. sputorum subsp. *sputorum*	NCTC 11528^T				—[c]

[a] RFLP, Restriction fragment length polymorphism.
[b] ND, No data available.
[c] —, No bands.

Chromosomal DNA was isolated and purified in agarose pellets as described previously (8). The DNA (1 to 2 μg) was digested with the restriction endonucleases *Hae*III and *Xho*I (5 U/μg of DNA) for 3 h at 37°C in the buffer recommended by the manufacturers [GIBCO Ltd., Paisley, United Kingdom; Boehringer (London) Ltd., Lewis, United Kingdom; and Amersham International, Amersham, United Kingdom]. The digested DNA (10- to 20-μl sample) was electrophoresed at 40 V for 18 h in a horizontal 0.7% (wt/vol) agarose gel (ultrapure, electrophoresis grade; GIBCO Ltd.) in a buffer containing 89 mM Tris hydrochloride, 89 mM boric acid, and 2 mM disodium EDTA (pH 8.3). All digests were electrophoresed on the same gel to eliminate between-gel reproducibility errors. After electrophoresis, the gels were stained and photographed (26).

Probe synthesis. A biotin-labeled cDNA probe synthesized from a mixture of 16S and 23S rRNA from *E. coli* (Sigma Chemical Co., Poole, United Kingdom) was prepared by using methods described previously (31).

Southern blot hybridization. The DNA in the gel was nicked further after UV illumination by treatment with 0.25 N HCl for 15 to 30 min and was then denatured in 0.5 M NaOH–1.5 M NaCl for 1 h and neutralized in 0.5 M Tris hydrochloride (pH 7.2)–1.5 M NaCl–1 mM disodium EDTA for 1 h. DNA transfer to a Hybond-N membrane (pore size, 0.45 μm; Amersham International) was done overnight (18 to 20 h) as recommended by the manufacturer. The membranes were washed once in 2 × SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate), air dried, and baked at 80°C for 2 h.

The nylon membranes were prehybridized (20) at 42°C for 3 to 4 h in a solution containing 50% (vol/vol) formamide, 5× SSC, 5× Denhardt solution (1× Denhardt solution is 0.02% each polyvinylpyrrolidone, Ficoll 400 [Pharmacia Fine Chemicals, Piscataway, N.J.] and bovine serum albumin), 25 mM sodium phosphate (pH 6.5), 5% (wt/vol) sodium dodecyl sulfate (SDS), and 500 μg of freshly denatured herring sperm DNA per ml. Nylon membranes were hybridized (20) at 42°C for 18 h in a solution containing 45% (vol/vol) formamide, 5× SSC, 1× Denhardt solution, 20 mM sodium phosphate (pH 6.5), 5% (wt/vol) SDS, 5% (vol/vol) dextran sulfate, 200 μg of freshly denatured sheared herring sperm DNA per ml, and 1 μg of heat-denatured biotinylated probe DNA. After hybridization, the nylon membranes were washed twice in 0.1% (wt/vol) SDS–2× SSC for 5 min at room temperature, twice in 0.1% (wt/vol) SDS–0.2× SSC for 5 min at room temperature, and twice in 0.1% (wt/vol) SDS–0.16× SSC for 15 min at 50°C. The hybridization reactions were visualized colorimetrically with the BluGENE (GIBCO Ltd.) nonradioactive nucleic acid detection system, which contained streptavidin-alkaline phosphatase conjugate and dyes, as recommended by the manufacturer.

