Evaluation of 10 Methods To Distinguish Epidemic-Associated Campylobacter Strains

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We compared four phenotypic and six genotypic methods for distinguishing Campylobacter jejuni strains from animals and humans involved in four epidemics. Based on a comparison with epidemiologic data, the methods that correctly identified all strains in three milkborne outbreaks and one waterborne outbreak were heat-stable and heat-labile serotyping; multilocus enzyme electrophoresis (MEE); DNA restriction endonuclease analysis (REA); electrophoresis, phage typing, plasmid analysis, and probing of BglII and XhoI DNA digests with C. jejuni 16S rRNA genes failed to correctly separate one or more strains. MEE, restriction endonuclease analysis, and ribotyping were the most sensitive methods and identified nine types among the 22 strains. These methods were also capable of further distinguishing strains within the same serotype. Data from MEE were also analyzed to calculate genetic relatedness among strains. Serotyping was the most discriminating phenotypic method, with eight and seven types distinguished by the heat-stable and heat-labile methods, respectively. MEE and ribotyping had several advantages over the other methods because they measure relatively stable and significant chromosomal differences and are applicable to other species and genera. These methods, however, are complex and not easily quantified; they are currently limited to specialized laboratories. When antisera are available, serotyping appears to be an effective and more practical approach to the identification of epidemic-related strains.

An understanding of the epidemiology of diarrheal disease caused by Campylobacter strains, especially C. jejuni and C. coli, depends on methods that can discriminate within species and distinguish strains from different sources. Several reservoirs and vehicles of transmission for Campylobacter strains have been identified by using strain marker systems (6). The various techniques used to characterize campylobacters over the past few years include serotyping, biotyping, phage typing, lectin typing, plasmid analysis, auxotyping, resistotyping, outer membrane protein analysis by gel electrophoresis, and bacterial restriction endonuclease analysis (REA) of chromosomal DNA. Each method or system has its advantages and disadvantages, and some investigators find two or more methods more useful for distinguishing strains (8, 17, 18, 24, 32). The most commonly used techniques to distinguish thermophilic Campylobacter strains are serotyping by using the methods of Penner and Hennessy (33) and Lior et al. (26) or a combination of both. Recently developed molecular techniques based on rRNA hybridization of restriction digests of bacterial chromosomal DNA (ribotyping) and multilocus enzyme electrophoresis (MEE) have been applied to epidemiologic investigations of Salmonella species (34, 46), Legionella species (11, 39, 47), Haemophilus influenzae (9, 42), and Escherichia coli (38). Ribotyping was shown to be useful in discriminating among several different Campylobacter species (30, 35); C. jejuni and C. coli species can also be distinguished by MEE (1). The value of these techniques for studies in the epidemiology of Campylobacter species has not been determined.

A multicenter collaborative study was designed to determine the most sensitive, reproducible, and practical method to characterize C. jejuni and C. coli strains isolated during well-documented epidemic investigations. The typing systems evaluated fell into two categories: (i) phenotypic methods that can be applied only to Campylobacter species, i.e., serotyping by the schemes of Penner and Hennessy (33) and Lior et al. (26), biotyping, and phage typing; and (ii) genotypic methods that can be used for any bacterial genus or species, i.e., MEE, REA, ribotyping, and plasmid profile analysis. The results of a comparison of 10 methods with strains from four epidemics are described.

MATERIALS AND METHODS

Bacterial strains. The study strains were 22 Campylobacter isolates from three milkborne outbreaks and one waterborne outbreak. The raw milk-associated outbreaks occurred in Kansas in 1981 (20) and in Vermont in 1982 and 1983 (16, 48). Florida was the site of the 1983 waterborne outbreak (36). The strains included fecal isolates from seven humans, seven cows, one calf, and one pig; cloacal isolates from four birds; and one isolate from the milk of a cow.

The strains were identified by conventional biochemical and phenotypic tests and by cellular fatty acid analysis (22, 29) and included 21 C. jejuni and 1 C. coli isolate. Two strains from birds in the waterborne outbreak, D472 and D473, were originally identified as C. jejuni; but they did not grow on Mueller-Hinton agar without or with 5% sheep blood, were glycine negative (did not grow in brucella media with 1% glycine), and were delayed H2S positive in brain heart infusion agar with 0.02% cysteine, as detected by lead

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acetate paper (required 7 days of incubation instead of ≤3 days). Their fatty acid compositions differed from those of C. jejuni and C. coli strains by the absence or presence of only trace amounts of hexadecanoic acid (C16:0).

