Comparing the Utilities of Different Multilocus Sequence Typing Schemes for Identifying Outbreak Strains of *Mycobacterium abscessus* subsp. *massiliense*

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ABSTRACT Outbreaks of infections by *Mycobacterium abscessus*, particularly subspecies *massiliense*, are increasingly reported worldwide. Several multilocus sequence typing (MLST) protocols for grouping international outbreak strains have been developed but not yet directly compared. Using the three-gene (*hsp65*, *rpoB*, and *secA1*), seven-gene (*argH*, *cya*, *glpK*, *gnd*, *murC*, *pta*, and *purH*) and thirteen-gene (all of the preceding genes plus *gdhA*, *pgm*, and *pknA*) MLST schemes, we identified 22, 38, and 40 unique sequence types (STs), respectively, among a total of 139 nonduplicated *M. abscessus* isolates. Among subspecies *massiliense*, three-gene MLST not only clustered all outbreak strains together (in 100% agreement with the seven-gene and thirteen-gene schemes), but it also distinguished between two new STs that would have been grouped together by the seven-gene MLST but were distinct by the thirteen-gene MLST owing to differences in *hsp65*, *rpoB*, and *pknA*. Here, we show that an abbreviated MLST may be useful for simultaneous identification of *M. abscessus* the subspecies level and screening *M. abscessus* subsp. *massiliense* isolates with outbreak potential.

KEYWORDS multilocus sequencing, *Mycobacterium abscessus*

The *Mycobacterium abscessus* complex comprises three closely related genomospecies—*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*—that cannot be reliably discriminated by single gene sequencing (1–3). Previous studies have indicated great diversity within *M. abscessus* among cystic fibrosis patients, suggesting independent acquisitions from the environment (4, 5). However, suspicion of patient-to-patient transmission arose after two reports of respiratory outbreaks with *M. abscessus* subsp. *massiliense* at different cystic fibrosis centers across the Atlantic (6–8). One outbreak occurred in Seattle, WA, wherein the index case-patient and four additional patients were infected with nearly identical *M. abscessus* subsp. *massiliense* isolates with resistance to amikacin and clarithromycin and indistinguishable by repetitive unit sequence-based PCR (rep-PCR) patterns and pulsed-field gel electrophoresis (PFGE) analysis (9). The second outbreak occurred in Cambridge, United Kingdom, and involved 11 patients who all had *M. abscessus* subsp. *massiliense* infections sharing the same constitutive resistance to amikacin and clarithromycin, despite some individuals being naive to long-term macrolide or aminoglycoside therapy (6).

By whole-genome sequencing (WGS), isolates from these two cystic fibrosis centers were subsequently found to be highly related, belonging to sequence type 23 (ST23)

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and clonal cluster 3 (CC3) according to a multilocus sequence typing (MLST) protocol (7). Meanwhile, an epidemic of at least 2,032 postsurgical infections between 2004 and 2011 across Brazil was also due to *M. abscessus* subsp. *massiliense* ST23 (CC3), thereafter referred to as the “globally successful clone” (10, 11). Other outbreaks occurring after ultrasound-guided procedures, acupuncture, injections, dental, ophthalmological, cardiac, obstetric, and cosmetic surgeries due to *M. abscessus* continue to be reported worldwide (12–20).

However, outbreak investigations and comparisons of interrelatedness of outbreak strains by WGS and PFGE are too costly, lengthy, and labor-intensive for routine infection control surveillance. Hence, the aim of the present study was to identify a molecular typing method for *M. abscessus* that would be feasible in the context of epidemiology, postoutbreak surveillance, and the validation of new infection control measures. Although the optimal research method may be in evolution, such as 65-kDa heat shock protein analysis, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), next-generation sequencing, and WGS, we chose here to characterize MLST, since the MLST approach has been validated for determining subspecies of the *M. abscessus* complex (11, 21, 22). Three different MLST schemes, with three-gene, seven-gene, and thirteen-gene targets have been proposed in recent decades by different investigators for typing collections of clinical and environmental isolates of the *M. abscessus* complex, but these methods have not been directly compared (7, 11, 21).