Computation of strain similarities. Photographs of the *Hae*III digests were scanned with a laser densitometer (Ultroscan XL model 2222; Pharmacia-LKB Biotechnology, Uppsala, Sweden), and each of the traces was compared as follows. A_{633} was recorded at intervals along the gel, yielding 625 points per 10-cm gel. A rectangular line beam was used to scan each track in triplicate, resulting in a 2.4-mm multiple track scan. The peak area output for each zone in the same track was the mean absorbance of the area scanned, and the resulting values were recorded in absolute ASCII as raw data on disc. The analyses were performed on the basis of a 60-mm-long scan (Fig. 1, region a-b), which excluded the high- and low-molecular-weight regions containing partly digested, unresolved, or low-intensity bands. The background cutoff was set at 0.5 (the fraction of total absorbance). Similarities between all possible pairs of fingerprints were expressed as the Pearson product-moment correlation coefficient (r) (34) converted to a percentage value for convenience. The best fit between each pair of traces was obtained by laterally shifting one trace with respect to the other in single-point steps of 160 μm up to five points either side of the initial alignment. Strains were then clustered by the method of unweighted pair-group average (UPGMA) linkage. Recording of the raw data, background trend removal, calculation of similarity, and clustering were all carried out by using a program package in Turbo Pascal as previously described (12, 27).

The *Xho*I digest patterns, which were less complex, were screened for 31 different bands, and positive (presence) and negative (absence) results were coded as 1 and 0, respectively. Similarity among strains was estimated by means of the simple matching coefficient (Ssm), which included negative matches (34), and the Jaccard coefficient, which excluded negative matches (34). Clustering was based on the UPGMA algorithm described above.

Band size estimation. Fragment sizes in the total digests and in the Southern blot hybridization patterns were calculated from migration distances by the DNA SIZE program as described previously (25).

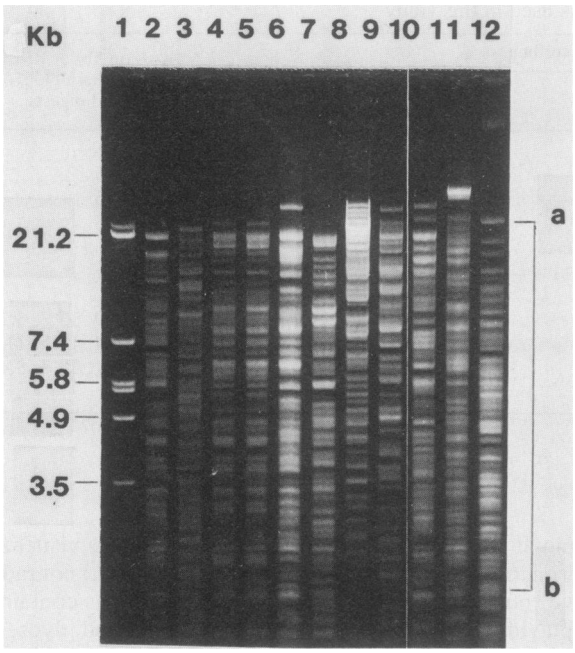

FIG. 1. Agarose gel electrophoresis of *Hae*III digests of *Campylobacter* chromosomal DNA. Lanes: 1, *Eco*RI digest fragments of bacteriophage λ DNA; 2, *C. jejuni* A174/81; 3, *C. fetus* NCTC 10842; 4, *C. jejuni* NCTC 10983; 5, *C. jejuni* NCTC 11168; 6, *C. jejuni* NCTC 11351; 7, *C. jejuni* NCTC 11392; 8, *C. sputorum* NCTC 11528; 9, *C. hyointestinalis* NCTC 11608; 10, *C. jejuni* NCTC 11950; 11, *C. jejuni* NCTC 12109; 12, *C. coli* NCTC 12110. The region between a and b on the right is the region of the gel scanned for the numerical analysis (60 mm). Fragment sizes (in kilobases) are indicated on the left.

