

Development of a Canadian Standardized Protocol for Subtyping Methicillin-Resistant *Staphylococcus aureus* Using Pulsed-Field Gel Electrophoresis

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A panel of 24 methicillin-resistant *Staphylococcus aureus* strains was distributed to 15 laboratories in Canada to evaluate their in-house pulsed-field gel electrophoresis (PFGE) protocols and interpretation criteria. Attempts to compare fingerprint images using computer-aided analysis were not successful due to variability in individual laboratory PFGE protocols. In addition, individual site interpretation of the fingerprint patterns was inadequate, as 7 of 13 sites (54%) made at least one error in interpreting the fingerprints from the panel. A 2-day standardized PFGE protocol (culture to gel image) was developed and distributed to all of the sites. Each site was requested to use the standardized protocol on five strains from the original panel. Thirteen sites submitted gel images for comparisons. The protocol demonstrated excellent reproducibility and allowed interlaboratory comparisons with Molecular Analyst DST software (Bio-Rad) and 1.5% band tolerance.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in England in 1961, shortly after the introduction of methicillin in 1959 (3, 9). Since that time, MRSA has become a worldwide problem, causing nosocomial infections in many countries (10, 13, 14, 17, 19, 21, 22). Surveillance programs in the United Kingdom, Spain, Brazil, Germany, the United States, and Canada have identified epidemic MRSA strains which appear to possess the ability to spread rapidly among patients within and between hospitals and nursing homes (1, 4, 7, 12, 13, 16, 22, 25).

Pulsed-field gel electrophoresis (PFGE) has become the gold standard for the epidemiological typing of a number of bacterial species (2). However, multicenter studies comparing PFGE fingerprints for MRSA clearly have demonstrated the requirement for standardized conditions (5, 6, 23). Differences in cell numbers, lysis conditions, PFGE equipment, electro-

phoresis conditions, run times, and analytical methods all potentially affect the intercenter reproducibility of this technique.

The Canadian Committee for the Standardization of Molecular Methods (CCSMM) is a Health Canada initiative established in 1998 to deal with issues regarding the standardization of molecular techniques (11). All known Canadian laboratories currently using molecular techniques for routine epidemiologic surveillance as well as all provincial public health laboratories were invited to join the CCSMM. The mission statement, "To standardize molecular methodologies to ensure quality testing and reporting of results for epidemiologic purposes," reflected the requirement by Canadian laboratories for standardized molecular protocols to aid in the molecular surveillance of many organisms. Realizing the strong potential for monitoring molecular subtypes of organisms using DNA restriction fragment length polymorphisms in PFGE, the committee has initially focused its efforts on this area. One of the subcommittees undertook the task of developing and implementing protocols for data collection of PFGE fingerprints for MRSA.

In this report, we describe the process involved in establishing a Canadian standardized PFGE protocol for macrorestriction analysis of *Sma*I-digested MRSA. The protocol is rapid, and the quality assurance program initiated will allow intercenter comparisons of MRSA DNA fingerprints for national surveillance studies.

MATERIALS AND METHODS

Bacterial strains. Strains 1 and 13 were NTCC 8325, and the remaining strains used in this study were kindly provided by the Canadian Nosocomial Infection Surveillance Program (CNISP). Stock cultures were stored at -70°C in Microbank vials (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). Strains were distributed on nutrient agar stabs to participating laboratories.

Study design. (i) Phase 1: initial PFGE evaluation A panel of 24 well-characterized MRSA strains collected by the CNISP and NTCC 8325 (in duplicate) was distributed to each site. Participants were requested to type the strains on

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two gels in a specific order using their in-house PFGE protocol. Laboratories were asked to submit their protocol, gel images, and interpretation for evaluation.

