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Two Distinct Clones of Methicillin-Resistant *Staphylococcus aureus* (MRSA) with the Same USA300 Pulsed-Field Gel Electrophoresis Profile: a Potential Pitfall for Identification of USA300 Community-Associated MRSA[▽]

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Analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) characterized as USA300 by pulsed-field gel electrophoresis identified two distinct clones. One was similar to community-associated USA300 MRSA (ST8-IVa, t008, and Panton-Valentine leukocidin positive). The second (ST8-IVa, t024, and PVL negative) had different molecular characteristics and epidemiology, suggesting independent evolution. We recommend *spa* typing and/or PCR to discriminate between the two clones.

The methicillin-resistant *Staphylococcus aureus* (MRSA) clone USA300, having multilocus sequence type (MLST) ST8 and staphylococcal protein A (*spa*) type 008 and carrying staphylococcal cassette chromosome (SCC*mec*) IVa, has disseminated in the United States, as well as to other parts of the world (16, 26, 27). USA300 carries the *luk-PV* genes encoding Panton-Valentine leukocidin (PVL), has been identified in a variety of community populations, and has been associated with skin and soft tissue infections (SSTI), as well as more severe infections, such as sepsis, pneumonia, and necrotizing fasciitis (7, 22, 28).

The identification of USA300 isolates is primarily based on pulsed-field gel electrophoresis (PFGE) (10). Other genetic markers have also been suggested for identification of USA300 isolates, including (i) the *arcA* gene of the arginine catabolic mobile element (ACME) (3, 8, 26, 29), (ii) sequencing of the direct repeat unit (*dru*) region (9), and (iii) different USA300-specific multiplex PCRs targeting *luk-PV* and a "signature" six-AT-repeat sequence within the conserved hypothetical gene SACOL0058 (2).

In Denmark, MRSA isolates have been consecutively typed by PFGE since 1999, with the addition of sequence-based methods, such as MLST and *spa* typing, on selected isolates since 2001 (6). This process identified some of the first USA300 isolates in Europe but, surprisingly, also identified isolates with USA300 PFGE banding patterns but a different *spa* type (1, 15, 16). In this study, we investigated the epidemiology and genetic diversity of these isolates and USA300 and USA500 reference strains (20) using PFGE (23), *spa* typing (11), MLST (5), SCC*mec* typing (21, 24), *dru* typing (9), ACME (26), the six-AT signature sequence (2), detection of *luk-PV* (4), and antimicrobial susceptibility testing (Neo-Sensitabs), as well as microarray analysis (18, 19).

Clinical and epidemiological information was obtained consecutively (17). Infections were categorized into four different groups: import, hospital associated, community associated, and health care associated with community onset (14).

Where appropriate, statistical significance ($P < 0.05$) was assessed using the Mann-Whitney test or Fischer's exact test.

Between 1999 and 2006, 80 MRSA isolates from Denmark had USA300 PFGE profiles (50 representative PFGE profiles are shown in Fig. 1). However, by *spa* typing, two different *spa* types, t008 (11-**19**-12-21-17-34-24-34-22-25 [$n = 38$]) and the single repeat variant t024 (11-12-21-17-34-24-34-22-25 [$n = 42$]), both belonging to ST8, were identified (the extra repeat [19] is shown in boldface). All isolates typed as SCC*mec* IVa. However, significant genetic, epidemiological, and clinical differences were found, as shown in Tables 1 and 2. Patients infected with t008 isolates were significantly younger than patients infected with t024 isolates ($P < 0.01$). Patients acquired t008 MRSA in the community (50%) or through travel abroad (21%), and infections were predominantly SSTIs (94%). In contrast, the majority of patients with t024 MRSA were either hospitalized (26%) or had health care-associated risk factors (38%), and they presented with SSTIs (64%) but, also, a larger variety of infections, including fatal respiratory tract infections (11%) and operation- and procedure-related infections (11%). Remarkably, no t024 MRSA cases were imported.