**Serotyping.** Strains were serotyped at the Centers for Disease Control, Atlanta, Ga., by the methods of Penner and Hennessy (33) and Lior et al. (26) to identify heat-stable (HS) and heat-labile (HL) antigens, with results interpreted as described previously (32). The thermostable and heat-labile (HL) antigens, with results interpreted as described by Hennessy (33) and Lior et al. (26) to identify heat-stable antigens, were identified by slide agglutination by using 53 absorbed antisera to strains HL1 to HL60, except for strains HL34, HL41, HL48, and no antisera for strains HL37, HL49, HL50, and HL58. Strains nontypeable for HL antigens were confirmed at the Laboratory Centre for Disease Control, Ottawa, Canada, by using 108 antisera (63 C. jejuni, 37 C. coli, and 8 C. lari, formerly C. lari). The serotypes of the other study strains were also confirmed at the Laboratory Centre for Disease Control.

**Biotyping.** The extended biotyping scheme of Lior (24) based on biphasic hydrolysis, rapid H₂S production, and DNA hydrolysis with toluidine blue-DNA agar (25) was used at the Laboratory Centre for Disease Control to separate strains into biotypes. Thermophilic Campylobacter species can be subdivided into four C. jejuni, two C. coli, and two C. lari biotypes by this system (24).

**Bacteriophage typing.** The bacteriophage type (phage) of each strain was identified at the Laboratory Centre for Disease Control by using the 14 phages selected by Grajewski et al. (13) and expanded to 24 phages by Khakhria and Lior (19a). An additional 5 phages were isolated and added to the battery, for a total of 29 phages.

**MEE.** To determine the multilocus enzyme electrophoretic type (ET), strains were tested at the Centers for Disease Control as described by Selander et al. (37) and modified by Reeves et al. (34). Protein extracts were electrophoresed through starch gels and screened for 40 enzymes. All strains were assayed for the following 11 enzymes: malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, threonine dehydrogenase, indophenol oxidase, NADH dehydrogenase, adenylate kinase, phenylalanine-leucine peptidase, alkaline phosphatase, fumarase, and acetyl-CoA synthetase. Each strain was incubated for maximum growth on 20 plates (15 by 150 mm) of either Mueller-Hinton agar with 5% sheep blood or heart infusion agar with 5% rabbit blood, and an extract was prepared by lysing cells as described by Reeves et al. (34). Buffer systems were prepared, and electrophoresis and enzyme analysis were performed as described previously (34), except that a modified Bio-Phorex horizontal electrophoresis cell (Bio-Rad Laboratories, Rockville Centre, N.Y.) was used and run at 170 V. The mobilities of an enzyme from different isolates on the same gel were compared with each other, and the variants were considered the alleles of that enzyme. Alleles were given different ascending numbers based on their migration toward the anode.

The data were analyzed by the following statistical methods. Genetic diversity at each enzyme locus (the degree to which each enzyme locus varied) was calculated as described by Selander et al. (37). Mean genetic diversity per locus was calculated as the arithmetic average of the values for all of the loci. Genetic distances between pairs of strains were calculated as the proportion of weighted mismatches of alleles (37, 39), and a dendrogram showing the clustering or relatedness of the ETs was generated by the unweighted pair group method for arithmetic averages (40) by using the commercially available program CLUSTER AN IC (51). Genetic distances were also examined by principal coordinate analysis (12) to provide a more visual representation of the spatial relationships among the ETs.

**REA.** Strains were studied by bacterial restriction endonuclease DNA analysis (REA-1 and REA-2) and genetic probing of Southern blots (Blot-1 and Blot-2) at Stanford University and the Centers for Disease Control, respectively, by using various enzymes and probes. For REA-1 patterns, each isolate was grown on Columbia agar base or in heart infusion broth in a microaerobic atmosphere; and whole-cell DNA was isolated, purified, and digested with HindIII, BglII, and XhoI as described previously (21). Cells for use in producing REA-2 patterns were grown on heart infusion agar with 5% rabbit blood in an atmosphere of 5% O₂-7.5% CO₂-7.5% H₂-80% N₂. DNA was extracted by the method of Owen and Boreman (31) as modified by Kiehlbauch et al. (19b). Whole-cell DNA was digested with enzymes PvuII and PstI according to the instructions of the manufacturer (New England BioLabs, Beverly, Mass.). Fragments were electrophoresed as described by Maniatis et al. (28), and REA-1 and REA-2 patterns were observed by UV illumination following ethidium bromide staining (1 μg/ml).