The three-gene MLST scheme was developed in 2011 for the accurate determination of *M. abscessus* subspecies after the failure of single-gene targets, such as *16srRNA*, to reliably distinguish between *M. chelonae* and *M. abscessus*, followed by the failure of *rpoB*, *hsp65*, and *secA1* individual gene sequencing to reliably distinguish between subspecies due to the inferred horizontal transfer of genes between the closely related subspecies, especially from the more ancestral *M. abscessus* subsp. *abscessus* to the more recently emerged, *M. abscessus* subsp. *massiliense* (22–24). Shortly after publication, we used three-gene MLST to identify a clone of *M. abscessus* subsp. *massiliense*, TPE 101, that was determined to be identical by PFGE and rep-PCR as the cause of a multicenter outbreak of postprocedural infections related to the use of contaminated ultrasonography gel between 2010 and 2012 island-wide across Taiwan (12, 25).

The more usual seven-gene MLST scheme in bacterial taxonomy was developed to delineate microbial species within various taxonomic groups, including groups of highly recombinant bacteria, such as *Neisseria* spp., and to allow the assignment of unknown strains to species clusters over the Internet in a global collective electronic taxonomy (26). Typically, the seven-gene MLST scheme concatenated the sequences of between six to eight housekeeping genes that are present as a single copy within the genome and are not subject to selective pressure (26). For the *M. abscessus* complex, a seven-gene approach using the housekeeping genes *argH*, *cya*, *glpK*, *gnd*, *murC*, *pta*, and *purH* was published for nonoutbreak molecular epidemiological studies of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* in 2014 (with different numbering systems and protocols in two different databases (<https://bigsdbs.pasteur.fr/mycoabscessus/mycoabscessus.html> and <https://pubmlst.org/mabscessus/>) (11, 27). Notably, no strains of *M. abscessus* subsp. *bolletii* were included in these MLST databases. According to the publication of this MLST scheme, we used the same set of primers and conditions to identify our TPE 101 outbreak strain as ST48 by the former Pasteur Institute’s system, which differed by only one of seven MLST loci (*MurC* gene) from ST23.

However, due to the recognized differences between mycobacteria and more rapidly evolving bacteria, investigators studying trans-Atlantic cystic fibrosis outbreaks proposed simultaneously in 2014 an extended 13-gene MLST approach (incorporating the loci *cya*, *gdhA*, *argH*, *glpK*, *gnd*, *murC*, *pgm*, *pknA*, *pta*, *pur*, *rpoB*, *hsp65*, and *secA1*) alongside WGS to better characterize strains similar to the Seattle and Papworth outbreak strains (7). As far as we know, the merits of increasing the number of loci in the MLST approach from three mycobacterium-specific genes, seven generic housekeeping genes, and three mycobacterial and ten housekeeping genes have not been directly compared. In this study, we sought to study the

