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Differentiation between persistent infection/colonization and re-infection/re-colonization of *Mycobacterium abscessus* isolated from patients in Northeast Thailand

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ABSTRACT

Mycobacterium abscessus can cause true infection or be present in the host as a harmless colonist. The ability of *M. abscessus* to cause disease and develop drug resistance is known to have a genetic basis. We aimed to differentiate between persistent infection and reinfection using multilocus sequence typing (MLST) and to study the genetic diversity of *M. abscessus* relative to multi-organ infection and drug resistance in Northeast Thailand. DNA was extracted from 62 *M. abscessus* isolates (24 cases). The following genes were sequenced: *argH, cya, glpK, gnd, murC, pta, purH* and *rpoB*. Drug susceptibility tests were performed using broth microdilution. Subspecies classification and phylogeny were determined. Among the 24 cases (62 isolates), 19 cases (49 isolates) were of true NTM infection and 5 cases (13 isolates) examples of colonization. Two subspecies, *M. abscessus* subsp. *massiliense* (12 cases, 32 isolates) and *M. abscessus* subsp. *abscessus* (12 cases, 30 isolates) were identified. The major sequence type (ST) was ST227. Two clonal groups among patients were found; clonal cluster I (5 cases, 8 isolates) and clonal cluster II (2 cases, 4 isolates) but no epidemiological link was apparent. Reinfection (2 cases with different clones of *M. abscessus* strains; > 9 SNPs different) and persistent infection (14 cases with the same clone; < 6 SNPs) were distinguished based on a phylogeny. Based on these SNP cutoff values, 3 cases of persistent colonization (same strain through time) and 2 cases of re-colonization (different strains through time) were identified. *M. abscessus* subsp. *abscessus* was significantly associated with clarithromycin resistance ($p < .001$) and multi-organ infection ($p = .03$). Molecular epidemiology based on MLST can be used to differentiate between reinfection vs persistent infection, persistent colonization vs re-colonization. ST227 was the main epidemic strain in Northeast Thailand.

1. Introduction

Since the early 1950s, it has been recognized that non-tuberculous mycobacteria (NTM) are causative agents of various human diseases. Although common in the environment and often present in immunocompromised patients, NTM are an important cause of morbidity and mortality in humans (Cassidy et al., 2009). NTM disease can be classified into four clinical entities; pulmonary disease (which is the most common), skin and soft tissue disease, lymph node disease and disseminated disease (Wu and Holland, 2015). The incidence and the prevalence of NTM disease are increasing worldwide (Ide et al., 2015; Shah et al., 2016).

In Southeast Asia including Thailand, the *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* are the most prevalent NTM species causing human diseases (Ide et al., 2015; Simons et al., 2011). The latter is associated with serious drug resistance problems and treatment failure (Nessar et al., 2012). Outbreaks of such a pathogen obviously present an important public health threat (Nunes Lde et al., 2014; Smith et al., 2016).

NTM can be found in the environment and can colonize the human body without causing disease. Acquisition of *M. abscessus* might be from the environment or from other hosts. A single strain of *M. abscessus* can persist in a human host with accumulation of spontaneous mutations through time (Sapriel et al., 2016), or might be replaced by another

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strain. However, the replacement of one strain by another within a single host has never been investigated. Human-to-human transmission of *M. abscessus* has been reported (Bryant et al., 2013) but is controversial and needs further investigation.

Like other NTM, *M. abscessus* causes chronic infectious disease requiring treatment for up to 2 years. Over the course of such a lengthy treatment, the pathogen might be derived from the same clone that initially infected the patient or reinfection may have occurred with different strains of *M. abscessus* from other sources. However, this possibility has never been investigated. The rate of *M. abscessus* reinfection in Asia, including Thailand, is unknown.