**Southern blot and hybridization.** DNA fragments obtained by electrophoresis in the REA-1 procedure were transferred to nitrocellulose sheets by the methods of Southern (41) and hybridized with a 32P-labeled (nick translation) C. jejuni DNA probe for 16S rRNA (Blot-1) as described by Maniatis et al. (28). Digested DNA from the REA-2 procedure was transferred to nitrocellulose or nylon membranes (MSI Manigraph; MSI, Tozer, Md.) as described by Southern (41) and probed with 32P-labeled (5'-end-labeled) E. coli 16S and 23S rRNA (Blot-2) by the method of Grimont and Grimont (14), as modified by Altweg et al. (2). Nitrocellulose and nylon membranes (Blot-1 and Blot-2) were autoradiographed with XAR-Omat Kodak film and an intensifying screen at −70°C.

**Plasmid analysis.** Plasmid analysis was done by the technique of Birnboim and Doly (5) by using the modification of Baig (4a). Briefly, overnight growth from a heart infusion agar with 5% rabbit blood plate was suspended in Mueller-Hinton broth to the density of a no. 2 McFarland standard. One milliliter of this suspension was centrifuged, and the pellet was treated with lysozyme followed by alkaline denaturation and ethanol precipitation as described by Birnboim and Doly (5). Following treatment with RNase A (Sigma Chemical Co., St. Louis, Mo.) and phenol-chloroform extraction, the DNA was again ethanol precipitated. Electrophoresis was carried out on a 0.66% horizontal agarose gel in TBE (89 mM Tris base, 0.25 disodium EDTA, 8.9 mM boric acid) at 50 mA for approximately 3 h. An E. coli strain, V157, containing plasmids with known molecular weights (27) was included in each gel.

**RESULTS**

**Correlation of methods with epidemic data.** We considered epidemiologic evidence as the "gold standard" for determining the source of Campylobacter infections for each of the four outbreaks. The ability of a method to distinguish strains within each outbreak was compared with the epidemiologic evidence linking or incriminating strains.
Epidemiologic data. The four outbreak investigations have been described previously (16, 20, 36, 48). Brief summaries of the aspects of each investigation that are pertinent to this study follow.

(i) Outbreak 1. Raw milk from a dairy (dairy A) was implicated as the vehicle of transmission for outbreak 1 in Kansas in 1981 (20). An inspection of three dairies that sold raw milk in the local area of the epidemic revealed the following discrepancies at dairy A: (i) a higher incidence of mastitis than that at the two other dairies, (ii) several cows with mastitis were not excluded from the milking line during the epidemic period, and (iii) unacceptable standard plate count and direct microscopic somatic cell count levels were recorded for milk samples during the time of the epidemic. Counts fell to acceptable levels after the cows with mastitis were removed from the milking line. C. jejuni was also isolated from rectal swabs of cows from one of the other dairies under inspection (dairy B). No further cases of raw milk-associated Campylobacter enteritis were reported after dairy A suspended operation.

(ii) Outbreak 2. Epidemiologic evidence implicated raw milk produced at a commercial dairy farm as the cause of outbreak 2 in Vermont in 1982 (48). Raw milk production included milk taken from one cow during and after it had a diarrheal illness. Consumption of this raw milk was associated with the development of Campylobacter gastroenteritis. Two C. jejuni isolates, one from the cow with diarrhea and one from an ill patient who consumed raw milk from the commercial dairy, were indistinguishable in laboratory tests.

(iii) Outbreak 3. Unpasteurized cow's milk from a local farm was identified as the source for outbreak 3 in Vermont in 1983 (16). Campylobacter diarrhea developed in humans after they drank raw milk from the farm and used this milk for consumption in coffee. C. jejuni was isolated from separate milk samples of cow A (an apparently healthy cow, but with low-grade mastitis), from a fecal sample of cow A, and from a child with diarrhea who drank milk from cow A. Calves that drank milk from cow A were also fecal culture positive for C. jejuni. A single isolate from a pig was identified as C. coli. Results of the investigation suggested that C. jejuni was present in the milk as a result of low-grade mastitis rather than as a result of fecal contamination.