TABLE 1 Primers used to amplify each gene in the MLST schemes

Gene locus	Primers, sequences (5'–3')	Size (bp)	Analyzed fragment (5'–3')	Reference
<i>hsp65</i>	HSP65-F, ACCAACGATGGTGTGTCAT; HSP65-R, CTTGTGGAACCGCATACCCT	401	CGCCAAGGAG...GAGCTCACCG	21
<i>rpoB</i>	RPOB-F, GGCAAGGTACCCGAAAGG; RPOB-R, AGCGGCTGCTGGGTGATCATC	711	TGARACCGAG...GBCCGTACTC	21
<i>secA1</i>	SECA1-F, GACAGYGATGGATGGYCGSGTGACCCG; SECA1-R, GCGGACGATGARTCTTGTCSCG	466	CTTCCTVGG...RCTRTTCMMS	21
<i>argH</i>	ARGHF, GACGAGGGCGACAGCTTC; ARGHSR1, GTGCGCGAGCAGATGATG	480	GTGAGCACYAACGAAGGCTC...CGATCATGCCGGGCAAGACT	11
<i>cya</i>	ACF, GTGAAGCGGGCCAAAGAAG; ACSR1, AACTGGGAGGCCAGGAGC	510	CTGGTGGGTCCACCCAGTT...TKGCGCGCCGCGTACGCGC	11
<i>glpK</i>	GLPKSF1, AATCTACCGGGGTGTC; GLPKSR2, GGACAGACCCACGATGGC	534	GTGACAAATGCCAGTCGCAC...TGTTGCGCGCGTACTGGCGR	11
<i>gnd</i>	GDNF, GTGACGTGGAGTGGTTGG; GNDSR1, CTTGCGCTCAGGTCAAGTC	480	CARTTCRTTGAAGAYGTGCG...WCCGYAACGAAGTWAGGCG	11
<i>murC</i>	MURCSF1, CGGACGAAAGCGACGGCT; MURCSR2, CAAAACCTGCTGAGCC	537	CCGAACCTGATCRTCCTSAC...AGGTGCGYACRGTTGCTGCAG	11
<i>pta</i>	PTASF1, GATCGGGCGTCATGCCCT; PTASR2, ACAGGCACTGCTCTCCC	486	GACGTMCTACTSGCCGTGGC...AAATCCGYTCCCGTGCYGCC	11
<i>purH</i>	PURHSF1, CGGAGGCTTACCCTGGA; PURHSR2, CAGGCCACCGCTGATCTG	549	AAGGTTYTRGTGCCAAGGC...GCAAGAAGAAGCTGCGGCTG	11
<i>gdhA</i>	GDHAF, GTCAGTGCCCGATCGCTGDHASR1, GGCTCTCGGAGTACGTCGA	542	GTGACGCGGDCMGAAGGGTC...GAGCTCCCCCGCGTGTYY	7
<i>pgm</i>	PGMSF1, CCATTGAACCCGACCGG; PGMsr2, GTGCCAACGATCTCTCGG	559	TACCTGATCAGCTCCGGC...TCACCGAGGCCAGCCGTCG	7
<i>pknA</i>	PKNAF, CAGGTGGACTCGGACATG; PKNASR1, AACGAGGCGCCACCATC	457	CCGCCATAGCCGAGGATCTC...GCAGCCGGCTCGCSCGGCT	7

TABLE 2 Source and subspecies distribution of 139 *M. abscessus* isolates included in this study

Source	No. (%) of subspecies	No. (%) of <i>M. abscessus</i> strains	
		Subsp. <i>abscessus</i>	Subsp. <i>massiliense</i>
Clinical isolates	121 (87.1)		
Pulmonary	65 (50.4)	40 (61.5)	25 (38.4)
Extrapulmonary	56 (46.2)	12 (21.4)	44 (78.6)
Environmental isolates	16 (11.5)		
Ultrasonography gel	13 (81.3)	0 (0.0)	16 (100)
Hospital water	3 (18.7)	1 (33.3)	2 (66.7)
Reference standard isolates ^a	2 (1.4)	1 (50.0)	1 (50.0)
Total		54 (38.8)	85 (61.2)

^a*M. abscessus* subsp. *abscessus* ATCC 19977 and *M. abscessus* subsp. *massiliense* BCRC 16916.

discriminatory power of these three MLST schemes in discerning isolates from the previous outbreak, clustering within the “globally successful clonal cluster 3” from sporadic clinical and environmental isolates.

MATERIALS AND METHODS

Mycobacterial isolates. A total of 139 *M. abscessus* nonduplicated isolates were included in this study, comprising 121 clinical isolates, 16 environmental isolates, and 2 reference isolates (*M. abscessus* subsp. *abscessus* ATCC 19977 and *M. abscessus* subsp. *massiliense* BCRC 16916). Of these, 57 *M. abscessus* isolates were outbreak strains of *M. abscessus* subsp. *massiliense* ST48 (CC3), as reported previously, and 81 *M. abscessus* isolates were sporadic isolates that were confirmed to be unrelated to the outbreak by epidemiological investigation, PFGE, and rep-PCR (12, 25). Of the 121 clinical isolates, 65 were pulmonary isolates cultured from sputum ($n = 61$), bronchoalveolar lavage ($n = 2$), pleural effusion ($n = 1$) and biopsied lung tissue ($n = 1$), and 56 were extrapulmonary isolates cultured from the blood ($n = 10$), surgical wound ($n = 21$), cerebrospinal fluid ($n = 1$), ascites ($n = 2$), cornea ($n = 5$), endocervical swab ($n = 1$), biopsied lymph node ($n = 2$), ear ($n = 4$), and other skin and soft tissue ($n = 10$). Of the 16 environmental isolates, 13 were obtained from contaminated ultrasonography transmission gel (different batches and lot numbers) implicated in the nationwide outbreak, and 3 were obtained from routine infection control surveillance of hospital water at the National Taiwan University Hospital, a 2,500-bed teaching hospital in Taipei, Taiwan.

