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High-Resolution Melting Analysis of the *spa* Repeat Region of *Staphylococcus aureus*

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BACKGROUND: The staphylococcal protein A (*spa*) locus of *Staphylococcus aureus* contains a complex repeat structure and is commonly used for single-locus sequence-based genotyping. The real-time PCR platform supports genotyping methods that are single step and closed tube and potentially can be carried out simultaneously with diagnosis. We describe here a method for genotyping *S. aureus* using high-resolution melting (HRM) analysis of the *spa* polymorphic region X.

METHODS: The conventional PCR spa assay was modified and optimized for the Rotor-Gene 6000 instrument (Corbett Life Science). HRM analysis on the Corbett Rotor-Gene 6000 instrument was used to test 22 known *spa* sequences obtained from 44 diverse methicillin-resistant *S. aureus* (MRSA) isolates. Criteria for calling pairs of melting curves "same" or "different" were developed empirically by converting the data to difference graph format with one curve defined as the control. HRM curve comparison between runs was done to determine the portability of the method. The assay performance was assessed by genotyping uncharacterized isolates, carrying out blind trials, and comparing HRM profiles from different runs.

RESULTS: HRM analysis of 44 diverse MRSA isolates generated 20 profiles from 22 *spa* sequence types. The 2 unresolved HRM *spa* types differed by only 1 bp. Two blind trials demonstrated complete reproducibility with respect to calling the different *spa* types. Interrun comparisons of HRM curves were successfully developed, indicating the robustness of the method.

CONCLUSION: Analysis of the *spa* locus by HRM resolves spa sequence variants. This single- and closed-tube single-step method for *S. aureus* genotyping can be easily combined with the interrogation of other genetic markers.

The hypervariable region X of staphylococcal protein A (*spa*) is used for single-locus sequence-based genotyping of *Staphylococcus aureus (1–4)*. This locus contains a complex repeat structure that is thought to rapidly evolve through slipped-strand mispairing and recombination *(5–7)*. Additionally, the *spa* extracellular domains are subject to immune surveillance, which is expected to increase its speed of evolution. Consequently, there are currently 2366 *spa* types in the Ridom SpaServer database (http://spaserver.ridom.de).

Although sequencing technology is now extremely effective, non–sequence-based genotyping methods are also advantageous. In particular, the real-time PCR platform supports genotyping methods that are single step and closed tube, can potentially be carried out simultaneously with diagnosis, and can interrogate different classes of genetic polymorphisms. These features provide real advantages for the clinical microbiology laboratory. A recent development in real-time PCR technology is high-resolution DNA melting (HRM) analysis *(8, 9)*. Although melt curves are predominantly used to determine the melting temperature (T_m) of amplified double-stranded DNA, it is recognized that the precise shape of a melting curve is a function of the DNA sequence, and this characteristic forms the basis of HRM analysis *(10)*. Accurate melting curves are derived using very small temperature increments, and normalization and comparison of melting curves allows sensitive determination of whether different amplicons have the same or different sequence *(9)*. The potential resolving power of this approach is much greater than conventional melting curve analysis because in HRM melting curves from different amplicons can be differentiated on the basis of shape, even when they define the same T_m values.

HRM has previously been applied to human mutation screening *(11–13)* and to differentiating the hypervariable *CRISPR* (clustered regularly interspaced short-palindromic-repeat) locus of *Campylobacter jejuni (14)*. We tested the hypothesis that HRM analysis on the Corbett Rotor-Gene 6000 instrument (Corbett Life Science) can be used to differentiate *spa* alleles. We also hypothesized that HRM curves can be compared between different runs of the Rotor-Gene 6000, a characteristic that would indicate that HRM analysis can be used as a library-based genotyping method instead of being strictly comparative. Initially, the HRM analysis was tested against 22 known *spa* sequences that were derived during the course of this study from 44 diverse MRSA isolates (Table 1) *(15–17)*. To rigorously test the discriminatory power of HRM, the collection included multiple isolates with identical multilocus sequence types (MLST), or MLST-derived single-nucleotide polymorphism profiles *(16)* thought to possess very similar *spa* sequences. The 22 *spa* se-

