JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2001, p. 1353±1359 Vol. 39, No. 4

0095-1137/01/$04.0010 DOI: 10.1128/JCM.39.4.1353±1359.2001

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Molecular Characterization of Invasive and Noninvasive

*Campylobacter jejuni* and *Campylobacter coli* Isolates

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Received 3 August 2000/Returned for modi®cation 30 October 2000/Accepted 9 February 2001

***Campylobacter jejuni* is one of the most common causes of bacterial diarrhea worldwide and is the primarybacterial cause of food-borne illness. Adherence to and invasion of epithelial cells are the most important pathogenic mechanisms of *Campylobacter* diarrhea. Molecular characterization of invasive and noninvasive *Campylobacter* isolates from children with diarrhea and symptom-free children was performed by randomampli®ed polymorphic DNA techniques (RAPD). A distinct RAPD pro®le with a DNA band of 1.6 kb was observed signi®cantly more frequently among invasive (63%) than among noninvasive (16%) *Campylobacter* isolates (*P*** 5 **0.000005). The 1.6-kb band was named the invasion-associated marker (IAM). Using speci®cally designed primers, a fragment of 518 bp of the *iam* locus was ampli®ed in 85% of invasive and 20% of non-invasive strains (*P*** 5 **0.0000000). Molecular typing with a PCR-restriction fragment length polymorphism assay which ampli®ed the entire *iam* locus showed a *Hin*dIII restriction fragment polymorphism pattern associated mainly with invasive strains. Although cluster analysis of the RAPD ®ngerprinting showed genetic diversity among strains, two main clusters were identi®ed. Cluster I comprised signi®cantly more pathogenic and invasive isolates, while cluster II grouped the majority of nonpathogenic, noninvasive isolates. These data indicate that most of the invasive *Campylobacter* strains could be differentiated from noninvasive isolates by RAPD analysis and PCR using speci®c primers that amplify a fragment of the *iam* locus.**

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*Campylobacter jejuni* is the most common cause of diarrheain children of developing countries (4) and the primary cause of food-borne enteritis in industrialized regions (21). Variabil-ity in the clinical expression of *Campylobacter* infection has been observed for many years. In a study on the natural history of this infection in children, the clinical picture ranged from asymptomatic infections to secretory diarrhea and, less frequently, in¯ammatory diarrhea (4). Other clinical presenta-tions of *Campylobacter* infection are meningitis (12), bactere-mia (32), localized extraintestinal infections (5), and immuno-reactive complications such as Guillain-BarreÂ syndrome (17,

1. and reactive arthritis (2). This wide range of clinical man-ifestations cannot be explained as pertaining only to the host's response; characteristics of the bacterial pathogen may con-tribute. Recently, some phenotypic traits of infecting strains have been associated with the clinical presentation. In enteri-tis, three pathogenic mechanisms have been proposed: produc-tion of a cholera-like enterotoxin (28), production of a cyto-toxin (35), and the ability to adhere to and invade epithelial cells, as demonstrated in vitro (6, 22, 30). The latter is consid-ered essential for intestinal infection and production of disease (14). There is a good correlation between the clinical presen-tation of diarrhea and the isolation of *Campylobacter* strains that adhere to and invade HEp-2 cells. In a study in Mexico, we found that 70% of *C. jejuni* and *Campylobacter coli* isolates

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from children with diarrhea were invasive, as determined by the HEp-2 cell chamber-slide monolayer method, while 83% of isolates from asymptomatic children were nonadherent and non-invasive (29).

Variability in the clinical expression and in the phenotypic traits of isolates may be related to genetic diversity of *Campy-lobacter* strains. Several studies have focused on the character-ization of *C. jejuni* adhesins and binding factors that enable some strains to adhere to and invade epithelial cells (10, 13, 31). Most of these genetic studies, however, have employed a single strain or reference strains, and to date no studies have examined genetic diversity in a population of *Campylobacter* isolated from symptomatic and symptom-free infections and its relation to adherence and invasion of epithelial cells.

Random ampli®ed polymorphic DNA (RAPD) is a PCR-based molecular method that has been widely used for bacte-rial inter- and intraspecies discrimination (36, 37). The RAPD methodology does not require previous knowledge of the DNA template to be analyzed, and only a small quantity of DNA is needed to generate a ®ngerprint. Single, short, arbitrary nu-cleotide sequences can be ampli®ed by PCR assay under low stringency conditions, thus generating polymorphic ®ngerprints that may be used for clustering pathogenic and nonpathogenic organisms and for demonstrating genetic diversity (25, 39).

The present study was designed to evaluate whether RAPD techniques could be applied to (i) identify genetic markers of pathogenicity in *Campylobacter* and (ii) generate ®ngerprints that distinguish invasive from noninvasive *Campylobacter* strains. Here we report the identi®cation of a new, chromosomal 1.6-kb genetic marker of *Campylobacter* strains that was preferen-tially associated with adherence to and invasion of HEp-2 cells in vitro and was named the invasion-associated marker (IAM).