MLST. As described previously, molecular typing of the *M. abscessus* isolates was done by concatenating the partial sequences of three genes (*hsp65*, *rpoB*, and *secA1*) according to the methods of Zelazny et al. and Cheng et al. (21, 25), seven genes (*argH*, *cya*, *glpK*, *gnd*, *murC*, *pta*, and *purH*) according to the primers and conditions pioneered by Macheras et al. and publicly available at <http://bigsd.b.pasteur.fr/mycoabscessus/mycoabscessus.html> (11, 25), and thirteen genes (the above-mentioned ten genes plus *gdhA*, *pgm*, and *pknA*) according to the method of Tettelin et al. (7). Subspecies determination was secondarily confirmed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) (28).

Briefly, the mycobacterial strains were stored at -80°C in GermBank (Creative Life Sciences, New Taipei City, Taiwan). Prior to use, the strains were subcultured onto sheep blood agar at 30°C (Creative Life Sciences). Mycobacterial DNA was extracted using Tris-EDTA, lysozyme, and proteinase K (Uni-Onward Corp., New Taipei City, Taiwan). PCRs using the primers listed in the Table 1 were performed to amplify fragments of the thirteen genes. Sequences were analyzed for their similarity with sequences in the GenBank database using the Basic Local Alignment Tool (BLAST; <https://www.ncbi.nlm.nih.gov/BLAST>) and compared to type strains of *M. abscessus* subsp. *abscessus* (ATCC 19977) and *M. abscessus* subsp. *massiliense* (CIP 108297). Phylogenetic analysis conducted by the minimum-spanning tree algorithms based on the p distance of concatenated sequence data were performed using BioNumerics v6.6 (Applied Maths, Austin, TX).

RESULTS

Of the 139 *M. abscessus* isolates characterized in the previously published outbreak and case-control studies, 54 belonged to the subspecies *abscessus*, and 85 belonged to the subspecies *massiliense* (Table 2). Figures 1 and 2 and Table 2 show the results for 139 isolates discriminated based on the three-gene, seven-gene, and thirteen-gene MLST schemes. The 54 *M. abscessus* subsp. *abscessus* isolates were grouped into 10 sequence types, MAB1 to MAB10 by the three-gene scheme and into 24 and 25 sequence types, respectively, by the seven-gene and thirteen-gene schemes. Forty isolates clustered together as determined by the three-gene scheme (MAB1). Of these 40 MAB1 isolates, 22, including the ATCC 19977 reference strain, belonged to ST1 according to the seven-gene MLST database of the Pasteur Institute (Table 3). These MAB1/ST1 isolates exhibited different PFGE/rep-PCR patterns and were not epidemio-

FIG 1 Minimum-spanning tree of 139 *M. abscessus* complex isolates based on the three-gene and seven-gene MLST schemes. Strains clustered together by the three-gene scheme were depicted as circles, segments within the circles depict more than one isolate clustered together by the seven-gene schemes. Different colors within one circle represent different sequence types determined by the seven-gene MLST that were indistinguishable by the three-gene MLST.

logically linked (published previously) (12, 25). One ST1 isolate based on seven-gene scheme did not fall into the MAB1 main cluster due to a difference in the internal sequencing of the *secA1* gene alone (labeled MAB5 for the three-gene approach and ST1a for the thirteen-gene approach) (Table 4). This was the only additional sequence type gained from extending the MLST schemes from seven to thirteen genes (Fig. 1 and 2). For the remaining isolates, there was full agreement between the seven-gene and thirteen-gene schemes, i.e., the addition of *gdhA*, *pgm*, and *pknA* to the latter method did not increase its discriminatory power. Overall for *M. abscessus* subsp. *abscessus*, the agreement between the three-gene and seven-gene MLST schemes was 75%, the agreement between the three-gene and thirteen-gene MLST schemes was 77.5%, and the agreement between the seven-gene and thirteen-gene MLST schemes was 97.5%.