Table 1. *spa* **sequence types of the 44 MRSA isolates used in this study.**[a]

Isolate (*n* = 44)	PFGE	MLST[a] (*n* = 13)	*spa* genotype[b] (*n* = 22)	HRM genotype (*n* = 20)	*spa* repeat units[c]	T_m[e]	Repeat region GC%	Repeat region size, bp
SN39	ND[f]	1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
FMC53	ND	1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
SJOG 30	ND	1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
MD828534	E	1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
RHH10[e]	ND	~1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
FH53[e]	ND	~1	t127	HRM1	07-23-21-16-34-33-13	80.6	44	168
749	ND	1	t175	HRM2	07-23-21-16-16-33-21-16-33-13	82.1	46.3	240
RPH29	ND	~1	**NEW1**	HRM3	26-23-17-34-34-16-34-33-13	79.9	41.7	216
E804531	I	5	t002	HRM4	26-23-17-34-17-20-17-12-17-16	81.6	45.4	240
37[e]	ND	~5	t002	HRM4	26-23-17-34-17-20-17-12-17-16	81.6	45.4	240
CH 97	ND	73	t002	HRM4	26-23-17-34-17-20-17-12-17-16	81.6	45.4	240
24	ND	~5	**NEW2**	HRM4	**new**-23-17-34-17-20-17-12-17-16	81.6	45	240
56	ND	~5	**NEW2**	HRM4	**new**-23-17-34-17-20-17-12-17-16	81.6	45	240
IMVS 67	ND	8	t008	HRM5	11-19-12-21-17-34-24-34-22-25	80.6	43.8	240
251	ND	8	t008	HRM5	11-19-12-21-17-34-24-34-22-25	80.6	43.8	240
RPH 2	ND	8	t190	HRM6	11-17-34-24-34-22-25	80.0	44	168
CH69	ND	~22	**NEW3**	HRM7	26-23-13-17-31-29-17-25-17-25-16-28	81.4	44.4	288
68284/98	A5	30	t019	HRM8	08-16-02-16-02-25-17-24	80.7	44.3	192
62 (1)[e]	ND	30	t019	HRM8	08-16-02-16-02-25-17-24	80.7	44.3	192
PAH 1[e]	ND	30	t019	HRM8	08-16-02-16-02-25-17-24	80.7	44.3	192
PAH 58	ND	30	t019	HRM8	08-16-02-16-02-25-17-24	80.7	44.3	192
D828570	A0	~30	t019	HRM8	08-16-02-16-02-25-17-24	80.7	44.3	192
E822485	B	36	t018	HRM9	15-12-16-02-16-02-25-17-24-24-24	81.3	44.7	264
B8-31	K	~45	t123	HRM10	09-02-16-34-13-16-34-16-34	79.6	40.7	216
MJ710566	C	~45	t065	HRM11	09-02-16-34-13-17-34-16-34	79.7	40.7	216
337	ND	59	t437	HRM12	04-20-17-20-17-25-34	80.4	43.5	168
751	ND	59	t216	HRM13	04-20-17-20-17-31-16-34	81.0	44.3	192
IP01M1081	Q	59	t216	HRM13	04-20-17-20-17-31-16-34	81.0	44.3	192
IP01M2046	P1	78	**NEW4**	HRM14	08-21-17-13-13-**new**-34-33-34	79.7	40.7	216
MC801535	D	88	t325	HRM15	07-12-21-17-34-13-34-34-33-34	79.7	40.4	240
F829549	D	88	t186	HRM16	07-12-21-17-13-13-34-34-33-34	79.8	40.8	240
RBH 98	ND	93	t202	HRM17	11-17-23-17-17-16-16-25	82.2	47.9	192
15808-0020	ND	~93	t202	HRM17	11-17-23-17-17-16-16-25	82.2	47.9	192
15814-9852	ND	~239	t631	HRM18	15-12-16-17-24	81.1	47.5	120
RPAH 18[e]	ND	239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
AH 13	ND	239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
IMVS 20[e]	ND	239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
K714372[e]	F4	~239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
K711532	F3	~239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
K704540[e]	F	~239	t037	HRM19	15-12-16-02-25-17-24	80.9	45.2	168
E812560	J	~239	t1155	HRM19	15-12-16-02-25-17-16	80.9	45.2	168
IPOOM14235[e]	O	~239	t1155	HRM19	15-12-16-02-25-17-16	80.9	45.2	168
MK703484	G1	~239	t1155	HRM19	15-12-16-02-25-17-16	80.9	45.2	168
14176-5710	ND	~239	**NEW5**	HRM20	15-21-12-16-02-25-17-16	81.2	45.3	192