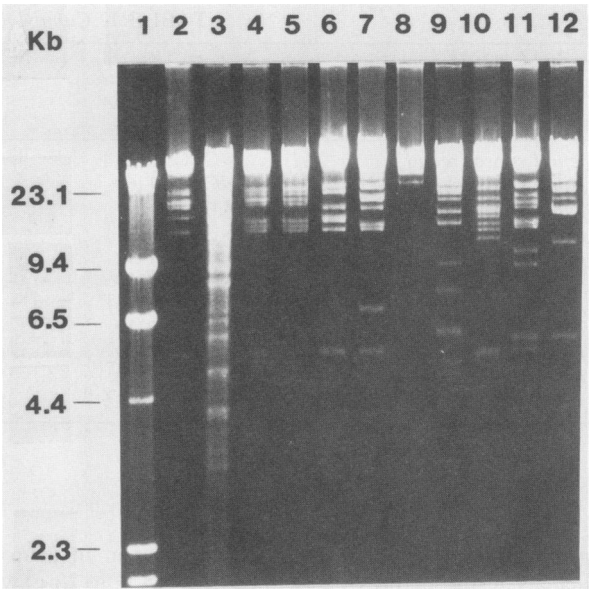

FIG. 2. Agarose gel electrophoresis of *Xho*I digests of *Campylobacter* chromosomal DNA. Lane 1, *Hin*dIII digest fragments of bacteriophage λ DNA; lanes 2 to 12 contain DNA from the *Campylobacter* strains indicated for lanes 2 to 12 in the legend to Fig. 1. Fragment sizes (in kilobases) are indicated on the left.

RESULTS

Restriction endonuclease digest patterns. Chromosomal DNAs from the 11 strains of *Campylobacter* were digested with the restriction endonucleases *Hae*III and *Xho*I. All the DNA samples were cut by *Hae*III (recognition sequence, GGCC) with a high frequency, giving complex but well-resolved patterns of 25 or more bands with sizes up to about 30 kilobases (kb) in 0.5% agarose gels (Fig. 1). There were clear differences between all the strains except for between *C. jejuni* NCTC 10983 and NCTC 11168, which were identical. In contrast, *Xho*I (recognition sequence, CTCGAG) cut the DNA samples less frequently, giving relatively simple patterns of between two bands for *C. sputorum* to about 15 bands for *C. fetus*, most of which were within the 4- to 24-kb size range (Fig. 2). A significant proportion of the DNA in each of the samples (23- to 25-kb molecular weight region) was not completely cut by *Xho*I, and the fragments in the *C. fetus* DNA sample were less clearly focused due to DNA overloading. Even so, each of the species and subspecies had unique digest patterns except for the two identical strains (*C. jejuni* NCTC 10983 and NCTC 11168 [serotype, PEN 02]).

Numerical analysis of *Hae*III digest patterns. The *Hae*III digest patterns were analyzed by numerical methods (Pearson product-moment correlation coefficient and UPGMA clustering), and the analysis results are illustrated by the dendrogram in Fig. 3. It shows that the strains of *C. fetus*, *C. coli*, *C. sputorum*, and *C. hyointestinalis* were clearly separated at the 72% similarity level from each other and from the set of *C. jejuni* strains. The analysis revealed a single phenon at the 72% similarity level comprising five strains of *C. jejuni* (NCTC 11351^T, NCTC 11950, NCTC 11168, NCTC 10983, and A174/81). All of these were *C. jejuni* subsp. *jejuni* Lior biotype I except for NCTC 11950, which was nitrate negative and was recently classified as *C. jejuni* subsp. *doylei* (36). Two strains (NCTC 11168 and NCTC 10983) with the highest percent similarity (96%) to each other were also of the same serotype (PEN 02), but the other strains in the *C. jejuni* cluster had lower similarities and were of different serotypes (PEN 03, 06, and 023). The main cluster did not include *C. jejuni* NCTC 11392 (Lior biotype, III; serotype, PEN 06), which was linked to the above strains at a slightly lower similarity level (70%). The remaining *C. jejuni* strain (NCTC 12109; Lior biotype, IV; PEN serotype, unknown) was linked at a significantly lower level (58% similarity) to the main cluster.

Numerical analysis of *Xho*I digest patterns. In the analysis of *Xho*I digest patterns, similarities were based on a simple matching coefficient using data on the presence or absence of bands, and the dendrogram obtained is illustrated in Fig. 4. At the 85% level each species was clearly distinguishable, and the *C. jejuni* strains formed a homogeneous cluster, except for NCTC 12109 (Lior biotype IV), which clustered quite separately from the other *C. jejuni* strains. No distinctions could be made in this numerical analysis between strains of Lior biotypes I and III or between strains of different serotypes. Similar groupings were obtained in a second analysis, in which similarities were determined by using the Jaccard coefficient (negative matches were excluded), although the cutoff level between species was reduced to about 45% (data not shown). However, visual inspection of these DNA patterns revealed minor but clear differences between the strains of different Lior biotypes.

