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High-Resolution DNA Melt Curve Analysis of the Clustered, Regularly Interspaced Short-Palindromic-Repeat Locus of *Campylobacter jejuni*▽†

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A novel method for genotyping the clustered, regularly interspaced short-palindromic-repeat (CRISPR) locus of *Campylobacter jejuni* is described. Following real-time PCR, CRISPR products were subjected to high-resolution melt (HRM) analysis, a new technology that allows precise melt profile determination of amplicons. This investigation shows that the CRISPR HRM assay provides a powerful addition to existing *C. jejuni* genotyping methods and emphasizes the potential of HRM for genotyping short sequence repeats in other species.

Clustered, regularly interspaced short palindromic repeats (CRISPRs) are a class of short sequence repeats that have been found in many bacterial genomes (7, 10, 11). CRISPRs comprise near-perfect direct repeats (DRs) interspersed with similarly sized nonrepetitive spacer sequences. The *Campylobacter jejuni* CRISPR locus harbors few DRs but extensive spacer variation (18). The aim of this study was to develop a high-resolution melt (HRM)-based assay for interrogating the hypervariable CRISPR locus of *C. jejuni.* It was hypothesized that HRM would be effective for discriminating CRISPR variants and that this assay would efficiently add resolution to existing single nucleotide polymorphism (SNP) and binary gene-based typing methods for *C. jejuni* (5, 6, 15–17, 19).

CRISPR detection in *C. jejuni*. Schouls and coworkers (18) have previously demonstrated the absence of the CRISPR locus in 10% of *C. jejuni* and *Campylobacter coli* strains, with a further 15% harboring a single repeat without a spacer. However, due to polymorphisms between the original CRISPR primers and the genome-sequenced *C. jejuni* strain RM1221, the distribution of CRISPRs in *C. jejuni* and *C. coli* may have been underestimated. Therefore, new CRISPR primers were designed: CRISPR-For (5′-GCAACCTCCTTTTAGTGGAG TAATTAG-3′) and CRISPR-Rev (5′-AAGCGGTTTTAGG GGATTGTAAC-3′). A total of 210 Australian *C. jejuni* and *C. coli* isolates, including 181 that have been previously described (13, 15, 16), were tested for the presence of the CRISPR locus using the redesigned primers (Sigma-Proligo, Lismore, Australia) and the manufacturer's PCR reagent and thermocycling schedule (Invitrogen).

Twelve (6%) isolates did not yield a CRISPR PCR product, including five *C. jejuni* (2%) and seven *C. coli* (100%) isolates. Seventy-four (35%) strains contained a single DR lacking a spacer unit. The remaining 138 isolates contained between 2 and 11 DRs, consistent with the data set of Schouls et al. (18), in which between 1 and 13 DRs were identified. All four sequence type 42 (ST-42) isolates were CRISPR negative, in agreement with the study by Schouls and colleagues in which six of seven ST-42 isolates were absent for the CRISPR locus (18). The *C. jejuni* isolate F079, an ST-536 isolate belonging to the ST-21 multilocus sequence typing (MLST) clonal complex (CC), was also CRISPR negative. The SNP genotype consistent with ST-48 (SNP group 10) was numerically dominant in all isolate collections used in this study, represented by 43 (20%) strains. The dominance of this genotype is not seen in other countries, such as the United Kingdom and United States, in which the ST-21 and ST-828 CCs, respectively, are most commonly isolated (8). These results suggest that the ST-48 genotype is common in the Australian *C. jejuni* population and contributes substantially to human gastroenteritis in this country. All 43 isolates with this SNP profile possess a single DR. This finding is consistent with a previous study which found that all ST-48 isolates contained a single DR (18).