The 85 *M. abscessus* subsp. *massiliense* isolates were grouped into 12 sequence types, MMA1 to MMA12 by the three-gene scheme and consisted of 14 and 15 sequence types, respectively, as determined by the seven-gene and thirteen-gene schemes. Of 85 isolates evaluated, 57 isolates belonged to MMA1/ST48, with 100% agreement between the three MLST schemes (Table 3). Clear epidemiological links to

FIG 2 Minimum-spanning tree of 139 *M. abscessus* complex isolates based on the three-gene and thirteen-gene MLST schemes. Strains clustered together by the three-gene scheme were depicted as circles; segments within the circles depict more than one isolate clustered together by the thirteen-gene schemes. Different colors within one circle represent different sequence types determined by the thirteen-gene MLST that were indistinguishable by the three-gene MLST.

the 2010-2012 outbreak by case definition of contaminated ultrasonography gel or invasive procedures were established for 51 of these MMA1/ST48 isolates (as published previously) (12, 25, 29). The remaining six MMA1/ST48 isolates exhibited identical PFGE patterns but did not fit the case definition of the contaminated ultrasonography gel or invasive procedures (Table 3).

Ten of the eleven isolates grouped together in the second largest subspecies *massiliense* group (MMA2) by the three-gene scheme belonged to ST117 based on the seven-gene and thirteen-gene schemes (Fig. 1 and 2). The remaining isolate differed from ST117 in the *glpK* loci (Tables 2 and 3). The reference strain, *M. abscessus* subsp. *massiliense* BCRC 16916, was typed as MMA12/ST37 and was closely related to MMA2/ST117 (Fig. 1 and 2).

The overall rates of agreement between the three-gene and seven-gene MLST schemes, the three-gene and thirteen-gene MLST schemes, and the 7-gene and 13-gene MLST schemes for *M. abscessus* subsp. *massiliense* were 95.3, 96.5, and 98.8%, respectively. Two isolates of a new sequence type (ST273) based on the seven-gene scheme were discriminated into two clones by the three-gene scheme (MMA9 and

TABLE 3 Comparison of three-, seven-, and thirteen-gene MLST schemes for 139 *M. abscessus* isolates

Subspecies (n)	Subspecies and sequence type detection (n)		
	Three-gene MLST	Seven-gene MLST	Thirteen-gene MLST
<i>M. abscessus</i> subsp. <i>abscessus</i> (54)	MAB1 (40)	ST1 (22)	ST1 (22)
		ST22 (1)	ST22 (1)
		ST40 (1)	ST40 (1)
		ST63 (3)	ST63 (3)
		ST127 (5)	ST127 (5)
		ST276 ^c (1)	ST276 ^c (1)
		ST280 ^c (1)	ST280 ^c (1)
		ST272 ^c (1)	ST272 ^s (1)
		ST289 ^c (1)	ST289 ^c (1)
		ST277 ^c (1)	ST277 ^c (1)
	MAB2 (2)	ST283 ^c (1)	ST283 ^c (1)
		ST284 ^c (1)	ST284 ^c (1)
	MAB3 (4)	ST288 ^c (1)	ST288 ^c (1)
		ST126 (2)	ST126 (2)
	MAB4 (2)	ST33 (1)	ST33 (1)
		ST49 (2)	ST49 (2)
	MAB5 (1)	ST286 ^c (1)	ST286 ^c (1)
		ST137 (1)	ST137 (1)
	MAB6 (1)	ST278 ^c (1)	ST278 ^c (1)
		ST1 ^a (1)	ST1 ^a (1)
<i>M. abscessus</i> subsp. <i>massiliense</i> (85)	MMA1 ^b (57)	ST61 (1)	ST61 (1)
		ST282 ^c (1)	ST282 ^c (1)
	MMA2 (11)	ST281 ^c (1)	ST281 ^c (1)
		ST290 ^c (1)	ST290 ^c (1)
	MMA3 (1)	ST274 ^c (1)	ST274 ^c (1)
		ST48 ^b (57)	ST48 ^b (57)
	MMA4 (4)	ST117 (10)	ST117 (10)
		ST275 ^c (1)	ST275 ^c (1)
	MMA5 (3)	ST271 ^c (1)	ST271 ^c (1)
		ST23 (4)	ST23 (4)
	MMA6 (3)	ST176 (2)	ST176 (2)
		ST287 ^c (1)	ST287 ^c (1)
	MMA7 (1)	ST115 (2)	ST115 (2)
		ST285 ^c (1)	ST285 ^c (1)
	MMA8 (1)	ST279 ^c (1)	ST279 ^c (1)
		ST34 (1)	ST34 (1)
	MMA9 (1)	ST273 ^c (1)	ST273 ^c (1)
		ST291 ^c (1)	ST291 ^c (1)
	MMA10 (1)	ST279 ^{a,c} (1)	ST279 ^a (1)
		ST37 (1)	ST37 (1)