The ability of *M. abscessus* to cause disease in humans and to exhibit drug resistant phenotypes is known to have a genetic basis. Three subspecies of *M. abscessus* (subsp. *abscessus*, subsp. *bolletii* and subsp. *massiliense*) are generally recognized (Tortoli et al., 2016). Previously, *M. abscessus* subspecies associated with stable progressive disease of the nodular bronchiectatic type (Shin et al., 2013). However, any association between genotype and organ-specific infection has never been investigated. Several studies have identified strains/subtypes of *M. abscessus* associated with drug resistance phenotypes (Jeong et al., 2017; Kim et al., 2016; Kim et al., 2015). However, genetic variants of *M. abscessus* associated with drug resistance in Thailand have never been investigated.

To address some of these research gaps, we aimed to differentiate between persistent infection and reinfection using the multilocus sequence typing (MLST) technique and to identify genetic variants of *M. abscessus* associated with specific sites of infection and drug-resistant phenotypes. This study could provide information relating to association between genotype (strain/subspecies), organ-specific infection and drug resistance, as well as demonstrating a way to differentiate between reinfection and persistent infection.

2. Methods

2.1. Study population

All consecutive patients receiving medical care for NTM at Srinagarind Hospital, Khon Kaen Province, Thailand during 2012–2016 were recruited. Srinagarind Hospital is a super-tertiary University Hospital and the largest hospital in Northeast Thailand, serving patients from several provinces there. Clinical specimens (totaling 62) from 24 patients (from single or multiple organs) were included in the study. Nineteen of 24 patients were defined as having NTM disease according to case-definition criteria. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE591454).

2.2. Case definitions

True cases of *M. abscessus* infection were defined as symptomatic patients receiving medical care with the isolation of NTM from sterile sites (i.e. bone and joint samples, blood/ bone marrow samples, eye, lymph node and pleural fluid samples). In the case of *M. abscessus* isolated from non-sterile sites (especially pulmonary sites), the criteria in the ATS/IDSA guidelines 2007 were adopted (Griffith et al., 2007), including exclusion of active tuberculosis; radiological data: a medical record with a specific diagnosis of *M. abscessus* infection made by physician and/or a record of receiving appropriate relevant antibiotics (i.e. clarithromycin, azithromycin, amikacin, cefoxitin, ciprofloxacin, doxycycline, ethambutol, isoniazid, imipenem, moxifloxacin, levofloxacin, ofloxacin, rifabutin, rifampicin, trimethoprim/sulfamethoxazole and tobramycin) (Burgess et al., 2014). Cases from which *M. abscessus* was isolated, but which did not otherwise match the definition (above) of true cases of *M. abscessus* infection, were defined as examples of colonization.

Multi-organ infections are here defined as infections occurring in different organs. Reinfection is defined as the isolation of different strains from the patient through time. Re-colonization is the isolation of different colonizing strains from an individual at different times. Persistent infection is isolation of the same strain in a patient through time. Persistent colonization is isolation of the same colonizing strain through time.

2.3. Culture, identification and DNA extraction from M. abscessus

All 62 *M. abscessus* isolates from the 24 patients were retrieved from archived stock cultures. The species identification of *M. abscessus* was performed using INNO-LiPA Mycobacteria v2 (Innogenetics GmbH, Heiden, Germany), Genotype *Mycobacterium* CM/AS assay (Hain Lifescience GmbH, Nehren, Germany) or Molecutech REBA Myco-ID (YD Diagnostics CORP, Gyeonggi-do, Korea). All *M. abscessus* isolates were re-subcultured on Lowenstein-Jensen (LJ) solid medium and then incubated at 37 °C for 7 days. Genomic DNA of all bacterial isolates was extracted from multiple loopfuls of colonies using the cetyl-trimethylammonium bromide-sodium chloride (CTAB) method (De Almeida et al., 2013).