CRISPR sequencing. Thirty-two PCR-positive CRISPRs of various sizes were subjected to DNA sequencing using the CRISPR-For and CRISPR-Rev primers. Sequencing of CRISPR loci enabled (i) correct size determination of all CRISPR products, (ii) comparison of spacer sequences between the present study and that of Schouls et al. (18), and (iii) assessment of the performance and resolving power of HRM analyses. DNA sequencing revealed 22 different CRISPR types (CTs) within the 32 isolates, of which 8 isolates contained a single DR (Table 1). Schouls et al. (18) identified 170 unique spacers among 137 *Campylobacter* isolates, and 55 unique spacers from 32 *C. jejuni* strains were found in the present study. While the DR sequence was strongly conserved, the spacer sequences are all novel. This confirms the highly polymorphic nature of the CRISPR spacers.

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TABLE 1. CRISPR spacer sequences of 32 *C. jejuni* isolates from the present study

Isolate(s)	ST (CC)	No. of DRs	Spacer allele(s)[a]	CT
F381	529 (ST-45)	2	217	102
F470	532 (ST-257)	2	223	103
F431	50 (ST-21)	2	239	104
RM1221	354 (ST-354)	4	202-203-204	105
F162	53 (ST-21)	6	205-205-257-257-258	106
NCTC 11168	43 (ST-21)	5	205-206-207-208	107
F475, F395, F228	528 (ST-354)	8	209-210-211-212-213-214-215	108
F459	530 (NA)[b]	5	209-210-222-211	109
F050, F119	354 (ST-354)	11	210-211-235-212-212-213-214-236-237-238	110
F458	538 (ST-45)	7	216-217-218-219-220-221	111
F007	535 (ST-460)	7	224-225-226-227-228-229	112
F009	52 (ST-52)	3	230-231	113
F041	52 (ST-52)	4	232-233-234	114
F226	52 (ST-52)	7	232-248-249-250-251-252	115
F014	50 (ST-21)	6	239-240-241-242-243	116
F053, F087	257 (ST-257)	5	244-245-246-247	117
F042	197 (ST-257)	4	244-246-247	118
F001	227 (ST-206)	3	255-256	119
F025	161 (ST-52)	3	253-254	120
F451, F501	161 (ST-52)	4	253-254-254	121
F280	51 (ST-443)	6	259-224-260-261-262	122
F486, F509, F100, F168, F448, 01M27530,[c] F421, F492	531 (NA), 523 (ST-658), ST-312 (ST-658), 48 (ST-48), 5 (ST-353)	1	NA	12

[a] Each unique spacer sequence was given an allele number according to previous spacer alleles designated by Schouls et al. (18). RM1221 (1) and NCTC 11168 (14) CRISPR spacers are included for comparison.
[b] NA, not applicable.
[c] MLST not performed on this isolate.

HRM analysis of the CRISPR locus. Real-time PCR is an attractive platform for high-throughput bacterial genotyping, as it is single step, closed tube, and cost-effective, and real-time PCR devices are used extensively in both research and analytical laboratories. Recently, real-time PCR devices that contain HRM capabilities have emerged (9). HRM differs from conventional PCR product melting temperature (T_m) measurement in two ways. First, the accuracy of the melt curve is maximized by acquiring fluorescence data over small temperature increments (as low as 0.01°C). Secondly, the precise shape of the HRM curve is a function of the DNA sequence being melted, allowing amplicons containing different sequence to be discriminated on the basis of melt curve shape, irrespective of whether amplicons share the same T_m. HRM analysis makes use of melt curve normalization and comparison software that allows the user to determine whether two similar melt curves differ from one another.

HRM analysis of the *C. jejuni* CRISPR was achieved by transferring the conventional PCR procedure to the Rotor-Gene 6000 (Corbett Research, Sydney, Australia) platform. First, the levels of effectiveness of different double-stranded nucleic acid-specific fluorescent dyes in performing HRM were compared. SYTO9 (Invitrogen-Molecular Probes) has recently been reported as a suitable chemistry for HRM analysis, and due to its lack of PCR inhibition activity, it can be used at higher concentrations than SYBR green I (3, 12). However, there is currently limited information on the comparative performance of intercalating DNA dyes for HRM analysis, particularly for examination of complex amplicons. SYTO9 reaction mixtures contained 5 pmol (0.5 μM) of each primer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 U Platinum *Taq* DNA polymerase and the relevant PCR buffer, 2.5 μM SYTO9, and 1 μl genomic DNA, made to a volume of 10 μl with double-distilled H_2O. SYBR green I reactions were performed as previously detailed (15). Despite altering several parameters, including dye and genomic DNA concentration, efficient and reproducible amplification using SYBR greenER (Invitrogen) could not be achieved, and this dye was not used further.