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Subsequently, using RAPD-generated ®ngerprints to construct dendrograms, we identi®ed clusters of *Campylobacter* isolates which carried the IAM and were invasive and diarrhea associ-ated.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** A total of 119*Campylobacter*strainsfrom Mexico were studied, 60 from children with diarrhea (56 *C. jejuni* and 4 *C. coli* isolates) and 59 from asymptomatic children (53 *C. jejuni*, 4 *C. coli*, and2 *Campylobacter* sp. isolates) (4, 29). *Campylobacter* strains were routinely sub-cultured at 42°C under microaerophilic conditions on brain heart infusion agar plates supplemented with 0.4% activated charcoal. *C. jejuni* 287ip (invasive and IAM positive) and 49sp (noninvasive and IAM negative) were used as prototype strains.

**Adherence and invasion assays.** All*Campylobacter*strains were tested foradherence and invasion in HEp-2 cell monolayer chamber-slide assays, as pre-viously described (18, 29). This technique allowed discrimination between strains with high and low indices of adherence to and invasion of HEp-2 cells; *Campy-lobacter* strains with\_20% association were considered invasive. Using thismethod, 70 strains were invasive (66 *C. jejuni* and 4 *C. coli*) and 49 were noninvasive (45 *C. jejuni* and 4 *C. coli*).

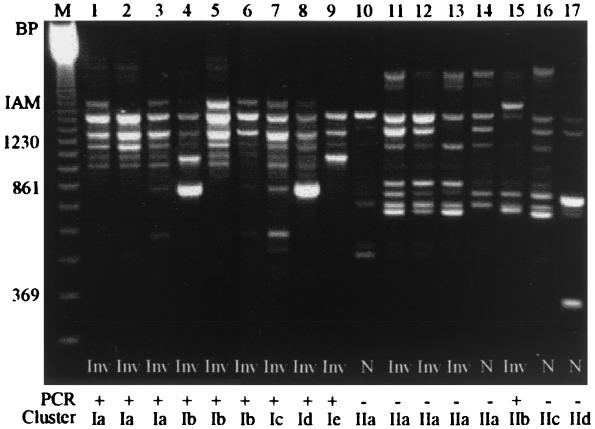
**DNA extraction and RAPD ®ngerprinting.** Genomic DNA was isolated usingthe guanidinium thiocyanate method (27). For initial screening, RAPD ®nger-prints were generated for 21 strains by using seven different arbitrary primers of 10-mers each, with different G1C contents: R2, 59-AGTACAGGTC (11); 1290, 59-GTGGATGCGA; 1283, 59-CGATCCCCA; 1247, 59-AAGAGCCCGT (1); HLWL74, 59-ACGTATCTGC; HLWL85, 59-ACAACTGCTC (20); and Wil2, 59-TCACGATGCA (38). Approximately 10 ng of puri®ed *Campylobacter* geno-mic DNA was used as a template for RAPD ampli®cation in a volume of 20 ml containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate, 1 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), and 30 pmol of 10-mer primer (Operon Technologies, Alameda, Calif.). This reaction was overlaid with 1 drop of mineral oil and placed in a thermal cycler (model 9600; Perkin-Elmer, Norwalk, Conn.). The ampli®cation program consisted of 35 cycles of 15 s at 92°C, 45 s at 36°C, and 1 min at 72°C. The PCR-ampli®ed products were analyzed in a horizontal 1.4% agarose gel by electrophoresis in 0.53 Tris-borate±EDTA buffer and then visu-alized under UV light after ethidium bromide staining.

**Ampli®cation of a fragment of the *iam* locus.** PCR assay for ampli®cation ofa 518-bp DNA fragment (nucleotides 316 to 834) of the 1.6-kb band was stan-dardized using a pair of primers selected from the *iam* locus sequence of the *C. jejuni* 287ip strain. A 19-nucleotide forward primer, 1.6F (GCG CAA AATATT ATC ACC C), corresponding to positions 316 to 334 of the *iam* locus, and an 18-nucleotide reverse primer, 1.6R (TTC ACG ACT ACT ATG CGG), corresponding to positions 817 to 834, were selected. PCR ampli®cation was carried out with a DNA thermal cycler (model PTC 200 thermocycler; MJ Research, Cambridge, Mass.) using ®nal volumes of 40 ml containing 30 pmol each of speci®c primers 1.6F and 1.6R. The ampli®cation program consisted of 30 cycles of 30 s at 92°C, 1 min at 52°C, and 1 min at 72°C, with a ®nal extension step at 72°C for 5 min. Five-microliter volumes of the products were analyzed in horizontal 1.2% agarose gels stained with ethidium bromide.