[a] "~" denotes isolates genotyped using MLST SNP typing *(16)*.
[b] **NEW** = new *spa* type.
[c] **new** = new repeat unit.
[d] T_m calculated by the Rotorgene 6000 using SYBR Green dye and rounded to one decimal place.
[e] Ten blind tested isolates.
[f] ND = Not done.

quences included 5 novel types; 3 with novel combinations of known repeat units, and 2 including novel repeat units. As anticipated, these included sequences that differed from each other at a single base, thus allowing rigorous testing of the HRM resolving power.

The conventional PCR *spa* assay using primers 1095F (5′-AGACGATCCTTCGGTGAGC-3′) and 1517R (5′-GCTTTTGCAATGTCATTTACTG-3′) *(4)* was modified and optimized for the Rotor-Gene 6000 instrument (Corbett Life Science). Each 10-μL reaction contained 5 μL Platinum® SYBR® Green qPCR SuperMix-UDG (2x, Invitrogen Life Technologies), 0.25 μL of each primer (20 μM stock, final concentration 0.5 μM), 3.5 μL ddH20, and 1 μL DNA template (20 fg/L). The real-time PCR thermocycling parameters were: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of (95 °C for 5s, 60 °C for 30s), 72 °C for 2 min, and 50 °C for 20 s, followed by HRM ramping from 75–87 °C with fluorescence data acquisition at 0.05 °C increments. Reactions were routinely carried out in duplicate.

The Rotor-Gene 6000 proprietary software (version 1.7.34) enables the user to visualize HRM data in multiple ways. The negative derivative of fluorescence (F) over temperature (T) (*df/dt*) curve primarily displays the T_m, the normalized raw curve depicts the decreasing fluorescence vs increasing temperature, and difference curves *(9)*, which display a user-defined curve as the baseline (i.e., the x-axis), and depicts other normalized curves in relation to that baseline. Criteria for calling melting curves as "same" or "different" using difference graphs were developed empirically. Melting curves are called the same as the defined control if the difference graph lies within ±4 U relative to the x-axis, and does not display reproducible differences such as double peaks or crossing the x-axis more than twice in both replicates (Fig. 1). In addition, a 2-step procedure was followed to determine if an unknown HRM curve was the same as a known curve. First, the normalized HRM curve for the unknown type was compared to known normalized HRM profiles. These profiles are either generated together with the unknown samples, or more practically, have been previously produced. Second, the closest known HRM profile was selected as the difference graph control, and comparison of the difference curves was use to determine whether the unknown isolate was the same as or different from the known. For each data analysis, the digital filter was set to "heavy" and the replicate grouping option was selected. When conflicts between replicates occurred, repeat or sequence analysis was performed. According to the above criteria, 22 known *spa* sequences generated 20 reproducibly different HRM curves. As expected, this result was a higher degree of resolution than could be obtained from T_m determination. For example, HRM curves 1 and 5 were derived from different *spa* sequences but defined identical T_m values, as did HRM curves 11, 14, and 15. Two pairs of *spa* sequences were not resolved by HRM analysis. These were t002 and the NEW2, and t037 and t1155. Both pairs of sequences differed at a single base. We concluded that *spa* sequences can be differentiated by HRM even when they are closely related, but the resolving power using SYBR Green dye does not reliably extend to sequences that differ by a single base pair.