^aDenotes where typing results obtained by seven- and thirteen-gene MLST were not in agreement.

^bFor MMA1/ST48, all 57 outbreak isolates shared the same PFGE and rep-PCR patterns. Of the 57 isolates, 51 were obtained from contaminated ultrasonography or patients with documented exposure to invasive procedures following ultrasonography. Two isolates were from hospital water supplying two bronchoscopic units, three were from three patients' sputum, and one was from a wound without documented exposure to contaminated ultrasonography gel.

^cNew STs from this study that were submitted to the Pasteur Institute and assigned a new sequence number (ST271 to ST291).

MMA11) and by the thirteen-gene scheme (ST273 and ST273a) owing to single nucleotide polymorphisms (SNPs) in *hsp65*, *rpoB*, and *pknA* (Table 4).

In addition, the hot spots for genetic variation within the internal sequences of the 10 housekeeping genes differed between subspecies (Table 4). For subspecies *abscessus*, maximum sequence divergence was observed at the *pta*, *purH*, and *gdhA* loci (11 different allelic types). In contrast, the *glpK* loci were highly conserved, and 53 of 54 subspecies *abscessus* strains had the same allelic type for *glpK* loci. For subspecies *massiliense*, there were fewer sequence divergences overall, the greatest being for *purH* loci (seven allelic types), followed by *gdhA* loci (six allelic types). Unlike the subspecies *abscessus*, there was also significant variation at the *murC* loci (six allelic types) and for the *glpK* and *pknA* loci (five allelic types each).

In summary, among the subspecies *abscessus*, the three-gene scheme was only modestly discriminative compared to the standard seven-gene MLST (agreement rates of 75%); however, for the subspecies *massiliense* with identical PFGE and outbreak

TABLE 4 Key divergent loci for strains clustered together by the three-gene scheme^a

MAB or MMA ST	Locus no.												
	<i>hsp65</i>	<i>rpoB</i>	<i>secA</i>	<i>argH</i>	<i>cya</i>	<i>glpK</i>	<i>gnd</i>	<i>murC</i>	<i>pta</i>	<i>purH</i>	<i>gdhA</i>	<i>pgm</i>	<i>pknA</i>
MAB1 ST1	1	1	1	1	1	1	1	1	1	1	7	2	6
MAB5 ST1	1	1	3	1	1	1	1	1	1	1	7	2	6
MAB1 ST22	1	1	1	3	1	1	3	5	15	1	15	7	7
MAB1 ST40	1	1	1	3	11	1	3	3	18	3	11	9	8
MAB1 ST63	1	1	1	3	11	1	3	3	3	3	11	5	8
MAB1 ST127	1	1	1	3	23	1	3	3	3	3	11	5	8
MAB1 ST276	1	1	1	3	1	1	3	3	12	35	10	5	7
MAB1 ST280	1	1	1	3	14	7	3	3	6	36	11	5	8
MAB1 ST272	1	1	1	18	1	1	8	5	1	1	11	2	7
MAB1 ST289	1	1	1	18	32	1	3	5	1	1	17	9	7
MAB1 ST277	1	1	1	20	12	1	8	3	37	9	16	2	7
MAB1 ST283	1	1	1	18	32	1	1	5	1	1	17	9	7
MAB1 ST284	1	1	1	3	11	1	3	3	3	20	11	5	8
MAB1 ST288	1	1	1	3	11	1	3	3	32	3	11	5	8
MAB3 ST33	1	1	2	7	5	1	8	5	6	1	10	6	7
MAB3 ST49	1	1	2	3	11	1	3	3	5	10	11	8	8
MAB3 ST286	1	1	2	38	1	1	3	5	1	1	12	7	7
MAB4 ST137	1	2	2	3	12	1	3	5	1	10	9	2	7
MAB4 ST278	1	2	2	13	12	1	3	3	3	9	11	7	7
MMA2 ST117	m1	m2	m2	11	14	4	24	6	2	16	2	1	1
MMA2 ST275	m1	m2	m2	11	14	30	24	6	2	16	2	1	1
MMA5 ST176	m2	m4	m1	21	13	4	10	6	11	7	4	1	3
MMA5 ST287	m2	m4	m1	21	13	4	10	8	11	7	4	1	3
MMA6 ST115	m1	m4	m2	24	20	1	9	8	8	27	3	2	2
MMA6 ST285	m1	m4	m2	24	20	1	9	6	8	27	3	2	2
MMA9 ST273	m1	1	m2	11	20	1	9	8	8	27	3	2	2
MMA11 ST273a	1	m4	m2	11	20	1	9	8	8	27	3	2	1