**Degenerated PCR and PCR-RFLP analysis.** A degenerated PCR was devel-oped to amplify the *iam* locus of all invasive and noninvasive strains. The DNA sequence of the *iam* locus was aligned with the DNA sequence of ABC trans-porter proteins from *Helicobacter pylori* (GenBank accession no. AE000646) (33), *Haemophilus in¯uenzae* (U32744) (7), and *Escherichia coli* (D90705). After comparison of the DNA sequences of the ABC transporter proteins, a pair of degenerated primers, p77F [GG(A)CCT TTA GG(A)G AAG CTG] and p1415R [CTT TAA AT(A)T(G) GAA TC(G)A CG(T)GG], was designed and used to amplify an ;1,360-bp fragment from genomic DNA of invasive and noninvasive *Campylobacter* strains. DNA ampli®cation consisted of 30 cycles of 30 s at 92°C, annealing of primers at 49°C for 1 min, and extension at 72°C for 1 min. A ®nal extension cycle at 72°C for 10 min was included. To determine whether there were any differences in DNA polymorphisms between invasive and noninvasive strains, a PCR-restriction fragment length polymorphism (PCR-RFLP) of the *iam* locus was done by digesting PCR-ampli®ed products with *Hin*dIII endonuclease, and the pro®les were checked in horizontal agarose gels.

**Southern blot analysis of PCR-RFLP products.** PCR-RFLP products sepa-rated in agarose gels were transferred to nylon membranes by capillarity, using 63 SSC (13 SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were probed with the entire DNA sequence of the IAM fragment labeled with digoxi-

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| PCR ampli®cation and their location in cluster analysis is shown. | [http://jcm](http://jcm.asm.org/) |  |
| FIG. 1. RAPD ampli®cation products of invasive and noninvasive |  |  |
| *Campylobacter* strains using the arbitrary primer 1290. The IAM band |  |  |
| size was estimated as 1.6 kb, marked on the left side. Results of the *iam* |  |  |
| Lanes: M, 123-bp ladder marker; 1, *C. jejuni* 84sp; 2, *C. jejuni* 135ip; 3, | [.](http://jcm.asm.org/) |  |
| [asm](http://jcm.asm.org/) |  |
| *C. jejuni* 227sp; 4, *C. jejuni* 33K; 5, *C. jejuni* 287ip; 6, *C. jejuni* 151sp; 7, |  |
| *C. jejuni* 268ip; 8, *C. jejuni* 401ip; 9, *C. jejuni* 188K; 10, *C. jejuni* 63sp; |  |
| 11, *C. jejuni* 221sp; 12, *C. jejuni* 286sp; 13, *C. jejuni* 246sp; 14, *C. jejuni* | [.](http://jcm.asm.org/) |  |
| [org/](http://jcm.asm.org/) |  |
| 349K; 15, *C. jejuni* 180ip; 16, *C. jejuni* 128sp; 17, *C. coli* 49sp. Inv, in- |  |
|  |  |
| vasive strains; N, noninvasive strains. | on |  |
|  |  |
| genin-11±ddUTP (DIG-ddUTP) by random priming (DIG DNA labeling kit; | August |  |
|  |  |
| Boehringer). Hybridization was carried out at 45 to 48°C. Prehybridization, |  |  |
| hybridization, washes, solutions, and detection of the DIG-labeled probes were |  |  |
| done according to the manufacturer's recommendations (Boehringer). | 26, |  |
| **Computer-assisted analysis of RAPD ®ngerprints.** Numerical analysis of the |  |
|  |  |
| RAPD ®ngerprinting data was done to examine associations between the geno- | 2020 |  |
| typic heterogeneity of the *iam* locus, as detected by PCR and RAPD techniques, |  |
| and the host's status, invasive or noninvasive phenotypes, and PCR-RFLP pat- |  |
| terns. Primer 1290 RAPD ampli®cation gel images of all 119 *Campylobacter* |  |
| isolates were digitized with a Gel Doc 1000 system (Bio-Rad Laboratories, | at |  |
| Hercules, Calif.) and stored as tagged image ®le format ®les; images were later |  |
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| Associations were determined using the x2 test. Results were considered signif- |  |
| converted, normalized, and analyzed with GelCompar software, version 4.0 (Ap- |  |  |
| plied Maths, Kortrijk, Belgium). The similarity matrix and clustering dendro- |  |  |
| grams were calculated using the Jaccard coef®cient and Ward algorithm, respec- |  |  |
| tively. Bands with faint intensity and high molecular weights were not |  |  |
| reproducible and were excluded from the ®nal analysis. *Campylobacter* strains |  |  |
| with a level of similarity of \_95% were considered to have the same RAPD type. |  |  |
| **Statistical analysis.** A basic descriptive analysis was done using percentages. |  |  |
| icant when *P* was \_0.05. All statistical analyses were done using Stata 7 (Stata | OF |  |
| Corporation, College Station, Tex.). |  |
| **Nucleotide sequence accession number.** The*iam*locus, including the 518-bp | NEW |  |
| DNA fragment described in this study, was registered by us in GenBank with |  |
|  |  |
| accession number AF023133. | SOUTH |  |
| **RESULTS** |  |
|  |  |
| **Identi®cation of an RAPD marker associated with invasive** |  |  |
| ***Campylobacter* strains.**Seven primers were selected for initial | WALES |  |
| screening by RAPD ®ngerprinting of 21 *Campylobacter* strains |  |
|  |  |
| (15 invasive and 6 noninvasive). Four of these primers (Wil2, |  |  |
| 1290, 1283, and 1247) produced distinct ®ngerprints. Primer |  |  |
| 1290 produced the most distinct pattern, with up to 13 bands |  |  |
| ranging in size from 0.2 to 3.0 kb, and was selected to test the |  |  |
| remaining 98 invasive and noninvasive *Campylobacter* strains. |  |  |
| All strains were typeable (Fig. 1), displaying patterns that al- |  |  |
| lowed discrimination of strains with high adherence and inva- |  |  |
| sion indices from those with low indices. A 1.6-kb band was |  |  |
| predominantly found in invasive strains (44 of 70; 63%) but |  |  |

