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Identification of *Campylobacter jejuni* and *C. coli* by Gel Electrophoresis of the Outer Membrane Proteins

I. DERCLAYE, I. DELOR, M. VAN BOUCHAUTE, P. MOUREAU, G. WAUTERS, AND GUY R. CORNELIS*

Unité de Microbiologie, Université Catholique de Louvain, UCL 5490, B1200 Brussels, Belgium

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Analysis of the electrophoretic profiles of the outer membrane proteins could be used to differentiate *Campylobacter jejuni* (16 strains) from *Campylobacter coli* (10 strains). This observation was confirmed by the study of DNA homology obtained by a quantitative filter hybridization method. The hippurate hydrolysis test gave a poor correlation with the results of differentiation obtained by DNA homology studies and outer membrane protein profile.

Thermophilic *Campylobacter* spp., i.e., *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis*, are human enteropathogens (21, 23). *Campylobacter fetus* is generally recognized as an agent of systemic infection in humans, but recent studies suggest that it can also be implicated in enteritis (6, 10). The three thermophilic *Campylobacter* species differ from the other species of the genus, and in particular from *C. fetus*, essentially by two characteristics: optimal growth at 42°C and resistance to cephalothin. These species are selectively isolated from human stools on a selective medium containing several antibiotics and by incubation at 42°C in a microaerophilic atmosphere. Although atypical *C. fetus* strains which either grow at 42°C or are resistant to cephalothin have been described previously (7), the isolation of *C. fetus* by using these selective conditions has not been reported. The distribution of *C. jejuni* and *C. coli* in human isolates varies from one area to another. For instance, in Europe, the proportion of *C. coli* ranges from 2.5% in the United Kingdom (12, 22) to 53.9% in Yugoslavia (13). *C. laridis* is rarely present in human samples (23).

The differentiation of thermophilic *Campylobacter* spp. relies on two biochemical tests: the capacity of *C. jejuni* alone to hydrolyze hippurate and the resistance of *C. laridis* only to nalidixic acid. Recently, Totten et al. (24) showed that 20% of hippurate-negative strains are in fact *C. jejuni* and, hence, that hippurate hydrolysis does not always correlate with genetic classification. Moreover, some *C. jejuni* strains show resistance to nalidixic acid (1). The identification of thermophilic *Campylobacter* spp. on the basis of biochemical tests alone does not appear to be reliable (24). This paper describes an alternative method for *Campylobacter* identification.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The test strains were isolated from human stools that were sent for analysis to the University Hospital St Luc, Brussels, Belgium. The reference strains *C. jejuni* I Lior 4, *C. coli* Lior 1, and *C. laridis* Lior 34 were received from Y. Glupczynski (Brussels, Belgium). *C. coli* Lior 8 and *C. coli* Lior 44 were supplied by J. P. Butzler and H. Goossens (Brussels, Belgium). *C. laridis* NCTC 11352 was received from F. Megraud (Bordeaux, France). The *C. fetus* strain was from our own collection.

* Corresponding author.

Campylobacter strains were grown on *Campylobacter* medium comprising brain heart infusion agar (GIBCO Laboratories, Madison, Wis.) supplemented with 3 g of yeast extract (Gibco) liter^{-1} and 10% defibrinated horse blood (Ecobio, Genk, Belgium). Plates were incubated for 24 to 72 h at 37°C in a microaerophilic atmosphere generated by an Anaerocult C Gaspak (E. Merck AG, Darmstadt, Federal Republic of Germany). For *Campylobacter* isolation from feces, the medium was supplemented with the antibiotics of Butzler (Institute Virion Ltd., Rüschlikon, Switzerland) (5) and incubated at 42°C. Brucella broth (Difco Laboratories, Detroit, Mich.) was used to suspend *Campylobacter* organisms scraped from plates. Bacterial counts were determined by measuring the optical density at 600 nm. One A_{600} unit was determined to correspond to 2×10^9 CFU ml^{-1}.