(a) Development of a standardized MRSA PFGE protocol. The CCSMM MRSA subcommittee evaluated the gel images submitted from all 15 sites for band resolution, intensity, reproducibility, and complexity of the protocol. There was no one method which produced rapid, well-resolved MRSA fingerprints acceptable for national comparisons; however, the subcommittee used the information gained from the various sites to produce a standardized 2-day protocol (culture to gel image). The subcommittee evaluated growth and lysis conditions, agarose plug concentrations, wash conditions, restriction enzyme concentrations and digest time requirements, electrophoresis temperatures, agarose gel concentrations, electrophoresis buffer concentrations, and run times to elucidate the optimal conditions required to establish an effective 2-day procedure (culture to gel image) necessary for a Canadian standardized protocol. This protocol was evaluated for reproducibility between subcommittee sites, and the data suggested that it may serve as a reproducible method (data not shown). The protocol is detailed below.

Pick one isolated colony of the strain to be subtyped using a sterile needle or loop and inoculate 3 ml of brain heart infusion broth. Incubate with gentle agitation at 37°C for 16 to 18 h. On the next day, take the following steps. Turn on heating blocks or water baths. Two will be required, with temperatures set at 37 and 55°C. Label 1.5-ml microcentrifuge tubes with culture numbers. Place 150 µl of the overnight culture into a microcentrifuge tube. Pellet the cells by centrifugation at 18,000 × *g* in a microcentrifuge for 1 min. Store the remaining culture at 4°C if retesting of the strain is required. Resuspend each pellet in 150 µl of cell suspension buffer (see below).

To prepare casting plugs, take the following steps. Label wells of disposable PFGE plug molds (Bio-Rad, Hercules, Calif.) with the appropriate culture numbers. Cast two plugs for each sample, as follows. Prepare 2.0% low-melting-point (LMP) agarose in deionized-distilled H₂O (dd H₂O). Melt or dissolve the LMP and incubate it in a 50°C water bath until ready to use. Note that unused LMP agarose can be stored at room temperature and reused two or three times. Complete the remaining casting plug steps (up to solidification; see below) for each strain before proceeding to the next sample. Add 2 µl of lysostaphin (1 mg of lysostaphin per ml of H₂O) per tube and mix gently. Add 150 µl of 2.0% LMP agarose. Mix gently by pipetting up and down several times, but avoid forming bubbles. Immediately fill two disposable plug molds (approximately 100 µl per plug). Avoid forming bubbles in the plug molds. Discard the remaining 100 µl. Continue with the next sample until all are processed. Allow plugs to solidify for approximately 15 min at room temperature or 5 min at 4°C.

To lyse cells in LMP agarose, take the following steps. Label 1.5-ml microcentrifuge tubes with culture numbers. Add 500 µl of lysis buffer (see below) to each of the 1.5-ml microcentrifuge tubes. Transfer the two plugs to the labeled tubes by removing tape from the bottom of the molds and pushing the plugs into the tubes. Incubate the tubes at 37°C for 1 h. Remove the tubes from the 37°C water bath and decant or aspirate the lysis buffer with a 1-ml pipette tip, taking care not to damage the plugs. Calculate the total volumes of proteinase K (PK) and PK buffer required to process all samples. Add 500 µl of PK-PK buffer (50 µg/ml) solution to each microcentrifuge tube. Incubate the tubes at 50°C for 0.5 h.

To wash LMP agarose plugs after lysis, take the following steps. Remove the tubes from the 50°C water baths and aspirate the PK-PK buffer solution. Rinse the plugs once with 1.4 ml of wash buffer (see below), and then wash them three additional times with 1.4 ml of wash buffer for 30 min each wash. All washes should be conducted at room temperature. Remove the wash buffer from the final wash of the preceding step. For long-term storage, resuspend the plugs in 1.4 ml of wash buffer. The plugs are stable for at least 6 months at 4°C.

For restriction enzyme digestion of LMP agarose plugs, take the following steps. Label 0.5-ml microcentrifuge tubes with culture numbers. Remove one plug from a microcentrifuge tube. Cut approximately one-third of the plug and place it into a new 1.5-ml microcentrifuge tube. Calculate the volume of restriction enzyme buffer (REB) required to digest the plugs. The restriction buffer for *Sma*I is usually sold as a 10× solution and must be diluted to 1× before use. (Calculate the total amount of 1× REB required. A total of 450 µl will be required per sample [300 µl for the equilibration and 150 µl for the digest]. Prepare 1× REB by diluting the appropriate amount of 10× REB stock with ddH₂O.) Equilibrate the plugs by adding 300 µl of 1× REB to the tube containing one-third of a plug and incubate the tube at room temperature or 25°C for 10 min. Remove the 1× REB from the tube with a 1-ml pipette tip, taking care not to damage the plug slice. Add 150 µl of 1× REB containing 25 U of *Sma*I to each tube and incubate the tube at 25°C for 2 h. Prepare a 1% agarose gel in 120 ml of 0.5× TBE (see below) while the DNA is being restriction digested. Pour the dissolved agarose into a casting tray. Use a 15-well 0.75-mm