In contrast to most t008 isolates, t024 isolates were often constitutively resistant to clindamycin and susceptible to kanamycin (Tables 1 and 2). Furthermore, t024 isolates did not

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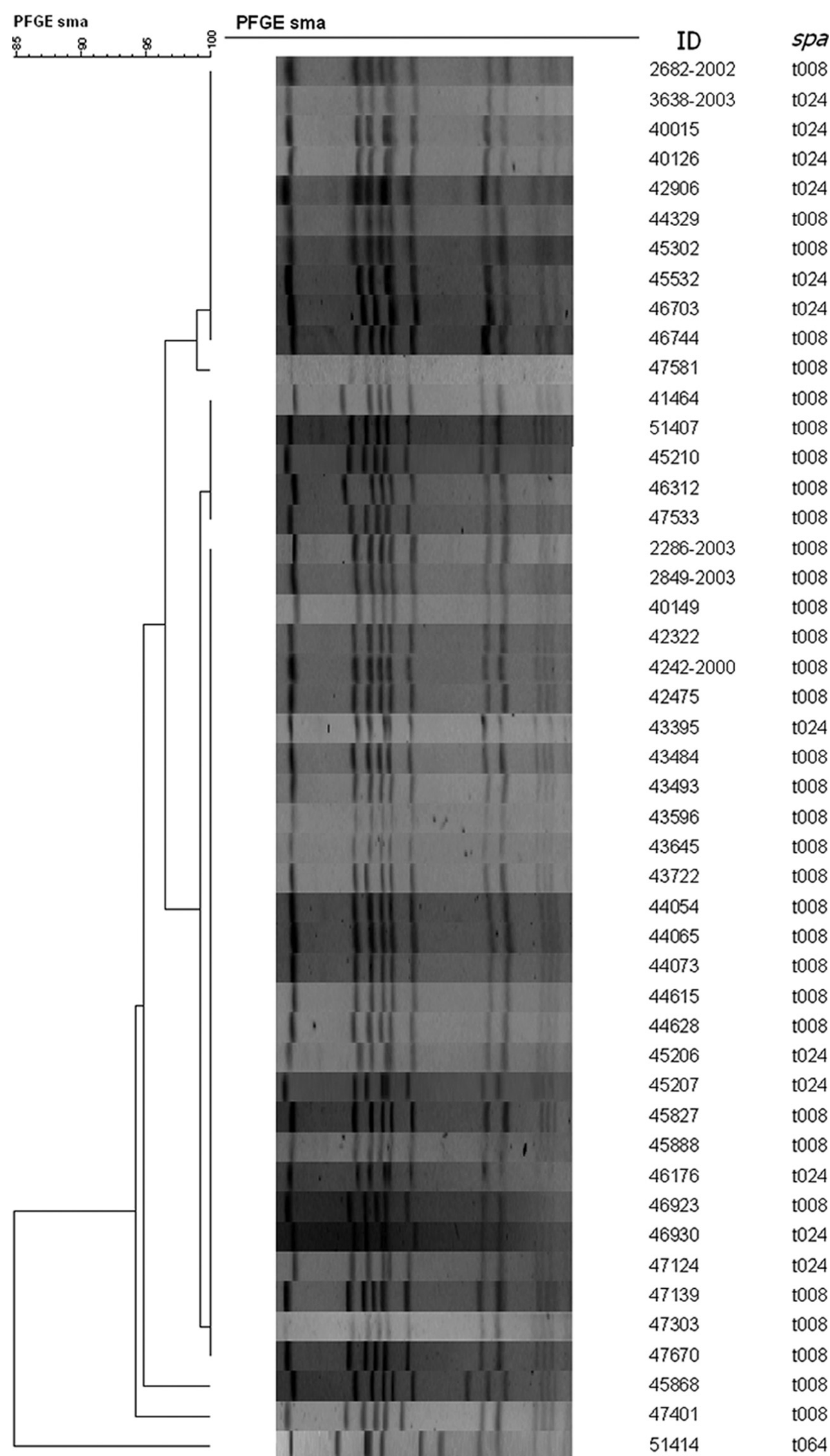

FIG. 1. *U*nweighted *p*air *g*roup *m*ethod with *a*rithmetic *m*ean (UPGMA) dendrogram of SmaI PFGE profiles from Danish MRSA isolates (2000 to 2005) constructed by the use of Dice determinations (optimization, 1.0%; tolerance, 2.1 to 3.1%). The reference strains are USA300 and USA500. Note that t008 and t024 isolates do not cluster separately by PFGE.

carry the *luk-PV* genes, exhibited different *dru* types, and inconsistently carried the ACME-related *arcA* gene and the conserved hypothetical gene SACOL0058 containing the six-AT-repeat sequence characteristic of typical t008 USA300 isolates (Table 1). Whole-genome microarray analysis of the CDC USA300 and USA500 reference strains and four clinical isolates, including two t008 (2849-2003 and 44073) and two t024 (45532 and 46703) isolates, revealed additional genetic differ-