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was based on the broth microdilution method using SENSITITRE™ plate (TREK Diagnostic Systems, Ohio, USA). The manufacturer's protocol was followed. Briefly, a cell suspension was prepared by transferring multiple colonies of *M. abscessus* into demineralized water and dispersing cells by shaking. Cell density was adjusted to a 0.5 McFarland Standard. Fifty µl of the suspension was transferred into a tube of cation-adjusted Mueller-Hinton broth with TES buffer to give an inoculum of 5×10^5 CFU/ml. Then, 100 µl of inoculum broth was added to the 96-well plates containing different concentrations of antibiotics. The plates were covered using adhesive seal and incubated at 30 °C for 72 h. The results were interpreted according to CLSI M24 guidelines (CLSI, 2011).

2.5. Multilocus sequence typing (MLST)

Sequences from the seven standard housekeeping genes were used for MLST (Table 1). In addition, a specific region (positions 2556 to 3278; 723 bp) of the *rpoB* gene (RNA polymerase β subunit), located outside the drug resistance-associated region, was included. All genes were amplified using the primer sets shown in Table 1.

The PCR reactions (final volume 50 µl) were prepared with 25 µl 2× Quick Taq HS DyeMix (TOYOBO.CO., LTD, Osaka, Japan), 18 µl distilled water, 10 pmol/µl of each primer and 5 µl DNA templates (40 ng/µl). The PCR conditions were modified according to conditions outlined at the MLST website (http://bigsdb.pasteur.fr/mycoabscessus/primers_used.html). The PCR conditions for amplification of *rpoB* were as follows; pre-denaturation at 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 67 °C for 1 min, 72 °C for 1 min and final elongation at 72 °C for 10 min. The PCR products were visualized using 2% agarose gel electrophoresis and sent for gene sequencing (Bio Basic Inc., Ontario, Canada). For each gene, an alignment was produced using the Seaview4 package (Davidson et al., 2014). Included were reference sequences from the MLST database (http://bigsdb.pasteur.fr/mycoabscessus/mycoabscessus. html) The sequence data were submitted to the MLST Sequence Query website (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db = pubmlst_mycoabscessus_seqdef_public&page = sequenceQuery). The allelic number and sequence type (ST) were assigned.

2.6. Phylogenetic analysis

Phylogenetic relationship analysis was analyzed using the maximum likelihood method with the general time reversible (GTR) model and gamma distribution implemented in Seaview4 package software

Table 1
Primers used for multilocus sequence typing (MLST).

Genes	Primers	Sequences (5′ to 3′)	Sequence lengths (bp)	Genomic positions[a]	References
argH	ARGHF	GACGAGGGCGACAGCTTC	480	2,398,010–2,399,431	(Macheras et al., 2009)
	ARGHSR1	GTGCGCGAGCAGATGATG			
cya	ACF	GTGAAGCGGGCCAAGAAG	510	487,968–489,527	(Macheras et al., 2009)
	ACSR1	AACTGGGAGGCCAGGAGC			
glpK	GLPKSF1	AATCTCACCGGCGGTGTC	534	377,959–379,473	(Macheras et al., 2009)
	GLPKSFR2	GGACAGACCCACGATGGC			
gnd	GNDF	GTGACGTCGGAGTGGTTGG	480	2,473,091–2,474,545	(Macheras et al., 2009)
	GNDSR1	CTTCGCCTCAGGTCAGCTC			
murC	MURCSF1	CGGACGAAAGCGACGGCT	537	2,007,075–2,008,547	(Macheras et al., 2009)
	MURCSR2	CCAAAACCCTGCTGAGCC			
pta	PTASF1	GATCGGGCGTCATGCCCT	486	4,294,451–4,296,532	(Macheras et al., 2011)
	PTASR2	ACGAGGCACTGCTCTCCC			
purH	PURHSF1	CGGAGGCTTCACCCTGGA	549	1,073,939–1,075,519	(Macheras et al., 2011)
	PURHSR2	CAGGCCACCGCTGATCTG			
rpoB	MycoF	TCCGATAGGTGCTGGCAGA	940	3,915,755–3,919,270-	(Luo et al., 2016)
	MycoR	ACTTGATGGTCAACAGCTCC			