comb to make wells. Aspirate the enzyme-buffer solution and melt the plug at 65 to 70°C for 10 to 15 min. Add a thin slice of lambda molecular weight markers (NO340; New England Biolabs, Mississauga, Ontario, Canada) to lanes 1, 8, and 15. Load 30 µl of the melted plug into a well with a tip with approximately 3 mm cut from the end on an angle. Load the gel dry (on the benchtop) and place it into the buffer chamber after all of the samples and ladder have been loaded and solidified. Perform electrophoresis with a CHEF DR-III apparatus (Bio-Rad) using switch times of 5.3 to 34.9 for 18 h (or 20 h for Mapper or DR-II) (Genepath strain typing system program STA) at 6.0 V/cm and 14°C in 0.5× TBE. Stain the gel for 20 min with 0.5 mg of ethidium bromide per liter, and destain it with fresh ddH₂O for at least 30 min with three changes of water. Use UV light transilluminator to visualize samples.

Solutions should be made as follows: cell suspension buffer, 10 mM Tris-HCl (pH 7.2)–20 mM NaCl–50 mM EDTA; lysis buffer, 10 mM Tris-HCl (pH 7.2)–50 mM NaCl–50 mM EDTA–0.2% deoxycholate–0.5% Sarkosyl; wash buffer, 10 mM Tris-HCl (pH 7.6)–0.1 mM EDTA; PK buffer, 250 mM EDTA (pH 9.0)–1% Sarkosyl; and 10× TBE; 0.89 M Tris-HCl (pH 8.4)–0.89 M boric acid–0.02 M EDTA.

(b) Computer-aided analysis. DNA fingerprints were digitized using an in-house apparatus and saved as TIFF files. The fingerprints generated using the standardized protocol were evaluated using Molecular Analyst DST version 1.6 software (Bio-Rad) or BioNumerics version 2.0 (Applied-Maths, Sint-Martens-Latem, Belgium). DNA fragments on each gel were normalized using the molecular weight standard run on each gel to allow comparisons between different gels. A 1.5% band tolerance was selected for use during comparisons of DNA profiles. Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UPGMA), and DNA relatedness was calculated based on the Dice coefficient.

(ii) Phase 2: PFGE using the standardized protocol. Laboratories were requested to perform PFGE using the standardized protocol developed by the CCSMM MRSA subcommittee. A subset of the originally distributed panel of MRSA strains was used by participating sites for quality assurance and included strains 1, 2, 3, 9, and 17. These five strains were chosen in part to limit the workload of participating laboratories in phase 2 of the study; to test the ability of a site to differentiate slight band deviations (strains 2 and 3), to differentiate smaller bands (under 150 kb) (strain 9), and to resolve large bands (strain 17); and for comparability with other methods by using NCTC 8325 (strain 1). Laboratories were required to fingerprint these strains in order of the strain number and flank them with lambda markers.

RESULTS AND DISCUSSION

Assessment of protocols. A total of 15 laboratories representing 7 provinces participated in phase 1 of the study. A panel including 24 well-characterized MRSA strains collected by the CNISP and NTCC 8325 was distributed to each site (Table 1). Participants were requested to type the strains in a specific order on two gels using their in-house PFGE protocol and to submit the protocol, gel images, and interpretation for evaluation. A sample gel of the strains distributed and the order in which they were requested to be loaded is shown in Fig. 1. Protocols varied considerably between sites (data not shown) with respect to length of protocol, pulse times, and duration of electrophoresis. Not surprisingly, the fingerprints from the gel images submitted from the 15 sites could not be compared using Molecular Analyst DST version 1.6 software due to different variables described previously (5, 6, 23; data not shown). Thirteen of the 15 laboratories interpreted the fingerprints using current guidelines or a modification thereof (20). The intralaboratory fingerprint analysis was unreliable, as 7 of 13 sites (54%) made at least one error in interpreting the 24 fingerprints (Table 1). Fingerprint analyses for identical strains were correct for 76.6 and 76.9% of the profiles on different and same gels, respectively. For unrelated strains, analyses at the 13 sites were correct for 90.4% of the profiles on same or different gels (Table 1). Analysis of the interpretations revealed that the errors were primarily linked to failure