(iv) Outbreak 4. Epidemiologic evidence indicated that city water was the vehicle for an estimated 865 cases of gastrointestinal disease (outbreak 4) in Florida in 1983 (36). C. jejuni was recovered from ill persons, and Campylobacter-specific antibodies were also demonstrated in paired sera of patients. The city water plant had numerous deficiencies, and birds were observed perching and defecating on the open-top treatment tower. Wild birds, trapped 7 weeks after the outbreak and during a time of migration, carried C. jejuni, and the investigators postulated that at the time of the outbreak local birds contaminated the city water supply.

Strain discrimination within an epidemic. When compared with the epidemiologic evidence, the methods that were independently capable of correctly distinguishing all strains in outbreaks 1 to 4 were HS and HL serotyping, MEE, REA-1, REA-2, and Blot-2. The systems that failed to correctly separate the strains by epidemiologic data were biotyping in outbreaks 1, 3, and 4; phage typing in outbreaks 3 and 4; Blot-1 in outbreak 3; and plasmid analysis in outbreaks 2 and 3.

An analysis by outbreak showed that all methods except biotyping were comparable for distinguishing strains in outbreak 1. Each assay recognized the bovine source (dairy A) for the human infections; however, biotyping failed to identify multiple types in the implicated herd. All methods except biotyping identified strain SSU9896 as being unrelated to all other strains. The HS22 antigen was intermittently detected on serotype HS23,36; therefore, HS22,23,36 and HS23,36 in outbreak 1 were considered to be the same serotype. None of the marker systems separated the epidemiologically implicated strains from the herd at dairy A from the nonimplicated strains from the herd at dairy B (Table 1).

In the evaluation of methods for outbreak 2, all methods except plasmid analysis recognized the isolate from the ill human patient and the implicated source to be the same (Table 1). Plasmids were not detected in either isolate, confirming the original epidemiologic data (48).

The implicated source for the human and calf infections in outbreak 3 was identified as the isolate from milk of cow A and was recognized by all methods except plasmid analysis. Biotyping, phage typing, and Blot-1, however, could not distinguish the fecal isolate from cow A from the epidemic (milk) strain from cow A. The C. coli isolate from a pig was not implicated by any of the assays.

With the exception of biotyping of one isolate from a bird, all methods eliminated these birds as the epidemic source for outbreak 4. The avian and human isolates in the waterborne epidemics were different by all methods. HS serotyping, MEE, REA-1, REA-2, Blot-1, and Blot-2 were more discriminating than the other methods were and identified three types among the four isolates from birds. Also, each method except phage typing identified all strains from humans in outbreak 4 to be the same.

Evaluation of methods. The group of 22 isolates was analyzed by each method to determine the most effective method for distinguishing C. jejuni strains obtained from different epidemics and to determine the relationship of the strains to each other.

(i) Serotyping. The HS and HL serotyping systems identified eight and seven serotypes, respectively, among the 22 strains (Table 1). All strains were serotypeable in the two schemes except for one isolate from a bird (D473) by the system of Penner and Hennessy (33) and two isolates from birds (D472, D473) by the system of Lior et al. (26). These two isolates from birds were identified as C. jejuni; however, they demonstrated unusual biochemical and growth characteristics (see above).

(ii) Biotyping. C. jejuni biotypes I and II were identified among the 21 C. jejuni strains (Table 1). The single C. coli isolate, D1159, was found to be C. coli biotype I. Most C. jejuni isolates (n = 18) belonged to biotype I, which is the most common biotype among human and nonhuman strains (24). Of these 18 isolates, 7 were from humans and 11 were from nonhumans.

(iii) Bacteriophage typing. Among the 22 strains studied, five phage types were found. Four isolates from outbreak 4, two from humans (D445, D450) and two from birds (D472, D473), were untypeable. Nine strains, three from humans and six from nonhumans, were phage type 11, the most frequently identified phage type among campylobacters (19a).

(iv) HL serotyping-biotyping-phage typing. HL serotyping in combination with biotyping and phage typing (HL-B-P) recognized 10 different types. Two, one, three, and five types were found in outbreaks 1 to 4, respectively. The HL-B-P combination identified the most types among the 22 strains. The phage types, and therefore the HL-B-P type, of the epidemic strains in the two outbreaks in Vermont were found to be different. All other methods identified the case
TABLE 1. C. jejuni strains from four outbreaks distinguished by 10 methods and epidemiologic evidence

