

Molecular Evidence for Dissemination of Unique *Campylobacter jejuni* Clones in Curaçao, Netherlands Antilles

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Campylobacter jejuni isolates ($n = 234$) associated with gastroenteritis and the Guillain-Barré syndrome (GBS) in the island of Curaçao, Netherlands Antilles, and collected from March 1999 to March 2000 were investigated by a range of molecular typing techniques. Data obtained by pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, multilocus sequence typing (MLST), automated ribotyping, and sequence analysis of the short variable region of the flagellin gene (*flaA*) were analyzed separately and in combination. Similar groupings were obtained by all methods, with the data obtained by MLST and AFLP analysis exhibiting the highest degree of congruency. MLST identified 29 sequence types, which were assigned to 10 major clonal complexes. PFGE, AFLP analysis, and ribotyping identified 10, 9, and 8 of these clonal groups, respectively; however, these three techniques permitted subdivision of the clonal groups into more different types. Members of seven clonal groups comprising 107 isolates were obtained from November 1999 to February 2000, and no distinguishing characteristics were identified for two GBS-associated strains. The sequence type 41 (ST-41), ST-508, and ST-657 clonal complexes and their corresponding AFLP types have been rare or absent in the *Campylobacter* data sets described to date. We conclude that several clonal complexes of *C. jejuni* are associated with human disease in Curaçao, and some of these have not been reported elsewhere. Furthermore, given the observation that *C. jejuni*-associated diseases appear to be more severe from November to February, it can be speculated that this may be due to the presence of virulent clones with a limited span of circulation.

Campylobacter jejuni infections are the leading cause of acute bacterial gastroenteritis worldwide (9); it has been estimated, for example, that in Europe and the United States, the yearly incidence of *C. jejuni*-associated infections among the entire population is on the order of 1% (20). The clinical syndromes vary from mild and severe gastroenteritis to extraintestinal disease, including acute neuromuscular complications such as the Guillain-Barré syndrome (GBS) or Miller-Fisher syndrome (14). The reason for these variations in clinical presentations is not yet understood, but it may be due, at least in part, to differences among *C. jejuni* strains.

It is assumed that *C. jejuni* is transmitted to humans primarily by consumption of food and drinking water and to a lesser extent by handling of pets (20). This is consistent with the fact that the intestines of poultry, as well as those of other animals, are frequently asymptotically colonized with *C. jejuni*, leading to the widespread dissemination of the bacteria in the food chain. Tracing of the source of infection has proved to be complicated, given the ubiquity of the organism in the environment, the sporadic nature of the disease, and the fact that

outbreaks are rarely documented. A more detailed understanding of the epidemiology of human *C. jejuni* infections will enable the development of more effective public health measures for disease control.

The Caribbean island of Curaçao, Netherlands Antilles, is located near the coast of Venezuela and has approximately 150,000 inhabitants. The fact that it is an island with a limited population greatly facilitates epidemiological investigation of disease syndromes such as campylobacteriosis. It has been shown that the incidence of *C. jejuni*-associated GBS on this island increased from 1987 to 1999 and that patients from Curaçao had a more severe course of GBS than Dutch controls (22). Furthermore, 80% of the Curaçao GBS patients exhibited evidence of a recent infection with *C. jejuni*, and there was evidence for a seasonal trend in the severity of GBS (22).

We characterized the population of *C. jejuni* patient isolates in Curaçao (22) by robust, discriminatory typing methods suitable for the analysis of a large number of samples (24). These included pulsed-field gel electrophoresis (PFGE) (15), ribotyping (8), amplified fragment length polymorphism (AFLP) analysis (5), multilocus sequence typing (MLST) (3), and flagellin gene sequencing (13). All typing data were analyzed numerically, and the congruence of the typing data was determined to permit a comprehensive analysis of the *C. jejuni* strains causing

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disease on the island and the comparison of the different typing methods used.

MATERIALS AND METHODS

Bacterial isolates. Curaçao has only one central microbiology laboratory. Between 15 March 1999 and 15 March 2000 and following a local routine procedure, all stool samples were inoculated on Blaser-Wang *Campylobacter* selective medium and incubated at 37°C under microaerobic conditions for 48 h. Upon isolation, all *Campylobacter* strains were transported to the Erasmus Medical Center, Rotterdam, The Netherlands. Chromosomal DNA was extracted from each isolate and distributed to different laboratories for examination by AFLP analysis, MLST, and sequence analysis of the short variable region (SVR) of the flagellin gene (*flaA*).