TABLE 1. Individual site interpretation results obtained using nonstandardized PFGE protocols

Strains ^a	No. of strains with the following site interpretation:		% Correct interpretations
	Correct	Incorrect	
Identical (different gel)			
NTCC 8325 (1, 13)	11	2	84.6
7, 14 ^b	8	4	66.6
8, 24	9	4	69.2
9, 18	10	3	76.9
5, 16	11	2	84.6
11, 23	10	3	76.9
Overall	59	18	76.6
Identical (same gel)			
5, 11	11	2	84.6
16, 23	9	4	69.2
Overall	20	6	76.9
Unrelated			
10	12	1	92.3
12	12	1	92.3
20	11	2	84.6
22	12	1	92.3
Overall	47	5	90.4

^a Only identical or unrelated fingerprints were used in the evaluation.
^b One site did not report results for strain 14.

to recognize matching fingerprints (human error) or failure to resolve differences in the fingerprints due to run conditions. These data highlight the need for a standardized PFGE protocol and a quality assurance program to ensure data integrity.

Interlaboratory standardization. The standardized protocol was distributed to all participating laboratories. Five of the original 24 MRSA strains (1, 2, 3, 9, and 17) were selected as quality control strains for the purposes of evaluating the standardized protocol (Fig. 2A). Although it may have been more reliable to use all 24 MRSA strains used in phase 1, we decided to use a subset for quality control and certification purposes. Thirteen of the original sites submitted gel images for analysis using the standardized protocol and control strains. Comparisons of the normalized gel images with Molecular Analyst DST

version 1.6 software using the Dice coefficient and a UPGMA-derived dendrogram are shown in Fig. 2B for 12 sites. The fingerprints from the 13th site (site M) were not comparable due to poor resolution of the lambda markers in addition to problems with band resolution which may have been associated with plug washing conditions and overloading. Attempts are under way to modify that site's procedures to produce comparable fingerprints.

Using a band tolerance of 1.5% and an optimization of 4%, all strains from the 12 sites clustered in their respective strain groups; this result is not unexpected for strains 1, 9, and 17, since the patterns are not related (≥ 7 bands) to each other. However, strain 2 and strain 3 vary by only a single band shift, of less than 20 kb (Fig. 1 and 2), and they also correctly clustered in their respective strain groups.

Under the band tolerance and optimization settings applied, the site C patterns for strains 1 and 2, the site H pattern for strain 9, and the site A pattern for strain 17 did not show 100% similarity within each cluster. Percent similarities were as follows: 90.9% for strain 1 at site C with strains 1 at sites E and F; 90.9% for strain 2 at site A with strain 2 at site C; 90.9% for strain 9 at site B with strain 9 at site H; and 88.9% for strain 17 at site A with strain 17 at sites C, J, and K (Fig. 2B). All other comparisons yielded similarities of 100% under the conditions described. Identical results were obtained when the images were analyzed with BioNumerics version 2.0.