Southern blot hybridization of *Xho*I digests. Southern blots of the *Xho*I DNA digests were probed with biotin-labeled cDNA from *E. coli* 16S + 23S rRNA, and the hybridization band patterns obtained are illustrated in Fig. 5. Each strain

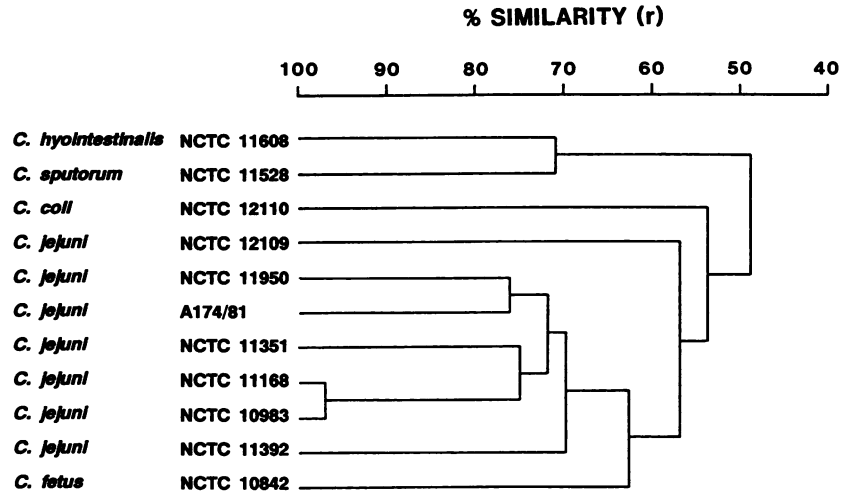

FIG. 3. Dendrogram of the cluster analysis based on *Hae*III digest patterns. The numbers on the horizontal axis indicate percent similarities as determined by the Pearson product-moment correlation coefficient, *r* (multiplied by 100), and UPGMA clustering.

pattern apart from that of *C. sputorum* NCTC 11528^T comprised between two and five discrete bands with sizes between 6 and about 24 kb. The area of unresolved partly digested DNA at the top of the pattern contained some homologous sequences, but these were not included in the present analysis. The approximate sizes of the bands are listed in Table 1. The results showed that the four *C. jejuni* Lior biotype I strains had a characteristic double-band pattern with band sizes of approximately 14 and 16 kb. The hybridization patterns of the Lior biotype III and IV strains also contained either a 14- or a 16-kb band, but differed from the Lior biotype I strains in having an additional large band. The DNA digest pattern of *C. jejuni* subsp. *doylei* was characterized by a single large band of about 30 kb. The other *Campylobacter* reference strains had different and unique patterns, except for *C. sputorum* for which there were no discreet hybridization bands apart from some homologous sequences in the partly digested DNA region. A distinctive feature of the *C. coli* pattern was a unique 6.2-kb band. These strain patterns were not subjected to numerical analysis because of the small number of bands available.

DISCUSSION

The development of DNA fingerprinting as an identification tool, especially for studying the epidemiology of *C. jejuni* infections, is limited by the difficulty of comparing large numbers of different and complex multiband fragment patterns. Although the technique has been used in a number of comprehensive studies of *Campylobacter* species from humans and animals (4, 6, 7, 14–16, 19, 26, 30), the criteria for defining pattern similarities remain highly subjective and imprecise. In the present study we used computer-assisted analysis of DNA digest patterns as well as visual methods to group strains. The feasibility of using numerical methods was previously demonstrated on DNA digests of *Campylobacter* species (6), *Neisseria* species (35), and cytomegalovirus strains (1). The type of numerical analysis required is largely determined by the complexity of the pattern, and we found it was necessary, depending on the endonuclease, to use two different analytical techniques. In the case of the complex *Hae*III digests (>25 bands), high-resolution scanning was combined with similarity estimation and hierarchi-