Biochemical characterization. The test for catalase production was performed as described by Sandstedt et al. (20). The rapid tube test for evaluating hippurate hydrolysis was performed as described by Harvey and Greenwood (11) with a single modification; the bacterial suspension in sodium hippurate was incubated 24 h at 37°C instead of 2 h at 35°C. Susceptibility to 30-μg disks of nalidixic acid and cephalothin (Biomérieux, Marcy l'Etoile, France) was tested by the diffusion method by inoculating a suspension adjusted to 5×10^8 CFU ml^{-1} on the *Campylobacter* medium. Plates were read after 24 h in a microaerophilic atmosphere. A strain was considered susceptible when the slightest zone of inhibition was observed. Optimal growth temperature was determined by streaking 10 μl of the same suspension on three plates of the *Campylobacter* medium incubated microaerophically at 28, 37, and 42°C after 24 h.

Preparation of OMPs and gel electrophoresis. Preparation of outer membrane proteins (OMPs) was adapted from the method of Wenman et al. (26). Whole *Campylobacter* cells from a 48-h culture were suspended in 0.001 M sodium phosphate buffer (pH 7.0). The concentration was adjusted to 10^{10} CFU ml^{-1}. Bacterial cells were disintegrated by sonication for 2 min (B12 sonicator; Branson Sonic Power Co., Danbury, Conn.) and 3-ml samples were centrifuged at $5,000 \times g$ for 20 min to remove cell debris. Total membranes were collected by centrifugation at $33,000 \times g$ for 90 min and suspended in 0.5 ml of distilled water. One milliliter of 2% sodium lauryl sarcosinate solution in 0.001 M phosphate buffer was added, and the mixture was gently shaken for 30 min at room temperature to dissolve inner membranes (8). The preparation was centrifuged at $33,000 \times g$ for 90 min. Sarkosyl-insoluble outer membranes were collected in 0.2 ml

of sample buffer (50 mM Tris hydrochloride [pH 6.8], 5% [vol/vol] β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 15% [vol/vol] glycerol, 0.01% bromophenol blue) and stored at −20°C. After being boiled for 5 min, OMP preparations were analyzed on a vertical 14 to 20% gradient (acrylamide-bisacrylamide ratio, 29:1) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Stacking gels were 3% acrylamide-bisacrylamide (ratio, 29:1). Samples (30 μl) containing the OMPs from 4.5×10^9 CFU were electrophoresed for 16 h at a constant current of 15 mA. The gels were stained with Coomassie brilliant blue (2). Molecular weight standards were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000) (Pharmacia, Uppsala, Sweden).

Total DNA preparation. Bacteria were cultivated on 12 plates of *Campylobacter* medium. Cells were collected and suspended in 50 mM Tris hydrochloride (pH 8.5)–50 mM EDTA–50 mM NaCl (THES). The cells were washed and suspended in THES supplemented with 10% sucrose. Ribonuclease (Serva, Heidelberg, Germany) and lysozyme (Serva) were added to final concentrations of 74 μg ml^{-1} and 1 mg ml^{-1}, respectively. The suspension was incubated for 20 min at 37°C, a 10% sodium lauryl sarcosinate solution in THES was added to a final concentration of 3.4%, and incubation was prolonged at 37°C for 5 min. The lysate was subjected to two chloroform-octanol (96:4) extractions, one phenol extraction, and one chloroform-isoamylic alcohol (99:1) extraction. DNA was collected on a glass rod by the addition of 0.5 volume of isopropanol and 0.1 volume of 0.5 M $NaClO_4$ to one volume of the aqueous phase. The DNA precipitate was air dried for 15 min prior to suspension overnight at 4°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]). DNA was finally purified by a CsCl-ethidium bromide isopycnic ultracentrifugation (100,000 × *g*, 40 h), dialyzed against 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0) (TE) and stored at −20°C. DNA concentration was estimated by comparing the fluorescence intensity of the sample DNA against a standard of lambda DNA (Bethesda Research Laboratories, Gaithersburg, Md.) on a ethidium bromide-stained agarose electrophoresis gel. The DNA concentrations of reference strains *C. jejuni* I Lior 4 and *C. coli* Lior 1 were accurately measured spectrofluorimetrically with 4′,6-diamidino-2-phenylindole · 2 hydrochloride essentially as described by Legros and Kepes (15). Excitation and emission wavelengths were 346 and 452 nm, respectively.