argH = argininosuccinate lyase, *cya* = adenylyl cyclase, *glpK* = glycerol kinase, *gnd* = 6-phosphogluconate dehydrogenase, *murC* = UDP *N*-acetylmuramate-L-Ala ligase, *pta* = phosphate acetyltransferase, *purH* = phosphoribosylaminoimidazole carboxylase ATPase subunit and *rpoB* = RNA polymerase β-subunit.
[a] Genomic position according to *M. abscessus* ATCC 19977 (GenBank accession no. NC_010397).

(Gouy et al., 2010). Bootstrap confidence values were based on 1,000 replications. Reference strains *M. abscessus* ATCC19977, *M. abscessus* FLAC047 and *M. abscessus* 50594 were used. Phylogenetic trees were constructed using *M. chelonae* CCUG47445 as an outgroup.

2.7. Data analysis

Minimum inhibitory concentration (MIC) results of antimicrobial susceptibility tests were calculated as mean and percentage. Comparison of the mean MIC level between two groups was performed using independent *t*-tests or Mann Whitney *U* tests. Antibiotic sensitivity analysis for *M. abscessus* infections in 24 cases was performed using chi-square or Fisher's exact tests. $P < .05$ was considered statistically significant. All statistical analyses were performed using SPSS version 17.0.

3. Results

3.1. Demographic data of patients

Nineteen of the 24 cases were defined as true NTM infections and 5 cases defined as examples of colonizations. In the 19 true *M. abscessus* infections, isolates were from tracheal suction and neck pus (1 case), eye (1 case), humerus tissue and other tissue sources (1 case), sputum (10 cases), blood and bone marrow (1 case), lymph node and blood (1 case), back pus and pus from other tissues (1 case), lymph node and sputum (2 cases), lymph node and pleural fluid (1 case). All 5 cases in the colonization group had been previously treated for TB; *M. abscessus* was isolated from their pulmonary specimens (tracheal suction and sputum). The distribution of the studied population in provinces within Northeast Thailand is shown (Fig. 1)

3.2. Subspecies classification based on rpoB gene sequence

Subspecies of *M. abscessus* based on *rpoB* gene sequences were determined. Two groups, corresponding to *M. abscessus* subsp. *massiliense* (12 cases, 32 isolates) and *M. abscessus* subsp. *abscessus* (12 cases, 30 isolates), were identified (Fig. 2). Based on *rpoB* gene sequences, the *M. abscessus* subsp. *bolletii* was absent from our studied population.

3.3. Genetic diversity of M. abscessus based on MLST

All 62 isolates (24 cases) were assigned into STs according to the MLST method based on 7 standard gene sequences (Fig. 3). Comparisons with the MLST database showed that the most common STs were ST227 (5 cases, 8 isolates), ST173 and its variants (4 cases, 11 isolates), ST243 and its variants (2 cases, 6 isolates) and ST242 and its variants (3 cases, 6 isolates).

3.4. Association between M. abscessus genotypes and multi-organ infection or colonization

Nineteen cases (49 isolates) were regarded as true *M. abscessus* infections. In 11 (30 isolates) and 8 (19 isolates) of these cases, the isolates fell into *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus*, respectively. In 8 cases (24 isolates), true *M. abscesses* infection was identified at > 1 organ site (multi-organ infection). In 6 of these cases (13 isolates), the isolates belonged to *M. abscessus* subsp. *abscessus* and in 2 cases (11 isolates), the isolates belonged to *M. abscessus* subsp. *massiliense*. A significantly higher proportion of *M. abscessus* strains causing multi-organ infection belonged to *M. abscessus* subsp. *abscessus* (13/19 isolates, 68.42%) rather than *M. abscessus* subsp. *massiliense* (11/30 isolates, 36.67%) (*p*-value = .030).