^aThe maximum sequence divergence observed in the *pta*, *purH*, and *gdhA* genes for *M. abscessus* subsp. *abscessus* and in the *murC*, *purH*, and *gdhA* genes for *M. abscessus* subsp. *massiliense*. The least sequence divergence was observed in the *glpK* gene for *M. abscessus* subsp. *abscessus* and in the *argH*, *cya*, and *pgm* genes for *M. abscessus* subsp. *massiliense*. A lowercase "m"—e.g., m1, m2, m2, m4, etc.—denotes allelic type numbering for *M. abscessus* subsp. *massiliense*.

potential, the three-gene scheme yielded very high agreement rates with the standard seven-gene scheme (95.3%) and with the extended 13-gene scheme (96.5%).

DISCUSSION

Most experts now recommend identifying *M. abscessus* complex isolates to the subspecies level due to differences in antimicrobial susceptibility and prognosis (30). However, there is even greater pressure on clinical laboratories to fully identify *M. abscessus* subsp. *massiliense* following the emergence of a globally successful clone, ST23 or CC3, causing outbreaks among cystic fibrosis patients and soft tissue infections in Brazilian patients, as well as a closely related clone, ST48 (also CC3), causing outbreaks among Taiwanese patients following invasive procedures (7, 8, 12, 25). Despite the emphasis in recent guidelines on the necessity of screening all isolates of subspecies *massiliense* recovered from patients with cystic fibrosis for relatedness to outbreak strains in an effort to prevent future outbreaks and patient-to-patient transmission, there has been little practical advice on how to do so within the limits of clinical rather than research facilities (31).

Our study is the first to compare the use of three different MLST schemes for a very well-characterized collection of *M. abscessus* isolates, notably including both outbreak and sporadic isolates. We showed that for the newly emerged subspecies *massiliense*, with potentially higher virulence and transmissibility (7, 8, 32), a three-gene MLST using partial sequences of the *hsp65*, *rpoB*, and *secA1* genes (1,578 bp) was sufficiently discriminatory. This mycobacterium-specific three-gene MLST scheme identified subspecies and accurately delineated dominant clusters to >95% agreement with the seven-gene MLST (3,576 bp) and thirteen-gene MLST (6,712 bp) schemes.

A limitation of this study is the lack of WGS for full genomic comparison. However, the clonality of the isolates clustered together by the three-gene MLST had previously been validated by PFGE and rep-PCR using a DiversiLab mycobacterium typing kit (12,

25). Another limitation of this study is the lack of *M. abscessus* subsp. *bolletii* isolates; hence, we cannot make any comparisons for this subspecies. However, in most clinical reports, *M. abscessus* subsp. *bolletii* appears to be less frequently encountered as a human pathogen, and this subspecies is not included in the publicly available MLST databases, nor has it been implicated in outbreaks (27, 33, 34). Misidentification of subspecies using the three-gene scheme might have led to the absence of *M. abscessus* subsp. *bolletii*; however, we attempted to exclude this possibility by cross-checking subspecies identity by using MALDI-TOF (28). As the latter technique is fine-tuned, this limitation will become less prominent in the future.