PFGE fingerprint analysis. PFGE fingerprint analysis was performed as described previously (21). In short, chromosomal DNA was embedded in agarose plugs and digested with *SmaI* (Boehringer Mannheim, Mannheim, Germany). Electrophoretic separation of the digestion products was carried out in a 1% SeaKem agarose gel in 0.5× TBE buffer (Tris-borate-EDTA) for 19 h in a Bio-Rad CHEF-DRIII system (Bio-Rad, Richmond, Calif.) with pulse times linearly ramped from 6.75 to 25 s. PFGE patterns were imported as TIFF files into Bionumerics software (version 3; Applied Maths, Sint-Martens-Latem, Belgium), and an automatic band search was performed on the normalized patterns. After visual control of the assigned bands, a band-based analysis was performed by using the Dice coefficient with 1% band position tolerance. For cluster analysis the unweighted pair group method with average linkages (UPGMA) was used.

MLST. Nucleotide sequence analysis of seven housekeeping genes was performed as described by Dingle et al. (3). The sequences were deposited in the Internet-accessible PubMLST *Campylobacter* database (<http://campylobacter.mlst.net>), enabling the identification and assignment of sequence types (STs) and clonal complexes. Related STs were imported to clonal complexes as described by Dingle et al. (3) by identification of central genotypes and the sequential assignment of variants that differed at one, two, or three loci (2). The clonal complexes were named after the ST of the central genotype (3). For analysis of congruency, MLST allele numbers were imported into Bionumerics software. The similarity between STs was calculated by using the categorical numerical similarity coefficient and the complete linkage clustering software of Bionumerics (version 3).

AFLP analysis. AFLP analysis was performed by the method by Duim et al. (6). In short, 20 ng of chromosomal DNA was digested with the restriction enzymes *HindIII* and *HhaI*, and in a simultaneous reaction the fragments were ligated with restriction site-specific adapters for 2 h at 37°C. Then, a preselective PCR with adapter-specific primers *HindIII* (5'-GACTGCGTACCAGCTT-3') and *HhaI* (5'-GATGAGTCTGATCGC-3') was performed. An aliquot was subjected to a selective PCR in which both primers contained an additional A nucleotide at the 3' end (5). The 5' end of the *HindIII* primer was labeled with the fluorescent dye 6-carboxyfluorescein. The products were run on a 7.3% denaturing acrylamide sequencing gel for 5 h by using an ABI 373 automated DNA sequencer (PE Applied Biosystems, Foster City, Calif.). After electrophoresis, the banding patterns were collected with ABI Genescan software (PE Applied Biosystems). Each lane of the gel was then imported into Bionumerics software. The gels were normalized by using the 6-carboxy-X-rhodamine-labeled size standard in each lane of the gel. After normalization and subtraction of the background readings with mathematical algorithms, the levels of similarity between the AFLP patterns were calculated with the Pearson product-moment correlation coefficient, and cluster analysis was done with the UPGMA algorithm. Isolates with >90% similarity were considered identical (5).

Automated ribotyping. Prior to ribotyping, the isolates were grown for 48 h on Columbia agar with 5% sheep blood. Automated ribotyping was performed on a Qualicon RiboPrinter (Qualicon, Atlanta, Ga.) by using the RiboPrinter microbial characterization system in accordance with the recommendations of the manufacturer. Digestion of chromosomal DNA was performed with *PstI*, and the digested DNA was subsequently hybridized with an rRNA probe. Ribotypes were assigned by the RiboPrinter software, and for further analysis the RiboPrinter data were imported as normalized files into Bionumerics software. For calculation of similarity levels between ribotype patterns, the Pearson product-moment correlation coefficient was applied with UPGMA clustering.