TABLE 1. Bacteriological, epidemiological, and clinical data obtained for patients infected or colonized with either t008 or t024 in Denmark (1999 to 2005)

Characteristic[b]	t008 (n = 38)	t024 (n = 42)
Typing		
ST	8	8
SCC*mec*	IVa	IVa
luk-PV	+	−
ACME	+	+/−
dru type	7d, 7e, **9g**,[a] 9i, 10a	10a
Six-AT repeats	+	+/−
Resistance (%)		
Kanamycin	74	12
Tetracycline	8	5
Erythromycin	82	88
Clindamycin	13	86
Norfloxacin	42	55
Fusidic acid	5	12
Median ages of patients (yrs)*	31	72
Sources of isolates (%)		
Community associated#	50	2
Health care associated with community onset	18	38
Hospital associated#	5	26
Import#	21	0
Active surveillance culture#	5	33
Locations of infections (%)	n = 36	n = 28
Skin and soft tissue#	94	64
Respiratory tract	3	11
Deep seated	3	7
Operation and procedure related		11
Urinary tract		3.5
Other		3.5

[a] Boldface indicates the predominant *dru* type.
[b] *, #, statistically significant difference ($P < 0.05$) between t008 and t024 isolates with either Mann-Whitney U or Fischer's exact test, respectively.

ences. All isolates, including the USA500 reference strain, had a typical ST8 gene distribution pattern, including the genomic islands GIα and GIβ. However, a marked difference in the carriage of mobile genetic elements (MGEs) was observed, including resistance and putative virulence genes (Table 2). The t008 clinical isolates were very closely related by bacteriophage, *Staphylococcus aureus* pathogenicity island (SaPI), plasmid, transposon, and SCC*mec* element content and were virtually identical to the USA300 reference strain, except that the latter carried an additional tetracycline resistance cassette. In contrast, the t024 isolates, while very closely related in their core genomes, were distinct from the t008 isolates due to dissimilar bacteriophages, SaPI's, SCC*mec* elements, plasmids, and transposons (Table 2). These differences represent multiple horizontal gene transfer and/or rearrangement events, suggesting a substantial difference between t024 and t008 isolates. The USA500 reference isolate was distinct from the t008 and t024 isolates by PFGE profile (Fig. 1), *spa* type (t064), and MGE profile, especially regarding phage, SaPI, and transposon carriage.

These results suggest that two different clones with a typical USA300 PFGE pattern and SCC*mec* IVa are common in Denmark. Half of the isolates seem to be "classic" USA300 both epidemiologically and genetically (*spa* type t008, *luk-PV* positive, ACME positive, and with six or more AT repeats within SACOL0058), whereas the other half belong to a genetically and epidemiologically different clone principally characterized as *spa* type t024. This latter clone has recently been shown to be inadequately detected by the BD GeneOhm MRSA kit compared to the detection of t008 isolates, emphasizing sequence differences in the primer binding sites of the SCC*mec* right extremity junction (1). At present, t024 comprises 1.01% of the *spa* sequences deposited in the Ridom database (www.Ridom.de), originating from most of Europe, as well as the United States and Canada. However, not all t024 isolates are identical either, as a Dutch isolate with *luk-PV* has been identified (12).

The observations in this study suggest that reports of USA300 could include isolates with important genetic variations if PFGE, MLST, or SCC*mec* typing is the method used, as supported by findings of ACME- and Panton-Valentine leukocidin-negative USA300 isolates (10, 13).

Already, the need for more discriminatory methods has been debated in areas of high USA300 prevalence (25). The results of this study suggest that *spa* type t008 may identify the ST8

TABLE 2. Summary comparison of MGEs between isolates as detected by multistrain microarray

Strain	PFGE pattern	*spa* type	Variant; relevant gene(s) carried by indicated MGE[a]								
			Bacteriophage			SaPI1 (COL)	SCC*mec* IV (MW2)	Plasmid class		Tn*554*	Transposon
			ϕSa1	ϕSa2 (MW2)	ϕSa3			I (COL)	II (MW2)		
2849-2003	USA300	t008		v1; *luk-PV*	(MRSA252) *chp*, *scn*, *sak*	v1; *sek*, *seq*	v1		v1; *bla*, *cad*		
44073	USA300	t008		v1; *luk-PV*	(MRSA252) *chp*, *scn*, *sak*	v1; *sek*, *seq*	v1		v1; *bla*, *cad*		
45532	USA300	t024		v2		v2	v2		v2; *bla*, *cad*	*ermA*, *spc*	
46703	USA300	t024		v2		v2	v2		v2; *bla*, *cad*	*ermA*, *spc*	
USA300	USA300 reference	t008		v1; *luk-PV*	(MRSA252) *chp*, *scn*, *sak*	v1; *sek*, *seq*	v1	*tet*	v1; *bla*, *cad*		
USA500	USA500 reference	t064	(Mu50)	v3	(MW2) *scn*, *sak*	v3; *sek*, *seq*, *seb*, *ear*	v3		v3; *bla*, *cad*		(Mu50) *aacA*