The presence of *M. abscessus* in 5 cases appeared to be due to colonization only. In 2 cases ($n = 5$ isolates) and 3 cases ($n = 8$ isolates), the isolates belonged to *M. abscessus* subsp. *massiliense* and subsp. *abscessus*, respectively. There was no association between the subspecies in which an isolate fell and whether it caused true infection or colonization only (p-value = .141).

3.5. Distinguishing between reinfection and persistent infection of M. abscessus

Serially isolated *M. abscessus* were analyzed from patients with a long duration of treatment (16 cases, 43 isolates) and from individuals with colonizing strains (5 cases, 13 isolates). Out of 16 true infection cases, 2 cases (patient#8 and #14) had different clones of *M. abscessus* strains (> 9 SNPs and 1 case had a different ST) (Fig. 3). These were regarded as examples of reinfection. Isolates from each of the remaining 14 belonged to the same clone (< 6 SNPs), indicating persistent infection. In the reinfection cases, isolates were sampled > 82 days apart.

We also investigated the genetics of 5 *M. abscessus* colonization cases (13 isolates). In 3 cases, the clone present did not change through time (0 SNPs in patient#3, 1 SNP in patient#20 and 1–2 SNPs in patient#24), leading to a definition of persistent colonization. Interestingly, 2 cases (patient#15 and #18) did exhibit different strains through time, with 26 (different STs) and 9 different SNPs, respectively. These were defined as examples of re-colonization (Fig. 3).

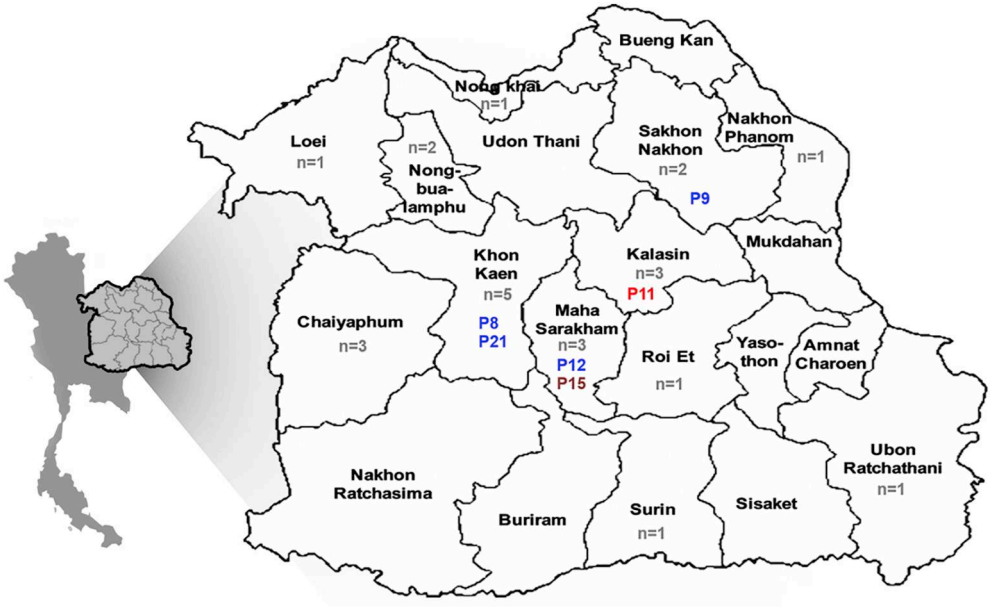

Fig. 1. Geographical distribution of the studied population (24 cases). Clonal clusters of patients based on a phylogeny of 7 genes (clonal cluster I; blue letters and clonal cluster II; red letters) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Cluster analysis of M. abscessus serially isolated from individual infected patients

Based on cluster analysis of 7 genes, 2 clonal clusters (based on almost-identical MLST patterns from different patients) were found. Clonal cluster I comprised of 5 patients (8 isolates) and clonal cluster II comprised of 2 patients (4 isolates) (Fig. 4). Most patients lived in different provinces within Northeast Thailand. Five patients had *M. abscessus* isolated from sputum specimens and one patient had strains isolated from blood. The putative relationship of strains from each of the two clonal clusters, according to the dates of isolation, is shown (Fig. 4). The longest time interval between sampling of strains within a clonal cluster was 3 years.