<table>
<thead>
<tr>
<th>Outbreak</th>
<th>Strain</th>
<th>Source</th>
<th>EI*</th>
<th>Type or pattern by the following methodb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HS</td>
</tr>
<tr>
<td>1</td>
<td>EDL 18</td>
<td>Human</td>
<td>Y 36, 23, 22 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td></td>
<td>EDL 22</td>
<td>Human</td>
<td>Y 23, 36, 22 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td></td>
<td>SSU9892</td>
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<td>Y 23, 36, 22 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td></td>
<td>SSU9894</td>
<td>Cow, dairy A</td>
<td>Y 23, 36, 22 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td></td>
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<td>Cow, dairy B</td>
<td>Y 2 4</td>
<td>I 3 2 8c</td>
</tr>
<tr>
<td></td>
<td>EDL 3</td>
<td>Cow, dairy B</td>
<td>N 36, 23 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td></td>
<td>EDL 4</td>
<td>Cow, dairy B</td>
<td>N 36, 23 5</td>
<td>I 11 3 3c</td>
</tr>
<tr>
<td>2</td>
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<td>Human</td>
<td>Y 2 4</td>
<td>I 3 1 leAc</td>
</tr>
<tr>
<td></td>
<td>D224</td>
<td>Cow, feces</td>
<td>Y 2 4</td>
<td>I 3 1 leAc</td>
</tr>
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<td>3</td>
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<td>Y 2 4</td>
<td>I 5 1 lec</td>
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<td></td>
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<td>I 5 1 lec</td>
</tr>
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<td></td>
<td>D1114</td>
<td>Calf, drunk cow A milk</td>
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<td></td>
<td>D1108</td>
<td>Feces, cow A</td>
<td>Y 4</td>
<td>I 5 6 5c</td>
</tr>
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<td></td>
<td>D1159</td>
<td>Pig, C. coli</td>
<td>N 46, 15</td>
<td>I 11 7 2c</td>
</tr>
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<td>4</td>
<td>D445</td>
<td>Human</td>
<td>Y 19 77</td>
<td>II UT 4 7c</td>
</tr>
<tr>
<td></td>
<td>D450</td>
<td>Human</td>
<td>Y 19 77</td>
<td>II UT 4 7c</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>D472</td>
<td>Bird</td>
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<td>NT</td>
</tr>
<tr>
<td></td>
<td>D473</td>
<td>Bird</td>
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<tr>
<td>Total no.</td>
<td></td>
<td></td>
<td>8</td>
<td>7</td>
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</table>

* EI, Epidemiologically implicated; Y, yes; N, no.
* UT, untypeable; NT, nontypeable.
* Whole-cell DNA was digested with BgIII and XhoI and hybridized with a C. jejuni DNA probe for 16S rRNA.
* DNA was digested with PvuII and PstI and hybridized with E. coli 16S and 23S rRNAs.
* Plasmid size (MDa).

and source strains from the 1982 and 1983 outbreaks in Vermont to be the same biochemically and genetically.

(v) MEE. The 22 isolates from the four outbreaks produced nine distinct ETs (Table 1). Three ETs were identified among the seven C. jejuni strains from humans, and seven ETs were identified among the 14 C. jejuni isolates from nonhumans. The C. coli strain (D1159) from outbreak 3 had an ET distinct from those of the C. jejuni isolates.

(vi) REA. By using BgIII and XhoI enzymes, the 22 isolates were sorted into nine different groups (REA-1 in Table 1). The 21 C. jejuni isolates produced eight distinct patterns. The isolates labeled leAc in Table 1 (outbreak 2) were virtually identical to the isolates labeled lec in Table 1 (outbreak 3), except for two minor band differences. The single C. coli isolate (D1159; Table 1, outbreak 3) could be distinguished from all C. jejuni isolates.

Digestion of the chromosomal DNA by the enzymes PvuII and PstI also produced nine restriction patterns among the 22 isolates (REA-2 in Table 1 and Fig. 1), eight patterns among the 21 C. jejuni strains, and a distinct pattern for the C. coli isolate (D1159).

(vii) Southern blot hybridization. Analysis of the DNA was done by hybridizing digests with a C. jejuni DNA probe for 16S rRNA. This method identified six Southern blot patterns among the 22 isolates (Blot-1 in Table 1), with fingerprints consisting of one to four bands of 1.96 to 27.98 kb. Five different patterns were observed in the 21 C. jejuni isolates, with 10 of the C. jejuni strains (7 from outbreak 1 and 3 from outbreak 4) all presenting the same pattern. The fecal isolate from cow A in outbreak 3 (D1108) could not be distinguished from other C. jejuni strains in that outbreak, and no difference was seen between those four C. jejuni isolates and isolate SSU9896 from outbreak 1. The single C. coli isolate (D1159 in outbreak 3) could be distinguished from all C. jejuni isolates.

