JOURNAL OF CLINICAL MICROBIOLOGY, June 2004, p. 2836–2839 Vol. 42, No. 6
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.6.2836–2839.2004

Identification of *Campylobacter jejuni* Multilocus Sequence Type ST-21 Clonal Complex by Single-Nucleotide Polymorphism Analysis

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Received 28 November 2003/Returned for modification 27 January 2004/Accepted 24 February 2004

Conserved single-nucleotide polymorphisms (SNPs) which characterize the allelic profile of the major epidemiological lineage ST-21 were identified from the alleles within the current *Campylobacter jejuni* multilocus sequence typing (MLST) database. Allelic discrimination assays were designed for the detection of SNPs, enabling rapid strain profiling for clonal complex ST-21. This method is suitable for epidemiological investigations and is complementary to full MLST.

Campylobacteriosis is the most commonly reported bacterial gastrointestinal infection throughout the United Kingdom; 41,406 laboratory-confirmed cases were reported in 2003 (www.hpa.org.uk/infections). The organisms are carried in the intestinal tracts of many animals and birds; epidemiological evidence has suggested this locale as a potential reservoir for human infections and as an entry point into the food chain (4, 6, 11). The majority of human infections are caused by *Campylobacter jejuni*, a highly diverse species that has been subdivided through current phenotyping and genotyping techniques (1, 2, 3, 6, 7, 8, 10, 12). Although these techniques have defined epidemiological groupings, they are unable to provide timely fingerprinting of isolates for robust early detection and case cluster recognition. Any effective intervention strategies for campylobacters would require the rapid identification of common sources of infection for clusters of cases, currently considered sporadic infections.

Multilocus sequence typing (MLST) (5), as described for *C. jejuni* by Dingle et al. (3), has the advantages of a method which provides a discriminatory molecular profile, is reproducible and easy to interpret (13), and provides data which are directly comparable between laboratories by use of the website http://pubmlst.org/campylobacter. Use of this method has resulted in the recognition of major genetic lineages or clonal complexes in *C. jejuni* populations from human infections and animal and environmental sources (2). Clonal complex ST-21 is one of the largest clonal complexes found to date, constituting 26% of all of the submitted isolates, with a total of 152 different sequence types (http://pubmlst.org/campylobacter). This clonal complex is frequently associated with cases of human disease and has been found in a wide range of food chain sources (3).

Conserved single-nucleotide polymorphisms (SNPs), which identify the allelic profile of major epidemiological lineages, such as ST-21, have been identified from all of the alleles within the current MLST database. Based upon this intelligence, the aim of this study was to identify informative SNPs within MLST alleles, develop rapid allelic discrimination assays to detect the SNPs, and verify the usefulness of SNPs in strain profiling for the ST-21 clonal complex.

Strain selection and preparation. A total of 236 isolates received by the Campylobacter Reference Unit, Health Protection Agency, London, United Kingdom, and independent from isolates reported in any other *C. jejuni* MLST publication (excluding reference isolates) were used. They included isolates from a United Kingdom-wide retail poultry survey (n = 88) (www.foodstandard.gov.uk/multimedia/pdfs/campsalmsurvey.pdf); human enteritis, referred as part of the Campylobacter Sentinel Surveillance Scheme (n = 90) (4); animals (dogs, birds, and cattle) (n = 22); and animal products (n = 21). Also included were the 15 reference isolates for *C. jejuni* MLST described by Wareing et al. (15).

C. jejuni isolates were used to inoculate Columbia blood agar (CM331; Oxoid, Basingstoke, United Kingdom) containing 5% defibrinated horse blood. The samples were incubated for 24 h at 37°C in anaerobic jars (Don Whitley Scientific, Shipley, United Kingdom) under microaerobic conditions (5% CO_2, 5% O_2, 3% H_2, 87% N_2). DNA was isolated by using MagNApure with a bacterial DNA isolation kit according to the manufacturer's instructions (Roche, Lewes, United Kingdom).

MLST. MLST was carried out as described by Dingle et al. (3), and sequenced products were separated and detected by using an ABI Prism 3700 or a Beckman CEQ 8000 sequencer. Contigs were assembled and edited by use of a sequence typing analysis and retrieval system (Man-Suen Chan and Nicki Ventress, University of Oxford), and allele numbers,

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TABLE 1. Association of alleles within clonal complex ST-21

Allele [SNP site(s)]	Specificity of allele for clonal complex	Specificity of allele for each clonal complex within MLST database	
		Assigned to clonal complex ST-21	Not assigned to clonal complex ST-21
glnA1 (bp 108 and 267)	0.93	0.9	0.04 (ST-49), 0.003 (ST-206, ST-22, ST-257, ST-353, ST-52), 0.002 (ST-177, ST-48)
tkt-1 (bp 330)	0.94	0.67	0.15 (ST-48), 0.12 (ST-206), 0.01 (ST-45), 0.003 (ST-257, ST-61, ST-42, ST-443, ST-573, ST-658)
Combined *glnA1* and *tkt-1*	0.88	0.98	0.004 (ST-206), 0.002 (ST-48)

sequence types, and clonal complexes were assigned by interrogation of the *Campylobacter* MLST website (http://pubmlst.org/campylobacter).