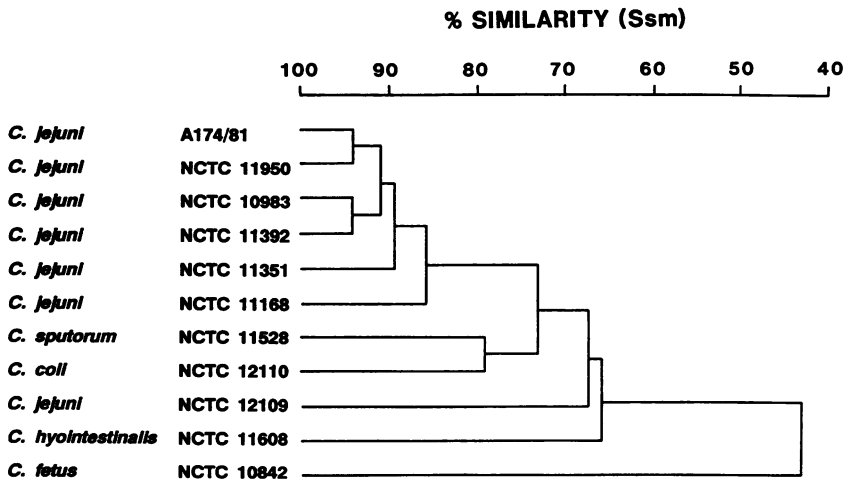

FIG. 4. Dendrogram of the cluster analysis based on *Xho*I DNA digest patterns. The numbers on the horizontal axis indicate percent similarities as determined by the simple matching coefficient (Ssm) with UPGMA clustering.

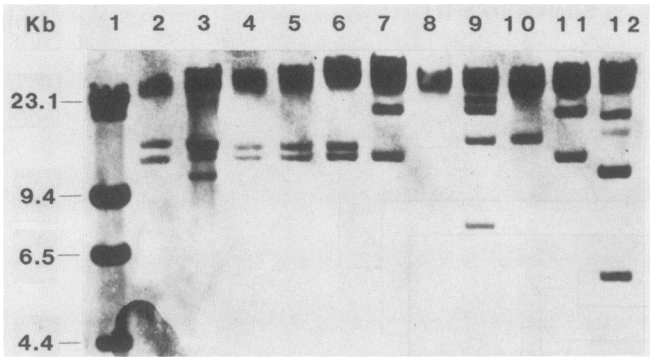

FIG. 5. Hybridization of biotin-labeled ribosomal cDNA synthesized from *E. coli* 16S + 23S rRNA with Southern-blotted *Xho*I restriction fragments of chromosomal DNA from different *Campylobacter* species. Lane 1, *Hind*III digest fragments of bacteriophage λ DNA; lanes 2 to 12 contain DNA from the *Campylobacter* strains indicated for lanes 2 to 12, in the legend to Fig. 1. Fragment sizes (in kilobases) are indicated on the left.

cal clustering as used previously for *Campylobacter* species (6). In contrast, the *Xho*I patterns, which were less complex (<10 bands), were used as the basis for calculating similarities with a simple matching coefficient.

Discrimination between species. The overall impression from visual comparison of the *Hae*III digest patterns of *Campylobacter* DNA was of considerable heterogeneity among the five species, and this was confirmed by the numerical analysis of the *Hae*III data in which all the species were linked at low (50% or less) similarity levels. Similar results with *Hae*III digests were reported previously (6), and they were consistent with the relatively low (≤20%) DNA-DNA sequence homologies that exist between most *Campylobacter* species (except for *C. jejuni* and *C. coli*, which are more closely related [≈40%] [23]). Our numerical analysis of *Hae*III data revealed that the seven *C. jejuni* strains were more similar to each other than to other campylobacters. The numerical analysis of the *Xho*I digest pattern data also clearly differentiated between the species. In both numerical analyses, *C. coli* was closely linked to the *C. jejuni* cluster. The two species have relatively high levels of sequence homology (30 to 50%) (23, 28), so the distributions of *Hae*III and *Xho*I recognition sequences within their genomes are likely to be very similar. However, there were more marked band differences between *C. jejuni* and *C. coli* in their *Xho*I Southern blot hybridization patterns, which in that case provided a more sensitive measure of genomic variation.