The software supplied with the Rotor-Gene 6000 does not allow the generation of difference graphs using curves from different runs of the device. To determine the practicality of HRM curve comparison between runs (and by extension, the development of a standard library of HRM curves), the normalized data were exported and converted to "difference" format in Microsoft Excel, and difference curves assembled using the freeware chart drawing program Teechart Office *(18)*. We found that the HRM curves were completely portable, and that the practice of comparing data from different real-time PCR runs caused no loss in the ability to differentiate different *spa* alleles (Fig. 1). To further confirm the robustness of this method, we carried out 2 blinded experiments. To increase the rigor of these tests, different batches of reagents were used. The first experiment entailed the analysis of 10 MRSA isolates of unknown *spa* sequence, as indicated in Table 1. Subsequent *spa* sequence determination revealed that all the *spa* sequences were identical to *spa* types found in isolates in Table 1, and all were correctly identified by HRM analysis. The second blinded experiment was the reanalysis of the original set of 34 isolates. Once again, the *spa* types were 100% consistent with the first 2 times these isolates were analyzed. Interestingly, because the melt curves were clearly visually distinct, we successfully differentiated *spa* sequences that differed by a single base and could not be separated in the first 2 analyses of these isolates owing to the very conservative sequence discrimination criteria. It is likely that these criteria will be refined as more data become available, thus increasing the resolving power of HRM analysis. In summary, HRM analysis was performed 3 times on the 34 isolates, and the results proved to be completely reproducible with respect to calling the *spa* types. There was one very minor reproducibility issue. When the Corbett 6000 runs were separated by several weeks, HRM curves from these runs could be offset by up to 0.2 °C, although there was complete consistency of curve shape. These results were likely attributable to very slight changes to the calibration of the instrument's thermometer and can easily be overcome by including a known control in all runs.

We concluded that HRM analysis of the *spa* locus is an effective method to easily and rapidly identify *spa* alleles. The major advantage of this method is that it is