Quantitative DNA-DNA filter hybridization. One microgram of heat-denaturated DNA from each strain was spotted on two nylon membranes (Hybond-N; Amersham, Little Chalfont, United Kingdom). One microgram of calf thymus DNA was also spotted in order to measure nonspecific binding. The membranes were air dried for 30 min and layered for 1 min on a filter paper saturated with 0.5 M NaOH–1.5 M NaCl. They were subsequently layered three times for 1 min on filter papers saturated with 0.5 M Tris hydrochloride (pH 7.5)–1.5 M NaCl–1 mM EDTA. The membranes were finally air dried for 30 min and irradiated for 30 s with 312-nm UV light.

DNA from *C. jejuni* I Lior 4 and from *C. coli* Lior 1 was radiolabeled with [α-^{32}P]dATP (3,000 Ci $mmol^{-1}$; Dupont, NEN Research Products, Boston, Mass.) by using a nick translation kit (Bethesda Research Laboratories). The unincorporated nucleotides were eliminated by passage through a Sephadex G-50 column (Pharmacia).

Both membranes were hybridized in parallel, each with one of the two radiolabeled DNAs. The specific activity was identical for the two labeled DNAs.

The membranes were incubated at 63°C in 2× SSC–0.1% bovine serum albumin (Merck)–0.1% Ficoll (Sigma Chemical Co., St. Louis, Mo.)–0.1% polyvinylpyrrolidone (Sigma)–0.5% SDS (Serva)–20 μg of heat-denaturated calf thymus DNA (Serva) ml^{-1}. After 2 h of incubation, a fresh solution of identical composition was added and the labeled DNA, previously heat denatured, was added to a final concentration of 10 ng ml^{-1}. After 16 h of incubation at 63°C, the membranes were washed four times for 15 min in 2× SSC at 63°C. Under these conditions, the T_m of the homologous DNA duplex was equal to 88°C by using the following relation: $T_m = 81.5 + 16.6 \log M + 0.41\% (G + C) - 0.72\%$ formamide, where M is the Na^+ concentration in moles $liter^{-1}$ (9). The average (G + C) percentage for *Campylobacter* was considered to be equal to 33 (3, 18, 25). Hybridization and washing were conducted at $T_m - 25$°C, i.e., 63°C. This corresponds to optimal conditions for hybrid formation (9).

After being washed, the membranes were air dried and autoradiographed. After autoradiography, the different DNA spots were cut off from the membranes and counted in a scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). The hybridization was measured in disintegrations per minute. Corrections for background were made by subtracting from each measured value the value (in disintegrations per minute) obtained for the hybridization of calf thymus DNA with the labeled DNA.

RESULTS

Biochemical characterization. Twenty-two strains were isolated as described in Materials and Methods. All of these strains were typical gram-negative curved rods. They were oxidase and catalase positive and did not grow at 37°C in normal atmosphere. The selective medium and the temperature of incubation used for their isolation from stools implied that all of these strains belong to one of the three species of thermophilic *Campylobacter*, i.e., *C. jejuni*, *C. coli*, and *C. laridis*. As expected, they grew at 42 and 37 but not at 28°C.

Table 1 shows the data from hippurate hydrolysis and antibiotic susceptibility. These results were generally unreliable for typing purposes.

Electrophoretic pattern of the OMPs. Analysis of the OMPs was performed by SDS-PAGE for the 22 isolated strains and for 7 reference strains. All of the strains but *C. fetus* showed a common general OMP profile dominated by a single protein band of 43 kilodaltons (kDa) with slight variation from strain to strain. It has been suggested that this major protein is a porin (16). The *C. fetus* OMP profile was characterized by the presence of two major proteins of 43 and 48 kDa (Fig. 1). In addition to these major proteins, the strains contained from 8 to 10 other prominent OMPs (Fig. 1 to 3).