3.7. Association between genotypes of M. abscessus and drug-resistant phenotypes

Sixty-two *M. abscessus* isolates from 24 patients were tested for drug susceptibility. Amikacin was the most effective antibiotic with a high susceptibility rate that did not significantly differ between the two subspecies (32/35 isolates susceptible in *M. abscessus* subsp. *massiliense*, 91.43% and 20/27 isolates susceptible in *M. abscessus* subsp. *abscessus*, 74.07%). Frequency of resistance to clarithromycin was significantly different between *M. abscessus* subsp. *massiliense* and subsp. *abscessus* (8/35 isolates, 22.86% vs 19/27 isolates, 70.37%) ($p \leq .001$) as was MIC level ($p \leq .001$) (Table 2). Isolates in both subspecies showed various degrees of susceptibility to doxycycline, cefoxitin, imipenem, minocycline, moxifloxacin, linezolid, trimethoprim/sulfamethoxazole and tobramycin (sensitivities ranging from 60% to 100%) (Table 2).

There was no significant difference in the proportion of strains resistant to clarithromycin that caused reinfection versus persistent infection (3/5 isolates, 60% vs 13/38 isolates, 34.21%) ($p = .344$). There was no significant difference in the proportion of strains resistant to clarithromycin that caused true infection versus colonization only (19/49 isolates, 38.78% vs 8/13 isolates, 61.54%) ($p = .141$).

4. Discussion

M. abscessus infection is a public health problem worldwide and an important cause of morbidity and mortality (Cassidy et al., 2009). The current drug-resistance problem and treatment failure of *M. abscessus* is a worldwide threat, including in Thailand (Imwidthaya et al., 1990; Phowthongkum et al., 2005). Molecular typing is a useful tool for outbreak investigation, allowing us to discriminate between reinfection and persistent infection cases and permitting investigation below the species level (Van Soolingen, 2001). The MLST technique has been successfully used for molecular epidemiology and for studies on evolution of various virulent bacterial species as well as analysis of population structure (Macheras et al., 2014). Here, we applied MLST for molecular epidemiology and to investigate the genetic variants that might be associated with disease characteristics.

A previous study suggested that molecular analysis of the 723-bp *rpoB* sequence is a rapid and accurate tool for identification of rapidly growing mycobacteria (Adekambi et al., 2003). Many studies have used this approach for subspecies identification (Macheras et al., 2011) (Macheras et al., 2009). Our phylogenetic trees based on this 723 bp *rpoB* gene sequence, which lies outside the drug resistance hotspot region (Nasiri et al., 2016), identified 2 subspecies corresponding to *M. abscessus* subsp. *massiliense* and subsp. *abscessus*. No *M. abscessus* subsp. *bolletii* was found in our studied population. We also performed MLST based on the 7 standard genes. Strains from one patient (patient#13) differed in placement between the *rpoB*-based tree and the 7-gene tree. This result supported a previous report of putative horizontal gene transfer of the *rpoB* gene between strains (Macheras et al., 2014): a tree based on a single gene might not accurately identify the subspecies of *M. abscessus*. We used the tree based on 7 genes for cluster analysis and association analysis.