[a] MGEs have been clustered into families according to the method of Lindsay and Holden (18), and the sequenced strain with the most closely matched MGE is given in parentheses (i.e., MRSA252, Mu50, COL, and MW2) (17). Note that the closest match is never identical, and there is substantial variation in MGEs between most *S. aureus* isolates. "v" indicates a variant compared to the other strains; strains with the same variant have the same v number. *aacA*, aminoglycoside resistance; *bla*, β-lactamase resistance; *chp*, chemotaxis inhibitory protein; *cad*, cadmium resistance; *ear*, putative β-lacatamase like protein; *ermA*, erythromycin resistance; *scn*, staphylococcal complement inhibitor; *sak*, staphylokinase; *seb*, enterotoxin B; *sek*, enterotoxin K; *seq*, enterotoxin Q; *spc*, spectinomycin resistance; *tet*, tetracycline resistance.

lineage related to community-associated MRSA infections more accurately than PFGE. A marker such as *luk-PV* is generally enough to identify "classic" USA300 isolates. Microarray analysis has revealed a range of other genes that could also be considered to discriminate isolates (Table 2). The results of this study clearly suggest that USA300 MRSA identified solely by PFGE should be confirmed by at least one PCR analysis, which could be for *luk-PV*, or a sequence-based typing method, such as *spa* typing.

Microarray data accession numbers. Fully annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-77; http://bugs.sgul.ac.uk/E-BUGS-77) and also ArrayExpress (accession number E-BUGS-77).