A more detailed analysis of the OMP SDS-PAGE patterns of the strains revealed that two groups of strains could be distinguished on the basis of four characteristic bands. One group featured protein bands of 55 and 37 kDa. This group contained strains 2, 3, 8, 46, 55, 78, 79, 81, 83, 84, 86, 88, 92, 95, and 96, as well as *C. jejuni* reference strain I Lior 4 no. 106 (Fig. 1 and 2). The second group is characterized by a protein of 25 kDa and a more marked OMP doublet at the level of 84 kDa. This second group contains strains 9, 80, 82, 85, 87, 89, and 90, as well as reference strains *C. coli* Lior 1

TABLE 1. Discrimination between *C. jejuni* and *C. coli* on the basis of hippurate hydrolysis, OMP patterns, and total DNA homology

Strain or isolate no. (strain)	Hippurate hydrolysis[a]	Susceptibility to[b]:		OMP (kDa)		Hybridization ratio (*C. coli*/*C. jejuni*)[c]
		NA	CF	37/55	25/84	
Reference strains						
1 (*C. fetus*)	−	R	S	−	−	0.95
106 (*C. jejuni* I Lior 4)	+	S	R	+	−	0.33
108 (*C. coli* Lior 1)	−	S	R	−	+	2.73
211 (*C. coli* Lior 44)	−	S	R	−	+	2.31
212 (*C. coli* Lior 8)	−	S	R	−	+	2.26
116 (*C. laridis* Lior 34)	−	R	R	−	−	0.62
195 (*C. laridis* NCTC 11352)	−	R	R	−	−	0.69
Clinical isolates						
2	+	S	R	+	−	0.26
3	−	S	R	+	−	0.34
8	+	S	R	+	−	0.33
9	v	S	R	−	+	1.72
46	+	S	R	+	−	0.26
55	+	S	R	+	−	0.23
78	+	S	R	+	−	0.29
79	+	S	R	+	−	0.32
80	v	S	R	−	+	1.86
81	+	S	R	+	−	0.21
82	v	S	R	−	+	1.92
83	v	S	R	+	−	0.31
84	+	S	R	+	−	0.38
85	v	S	R	−	+	1.90
86	+	S	R	+	−	0.20
87	v	S	R	−	+	1.92
88	+	S	R	+	−	0.42
89	−	S	R	−	+	1.82
90	−	S	R	−	+	2.0
92	+	S	R	+	−	0.18
95	+	S	R	+	−	0.35
96	v	S	R	+	−	0.39

[a] v, Variable; +, positive; −, negative.
[b] NA, Nalidixic acid; CF, cephalothin; S, susceptible; R, resistant.
[c] Hybridization ratio = Background corrected disintegrations per minute for hybridization of sample DNA with *C. coli* Lior 1 labeled DNA/background corrected disintegrations per minute for hybridization of sample DNA with *C. jejuni* I Lior 4 labeled DNA.

no. 108, *C. coli* Lior 8 no. 212, and *C. coli* Lior 44 no. 211 (Fig. 1 and 3; strains 211 and 212 are not shown).

Quantitative filter hybridization. Table 1 gives the ratio between the disintegrations per minute for the hybridization to reference strain *C. coli* Lior 1 and the disintegrations per minute for the hybridization to reference strain *C. jejuni* I Lior 4 for the 22 isolates and the 7 reference strains. The principle of this method was introduced by Totten et al. (24). It is clear from Table 1 that all of the isolated strains could be classified as *C. jejuni* or *C. coli* by this method. The mean hybridization ratio was 0.30 ± 0.07 for *C. jejuni* strains and 2.04 ± 0.30 for *C. coli* strains. The hybridization ratio was 0.95 for *C. fetus* and 0.62 or 0.69 for *C. laridis*.