An algorithm in Molecular Analyst DST version 1.6 software, called band tolerance statistics, allows the comparison of identical fingerprint from a number of different experiments to an averaged fingerprint. A comparison of the fingerprints using this algorithm is shown in Table 2. The numbers indicate the mean deviations of band positions from an averaged fingerprint for all the sites for a specific strain. The majority of the sites displayed average deviations of less than 1.7%, suggesting that the standardized PFGE protocol was reproducible. However, site C had a significantly higher deviation, 2.51%. Examination of the gel image from site C suggested that variability in the PFGE apparatus might have caused the deviation, assuming that the standardized protocol was followed. In addition, a similar explanation could explain the difficulties as-

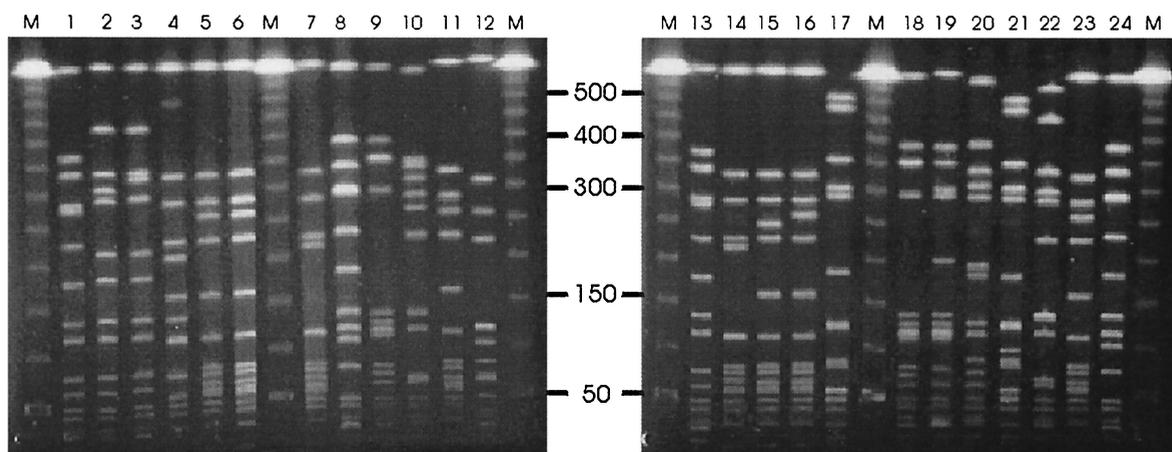


FIG. 1. DNA fingerprints obtained with PFGE for 24 MRSA strains distributed in the panel. Numbers above the lanes indicate strain numbers; M, lambda concatamers.