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TABLE 1. Detection of the 1.6-kb IAM by RAPD ®ngerprinting and by PCR assay for ampli®cation of the *iam* locus in invasive and noninvasive *Campylobacter* isolates

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | No. (%) of isolates with 1.6-kb IAM | | | | |  |
|  |  |  |  |  |  |  | |  |
| Strain type (*n*) |  |  | RAPD |  |  | PCR of *iam* locus | |  |
|  |  | *a* |  | (primers 1.6F | |  |
|  | (primer 1290) | |  |  |
|  |  |  |  | and 1.6R)*b* | |  |
|  | Positive | | Negative | |  | Positive | Negative |  |
|  |  |  |  | |  | |  |  |
| Invasive (70) | 44 | (63) | 26 (37) | | 60 (85) | | 10 (14) |  |
| Noninvasive (49) | 8 | (16) | 41 (84) | | 10 (20) | | 39 (79) |  |
| Total | 52 |  | 67 |  | 70 | | 49 |  |

1. x2 5 25.15; *P* 5 0.00000053.
2. x2 5 50.75; *P* 5 0.00000000.

was present in very few of the noninvasive isolates (8 of 49; 16%; x2 5 25.15; *P* 5 0.00000053) (Table 1). This is the fragment that was named the invasion-associated marker.

**Speci®c ampli®cation of the 518-bp fragment of the *iam***

**locus.** When the*iam*locus was ampli®ed by PCR using aninternal pair of primers, 1.6F and 1.6R, as expected a unique 518-bp PCR product (Fig. 2) was detected in 60 of 70 (85%) invasive strains, whereas only 10 of 49 (20%) of noninvasive isolates presented this band (x2 5 45.12; *P* 5 0.0000000) (Ta-ble 1). According to species, 64 of 109 (58%) *C. jejuni*, 5 of 8 (62%) *C. coli*, and 0 of 2 *Campylobacter* spp. were *iam* PCR positive. The sensitivity of the PCR was 85% and the speci®city 74%, with a false-positive rate for true negative (noninvasive) of 25%, and a false-negative rate for true positive (invasive) of 14%. In addition, *Campylobacter iam* PCR positivity was a signi®cant risk factor for intestinal infection associated with diarrhea (odds ratio, 18.53; 95% con®dence interval, 2.21 to 6.38). This 518-bp *iam* locus fragment could not be ampli®ed from the DNA of the following microorganisms: *Candida* spp., *Enterobacter aerogenes, E. coli, Klebsiella pneumoniae, Lacto-bacillus reuterii, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Salmonella enterica* serovar Typhimurium, *Shi-gella dysenteriae, Shigella sonnei, Staphylococcus aureus, Strep-tococcus faecalis, Vibrio cholerae*, and *Yersinia enterocolitica*. Tocon®rm that the 1.6-kb band was a homogeneous DNA frag-

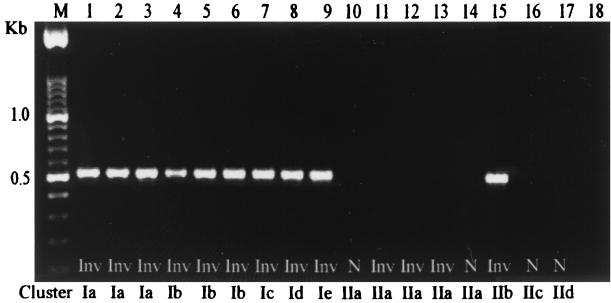
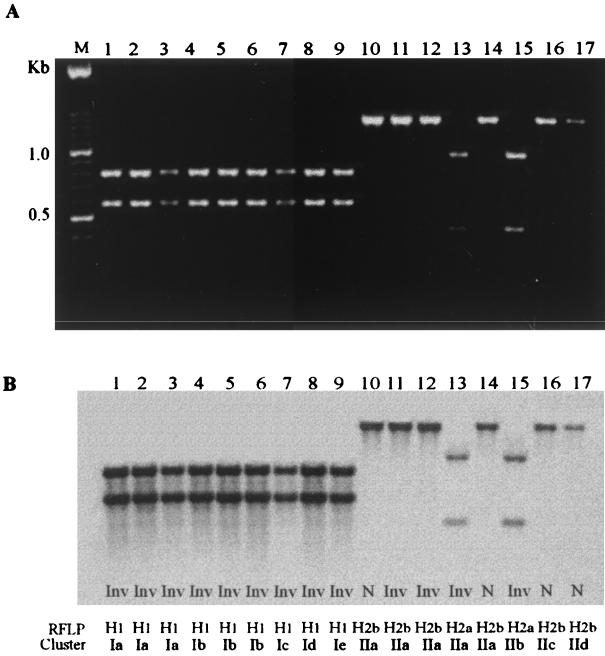