SYTO9 and SYBR green I cycling parameters were as follows: 95°C for 2 min, 40 cycles of 95°C for 3 s and 58°C for 45 s, and final extension at 72°C for 2 min. HRM analysis of all samples was undertaken postrun by incubation at 50°C for 20 s followed by ramping from 70 to 85°C, with fluorescence data acquisition at 0.05°C increments. HRM analyses were carried out using Rotor-Gene 6000 1.7.34 software. Both conventional and normalized dissociation plots were generated. The normalized dissociation plot was used to construct difference graphs, a feature of the Rotor-Gene 6000 software that allows the user to quantitatively determine the sample deviation relative to a control sample. Four controls were included in each run to facilitate inter-run comparisons, and all samples were tested in duplicate to ensure reproducibility of the melt curves and to determine appropriate cutoffs for "same" and "different" genotypes. Classification of "different" CRISPR HRM genotypes was determined by a cutoff of $\geq \pm 5$ U in the difference graph relative to a control sample of interest. "Different" genotypes inside the cutoff were further distinguished based on whether the curves traversed the midpoint of the difference graph two or more times (with a deviation of $\geq \pm 2$ U) or whether distinct peaks were evident in the difference graph (Fig. 1). Where appropriate, the normalized and conventional dissociation plots were also used to ascribe genotypes as the