Identification of alleles for SNP assay design. The most common alleles at each locus within every clonal complex were identified by searching the MLST database. Alleles which were most specific for the ST-21 clonal complex were selected.

Downloading of MLST alleles and identification of SNPs. All alleles were downloaded from the *Campylobacter* MLST website (http://pubmlst.org/campylobacter) into Bioedit Sequence Alignment Editor, version 4.0.9 (Tom Hall, North Carolina State University). SNPs unique for each chosen allele and in a location suitable to meet the primer and probe design parameters (Lightcycler probe design software; Roche) were identified from the alignments.

Design and application of allelic discrimination assays for the Lightcycler. Two reactions using Lightcycler probe design software were designed to detect the ST-21 clonal complex: one duplex reaction to detect the two SNPs (A→G at bp 108 and C→T/A at bp 267) within the *glnA1* allele and a separate, uniplex reaction to detect the one SNP (T→C at bp 330) within the *tkt-1* allele. The Roche Lightcycler 1.2 instrument with version 3.5 Lightcycler software was used for all reactions. MLST amplification PCRs were performed by using MLST amplification primers for *glnA1* and *tkt-1* (3); 1 μl of first-round PCR product was added to a 10-μl Lightcycler Mastermix reaction. The latter included 0.3 μM concentrations of forward and reverse primers (MWG Biotech, Milton Keynes, United Kingdom), 0.1 μM concentrations of sensor probes (Metabion, Planegg-Martinsried, Germany), 0.1 μM concentrations of anchor probes (Metabion), 2.5 μl of Lightcycler Faststart hybridization probe Mastermix (Roche), and 2 to 4 mM $MgCl_2$.

The primers (F, forward; R, reverse; S, sensor; A, anchor; phos, phosphate; fluo, fluorescein; LCred-640 and LCred-705, marker dyes) were as follows: *gln*F, GGATCAGGCGTAAAAGG; *gln*R, AACCCTTGAAAAAGTAGGTC; *gln*108S, LCred-640-GCCACTATTTTTAAGGTGTTCTATTGCT-phos; *gln*108A, TCTGGTCCAAAGTAAGCAGTATCAGCT-fluo; *gln*267S, LCRed-705-ATCGGTAAATTCTCTATCATCATTCCACT-phos; *gln*267A, TGTTTCTTGGCCTGTGTCCAGTATTGTAG-fluo; *tkt*F, CCATCTCCGCAAAGACA; *tkt*R, AGCACAAGGATTTGAAGT; *tkt*330S, LCRed-640-ATAGAGATATTGTTGCTATCATAAATAAGTATGAAGTTATCA-phos; and *tkt*330A, CGTTAAAGGCTAAACCTACATCGCCCTT-fluo.

Cycling consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 63°C for 10 s, and 72°C for 10 s. The melting steps consisted of denaturation at 95°C for 1 s, followed by 53°C for 1 min, and slow extension at a ramp rate of 0.1°C/s to 85°C, with continuous data acquisition. The highest melting temperature for each assay confirmed a perfect match between the probe and target sequences and hence was indicative of the SNP; melting temperatures that were 2 to 4°C lower indicated the absence of the SNP. Therefore, highest melting temperatures of 68°C for *glnA1* (bp 108), 68°C for *glnA1* (bp 267), and 66°C for *tkt*-1 (bp 330) would confirm the ST-21 clonal complex.

The *glnA1* and *tkt-1* alleles were selected for the ST-21 clonal complex due to their predicted specificities for ST-21 (0.88) and for this complex from the entire database (0.98) (Table 1). Additionally, both alleles had conserved informative SNPs (*glnA*, A→G at bp 108 and C→T/A at bp 267; *tkt*, T→C at bp 330), which enabled successful identification of the alleles.

Evaluation of the SNP assay for ST-21 with the MLST reference isolate panel (15) demonstrated specificity for ST-21 (isolate 13254) with the highest melting temperature for each SNP reaction; therefore, this isolate possessed the three SNPs (Table 2). Other reference isolates showed combinations of lower melting temperatures. The study isolates ($n = 221$) were found to be 100% concordant with all 51 clonal complex ST-21 isolates identified by full MLST and confirmed to be ST-21 by the SNP assay (Table 3).