FIG. 2. *iam* PCR ampli®cation, using primers 1.6F and 1.6R, of the genomic DNA of noninvasive and invasive *Campylobacter* strains. The location in cluster analysis is shown. Lanes: M, 100-bp ladder; 1, *C. jejuni* 84sp; 2, *C. jejuni* 135ip; 3, *C. jejuni* 227sp; 4, *C. jejuni* 33K; 5, *C. jejuni* 287ip; 6, *C. jejuni* 151sp; 7, *C. jejuni* 268ip; 8, *C. jejuni* 401ip;9, *C. jejuni* 188K; 10, *C. jejuni* 63sp; 11, *C. jejuni* 221sp; 12, *C. jejuni* 286sp; 13, *C. jejuni* 246sp; 14, *C. jejuni* 349K; 15, *C. jejuni* 180ip; 16, *C.* *jejuni* 128sp; 17, *C. coli* 49sp; 18, negative control.

FIG. 3. (A) Electrophoresis showing PCR-RFLP analysis of the *iam* locus. An;1,360-bp PCR product ampli®ed with degeneratedprimers (p77F and p1415R) was digested with *Hin*dIII, and three patterns were identi®ed: H1 (lanes 1 to 9), H2a (lanes 13 to 15), and H2b (lanes 10 to 12, 14, 16, and 17). (B) Southern blot of the same gel with the entire 1.6-kb probe labeled with DIG. Lanes: M, 100-bp ladder; 1, *C. jejuni* 84sp; 2, *C. jejuni* 135ip; 3, *C. jejuni* 227sp; 4, *C. jejuni* 33K; 5, *C. jejuni* 287ip; 6, *C. jejuni* 151sp; 7, *C. jejuni* 286ip; 8, *C. jejuni* 401ip; 9, *C. jejuni* 188K; 10, *C. jejuni* 63sp; 11, *C. jejuni* 221sp; 12, *C.* *jejuni* 286sp; 13, *C. jejuni* 246sp; 14, *C. jejuni* 349K; 15, *C. jejuni* 180ip;16, *C. jejuni* 128sp; 17, *C. coli* 49sp.



ment, a 518-bp *iam* locus DIG-labeled probe was hybridized in Southern blots with RAPD ®ngerprints from the 119 *Campy-lobacter* isolates. Only the 1.6-kb product from the RAPDampli®cation hybridized with the DIG-labeled probe (data not shown).

**PCR ampli®cation using degenerate primers and PCR-RFLP analysis of the *iam* locus.** To determine the genetic poly-morphism of the *iam* locus, we used degenerate primers (p77F and p1415R) to amplify a fragment of ;1,360 bp from the 119 strains. In 115 (96.6%), we were able to obtain PCR products. A unique band of the expected size was ampli®ed in 113 of 119 (95%); in addition, a PCR product of ;0.8 kb was observed in 2 noninvasive strains. Three main *Hin*dIII RFLP genotypes, named H1, H2a, and H2b, were observed for the 1.3-kb de-generated PCR products (Fig. 3). The majority (52 of 60) of the invasive and *iam* PCR-positive strains were typed as H1 (Table 2), which showed a characteristic RFLP pattern by digestion of the 1,360-bp PCR of two fragments, 0.78 and 0.58 kb, cut at the site expected for *Hin*dIII (Fig. 3A, lanes 1 to 9). On the other hand, the majority (30 of 39) of the noninvasive, PCR-negative strains were typed as H2b. None of the PCR products of the H2b pattern were cut with *Hin*dIII (Fig. 3A, lanes 10 to 12, 14, 16 and 17). Ten strains, four of which were invasive, were typed as H2a. This PCR-RFLP pattern yielded 0.4- and 0.96-kb fragments (Fig. 3A, lanes 13 and 15). PCR-RFLP analysis and hybridization with the DIG-labeled 1.6-kb

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TABLE 2. Correlation between invasive phenotype, *Hin*dIII-RFLP genotypes, and *iam* PCR in 119 *Campylobacter* isolates