Analysis of *flaA* SVR sequences. The nucleotide sequence of a 321-bp region of the *flaA* gene encompassing the SVR was performed as described recently (4, 13). Primers FLA4F (5'-GGATTTCGTATTAACACAAATGGT-GC-3') and FLA1728 (5'-CTGTAGTAATCTTAAAACATTTTG-3') or FLA625RU (5'-C AAG[AT]CCTGTTCC[AT]ACTGAAG-3', where the sequences in brackets in-

dicade sequence redundancy) were used for amplification of the SVR. Nucleotide sequence reactions were performed with the BigDye Ready Reaction Mix (PE Applied Biosystems) with primers FLA242FU (5'-CTATGGATGAGCAATT[A T]AAAAT-3') and FLA625RU. The reaction mixtures were analyzed with an ABI prism 3700 automated DNA sequencer (PE Applied Biosystems). The sequences of the SVRs were translated into amino acid sequences, and each unique amino acid sequence was assigned an FlaA SVR variant number in the order of discovery.

Data analysis. All typing data were collected in Bionumerics software (version 3.0); and the composite data set, which comprised banding patterns (for AFLP analysis, PFGE, and ribotyping) and allele numbers (for MSLT and FlaA SVR sequencing), was used to cluster the isolates by all methods combined. Clusters of banding patterns were generated by applying the Pearson product-moment correlation coefficient, whereas categorical clustering was used for sequence codes. The clusters obtained by UPGMA were represented as dendrograms. An internal weight was given to each data set to correct for the differences in lane lengths or band numbers between methods.

RESULTS

A total of 234 *C. jejuni* isolates were cultured from samples from patients with gastroenteritis, including two isolates from patients who subsequently developed GBS during the study period. One hundred sixty-three (70%) isolates were obtained during the rainy season (November to February). Full typing data were obtained for 205 isolates by five molecular techniques; MLST data for a total of 219 isolates are available at <http://campylobacter.mlst.net>.

MLST. A total of 29 distinct STs were defined, and these were assigned to 1 of 19 clonal complexes. Ten of the clonal complexes each contained more than 5 strains and appeared to be major clonal complexes, accounting for 191 (93%) of the isolates in the data set (Table 1). Of the 10 major clonal complexes, 7 (ST-21, ST-403, ST-48, ST-22, ST-42, ST-45, and ST-353) have been described previously; the others (ST-508, ST-41, and ST-657) are described here for the first time. In addition, some provisional clonal complexes, each of which contained between one and three isolates, were identified (ST-51, ST-52, ST-599, ST-650, and ST-663). The 41 isolates of ST-403 were obtained from November to February. Comparison of the MLST data with data for contemporary isolates from the United Kingdom in the PubMLST database showed three clonal complexes (ST-508, ST-41, and ST-657) that were present in Curaçao but absent in the United Kingdom. The two GBS-associated isolates were assigned to two different clonal complexes, ST-508 and ST-22.

PFGE. The *SmaI*-digested PFGE fingerprints contained six to nine bands, which, after cluster analysis with the band-based Dice coefficient, resolved into 10 major groups each with more than 5 members and 14 strains with unique PFGE patterns (Table 1). The majority of the isolates belonging to a given clonal complex also exhibited the same PFGE type, although PFGE fingerprint patterns containing one to two different bands (indicated in Table 1 with a prime symbol with the designation) and patterns with more band differences were observed. In some cases distinct PFGE patterns correlated with different STs within a clonal complex. PFGE groups A, D, and G accounted for 84 isolates (41%) and were isolated throughout the study period. PFGE groups L, M, P, T, V, Y, and X contained 107 isolates (79%) of the 163 isolates obtained from November to February. Group M accounted for 41 of the 163 isolates. Of the GBS-associated isolates, one was