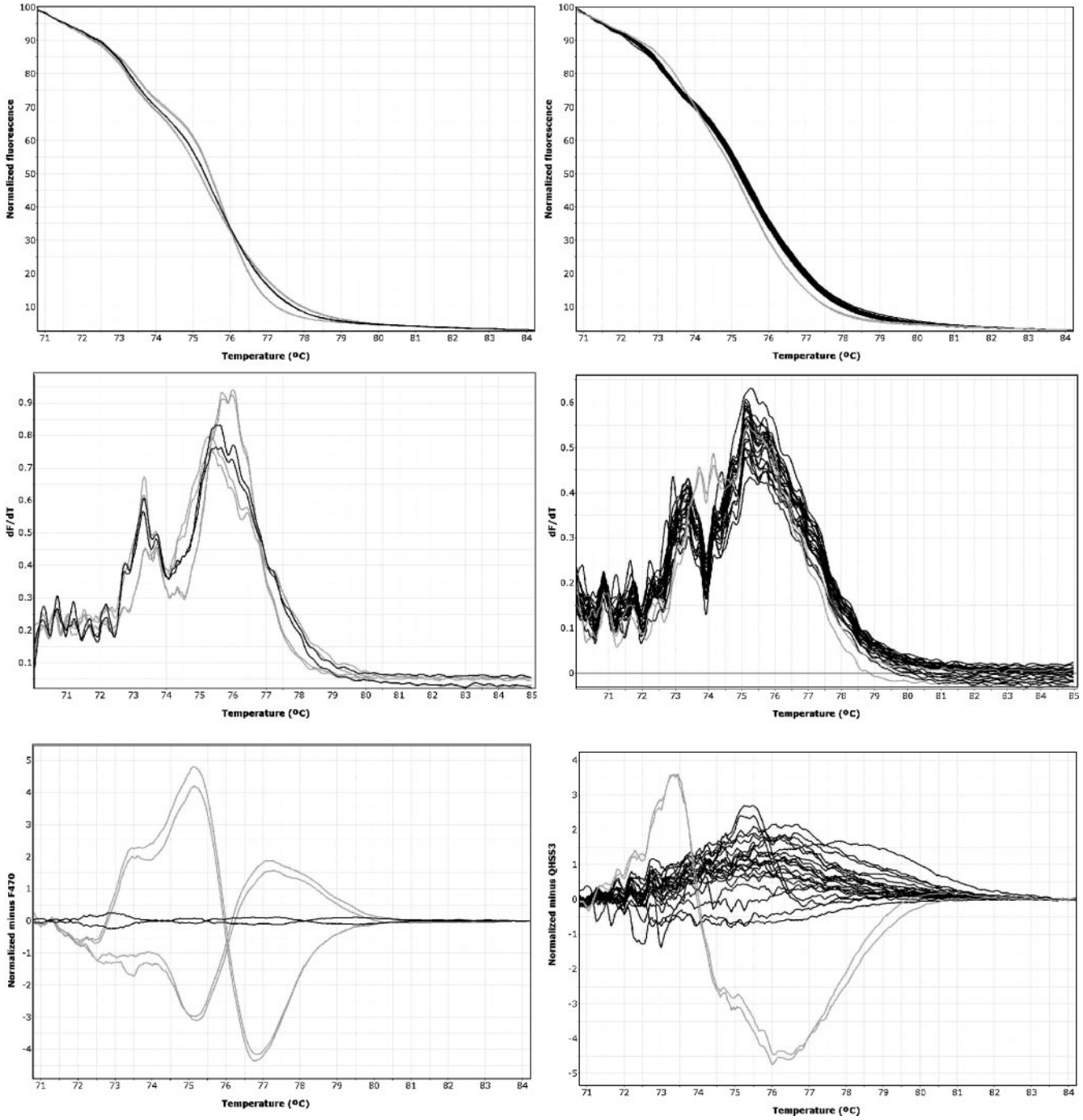

FIG. 1. Classification of "same" and "different" genotypes using HRM analysis of the *Campylobacter jejuni* CRISPR locus. Data that illustrate the limits of the technology were deliberately chosen: i.e., the alleles shown here were especially refractory to HRM discrimination. (Top) Normalized high-resolution melt curve. (Middle) Derivative of fluorescence with respect to temperature (dF/dT) dissociation curve. (Bottom) Difference graph. The left panel shows an example of two genotypes classed as "different" from a control sample (isolate F470, shown by the black lines) due to the erratic pattern in the difference graph, despite falling within the cutoff ($\leq \pm 5$ in the difference graph). The normalized and dF/dT melt curves confirmed that these genotypes are different from F470. The right panel shows genotypes classed as "same" (in black lines) compared with a control sample (isolate QHSS2). The difference graph, normalized, and dF/dT melt curves confirmed that these genotypes are indistinguishable from QHSS2. A "different" genotype (gray lines) is shown for comparison.

"same" or "different" by visual inspection of the curves relative to the control sample of interest.

The levels of performance of SYTO9 and SYBR green I were compared using CRISPR amplicons from a subset of 29 *C. jejuni* isolates. For all 29 isolates, the SYBR green I melt profiles were highly reproducible and easy to interpret, whereas the SYTO9 melt profiles were much less reproducible (Fig. 2). This was an unforeseen finding, as SYBR green I has