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *Hin*dIII-RFLP | No. of isolates by | |  |
| Phenotype (*n*) |  | *iam* PCR |  |
| genotypes |  |  |  |
|  | Positive | Negative |  |
|  |  |  |
|  |  |  |  |  |
| Invasive (70) | H1 | 52 | 1 |  |
|  | H2a | 2 | 2 |  |
|  | H2b | 2 | 7 |  |
|  | Nontypeable | 4 | 0 |  |
| Subtotal |  | 60 | 10 |  |
| Noninvasive (49) | H1 | 10 | 1 |  |
|  | H2a | 0 | 6 |  |
|  | H2b | 0 | 30 |  |
|  | Nontypeable | 0 | 2 |  |
| Subtotal |  | 10 | 39 |  |
|  |  |  |  |  |

fragment showed a strong homologous signal with H1 patterns and showed a less strong signal for H2a and H2b patterns (Fig. 3B). These ®ndings show that fragments ampli®ed with the degenerate primers were homologous to the *iam* locus.

**Cluster analysis of RAPD ®ngerprint patterns and relation-ship between host status, invasive phenotype, and genetic markers.** The genetic relationship between isolates based ontheir RAPD ®ngerprinting is represented in the dendrogram shown in Fig. 4. Using primer 1290, we found 42 RAPD types among the 119 isolates. Analysis using the Jaccard coef®cient followed by Ward algorithm revealed two major clusters. Clus-ter I, at a similarity level of 67%, contained 63 strains in ®ve subgroups (Ia to Ie), each of them with 6 to 21 isolates. Pro-totype invasive strain 287ip was clustered in Ib, with a similarity level of 89%. Subgroups Id and Ie were not distinguishable by RAPD ®ngerprinting. Cluster II, at a similarity level of 54.8%, comprised four subgroups (IIa to IId), each one containing 6 to 24 isolates. Subgroups IIc and IId showed distinctive ®nger-prints with clonal characteristics, including the prototype non-invasive strain 49sp.

The strains' denomination, host status, invasive phenotype, presence of the genetic marker, and PCR-RFLP patterns are also shown in Fig. 4. The number of strains isolated from patients with diarrhea was signi®cantly larger in cluster I (42 of

1. than in cluster II (18 of 56) (x2 5 14.02; *P* 5 0.00018). More signi®cantly, the majority of invasive strains were grouped in cluster I (52 of 63 versus 18 of 56; x2 5 30.83; *P* 5 0.00000000). PCR-RFLP genotypes were homogeneously dis-tributed between the two main clusters. Of 63 isolates from cluster I, 61 were genotype H1; the remaining 2 isolates could not be typed. All 49 isolates belonging to genotype H2 were grouped in cluster II; 10 were genotype H2a, 9 of which be-longed to cluster IIb. The remaining 39 were genotype H2b and were distributed in clusters IIa, IIc, and IId. The PCR fragment of *iam* was ampli®ed in 62 of 63 isolates from cluster I and in only 8 of 56 from cluster II (x2 5 85.90; *P* 5 0.00000). The fragment was ampli®ed in most of the invasive strains (52 of 56) of cluster I and in more than half of the invasive isolates of cluster II (10 of 18). None of the 38 noninvasive strains from cluster II carried the *iam* locus. The 1.6-kb band of RAPD ®ngerprinting was observed in 40 of 63 strains from cluster I and in 12 of 46 from cluster II (x2 5 21.14; *P* 5 0.0000043).