Ribotyping of the 22 digests produced nine Southern blot patterns (Blot-2 in Table 1), eight for the 21 C. jejuni strains and a distinct pattern for the C. coli isolate. These ribotypes contained three to eight bands of 2.5 to 23 kb (Fig. 2). Common bands were present in all C. jejuni PvuII digests at approximately 2.5 and 9.5 kb (Fig. 2, lanes A to E and G) and all PstI digests at approximately 5.0 kb (Fig. 2, lanes A to E and G), but they were not seen in the two atypical isolates from birds (Fig. 2, lanes H and I), providing further evidence that these isolates were probably not C. jejuni. In contrast to the C. jejuni DNA probe, the E. coli RNA probe could distinguish between isolates D1108 (cow A feces, outbreak
HL4 is a common serotype in sporadic cases and is also a common serotype among isolates from animals. This serotype was implicated in two milkborne outbreaks in Vermont (outbreaks 2 and 3) and was identified among isolates from cows in dairy A in outbreak 1. The HS2-HL4 strain in the two Vermont outbreaks, isolated from farms in different counties 1 year apart, were only slightly different (see REA-1 in Table 1). The HS2-HL4 strain associated with the Kansas outbreak (outbreak 1) is clearly unrelated to the strains involved in the Vermont outbreaks (outbreaks 2 and 3) (Fig. 3, ETs 2 and 1). The data suggest that the strains from Vermont could be part of a local clone, quite separate from the strain from Kansas.

Serotyping was the most sensitive phenotypic method. HS and HL serotyping identified eight and seven types, respectively. HS, HL, MEE, REA-1, REA-2, and Blot-2 provided additional sensitivities over those of the other methods and further distinguished isolates in outbreaks 1 and 3. These six methods identified more than one type in the isolates from the implicated cow herd in outbreak 1. These six methods also indicated that the milk and fecal isolates from cow A in outbreak 3 were different from each other and that the isolate from milk was the same as the isolates from ill humans. Similar data by all methods substantiate the suggestion "that the organism was shed in milk as a result of low-grade mastitis rather than as a result of fecal contamination" (16).

All four restriction enzymes, BglII, XhoI, PvuII, and PstI, showed the same strain discrimination among the strains examined in this study. Although banding differences were noted for each enzyme, all four enzymes were equally effective in identifying the same number of types and in further distinguishing strains of the same serotype.

Blot-2 (E. coli rRNA probe using PvuII and PstI) appeared to be more sensitive than Blot-1 (C. jejuni rRNA probe using BglII and XhoI) for isolates from outbreak 3 and outbreaks 1

3); SSU9896 (cow, outbreak 1); and D1117, D1118, and D1114 (similar C. jejuni strains, outbreak 3).

In the PvuII digests, the E. coli probe, like the C. jejuni probe, differentiated strains from humans and cows in outbreak 1 (Fig. 2, lane B) from strains from humans in outbreak 4 (Fig. 2, lane C) solely by a minor shift in the position of the uppermost band. After PstI digestion and probing (Fig. 2, lanes B and C), the same strains were clearly shown to be different. These data imply that PstI was more discriminating than PvuII; however, PstI failed to digest strain D473, an atypical C. jejuni isolate from a bird in outbreak 4 (Fig. 1 and 2, lanes I). These data suggest that isolates that appear to be identical by PvuII digestion should be restricted by using a second enzyme such as PstI or ClaI.

(vii) Plasmid analysis. By plasmid analysis, four profiles were found in the 12 plasmid-containing isolates (Table 1). A single plasmid was detected in 11 strains, and C. coli D1159 from outbreak 3 contained three plasmids. Plasmids of the same size (25 MDa) were isolated from the strains from humans and strains from the implicated herd and the control herd in outbreak 1. In that same outbreak, isolate SSU9896 from the implicated herd carried a plasmid of a different size (36 MDa). All strains from humans, but none of the isolates from birds, in outbreak 4 harbored a 23-MDa plasmid. Plasmids were not found in the case and control isolates involved in either Vermont outbreak (outbreaks 2 and 3).