The sensitivity and specificity of the three-gene scheme for simultaneous subtyping and screening of *M. abscessus* subsp. *massiliense* cannot be established by this preliminary study. Validation using larger collections of known outbreak versus sporadic collections is warranted. However, our collection was sufficiently diverse, including 21 novel sequence types submitted to the Pasteur Institute, each were recently assigned a new number from ST271 to ST291.

The three-gene MLST approach may offer time, costs, and labor savings over the seven-gene MLST scheme. In addition, accurate determination of subspecies (either by three-gene MLST scheme or by MALDI-TOF-MS) is required as an initial step before sequences can be compared to the publicly available MLST database at the Institute of Pasteur. Due to the minimal gains of extending the MLST scheme from seven to thirteen genes (only two additional sequence types were identified without a significant impact on the phylogenetic tree), our study supports the omission of *gdhA*, *pgm*, and *pknA* from current MLST schemes for *M. abscessus*. For confirmation of clonality or dominant clusters, the thirteen-gene MLST did not perform better than the seven-gene MLST. This may be due to extensive horizontal gene transfer of genomic blocks of housekeeping genes through distributive conjugal transfer (35). Taking into account the slow mutation rate of *M. abscessus*, only WGS or PFGE are recognized as having sufficient resolution to confirm an outbreak or person-to-person transmission.

Tettlin et al. previously identified signature SNPs in *rpoB* and *secA1* genes that were typical but not exclusive to the globally successful clonal cluster of subspecies *massiliense* outbreak strains (4). By using the *rpoB* gene MAB_3869c from the subspecies *abscessus* type strain described in the BRA-00 outbreak from Brazil, they showed that the Seattle and Papworth cystic fibrosis isolates carried two-*rpoB*-SNP signature (C→T at position 2569 and T→C at position 2760) and a *secA1* SNP signature (G→T substitution at position 820) by using the *secA1* gene MAB_3580c from the *M. abscessus* subsp. *abscessus* type strain). Similar to our findings wherein MMA1 by the three-gene MLST clustered all outbreak strains but also included six strains without clear epidemiological links but exhibiting the same PFGE patterns, the SNPs described for *rpoB* and *secA1* were not 100% specific markers for the outbreak strains and were found in four unrelated *M. abscessus* subsp. *massiliense* strains. Hence, we are in agreement over the value of the *rpoB* and *secA1* genes over other housekeeping genes for first-level identification of newly isolated strains as possibly being related to cystic fibrosis clusters or soft tissue outbreak strains, to be confirmed by a second assay.

Although Tettlin et al. suggested that partial sequencing of *rpoB* and *secA1* genes, should be followed by thirteen-target MLST analysis to rule out isolates as belonging to these two cystic fibrosis clusters, we have shown that a second assay based on another MLST approach with more routine loci targets did not outperform the first approach with mycobacterium-specific targets. Further studies are needed to elucidate a more appropriate second confirmatory assay that offers labor, time, and cost-savings over WGS and PFGE.

In conclusion, this preliminary study supports the utility of the three-gene MLST based on the partial sequences of the *hsp65*, *rpoB*, and *secA1* genes as a screening tool for the routine microbiology laboratory struggling to implement the recommendations of recent guidelines recognizing the outbreak and person-to-person transmission potential of *M. abscessus* subsp. *massiliense*. The next step would require development of a publicly available three-gene MLST database to corroborate previously identified

signature SNPs and to identify new patterns. With our collective efforts, accurate identification of *M. abscessus* subsp. *massiliense* with potentially higher transmissibility might soon fall within the scope of clinical practice in many more parts of the world.

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A.C. designed the study, analyzed the results, wrote the manuscript. H.-Y.S. provided critical analysis and review of the manuscript. Y.-T.T. conducted the experiments and analyzed the results. S.-Y.C. collected the mycobacterial isolates and helped execute the study. U.-I.W. helped collect mycobacterial isolates and execution of the study. P.-R.H. helped collect and analyze the mycobacterial isolates and critically reviewed the manuscript. W.-H.S. provided technical expertise, critique, and review of the manuscript. Y.-C.C. conceived the study, coordinated the infection prevention and control program and the clinical and laboratory research teams, provided technical expertise, critique, funding, and research assistance, and reviewed the manuscript. S.-C.C. provided technical expertise, critique, and review of the manuscript.

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