TABLE 1. Clonal complexes of *C. jejuni*

Clonal complex	No. of isolates	Allelic profile of obtained ST:							No. of STs	Type(s) by sequencing SVR of <i>flaA</i>	PFGE type ^a	AFLP type ^a	Ribotype ^a
		<i>aspA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkl</i>	<i>uncA</i>					
ST-21	23	2	1	1	3	2	1	5	7	1, 10	T (T', K, F) ^c	H	22 (10, 17, 24)
ST-403	41	10	27	16	19	10	5	7	1	5	M (A, D, R, L)	K (A, F)	17
ST-508^b	40	1	6	60	24	12	28	1	2	1	A	F	7
ST-48	28	2	4	1	2	7	1	5	3	1, 10	G (Y, L)	G	4 (9, 20)
ST-41	16	16	2	16	62	3	9	8	1	3	D	C	11
ST-22	13	1	3	6	4	3	3	3	1	3	L (Y)	A	13
ST-42	10	1	2	3	4	5	9	3	1	9	Y	B	13
ST-45	7	4	7	10	4	1	7	1	4	1, 9, 91	X (X')	D	12 (3)
ST-353	8	7	17	5	2	10	3	6	2	1, 2, 8	P (P', S)	H (G')	17
ST-657	5	73	4	2	4	19	3	6	1	5	V	I	6
ST-51	3	7	17	2	15	23	3	12	1	2	P (A)	E	4, 7
ST-52	2	9	25	2	10	22	3	6	2	2, 4	I	H	4, 25
ST-56	2	2	4	27	25	11	3	5	1	2	Z	D, G	2
ST-61	2	1	4	2	2	6	3	17	1	5	V	I	6
ST-49	1	3	1	5	17	11	11	6	1	11	W	K	9
ST-206	1	2	21	5	37	2	1	5	1	8	L	H	24
ST-599	1	1	4	27	25	11	3	5	1	2	Z	G	2
ST-650	1	1	6	60	24	12	28	53	1	46	A	F	7
ST-663	1	24	30	2	2	89	93	6	1	1	F	J	1

^a The predominant PFGE and AFLP types and ribotypes determined after combined cluster analysis with Bionumerics software.

^b The MLST STs first reported in this study are indicated in boldface.

^c Other minor types that occur in a clonal complex are indicated in parentheses.

assigned to PFGE group A and the other showed a unique PFGE type.

AFLP analysis. The banding patterns obtained by AFLP analysis consisted of 40 to 80 bands. UPGMA clustering generated a dendrogram with 11 distinct AFLP clusters (Table 1). AFLP clusters (clusters A to K) were distinguished when the banding patterns contained less than 70% similarity. Nine AFLP clusters were concordant with the MLST clonal complexes, but MLST ST-21 and ST-353 were not differentiated by AFLP analysis (Table 1). The banding patterns for clusters A, B, C, and F showed more than 90% similarity. The GBS-associated isolates were found in AFLP groups A and F. After comparison of the patterns with those in a database containing approximately 800 AFLP patterns for *C. jejuni* strains isolated between 1996 and 2000 from chickens, cows, and patients with gastroenteritis or GBS, all in The Netherlands (5, 6), three AFLP clusters (clusters C, F, and I) were found to be unique to Curaçao. The patterns of cluster C showed no similarity to those of the Dutch isolates, whereas those of cluster F showed similarity to the pattern of only one Dutch GBS-associated isolate. It was also speculated that the patterns of cluster I were unique to Curaçao as they showed similarity to only two Dutch gastroenteritis-associated strains. The patterns for strains from cluster K could not be reliably reproduced. These patterns contained a substantially larger number of bands, which suggested that incomplete digestion of chromosomal DNA occurred. PFGE classified these strains in clonal group K, which indicated that the aberrant AFLP patterns are due to problems relating to AFLP analysis.

Ribotyping. Ribotype patterns with four to five bands were obtained by digestion with *Pst*I. Automatic assignment of ribotypes with the Qualicon RiboPrinter resulted in 25 different types. By subsequently importing the ribotype patterns into

Bionumerics software and using the parameters for calculation of similarity on the basis of the positions and intensities of the bands (Pearson product-moment correlation coefficient), only 16 types were identified due to deletion of types that showed minor shifts in the position of one band. Eight ribotype groups were concordant with the MLST clonal complexes. Ribotyping did not differentiate between the ST-403 and ST-353 complexes or between the ST-22 and ST-42 complexes (Table 1). A type 18 ribotype pattern was obtained for one GBS-associated isolate. No reliable ribotype patterns were obtained for 13 strains belonging to AFLP cluster K.

Typing by *flaA* SVR sequencing. Sequence analysis of the *flaA* SVR regions of 205 strains identified 11 FlaA amino acid sequence variants. The ST-508, ST-41, ST-48, ST-403, ST-22, ST-21, and ST-657 clonal complexes each contained a single strain with variations in the FlaA SVR amino acid sequence, whereas the other clonal complexes contained multiple strains with variations in the FlaA SVR amino acid sequence (Table 1).