(ix) Evaluation data summary. The greatest distinction among isolates was achieved by MEE, REA-1, REA-2, and Blot-2. Each method identified nine types among the 22 strains (Table 1). In addition, these methods were capable of further distinguishing strains of the same serotype. HS2-
and 4. Blot-2 was able to differentiate the milk and fecal isolates from cow A in outbreak 3, but Blot-1 was unable to make this distinction. Blot-2 also distinguished the isolates from humans and cows in outbreak 1 from isolates from humans in outbreak 4; these two groups of strains were also unrelated by other methods (Table 1) but were indistinguishable by Blot-1.

**Strain relatedness.** The genetic relatedness among the 22 isolates is indicated by the dendrogram in Fig. 3 derived from the MEE data. A total of nine ETs were identified among strains in the four outbreaks. Of the 22 isolates (all C. jejuni), 19 were grouped into a cluster at a genetic distance of approximately 0.55. The two unusual isolates from birds (ETs 8 and 9) were found at a genetic distance of about 0.87, and the C. coli strain (ET 7) separated at a genetic distance of 0.80. These results indicate that the latter three isolates are not highly related to the other study strains, and the two unusual isolates from birds are distantly related to other isolates from birds in outbreak 4.

An illustration of the spatial relationship of the nine ETs is shown by a three-dimensional principal-coordinate plot of the data (Fig. 4). The two atypical C. jejuni isolates and the C. coli strain were very distant from the group of closely related C. jejuni strains. Also, among the closely related isolates, strains of the same serotype (HS2-HL4) that could be distinguished further by MEE, REA-1, REA-2, and Blot-2 could be visualized in the three-dimensional plot in Fig. 4.

**FIG. 3.** Genetic relationship among nine ETs of C. jejuni (21 strains) and C. coli (1 strain) from four outbreaks. The dendrogram was generated as indicated in Materials and Methods.

**FIG. 4.** Principal-coordinate analysis of Campylobacter strains from outbreaks in Vermont (■), Kansas (○), and Florida (♦). Arrows indicate strains with the same serotype.

**DISCUSSION**

One of the technical shortcomings of the national Campylobacter surveillance system is the lack of a good strain marker system for widespread use by reference laboratories. The necessary antisera and reagents are not commercially available for the serotyping systems of Penner and Hennessy (33) and Lior et al. (26), which require as many as 60 and 108 antisera, respectively. The purpose of our study was to evaluate several strain marker systems based on epidemiologically defined strains and to identify those methods potentially most useful for either research or clinical microbiology laboratories. The data presented here indicated that certain genotypic methods are more discriminating. MEE; REA using BglII, XhoI, PvuII, and PstI; and probing of DNA digests with E. coli 16S and 23S rRNAs were best and separated the strains equally. These results correspond with those of other studies in which these methods showed that Legionella species have a high correlation with each other and were more discriminating than plasmid analysis (11, 47).

MEE provides an advantage over other genotypic methods in that data can be analyzed to determine the genetic distance (genetic relatedness) among the strains as well as to identify or type strains. On the basis of DNA-DNA hybridization studies, C. jejuni and C. coli are separate species with 25 to 49% homology (15). Our ET data (dendrogram) placed the single C. coli and the cluster of C. jejuni strains at a genetic distance of 0.82. Selander et al. (39) proposed a genetic distance of between 0.5 and 0.6 as a cutoff point for separating Legionella species. Our MEE data thus agree with DNA hybridization studies when this definition is applied. Although two isolates from birds, D472 and D473, shared enough phenotypic characteristics with C. jejuni to be identified as atypical C. jejuni, the MEE, REA, and ribotyping data did not support this classification. The correct identification of these strains will depend on DNA hybridization studies.

Although a small number of strains were used in the study, results suggest that any one of four restriction endonuclease
enzymes can be used to differentiate *C. jejuni* strains. The enzymes *XhoI* and *BglII* have also been used to distinguish between the different species of *Campylobacter*, and *XhoI* identified four types among a collection of 43 *C. fetus* subsps. *fetus* strains (10, 30, 35). One disadvantage of REA is the large number of chromosomal restriction fragments that must be analyzed visually. The method is also difficult to apply in a comparison of large numbers of isolates. REA fingerprinting can, however, be accomplished in 2 to 3 days, compared with the 3 or more weeks required for MEE.

We analyzed the whole-cell restriction enzyme pattern, regardless of the presence of plasmid DNA. The differences in patterns were treated in the same manner whether plasmid DNA was present or absent. It could well be that REA differences relate directly to plasmid DNA differences in certain strains (see strain SSU9896 and strains from outbreaks 2 and 3).