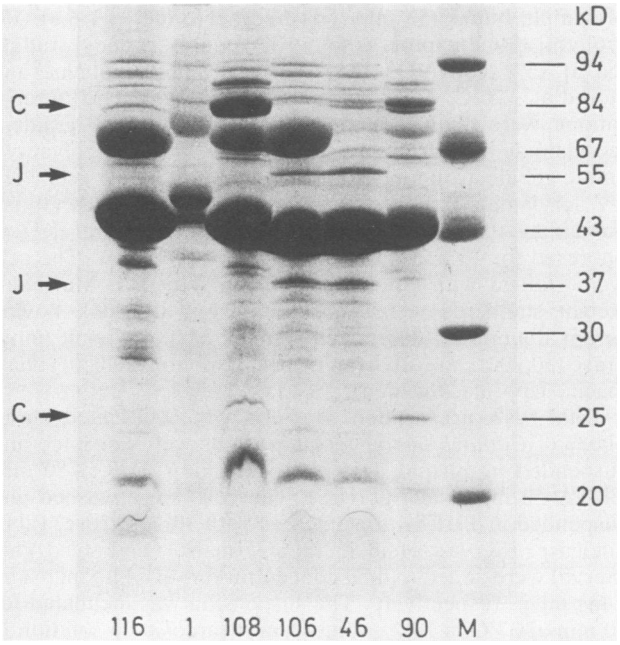

FIG. 1. Coomassie blue-stained SDS-PAGE (14 to 20% gradient) of OMPs from *Campylobacter* spp. The numbers at the bottom of each lane identify the strains: 116, *C. laridis* Lior 34; 1, *C. fetus*; 108, *C. coli* Lior 1; 106, *C. jejuni* I Lior 4; 46 and 90 are human isolates. Lane M, molecular mass standards for proteins. Molecular masses are expressed in kilodaltons (kD). C, Characteristic OMPs of *C. coli*; J, characteristic OMPs of *C. jejuni*.

DISCUSSION

Totten et al. (24) recently showed that hippurate hydrolysis does not always correlate with genetic classification. In this study, the hippurate hydrolysis tests were repeated several times and the incubation time was extended up to 24 h because these conditions gave the best results for the reference strains. Our major problem was a lack of reproducibility. As pointed out by Totten et al. (24), the association of genetically defined *C. jejuni* and *C. coli* with human disease has to be reevaluated since most of the previous epidemiological studies on thermophilic *Campylobacter* spp. rely on hippurate hydrolysis to discriminate between *C. jejuni* and *C. coli*.

In this study, we investigated the OMP-PAGE profile for the identification of *C. coli* and *C. jejuni*. The comparison of protein profiles has indeed become a widely used tool to differentiate species or subspecies (14, 17, 19).

By using the OMP-PAGE profile, the 22 tested strains fell into two groups. The first group contained 15 strains characterized by the presence of two OMPs of 37 and 55 kDa that were found only in the reference strain *C. jejuni* I Lior 4. These two OMPs were absent from other species, i.e., *C. coli*, *C. laridis*, and *C. fetus*. The second group contained seven strains with protein bands of 25 kDa and more marked protein doublets of 84 kDa. This OMP profile was presented only by the reference strains *C. coli* Lior 1, *C. coli* Lior 8, and *C. coli* Lior 44 (Table 1).

The isolated strains could thus be classified into two groups on the basis of the OMP profile. This result was at variance with that of Blaser et al. (4) and that of Logan and Trust (16). However, these authors used other electrophoresis conditions.