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REFERENCES

1. **Bartels, M. D., K. Boye, A. R. Larsen, R. L. Skov, and H. Westh.** 2007. Rapid increase of genetically diverse methicillin-resistant *Staphylococcus aureus*, Copenhagen, Denmark. Emerg. Infect. Dis. **13:**1533–1540.
2. **Bonnstetter, K. K., D. J. Wolter, F. C. Tenover, L. K. McDougal, and R. V. Goering.** 2007. Rapid multiplex PCR assay for identification of USA300 community-associated methicillin-resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45:**141–146.
3. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **367:**731–739.
4. **Dufour, P., Y. Gillet, M. Bes, G. Lina, F. Vandenesch, D. Floret, J. Etienne, and H. Richet.** 2002. Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. Clin. Infect. Dis. **35:**819–824.
5. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
6. **Faria, N. A., D. C. Oliveira, H. Westh, D. L. Monnet, A. R. Larsen, R. Skov, and H. de Lencastre.** 2005. Epidemiology of emerging methicillin-resistant *Staphylococcus aureus* (MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. J. Clin. Microbiol. **43:**1836–1842.
7. **Francis, J. S., M. C. Doherty, U. Lopatin, C. P. Johnston, G. Sinha, T. Ross, M. Cai, N. N. Hansel, T. Perl, J. R. Ticehurst, K. Carroll, D. L. Thomas, E. Nuermberger, and J. G. Bartlett.** 2005. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. Clin. Infect. Dis. **40:**100–107.
8. **Goering, R. V., L. K. McDougal, G. E. Fosheim, K. K. Bonnstetter, D. J. Wolter, and F. C. Tenover.** 2007. Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45:**1981–1984.
9. **Goering, R. V., D. Morrison, Z. Al Doori, G. F. S. Edwards, and C. G. Gemmell.** 2008. Usefulness of *mec*-associated direct repeat unit (*dru*) typing in the epidemiological analysis of highly clonal methicillin-resistant *Staphylococcus aureus* in Scotland. Clin. Microbiol. Infect. **14:**964–969.
10. **Goering, R. V., R. M. Shawar, N. E. Scangarella, F. P. O'Hara, H. Amrine-Madsen, J. M. West, M. Dalessandro, J. A. Becker, S. L. Walsh, L. A. Miller, S. F. van Horn, E. S. Thomas, and M. E. Twynholm.** 2008. Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials. J. Clin. Microbiol. **46:**2842–2847.
11. **Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel.** 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. **41:**5442–5448.
12. **Huijsdens, X. W., M. Janssen, N. H. Renders, A. Leenders, P. van Wijk, M. G. Santen Verheuvel, J. K. van Driel, and G. Morroy.** 2008. Methicillin-resistant *Staphylococcus aureus* in a beauty salon, the Netherlands. Emerg. Infect. Dis. **14**:1797–1799.
13. **Kennedy, A. D., M. Otto, K. R. Braughton, A. R. Whitney, L. Chen, B. Mathema, J. R. Mediavilla, K. A. Byrne, L. D. Parkins, F. C. Tenover, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo.** 2008. Epidemic community-associated methicillin-resistant *Staphylococcus aureus*: recent clonal expansion and diversification. Proc. Natl. Acad. Sci. USA 29. **105:**1327–1332.
14. **Klevens, R. M., M. A. Morrison, S. K. Fridkin, A. Reingold, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, G. Fosheim, L. K. McDougal, and F. C. Tenover.** 2006. Community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors. Emerg. Infect. Diseas. **12:**1991–1993.
15. **Larsen, A., R. Goering, M. Stegger, K. Boye, M. Sorum, H. Westh, and R. Skov.** 2007. Characterisation of an MRSA clone in Denmark similar to USA300 in PFGE pattern but with different properties: a potential pitfall when identifying USA300 isolates. Int. J. Antimicrob. Agents **29:**S441.
16. **Larsen, A., M. Stegger, R. Goering, M. Sorum, and R. Skov.** 2007. Emergence and dissemination of the methicillin resistant *Staphylococcus aureus* USA300 clone in Denmark (2000–2005). Euro. Surveill. **12:**22–24.
17. **Larsen, A. R., M. Stegger, S. Bocher, M. Sorum, D. L. Monnet, and R. L. Skov.** 2009. Emergence and characterization of community-associated methicillin-resistant *Staphylococcus aureus* infections in Denmark, 1999 to 2006. J. Clin. Microbiol. **47:**73–78.
18. **Lindsay, J. A., and M. T. Holden.** 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct. Integr. Genomics **6:**186–201.
19. **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds.** 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J. Bacteriol. **188:**669–676.
20. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J. Clin. Microbiol. **41:**5113–5120.
21. **Milheiriço, C., D. C. Oliveira, and H. de Lencastre.** 2007. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **51:**3374–3377.
22. **Miller, L. G., F. Perdreau-Remington, G. Rieg, S. Mehdi, J. Perlroth, A. S. Bayer, A. W. Tang, T. O. Phung, and B. Spellberg.** 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N. Engl. J. Med. **352:**1445–1453.
23. **Murchan, S., M. E. Kaufmann, A. Deplano, R. De Ryck, M. Struelens, C. E. Zinn, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, N. El Solh, C. Cuny, W. Witte, P. T. Tassios, N. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, I. Laconcha, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, G. Coombes, and B. Cookson.** 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J. Clin. Microbiol. **41:**1574–1585.
24. **Oliveira, D. C., and H. de Lencastre.** 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46:**2155–2161.
25. **Patel, M., K. B. Waites, C. J. Hoesley, A. M. Stamm, K. C. Canupp, and S. A. Moser.** 2008. Emergence of USA300 MRSA in a tertiary medical centre: implications for epidemiological studies. J. Hosp. Infect. **68:**208–213.
26. **Tenover, F. C., L. K. McDougal, R. V. Goering, G. Killgore, S. J. Projan, J. B. Patel, and P. M. Dunman.** 2006. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J. Clin. Microbiol. **44:**108–118.
27. **Tietz, A., R. Frei, and A. F. Widmer.** 2005. Transatlantic spread of the USA300 clone of MRSA. N. Engl. J. Med. **353:**532–533.
28. **Young, L. M., and C. S. Price.** 2008. Community-acquired methicillin-resistant *Staphylococcus aureus* emerging as an important cause of necrotizing fasciitis. Surg. Infect. **9:**469–474.
29. **Zhang, K., J. A. McClure, S. Elsayed, T. Louie, and J. M. Conly.** 2008. Novel multiplex PCR assay for simultaneous identification of community-associated methicillin-resistant *Staphylococcus aureus* strains USA300 and USA400 and detection of *mecA* and Panton-Valentine leukocidin genes, with discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. J. Clin. Microbiol. **46:**1118–1122.