Analysis of the restriction patterns was made easier by probing the chromosomal DNA. By analyzing only those DNA restriction fragments that hybridized with rDNA or rRNA, the number of bands for analysis was reduced to three to eight. Our results indicate a difference in the *C. jejuni* DNA probe for 16S rDNA sequence of *XhoI* and *BglII* and the *E. coli* 16S and 23S rRNA probe using *PvuII* and *PstI* for separating isolates. Several apparently unrelated isolates collected from different geographic areas (Kansas and Florida) more than 2 years apart had similar chromosomal fingerprint patterns with the *C. jejuni* probe and distinctly different patterns with the *E. coli* probe. The difference in the two probes may be explained by the presence of additional genes for 23S rRNA in the *E. coli* probe or the difference in enzymes used with each probe.

The results of our study show that plasmid analysis, another genotypic marker system, is only infrequently useful for differentiating strains of *C. jejuni*. Case and control strains from two outbreaks did not carry plasmids. These results were not unexpected, since previous studies have indicated that only about 20 to 30% of *C. jejuni* and *C. coli* isolates carry plasmid DNA (3, 4, 7, 23, 45). The plasmid results are in contrast to other enteric pathogen studies, in which plasmid profiles alone or in combination with other strain marker systems have proved to be useful epidemiologic tools (44, 49, 50). In fact, plasmid profile analysis and phage typing were significantly more efficient than MEE and REA in differentiating *Salmonella typhimurium* isolates associated with contaminated chocolate products (19). However, *HindIII* was the only restriction enzyme used for the *S. typhimurium* strains, and in our experience *HindIII* provides poor discrimination among strains of other *Salmonella* species.

The HS and HL serotyping methods were as successful as the most sensitive genotypic procedures in determining the relationship among isolates within each epidemic. Passive hemagglutination and slide agglutination methods are simple and rapid and could easily be applied by clinical as well as reference laboratories. The main disadvantage of these methods is the lack of commercially available, high-quality antisera. Production of antisera to the large numbers of strains for either one or both systems would be too time-consuming, costly, and impractical for most clinical or reference laboratories. An alternative approach for laboratories interested in serotyping is to produce antisera to the most common serotypes. Ten to 20 antisera from either system should serotype 70 to 90% of *C. jejuni* and *C. coli* isolates (18, 32). The battery of common antisera designated by Penner and Lior (33a) would serotype 95.5 and 77.3% of these study strains, respectively.

The biotyping scheme is simple and available to most laboratories, although it produces only a few markers among strains when used alone. In recognition of this, biotyping is suggested for use in conjunction with serotyping (24).

Phage typing produced additional discrimination over that provided by biotyping. Of the 22 strains, however, 4 were untypeable, indicating that additional phages are needed for the system to be practical as an independent marker system. This substantiates a previous observation that 58 of 235 *C. jejuni* isolates (19.3%) were untypeable with a battery of 24 phages (19a). When phage typing is combined, as recommended, with serotyping and biotyping, it offers additional markers for epidemiologic studies. In our study this combination was the most sensitive of all methods, as evidenced with isolates involved in the two Vermont outbreaks. Case and epidemic source isolates from the raw milk outbreaks in Vermont in 1982 and 1983 had similar serotypes, biotypes, and genotypes by ET, REA, and Blot analysis. The data suggest that strains from locations in close proximity may be derived from a common ancestor. The phage typing data, however, indicated that the two epidemic strains were different, suggesting that either the phage typing system is too sensitive or that this theory may not be correct. From a practical standpoint, the combination of serotyping, biotyping, and phage typing may be too time-consuming and costly for most clinical or reference laboratories.

In conclusion, MEE, REA, and ribotyping (REA-2) were the most sensitive marker systems, followed by HS and HL antigen serotyping. The genotypic methods have an important advantage over phenotyping tests. They measure stable chromosomal differences, whereas phenotyping methods measure characteristics, such as enzymes and antigens, which may not be stably expressed. Genotypic results have the potential of providing more consistent, reproducible answers and are applicable to other species and genera. These methods, however, are complex procedures that require specialized reagents and equipment. At present, they are most applicable to epidemiologic research, with ribotyping being the method of choice. For routine use in clinical laboratories, genotypic methods would have to be more rapid, have fewer complex steps, use a nonradioactive signal, and optimally, result in an objective or quantitative value for comparative purposes. When the necessary reagents are available, serotyping offers a more rapid and simple method for distinguishing epidemic-related strains. However, until antisera are commercially available, most laboratories must forward epidemic-related cultures to reference laboratories for serotyping.

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