To confirm the validity of the OMP-PAGE analysis, the 22 strains were tested by a quantitative filter DNA hybridiza-

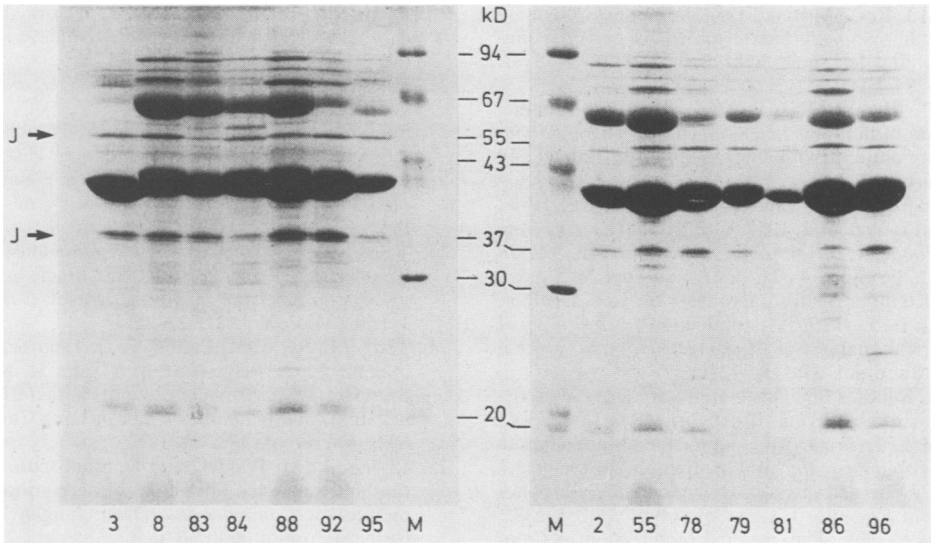

FIG. 2. Coomassie blue-stained SDS-PAGE (14 to 20% gradient) of OMPs from *C. jejuni*. The numbers at the bottom of each lane identify the human isolates. Lane M, Molecular mass standards for proteins, expressed in kilodaltons (kD). J, Characteristic OMPs of *C. jejuni*.

tion technique, and the DNA-DNA homology results correlated perfectly with the OMP-PAGE analysis (Table 1).

This classification based on OMP-PAGE profile and DNA hybridization was compared with the identification by hippurate hydrolysis. When the latter technique was used, 7 of 22 strains were unidentifiable. Moreover, strain 3 gave contradictory results; this strain, classified as a *C. jejuni* by our approach, was repeatedly hippurate negative. These results underline the problem of the discrimination of *C. jejuni* from *C. coli* by hippurate hydrolysis, as already pointed out by Totten et al. (24). We thus propose the analysis of the OMPs as an alternative and reliable method for *Campylobacter* identification.

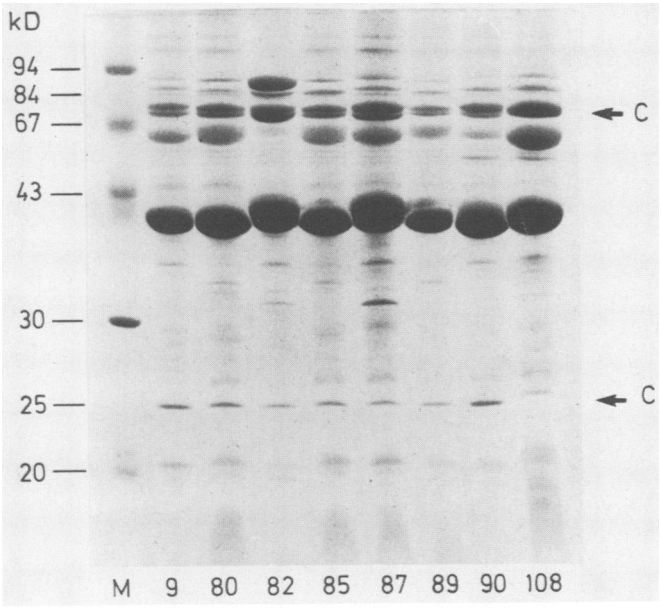

FIG. 3. Coomassie blue-stained SDS-PAGE (14 to 20% gradient) of OMPs from *C. coli*. The numbers at the bottom of each lane identify the human isolates. Strain no. 108 is a reference strain of *C. coli* Lior 1. Lane M, Molecular mass standards for proteins, expressed in kilodaltons (kD). C, Characteristic OMP of *C. coli*.

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