Using melting curve analyses with the Lightcycler, it was possible to design an assay to recognize the presence of informative SNPs that can identify alleles specific to clonal complex ST-21. The melting peak analyses are sufficiently robust, since each SNP reaction showed an obvious shift in melting temperatures between different alleles on the basis of sequences. The assignment of a melting temperature profile of 68, 68, and 66°C with the SNP assay identifies isolates belonging to clonal complex ST-21 in less than 4 h at a cost of US$2.40 per isolate.

The drawbacks of the SNP approach are that the data in the current MLST database are representative only of isolates that have been both typed by MLST and submitted; therefore, as the database expands, there is the potential for new alleles to be missed. The process of assigning clonal complexes from SNP analysis might not suit all areas of research where MLST might be considered. For population biology, the full MLST data set would be required; alternative strategies for MLST, such as the use of high-density DNA arrays like those described for MLST of *Staphylococcus aureus*, might be more applicable (14). Also, MLST or the SNP approach might not be suitable in certain situations. A supplementary technique,

TABLE 2. Results of SNP assay for ST-21 for MLST reference isolates (n = 15) and identification of isolates assigned to ST-21 by MLST and SNP analysis

Isolate	Source	Allele profile							ST (clonal complex)	Melting temp (°C) in SNP assay for[a]:			Clonal complex assigned by SNP analysis[b]
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		*glnA1* (bp 108)	*glnA1* (bp 267)	*tkt-1* (bp 330)	
13254	Beef offal	2	1	1	3	2	1	5	21	**68**	**68**	**66**	21
13257	Human stool	4	7	10	4	1	7	1	45	66	**68**	64	NA
13263	Human stool	2	21	5	37	2	1	5	206	66	**68**	**66**	NA
13261	Beef offal	1	4	2	2	6	3	17	61	62	64	64	NA
13258	Lamb offal	2	4	1	2	7	1	5	48	62	64	**66**	NA
13264	Human stool	9	2	4	62	4	5	6	257	66	**68**	60	NA
13256	Human stool	1	2	3	4	5	9	3	42	66	**68**	64	NA
13262	Sand	17	2	8	5	8	2	4	177	66	**68**	62	NA
13260	Lamb offal	9	25	2	10	22	3	6	52	62	**68**	64	NA
13265	Human stool	8	10	2	2	11	12	6	354	66	**68**	64	NA
13255	Human stool	1	3	6	4	3	3	3	22	62	64	64	NA
13266	Human stool	1	2	49	4	11	66	8	362	66	**68**	64	NA
13259	Human stool	3	1	5	17	11	11	6	49	**68**	**68**	64	NA
13268	Unknown	10	27	16	19	10	7	5	403	66	64	64	NA
13267	Unknown	7	17	5	2	10	6	3	353	62	**68**	62	NA

[a] Bold type indicates the highest melting temperatures. The highest melting temperature profile for all three SNPs was detected only for the isolate assigned to ST-21.
[b] NA, not applicable.

such as sequencing of the short variable region of the *flaA* gene, was described as being necessary for adequate discrimination in *C. jejuni* outbreak investigations (9).

Nevertheless, we have established a timely and discriminatory method for the identification of isolates belonging to the ST-21 clonal complex, the greatest benefit being its applicability for rapid screening. This work is currently being extended to include the other major important clonal complexes of *C. jejuni* and an additional real-time platform to achieve high-volume throughput. Furthermore, this method has the potential for rapid allelic discrimination of *C. jejuni* directly from clinical specimens and food and environmental samples. This approach is complementary to full MLST characterization of *C. jejuni*.

TABLE 3. Results of SNP assay for the ST-21 clonal complex for 221 isolates from different clonal complexes

MLST clonal complex[a] (no. of isolates)	Melting temp (°C) in SNP assay for[b]:			Clonal complex assigned by SNP analysis[c]
	glnA1 (bp 108)	*glnA1* (bp 267)	*tkt-1* (bp 330)	
ST-21 (51)	**68**	**68**	**66**	ST-21
ST-257 (29)	66	**68**	60	NA
ST-61 (3)	62	64	**66,** 64	NA
ST-48 (21)	62, 61	64, **68**	**66,** 62, 60	NA
ST-45 (28)	66	**68**	**66,** 64, 60	NA
ST-49 (4)	**68**	**68**	64, 62	NA
ST-206 (5)	66, 61	**68,** 64	**66**	NA
ST-22 (7)	62, 61	64	**66,** 64	NA
ST-177 (2)	66	**68**	64, 62	NA
ST-353 (4)	66, 62	**68**	65, 62	NA
ST-42 (10)	66	**68**	64, 62, 60	NA
ST-52 (7)	62, 61	**68**	**66,** 65, 64	NA
ST-354 (10)	66, 62	**68**	**66,** 64, 62	NA
ST-403 (4)	66	64	64, 62	NA
ST-362 (1)	66	**68**	64, 61	NA
Other clonal complexes and unassigned isolates (35)	ND	ND	ND	NA

[a] Determined by full MLST (3).
[b] Bold type indicates the highest melting temperatures.
[c] NA, not applicable.