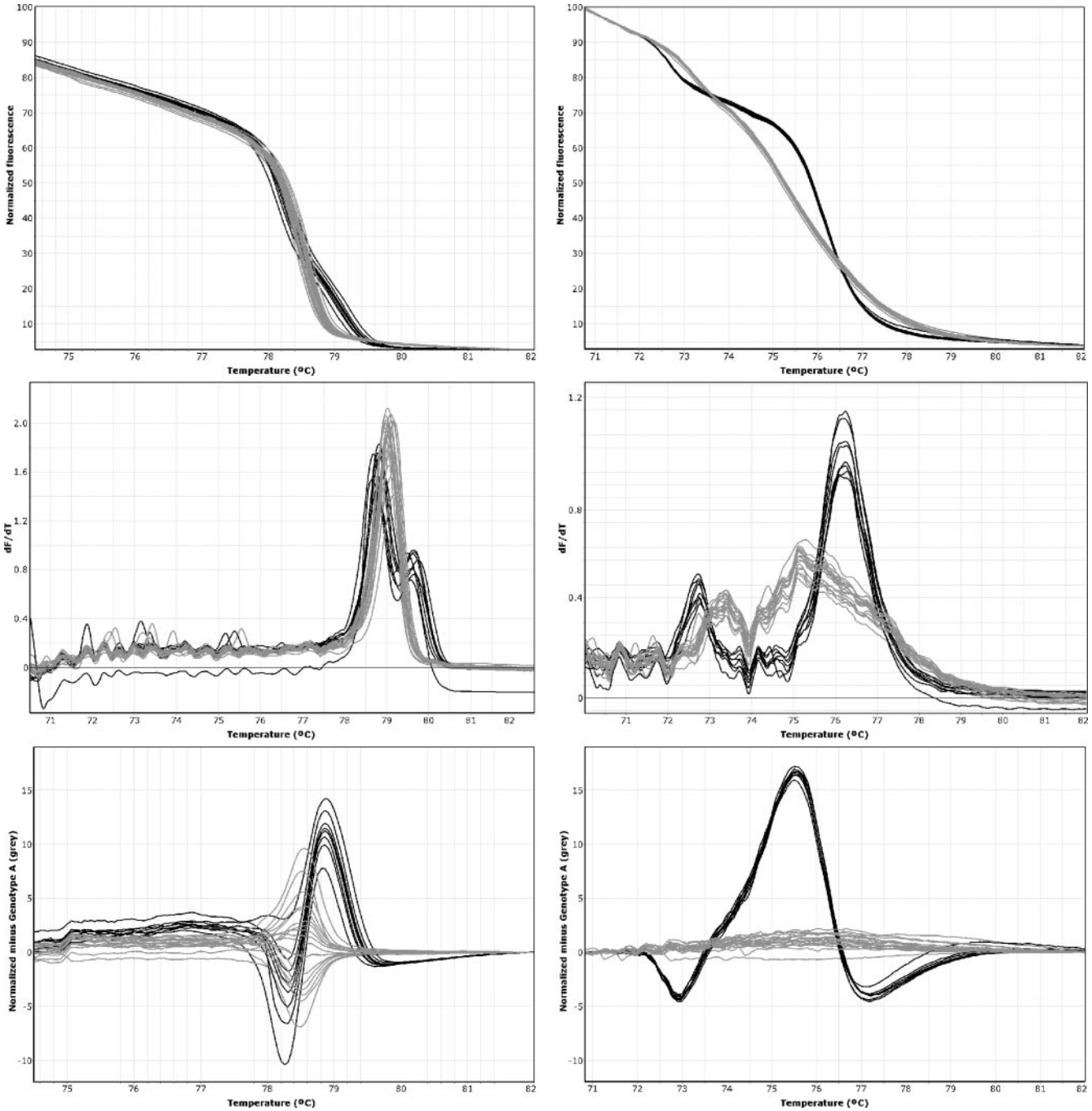

FIG. 2. Comparison of SYBR green I and SYTO9 by HRM analysis of the CRISPR locus of *Campylobacter jejuni*. Two genotypes (gray and black lines) are shown. (Left panels) SYTO 9. (Right panels) SYBR green I. (Top) Normalized high-resolution melt curve. (Middle) Derivative of fluorescence with respect to temperature (dF/dT) dissociation curve. (Bottom) Difference graph.

been shown to translocate between amplicons during melt curve analysis to higher-T_m products (2, 4, 20), suggesting instability of this dye during HRM analysis. A previous T_m study comparing the two chemistries demonstrated that SYBR green I melt profiles were more greatly influenced by dye, $MgCl_2$, and starting DNA concentrations than melt profiles with its counterpart (12). In addition, SYTO9 has been shown to generate more reproducible DNA melting curves over a larger range of concentrations than SYBR green I, as the dye is less inhibitory to PCR at higher concentrations and does not selectively detect amplicons in multiplex reactions (2, 12).