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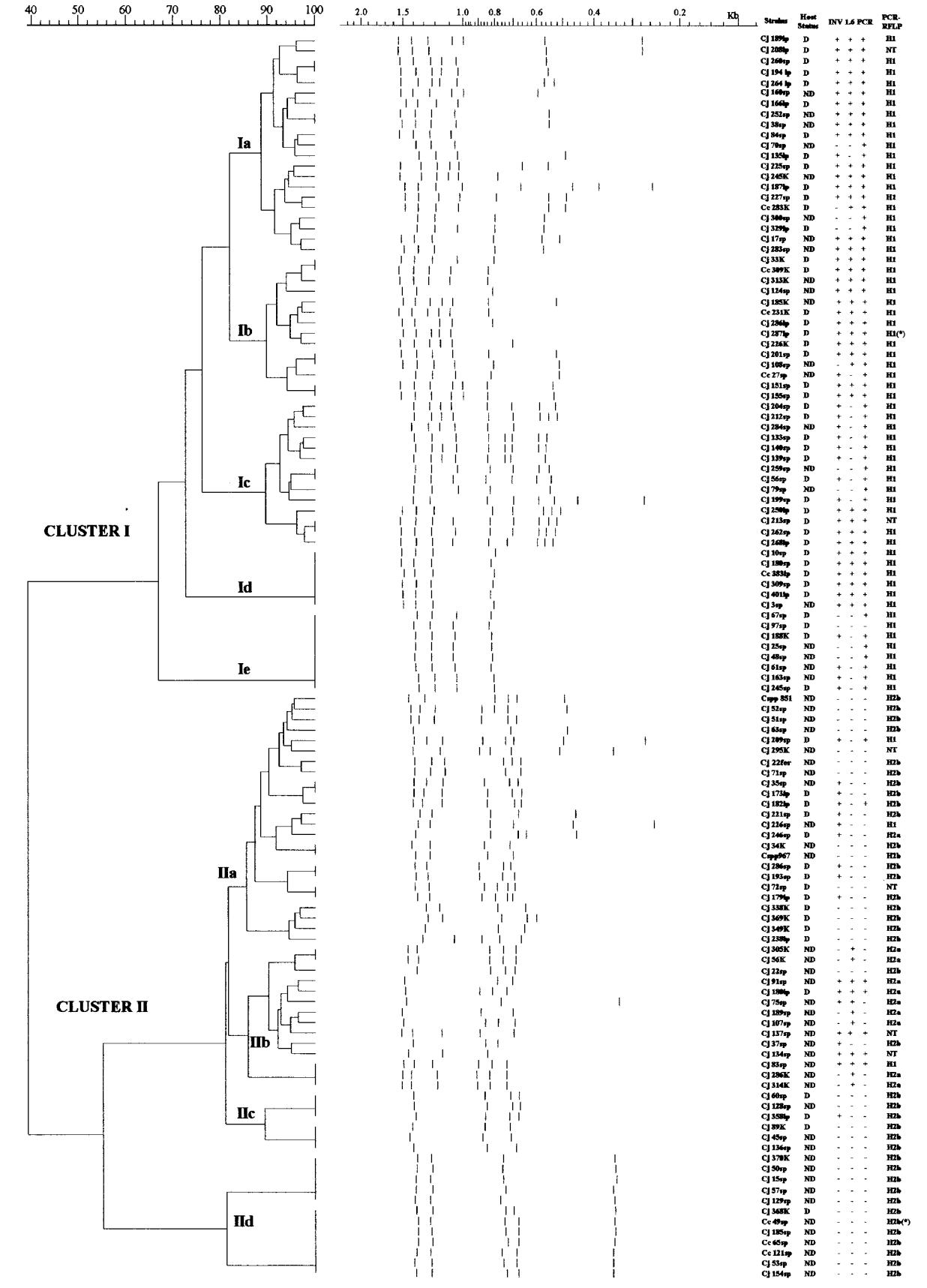
**DISCUSSION**

An undeniable bene®t of studying well-characterized popu-lations of *Campylobacter* isolates selected from symptomatic and symptom-free individuals and phenotypically de®ned as invasive or noninvasive is that when using methods that can screen complete bacterial genomic DNA, such as RAPD, it is possible to group strains according to their genotypic and phe-notypic features and to identify genetic markers of virulence. Results of the present study demonstrate genetic differences between invasive and noninvasive *Campylobacter* strains. A strong association was found between the invasive phenotype, a par-ticular RAPD ®ngerprint, and the presence of a speci®c DNA region of 1.6 kb. This DNA fragment was identi®ed signi®-cantly more frequently among invasive strains than among noninvasive strains and was equally distributed among *C. jejuni* and *C. coli* isolates.

RAPD has been used to identify speci®c DNA regions as-sociated with a given phenotype of different microorganisms (11, 25, 38). A novel DNA marker, with signi®cant similarity to some negative transcriptional regulators, was identi®ed in epi-demic clinical strains of *Burkholderia cepacia* isolated from patients with cystic ®brosis (19); this DNA marker was absent in nonepidemic and environmental strains. Recently, using random ampli®cation of different O serotypes of *C. jejuni* iso-lated from patients with Guillain-BarreÂ syndrome, an associa-tion of a clonal population with virulence was observed (9). Moreover, an RAPD marker of 1.4 kb that differentiated O19-positive from O19-negative *C. jejuni* strains was cloned, se-quenced, and characterized (23). In our study, RAPD tech-niques proved to be excellent molecular tools for typing *Campylobacter* strains, consistent with other reports (9, 20, 23).Although several investigators have successfully used RAPD in *Campylobacter* isolates as an epidemiological tool to identifythe source of infection (20), we are unaware of studies that apply this method to discriminate between invasive and non-invasive clinical isolates.