For the sequence types (STs) analysis based on the standard 7-gene-MLST pattern, we found that the most common ST was ST227 (exact match to MLST database), followed by ST173 and its variants, ST243 and variants and ST242 and variants. The sequence variants were similar to, but not identical with, *M. abscessus* strains to which ST numbers had been assigned in the MLST database (https://pubmlst.org/mabscessus/). Previously, ST1 and ST23 of *M. abscessus* were the main epidemic strains in Europe and Brazil (Macheras et al., 2014) as well as in Shanghai, China (Luo et al., 2016). A study from Ireland reported the commonest STs there were ST1, ST26, ST126 and ST22 (*M. abscessus* subsp. *abscessus*) and ST23 (*M. abscessus* subsp. *massiliense*) (O'Driscoll et al., 2016). In Korea, ST6 (*M. abscessus* subsp. *abscessus*) and ST10 (*M. abscessus* subsp. *massiliense*) were predominant (Kim et al., 2013). The

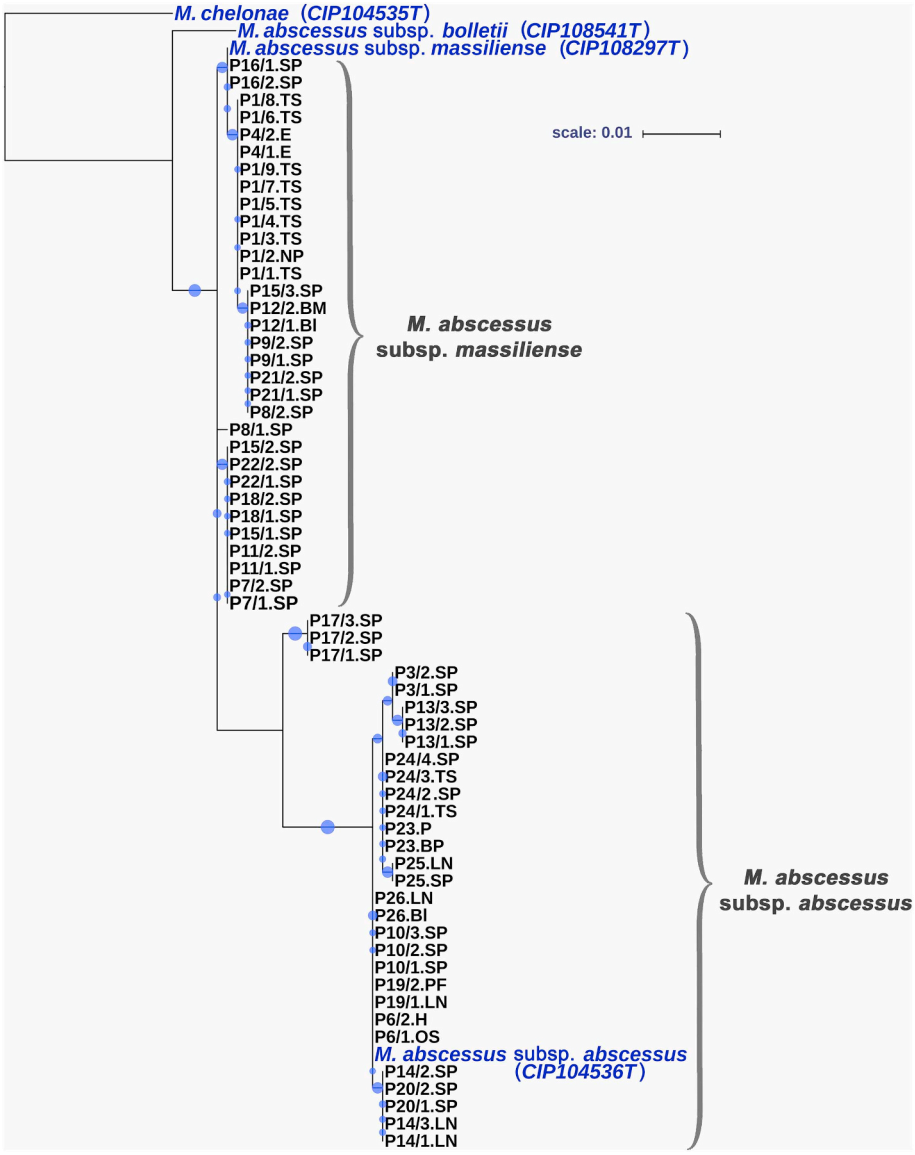

Fig. 2. Phylogenetic tree inferred from a 723-bp *rpoB* conserved region of 62 *M. abscessus* isolates using the maximum likelihood method. All 62 strains were classified into either *M. abscessus* subsp. *massiliense* or subsp. *abscessus*. This bootstrap consensus tree was inferred from 1000 replicates. Blue circles refer to bootstrap values and the size of each circle is proportional to its value (the largest blue circle indicates a value of 100%). TS (tracheal suction), E (eye), SP (sputum), H (humerus tissue), Bl (blood), BM (bone marrow), LN (lymph nodes), PF (pleural fluid), BP (back pus) and P (pus from other tissues). *Mycobacterium abscessus* subsp. *abscessus* CIP104536^{T}, *M. abscessus* subsp. *massiliense* CIP108297^{T} and *M. abscessus* subsp. *bolletii* CIP108541^{T} (accession numbers EU109308, EU109307 and EU109306, respectively) were included as reference strains. The reference strains were obtained from previous studies (Adekambi et al., 2003; Adekambi and Drancourt, 2009; Adekambi et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epidemic strain in Thailand is different from those in Europe and from elsewhere in Asia. The STs of *M. abscessus* might be associated with population and geographical region.