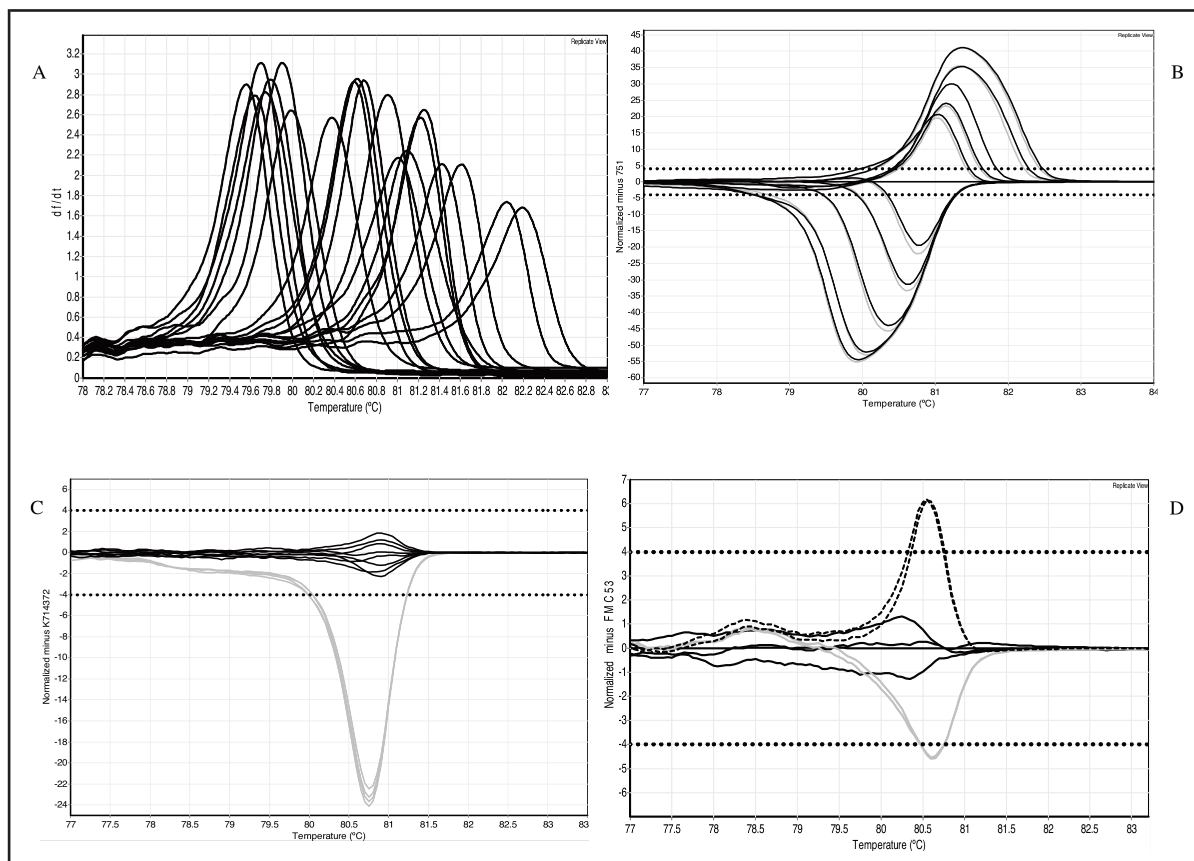

Fig. 1. Comparison of different *spa* polymorphic region X HRM curves obtained from *S. aureus*.

(A), negative derivative of fluorescence over temperature (df/dt) plots displaying the 20 HRM profiles. (B), difference graph demonstrating the accurate reproduction of 11 *spa* HRM profiles from separate experiments (grey and black traces). (C), a typical difference graph comparison of 2 unrelated *spa* HRM genotypes. From the 6 isolates displayed, 3 fall within the ±4 U of the baseline profile (HRM 19) and are called "same", the remaining 2 isolates lie outside of the ±4 cutoffs and therefore are denoted as "different" (HRM 1). In this instance, to highlight the HRM reproducibility within a run, the replicates were not grouped together. (D), Differentiation of 3 similar *spa* HRM curves using difference graphs. Three isolate genotypes are identical to the baseline *spa* profile (HRM 1); 2 isolates rise above +4 (HRM 8), 2 isolates fall beneath −4 (HRM 5) and are each denoted as "different." These *spa* HRM profiles correspond to the 3 df/dt curves displayed at 80.6 °C on graph A. Y-axis labels for (B), (C), and (D) indicate the isolate numbers against which unknown spa HRM types were normalized.

a single-step closed-tube procedure performed on a moderately priced and generic piece of laboratory equipment. This technique offsets the occasional inability to differentiate *spa* alleles, because *spa* interrogation could simply be combined with the real-time PCR interrogation of, for example, clonal-complex–specific single-nucleotide polymorphisms *(16)*, toxin-encoding genes *(16)*, or binary markers that subtype SCC*mec (19)*. In this context, HRM-mediated *spa* interrogation may be regarded as a facile means of adding resolving power to other genotyping methods for interrogating less polymorphic markers that more reliably define population structures. The portability of the HRM curves is very significant, because this characteristic makes the method potentially library based rather than comparative. A resource that would be very useful is a library of *spa* HRM curves for the major MRSA epidemic- and community-acquired clones, and this library has been provided in part by this study. Similar genotyping approaches could be developed for other bacteria.

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