To confirm the improved performance of SYBR green I over SYTO9, a further 125 *C. jejuni* isolates were compared. Inter-run variability was monitored by including four control strains per run. As previously observed, the SYBR green I amplicons generated highly reproducible and sensitive melt

TABLE 2. QHSS isolate genotyping results

Isolate no.	SNP group[a]	Binary type	CRISPR HRM type	No. of CRISPR repeats	Typing result by[b]:		Epidemiology	Date isolated
					flaA RFLP with DdeI	PFGE with SmaI		
QHSS1	8	12	1	4	2	PT4	Resort, waterborne	June 1997
QHSS5	8	12	1	4	2	PT4	Resort, waterborne	June 1997
QHSS6	8	12	1	4	2	PT4	Resort, waterborne	June 1997
QHSS7	8	12	1	4	2	PT4a	Resort, waterborne	June 1997
QHSS4	5	21	11	4	NT	PT8	Resort, waterborne	June 1997
QHSS13	25	24	10	1	NT	PT7	Blood, same patient	June 2000
QHSS14	25	24	10	1	NT	PT7	Feces, same patient	June 2000
QHSS18	5	21	12	4	3	PT5	Cairns	July 2000
QHSS20	5	21	12	4	1	PT5	Cairns	July 2000
QHSS2	10	22	2	1	1	PT6	Sporadic	1996
QHSS3	10	22	2	1	1	PT6	Sporadic	1997
QHSS9	10	22	2	1	1	PT6	Cairns, from chicken	2000
QHSS10	10	22	2	1	1	PT6	Cairns, from chicken	2000
QHSS11	10	22	2	1	1	PT6	Cairns, from chicken	2000
QHSS12	10	22	2	1	1	PT6	Cairns, from chicken	2000
QHSS15	10	22	2	1	3	PT6	Cairns, from chicken	2000
QHSS16	10	22	2	1	5	PT6	Cairns, from chicken	2000
QHSS22	10	22	2	1	1	PT6A	Sporadic	2002
QHSS23	8	12	57	2	NT	PT11	Cairns, same family	February 2003
QHSS24	8	12	57	2	NT	PT11	Cairns, same family	February 2003
QHSS26	12	14	13	4	4	PT1	Rockhampton, same family	May 2005
QHSS27	12	14	13	4	4	PT1	Rockhampton, same family	May 2005
QHSS28	12	14	13	4	4	PT1	Rockhampton, same family	May 2005
QHSS29	12	14	13	4	4	PT1	Rockhampton, same family	May 2005
QHSS25	16	26	39	3	NT	PT2	Rockhampton, same family non-food borne	May 2005
QHSS21	5	21	58	2	1	PT5	Sporadic	2002
QHSS17	26	14	14	4	NT	PT9	Sporadic	2000
QHSS19	8	22	2	1	1	PT10	Sporadic	2000
QHSS8	27	22	2	1	1	PT3	Sporadic	1997

[a] SNP groups are numbered according to the corresponding seven-member SNP profile (15). The SNP profiles for QHSS13 and QHSS14 (G, A, C, A1, C, G, and T), QHSS17 (G, G, C, A1, C, A, and C), and QHSS8 (A, G, T1, A1, T1, G, and C) have not been previously identified in the OzFoodNet or Princess Alexandria Hospital (Brisbane, Australia) (sporadic) isolate collections (13, 15, 16).

[b] PT4a, one-band difference from PT4; PT6a, two-band difference from PT6; NT, nontypeable (did not amplify).

curves, whereas SYTO9 amplicons were difficult to interpret due to large differences between replicates, with many replicates called as "different" using a relaxed cutoff value of $\leq \pm 7$ U. As a result of the superior performance of the SYBR green I chemistry over SYTO9, the remaining strains were examined using SYBR green I only.