We thank Martin Maiden, Frances Colles, and Roisin Ure (University of Oxford) for help in setting up MLST and the use of sequencing facilities. We thank the staff of the Molecular Epidemiology Department, Health Protection Agency North West Laboratory, Manchester, and the Campylobacter Reference Unit, Laboratory of Enteric Pathogens, Health Protection Agency, London, United Kingdom, and Ankur Agrawal for help in setting up the sequence typing analysis and retrieval system software.

We acknowledge the Health Protection Agency (HPA) for funding through an HPA studentship. During the course of this study we used the *Campylobacter* MLST website (http://pubmlst.org/campylobacter) developed by Man-Suen Chan and Keith Jolley and located at the University of Oxford. Initial development of this website was funded by the Wellcome Trust; maintenance is funded by DEFRA.

REFERENCES

1. **Champion, O. L., E. L. Best, and J. A. Frost.** 2002. Comparison of pulsed-field gel electrophoresis and amplified fragment length polymorphism techniques for investigating outbreaks of enteritis due to campylobacters. J. Clin. Microbiol. **40:**2263–2265.
2. **Dingle, K. E., F. M. Colles, R. Ure, J. A. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D. R. Wareing, and M. C. Maiden.** 2002. Molecular characterization of *Campylobacter jejuni* clones: a basis for epidemiologic investigation. Emerg. Infect. Dis. **8:**949–955.
3. **Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. J. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14–23.
4. **Gillespie, I. A., S. O'Brien, J. A. Frost, G. K. Adak, P. Hornby, and A. V. Swan.** 2002. A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. Emerg. Infect. Dis. **8:**937–942.
5. **Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
6. **Moore, J. E., L. O'Riordan, D. R. Wareing, R. Doyle, J. Lanser, T. Stanley, M. Matsuda, T. Matsui, and P. G. Murphy.** 2003. Phenotypic and genotypic relationship between *Campylobacter* spp. isolated from humans and chickens in Northern Ireland—a comparison of three phenotyping and two genotyping schemes. Int. J. Hyg. Environ. Health **206:**211–216.
7. **Ribot, E. M., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. Barrett.**

2001. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. J. Clin. Microbiol. **39:**1889–1894.
8. **Sails, A. D., B. Swaminathan, and P. I. Fields.** 2003. Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. J. Clin. Microbiol. **41:**4058–4067.
9. **Sails, A. D., B. Swaminathan, and P. I. Fields.** 2003. Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by *Campylobacter jejuni*. J. Clin. Microbiol. **41:**4733–4739.
10. **Schouls, L. M., S. Reulen, B. Duim, J. A. Wagenaar, R. J. Willems, K. E. Dingle, F. M. Colles, and J. D. Van Embden.** 2003. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. J. Clin. Microbiol. **41:**15–26.
11. **Stanley, K., and K. Jones.** 2003. Cattle and sheep farms as reservoirs of Campylobacter. J. Appl. Microbiol. **94:**104S–113S.
12. **Suerbaum, S., M. Lohrengel, A. Sonnevend, F. Ruberg, and M. Kist.** 2001. Allelic diversity and recombination in *Campylobacter jejuni*. J. Bacteriol. **183:**2553–2559.
13. **Taylor, J. W., and M. C. Fisher.** 2003. Fungal multilocus sequence typing—it's not just for bacteria. Curr. Opin. Microbiol. **6:**351–356.
14. **van Leeuwen, W. B., C. Jay, S. Snijders, N. Durin, B. Lacroix, H. A. Verbrugh, M. C. Enright, A. Troesch, and A. van Belkum.** 2003. Multilocus sequence typing of *Staphylococcus aureus* with DNA array technology. J. Clin. Microbiol. **41:**3323–3326.
15. **Wareing, D. R. A., R. Ure, F. M. Colles, F. J. Bolton, A. J. Fox, M. C. J. Maiden, and K. E. Dingle.** 2003. Reference isolates for the clonal complexes of *Campylobacter jejuni*. Lett. Appl. Microbiol. **36:**106–110.