The number of bands detected in the *Xho*I hybridization patterns confirmed previous results (14) in indicating that up to four copies of the 16S + 23S rRNA genes were encoded in the *Campylobacter* genome. However, it appears that species may differ in the number of copies present, as the six strains of *C. jejuni* subsp. *jejuni* had two copies of the rRNA cistrons and *C. fetus* subsp. *fetus* had four copies, assuming that no *Xho*I recognition sites occurred within the rRNA cistrons.

Discrimination within species. In the *Xho*I Southern blot hybridizations, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* were clearly distinguishable but were not as unequivocally separated in the *Hae*III or *Xho*I restriction digest analyses. The latter results probably reflected the close DNA-DNA homology between these two subspecies (28, 36).

Biotyping is important in characterizing *C. jejuni* isolates for epidemiological purposes, and the most widely used scheme is that of Lior (17), which provides a higher level of discrimination than the earlier and simpler scheme of Skirrow and Benjamin (33). Our DNA fingerprinting results showed that strains negative in the rapid H_2S test (Skirrow biotype 1 and Lior biotype I) were genomically different from the rapid H_2S-positive strains (Skirrow biotype 2 and Lior biotypes III and IV). The Lior biotype III and IV strains were also different from each other in the total digest analyses but appeared more similar in the Southern blot hybridizations. The results of all three analyses indicated a marked difference between the Lior biotype IV strain (NCTC 11209) and the set of four Lior biotype I strains; Lior biotype I is the most commonly encountered *C. jejuni* biotype (13, 21). Our genomic DNA results support the validity of these biotypes on the basis of conventional tests, even though strains of different biotypes are closely related on DNA-DNA homology (23). Our results also agreed with previous studies in which it was shown that strains of Skirrow biotypes 1 and 2 could be distinguished by *Hae*III digestion pattern analysis (6) and in Southern blot hybridization patterns with a specific *Campylobacter* 16S rRNA oligonucleotide probe (32).

Biotyping combined with serotyping either by heat-labile surface antigens (18) or by heat-stable (lipopolysaccharide) antigens (29) can be used to improve discrimination between strains of *C. jejuni* (17). A problem of serotyping is that a proportion of strains (up to 7%) are ungroupable and others react with more than one antiserum (15). Our results for strains of known Penner serotype and Lior biotype I showed that the *Hae*III digest pattern analyses were sensitive enough to distinguish serologically different strains, whereas the two strains with the same serotype had a high level of DNA fingerprint similarity. Differences detected visually were also reflected in the dendrogram. In contrast, the *Xho*I total digest dendrogram and rRNA probe hybridization patterns distinguished biotypes but not serologically different strains, although minor differences between the latter were apparent on visual inspection of the total *Xho*I digest patterns.

In general, we found that *Hae*III digest patterns gave better discrimination than those of *Xho*I at serotype levels, but numerical analysis was essential to form reliable groupings using the complex *Hae*III pattern data. These patterns were less valuable for between-species comparisons because they were generally so different. However, further studies are needed on a wider range of strains of known serotypes to compare *Hae*III DNA fingerprinting and serotyping. In contrast, the *Xho*I digests contained fewer bands than the *Hae*III digests, and we found that visual comparisons of *Xho*I total patterns and Southern blot hybridizations were convenient and reliable methods of grouping strains at species, subspecies, and biotype levels.

We conclude that DNA fingerprinting is useful for identifying *Campylobacter* species but that the level of discrimination depends on the restriction enzyme and data analysis method used. The relative ease of such genetic analyses contrasts significantly with the difficulty of obtaining serotyping data and suggests that they could be applied to *Campylobacter* species for which there are no suitable typing schemes.

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