Application of Different Chromosomal DNA Restriction Digest Fingerprinting 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 HaeIII and XhoI digest patterns of chromosomal DNA and Southern blot hybridization patterns of XhoI digests obtained with an Escherichia coli 16S + 23S rRNA gene probe. Visual comparisons and numerical analyses of the HaeIII and XhoI 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 XhoI total digests and Southern blot hybridization patterns were that they were less complex than the HaeIII patterns and easier to compare visually. However, numerical analysis of XhoI data resulted in reduced discrimination. We conclude that DNA fingerprinting using either HaeIII or XhoI 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. lariatis (15), C. fetus subsp. fetus (7), and C. pylori (16, 19). The DNA digest patterns produced by restriction endonucleases such as HaeIII, HindIII, BstEII, and XhoI 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 differs 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 HaeIII and XhoI 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 XhoI digests using a broad-spectrum, unfractonated 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) debrinated 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% CO₂-90% H₂.

DNA preparation, digestion, and separation of fragments.

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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 HaeIII and XhoI (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 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 (pH 8.3) 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 HaeIII digests were scanned with a laser densitometer (Ultrascan XL model 2222; Pharmacia-LKB Biotechnology, Uppsala, Sweden), and each of the traces was compared as follows. An30 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 XhoI 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).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Biotype according to:</th>
<th>Serotype (PEN)</th>
<th>Sizes (in kb) of RFLPs* in Southern blots of XhoI digests</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>NCTC 10983</td>
<td>1</td>
<td>I</td>
<td>23.9, 18.8, 14.5</td>
</tr>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>NCTC 11168</td>
<td>I</td>
<td>I</td>
<td>23.1, 21.1, 18.8, 8.4</td>
</tr>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>A174/81</td>
<td>I</td>
<td>I</td>
<td>21.2, 16.4, 14.5</td>
</tr>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>NCTC 11351T</td>
<td>I</td>
<td>I</td>
<td>22.0, 13.7, 6.2</td>
</tr>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>NCTC 11392</td>
<td>II</td>
<td>III</td>
<td>19.4, 14.5</td>
</tr>
<tr>
<td>C. jejuni subsp. jejuni</td>
<td>NCTC 12109</td>
<td>II</td>
<td>IV</td>
<td>ND*</td>
</tr>
<tr>
<td>C. jejuni subsp. doyleei</td>
<td>NCTC 11950T</td>
<td>I</td>
<td>I</td>
<td>19.5</td>
</tr>
<tr>
<td>C. coli</td>
<td>NCTC 12110</td>
<td>I</td>
<td>I</td>
<td>23.1, 22.0, 13.7, 6.2</td>
</tr>
<tr>
<td>C. fetus subsp. fetus</td>
<td>NCTC 10842T</td>
<td>I</td>
<td>I</td>
<td>23.1, 16.4, 14.5, 11.7</td>
</tr>
<tr>
<td>C. hypointestinalis</td>
<td>NCTC 11608T</td>
<td>I</td>
<td>I</td>
<td>13.7, 9.2, 6.2</td>
</tr>
<tr>
<td>C. sputorum subsp. sputorum</td>
<td>NCTC 11528T</td>
<td>I</td>
<td>I</td>
<td>5.0, 6.2, 3.5</td>
</tr>
</tbody>
</table>

* RFLP, Restriction fragment length polymorphism.
* ND, No data available.
* —, No bands.
RESULTS

Restriction endonuclease digest patterns. Chromosomal DNAs from the 11 strains of Campylobacter were digested with the restriction endonucleases HaeIII and XhoI. All the DNA samples were cut by HaeIII (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, XhoI (recognition sequence, CTGGAG) cut the DNA samples less frequently, giving relatively simple patterns of between two bands for C. sporum 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 XhoI, 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 HaeIII digest patterns. The HaeIII 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. sporum, 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 11351T, 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 to a significantly lower level (58% similarity) to the main cluster.

Numerical analysis of XhoI digest patterns. In the analysis of XhoI 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 XhoI digests. Southern blots of the XhoI 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. 1. Agarose gel electrophoresis of HaeIII digests of Campylobacter chromosomal DNA. Lanes: 1, EcoRI 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. sporum 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 XhoI digests of Campylobacter chromosomal DNA. Lane 1, HindIII 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.
DNA fingerprinting in 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 different analytical techniques. In the case of the complex HaeIII digests (>25 bands), high-resolution scanning was combined with similarity estimation and hierarch-
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 H₂S test (Skirrow biotype 1 and Lior biotype I) were genomically different from the rapid H₂S-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 HaeIII 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 HaeIII 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 XhoI 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 XhoI digest patterns.

In general, we found that HaeIII digest patterns gave better discrimination than those of XhoI at serotype levels, but numerical analysis was essential to form reliable groupings using the complex HaeIII 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 HaeIII DNA fingerprinting and serotyping. In contrast, the XhoI digests contained fewer bands than the HaeIII digests, and we found that visual comparisons of XhoI 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.

LITERATURE CITED