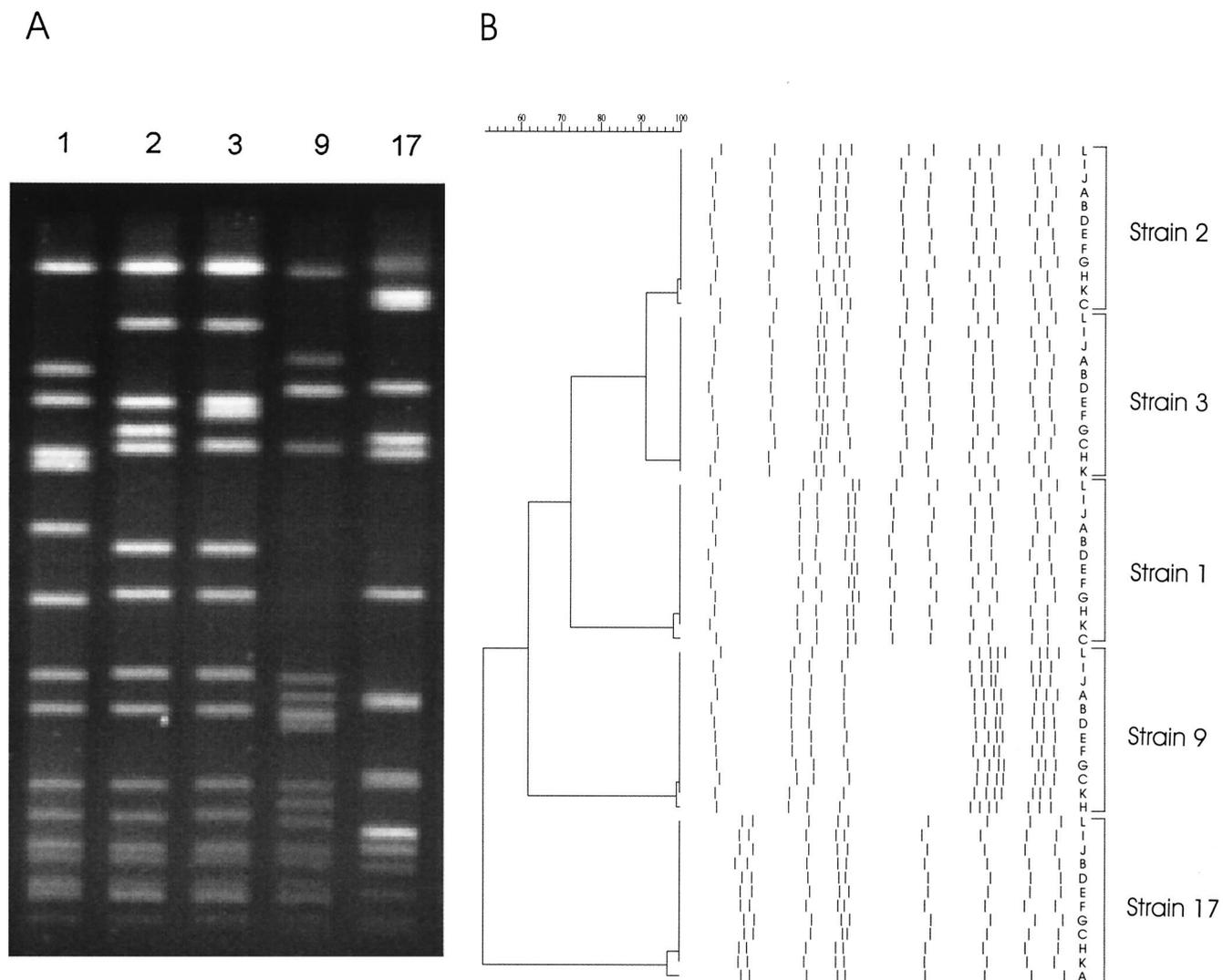


FIG. 2. PFGE analysis using the standardized protocol described in the text. (A) Sample gel showing five strains used in the quality control panel. Numbers above the lanes indicate strain numbers. (B) Dendrogram comparing fingerprints from 12 sites using the standardized protocol.

sociated with site M, the results from which could not be normalized using Molecular Analyst DST software. Efforts are under way to alter the electrophoresis conditions at these two sites to allow comparisons.

This procedure has been used to subtype over 2,000 MRSA isolates collected by the CNISP (data not shown). No isolates to date have been identified as nontypeable using this procedure. The advent of clinical MRSA isolates displaying reduced susceptibility to vancomycin in numerous countries raises the distinct possibility that vancomycin-resistant MRSA could emerge in patient populations (8, 15, 18, 24). This fact, along with the recent identification of epidemic strains in Canada (16), highlights the need for a rapid molecular-based surveillance mechanism in Canada to aid in infection control efforts. The recent development of server-based data collection systems for fingerprint information (BioNumerics) and the standardization of PFGE methods such as the methods described in this report, will be required to collect and disseminate accurate, timely information to infection control practitioners

TABLE 2. Variability of fingerprints compared with an averaged fingerprint

Site ^a	Deviation for strain:					Average deviation
	1	2	3	9	17	
A	1.64	1.09	1.00	1.73	1.89	1.47
B	1.45	0.82	1.09	1.09	1.67	1.22
C	1.55	3.18	2.45	2.82	2.56	2.51
D	1.73	2.09	1.73	0.91	1.78	1.65
E	1.45	1.36	1.55	1.55	1.89	1.56
F	1.45	0.73	0.82	0.82	1.67	1.10
G	1.09	1.36	1.09	1.00	0.78	1.06
H	1.73	1.36	1.27	1.55	2.11	1.60
I	0.91	1.91	1.73	2.45	2.67	1.93
J	1.36	1.00	1.18	1.91	1.67	1.42
K	1.27	1.09	1.45	1.91	2.33	1.61
L	2.09	1.27	1.27	1.73	1.67	1.94

^a Site M results could not be normalized using Molecular Analyst DST software.

and microbiologists to limit the spread of MRSA. A quality assurance program is currently being developed to ensure that accurate data are generated from participating sites as the standardized method develops into a national surveillance program.

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