RAPD techniques allowed us to compare polymorphisms of the entire bacterial genome in a population of invasive and noninvasive strains and to identify an invasion-associated DNA marker. However, we found that some of the invasive strains lacked this RAPD marker (Fig. 1, lanes 2 and 9). This could be explained by mismatching sequences at the binding site of random primer 1290 due to a greater polymorphism among invasive strains or by a low sensitivity of RAPD. To improve the sensitivity and more accurately differentiate invasive strains, we designed a PCR using speci®c primers that ampli-®ed a fragment of the *iam* locus. This PCR appears to be useful for the identi®cation of invasive strains, since it accurately classi®ed 82% of strains, with a sensitivity of 87%. However, this method misclassi®ed as positive 10 of 49 noninvasive strains (speci®city of 74%; false-positive rate for true negative of 25%). We do not have a clear explanation of why this DNA locus present in invasive strains also was ampli®ed in some noninvasive strains, but we could speculate that there may be internal mutations in the IAM fragment of noninvasive strains. It will be interesting to sequence the amplicons from these PCR-positive noninvasive strains and to compare them with amplicons from PCR-positive invasive strains. The fact that some invasive strains also were not identi®ed by PCR supports

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FIG. 4. Oligonucleotide 1290 RAPD ®ngerprinting dendrogram shows cluster analysis results of 119 invasive and noninvasive *Campylobacter* strains. Tracks show the band pattern after conversion, normalization, and GelCompar numerical analysis. Prototype strains 287ip and 49sp are marked with an asterisk (p). On the right side of the ®gure are columns describing strain denomination, host's status, invasive phenotype, presence of IAM, PCR ampli®cation of *iam* locus, and the PCR-RFLP pattern. D, diarrhea; ND, nondiarrhea; 1, positive; 2, negative.

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the existence of important polymorphism and high heteroge-neity in the *iam* locus or suggests that there may be other genetic markers of invasion in different loci. In previous studies on the molecular characterization of genes associated with the phenotype of adherence to and invasion of epithelial cells, several *Campylobacter* strains have been studied and some genetic loci have been identi®ed: *peb1* (26), *peb4A* (3), *cadF* (15), and *¯a* (13, 34), and more recently the gene that encodes antigen B (16) and the *galE* gene, involved in lipopolysaccha-ride synthesis and virulence (8).

When polymorphism of the *iam* locus was further explored in all *Campylobacter* strains by PCR-RFLP with *Hin*dIII endo-nuclease, it was possible to amplify this locus in most of the isolates by using degenerated primers. Invasive strains had a speci®c *Hin*dIII site (genotype H1), while most of the nonin-vasive strains lacked this site (genotype H2b), and a few inva-sive and noninvasive strains had this restriction site at a dif-ferent position (genotype H2a). These ®ndings con®rm the polymorphism of the *iam* locus and the genetic diversity of strains. It will be important to sequence the *iam* locus from invasive and noninvasive strains that have a different polymor-phism to determine whether there are changes in other sites of the locus which could have been overlooked by RAPD or PCR-RFLP.

Dendrograms constructed by numerical analysis of the RAPD ®ngerprints also con®rmed the genetic diversity of iso-lates. Two main clusters were clearly de®ned. Cluster I grouped most invasive strains from diarrhea cases, which cor-responded to the H1 genotype of RFLP and were *iam* PCR positive. By contrast, most strains from cluster II were nonin-vasive, were isolated from asymptomatic individuals, corre-sponded to genotype H2b of RFLP, and were *iam* PCR neg-ative. In cluster II there was an interesting subcluster that grouped invasive isolates from asymptomatic individuals; these strains had an H2a genotype, which has a *Hin*dIII site in a different position from H1, and some were *iam* PCR positive. These genetic differences with H1 genotype strains could ex-plain differences in virulence, since all but one of the strains were isolated from symptom-free individuals.

Another important ®nding was the presence of RAPD ge-notypes where strains had identical ®ngerprintings, i.e., clus-ters Id, Ie, and IId, which is a characteristic of clonal popula-tions. Clonality has also been observed in some *C. jejuni* strains isolated from patients with Guillain-BarreÂ syndrome (9, 23). It is then possible that some virulent *Campylobacter* strains are clonal populations. Finally, the presence of this molecular marker of invasion is not restricted to *C. jejuni* but is also present in *C. coli*. It would be interesting if this marker were carried by other less common *Campylobacter* species, such as *C. lari* or *C. jejuni* subsp. *doylei*.

We propose the use of RAPD or even more speci®c PCR assays as molecular tools for typing and studying dif-ferent populations of invasive and noninvasive *Campylo-bacter* strains. Using a single PCR, we were able to detectmost of the invasive isolates studied. These ®ndings suggest that RAPD and PCR assays are effective molecular methods to discriminate invasive from noninvasive *Campylobacter* strains.

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**ACKNOWLEDGMENTS**

This work was supported by U.S. Public Health Service grant PO HD 13021-22 from the National Institute of Child Health and Human Development and by a scholarship for A. C. T. Carvalho from Con-selho Nacional de Desenvolvimiento Cienti®co e TecnoloÂgico, Brazil.

We are indebted to B. R. Ruiz-Palacios, D. Newburg, A. Nieto, P. S. Cisalpino, D. M. M. Queiroz, and E. Calva for revision of the manu-script and for technical help. We thank the staffs of the Department of Infectious Diseases, National Institute of Medical Science and Nutri-tion Salvador ZubiraÂn, Mexico, and the Center for Pediatric Research, Eastern Virginia Medical School, Norfolk.

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