Congruence between typing methods. After cluster analysis by each method and construction of a composite data set comprising the MLST, PFGE, AFLP, and ribotype patterns, a similar clustering of the isolates was observed. The use of different weights to correct for differences in the numbers of bands (a weight of 1 for ribotyping patterns, a weight of 2 for PFGE patterns, and a weight of 4 for AFLP patterns) did not affect the clustering. Categorical clustering of the MLST and AFLP profiles according to the Pearson product-moment correlation coefficient resulted in 65% congruence. It may be possible to improve this level of congruence, as it has been shown that comparison of the sequences rather than the allelic profiles may increase the concordance by 25% (17).

DISCUSSION

Molecular typing identified the spread of clonal *C. jejuni* isolates in Curaçao. MLST identified 10 major clonal complexes, each of which was corroborated by PFGE, AFLP, and ribotyping. A poor correlation between the MLST clonal complexes and the FlaA amino acid sequence was detected, further indicating the instability of the latter parameter for epidemiological typing in the absence of other data. Isolates with variations in the FlaA SVR amino acid sequence occurred among isolates from all major MLST complexes, which supports the ongoing diversification selection and horizontal transfer of genes for flagellar antigens at a relatively high frequency (2, 3, 11, 17, 19). We conclude that MLST, PFGE, AFLP analysis, and ribotyping have the same value for epidemiological typing, as they equally established the genetic relatedness between strains with highly similar phylogenetic clustering. MLST has the advantages of interlaboratory portability, reproducibility, and a unified nomenclature that can be accessed via the Internet, enabling comparisons among studies.

The major clonal group was the ST-403 complex (PFGE type M, AFLP type K, and ribotype 17) which comprised 41 of the 205 (21%) fully characterized isolates (Table 1). Isolates in this complex exhibited aberrations in their AFLP patterns, probably due to incomplete digestion of the DNA. DNA methylation often results in incomplete restriction; and the first adenine of the recognition site of *Hind*III, one of the restriction endonucleases used in AFLP analysis, has been shown to be susceptible to methylation (12). The genome sequence of *C. jejuni* contains genes putatively encoding four adenine-specific methyltransferases (16), but it is unknown whether their gene products are functional. DNA methylation plays an important role in the virulence of several bacterial pathogens, and its contribution to the pathogenicity of the clonal *C. jejuni* isolates present in Curaçao may be worth investigating.

In Europe, human *Campylobacter* infections peak during the summer months (May to September) (1, 18). The epidemiological basis for this seasonality of infection is not well understood, but it is clear that the sources and vehicles for human infection are numerous (18). In Curaçao the incidence of infection is highest during the rainy season (November to February), and the course of *C. jejuni*-associated GBS was more severe in this period (22). The predominant distribution of certain clonal genotypes during this period is suggestive. Epidemiological studies of human infections in Europe have demonstrated the distribution of genetically diverse isolates in England (23) and The Netherlands (5, 17). In those studies the relative number of different STs was significantly higher than that obtained in the present study. Schouls et al. (17) identified 53 different STs among 83 human isolates. Dingle et al. (2) identified 72 different STs among 501 human strains. The present study has revealed an overrepresentation of clonal complexes among isolates from patients, although such an overrepresentation has not been identified in the United Kingdom or The Netherlands (2). However, in Finland predominant clonal groups of isolates have been found to be associated with human infections (10). In Curaçao, the 29 STs observed among 205 strains are suggestive of the clonal spread of effective pathogens as well. A commonality between Finland and Curaçao may be the relatively low numbers of inhabitants and

livestock, which may restrict the circulating pool of *C. jejuni* genotypes.

To date it has not proved possible to associate specific genotypes with the onset of GBS (4, 7). The GBS-associated isolates evaluated in this study were assigned to different clonal complexes, ST-22 and ST-508, which correlated with earlier data describing the heterogeneity of neuropathogenic *C. jejuni* (4, 7). However, the ST-22 complex, which is characteristically Penner serotype O:19, is overrepresented among GBS-associated isolates, suggesting the inclusion of virulent clones.

In conclusion, all of the molecular typing approaches identified similar clonal groupings of *C. jejuni*. These groups, some of which have not been reported outside of Curaçao, may exhibit differential virulence characteristics or may appear to have different geographical distributions. The possibility that the observed seasonality of clonal strains is related to strains with elevated pathogenic potentials and to the severity of human disease requires further investigation.

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