Comparison of the CRISPR sequence data with the HRM data revealed that SYBR green I enabled discrimination of CRISPR amplicons containing small sequence differences, independent of amplicon length. For example, CRISPR HRM discriminated between the single-DR (237 bp) amplicons of F448 and F509, which differ by an SNP at their 5′ end. Such sensitivity was not apparent in all cases, with F448 and F486 identical by HRM but harboring an SNP at the 3′ end of the amplicon. Overall, eight CRISPR HRM genotypes were identified within isolates containing a single DR. The larger (~900 bp) amplicons of F050 and F119, which differ at 1 base as well as having a length difference of 4 bp, were efficiently discriminated by the SYBR green I HRM method. Importantly, the HRM profiles of CRISPRs with identical sequences, such as F509, F168, 01M27530, F421, and F492, were indistinguishable. These results demonstrate the power of the HRM method for discriminating "same" or "different" CRISPR genotypes in *C. jejuni*.

Comparison of genotyping methods with CRISPR HRM. Previously, we have developed SNP and binary gene typing methods for *C. jejuni* and *C. coli* using the real-time PCR platform and have generated considerable data on the performance of these methods using the 181 Australian *C. jejuni* and *C. coli* isolates (15, 16). The present study addressed whether the HRM procedure provided comparable resolution to the current "gold standard" pulsed-field gel electrophoresis (PFGE) procedure when combined with SNP and/or binary gene interrogation of *C. jejuni* isolates.

The SNP-binary gene approach has previously been shown to provide comparable resolution to MLST-flagellin A short variable region (*flaA* SVR) sequencing. However, these combinatorial methods were unable to reach the discriminatory power of PFGE (16). One of the shortcomings of the study by Price et al. (16) was that the *Campylobacter* strains examined were obtained from sporadic gastroenteritis cases collected over a 3-year period, and therefore few definitive epidemiological data were available. To overcome this, 29 previously uncharacterized *C. jejuni* isolates from Queensland, Australia, were obtained from Queensland Health Scientific Services (QHSS). These isolates comprised six distinct outbreak clusters with confirmed epidemiological data, as well as seven sporadic gastroenteric isolates. The QHSS isolates were tested

blind by SNP typing, binary gene typing, and *flaA*-restriction fragment length polymorphism analysis using DdeI (Roche). PFGE was carried out on the QHSS isolates using SmaI (Roche) digestion. Digested DNA was electrophoresed using a CHEF DR III system (Bio-Rad) for 10 to 25 s at 6 V cm^{-1} in 0.5% Tris-borate-EDTA (pH 8.3) for 22 h at 14°C.

In the sporadic isolate collections (designated 84, 154, and PA isolates in Table S1 in the supplemental material), the binary-CRISPR HRM and binary-*flaA* SVR approaches provided the highest discriminatory power of two methods in combination. Significantly, the use of three methods (SNP-binary-*flaA* SVR/CRISPR HRM or MLST-binary-*flaA* SVR) enabled further delineation of the sporadic isolates and provided resolution comparable to or surpassing that of PFGE (see Table S1 in the supplemental material). In contrast, addition of CRISPR HRM to the SNP-binary profiles did not increase resolution of outbreak QHSS genotypes, most likely attributable to the high genetic similarity of these isolates; however, the discriminatory power remained comparable to that of PFGE. The PFGE and SNP-binary (with or without CRISPR HRM) genotypes for the 22 outbreak isolates corroborated in almost all instances, and both methods were effective in differentiating between outbreaks (Table 2). These results demonstrate that the SNP-binary-CRISPR HRM approach is a feasible alternative to PFGE in both sporadic and outbreak isolate investigations of *C. jejuni*.

To conclude, we have demonstrated the application of HRM as an alternative to DNA sequencing, using the CRISPR locus of *C. jejuni* and *C. coli* as a model. The CRISPR HRM assay, in combination with other established real-time PCR methods for *C. jejuni* and *C. coli*, provides a novel approach to bacterial genotyping that equals or surpasses the resolving power of PFGE. The value of HRM for characterizing complex DNA sequence is not limited to CRISPRs or *C. jejuni* and can potentially be applied to any polymorphic region, ranging from SNPs to entire genes.

Nucleotide sequence accession numbers. The nucleotide sequences of the CRISPRs from 32 *C. jejuni* isolates have been deposited in GenBank under accession no. EF017316 to EF017347.

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