M. abscessus has the ability to infect various organs and to cause disseminated multi-organ infection (Chetchotisakd et al., 2000; Moe et al., 2018; Tahara et al., 2016). To our knowledge, no study has investigated the association between genetic variant of *M. abscessus* and ability to cause multi-organ infection or true infection/colonization. We found that *M. abscessus* subsp. *abscessus* was more often associated with the ability to cause multi-organ infection compared to subsp. *massiliense*. It can be inferred that subspecies of *M. abscessus* differ in virulence and ability to cause the disseminated disease. We found no association between subspecies of *M. abscessus* and incidence of true infection (19 cases) versus colonization (5 cases).

No evidence of re-infection caused by *M. abscessus* has been reported previously. In our study, we serially isolated strains from 16 *M. abscessus* infection patients undergoing long periods of treatment. Based on the 7-gene-sequence tree, 14 cases were of persistent infection and 2 experienced reinfection. Some reinfection cases were supported by the distinctly different antibiogram. In the reinfection cases, sampling interval was > 82 days. Such an interval is adequate for the occurrence of reinfection.

NTM can colonize humans from the environment or other sources without causing disease. Theoretically, the colonizing strain might persist or might be replaced by others through time. The incidence rates of NTM colonization and disease have both increased significantly in recent times in Taiwan (Lai et al., 2011). Admixed (mosaic) subspecies of *M. abscessus* have been detected, significantly associated with cystic fibrosis patients with lung infections or chronic colonization (Sapriel et al., 2016). However, there have been no previous reports of one colonizing bacterial strain replacing another through time. In 2 of our 5 *M. abscessus* colonization cases, strains were replaced through time. These two re-colonization cases had different clones (9–26 SNPs difference and 1 case had a different ST). The 3 persistent colonization cases had the same clone of colonized strains with 0–2 SNPs difference. Our colonization cases therefore exhibited several possible scenarios: persistence of a single strain, replacement of one strain by another and presence of more than one strain simultaneously in the host.

There have been reports of possible human-to-human transmission of *M. abscessus* among patients with cystic fibrosis (Bryant et al., 2013; Harris et al., 2015). Eleven patients formed 2 clonal clusters (based on whole-genome sequencing) of *M. abscessus* subsp. *massiliense* (Bryant et al., 2013). Another study also reported 2 clonal clusters; the first cluster was a sibling pair and the second cluster consisted of 2

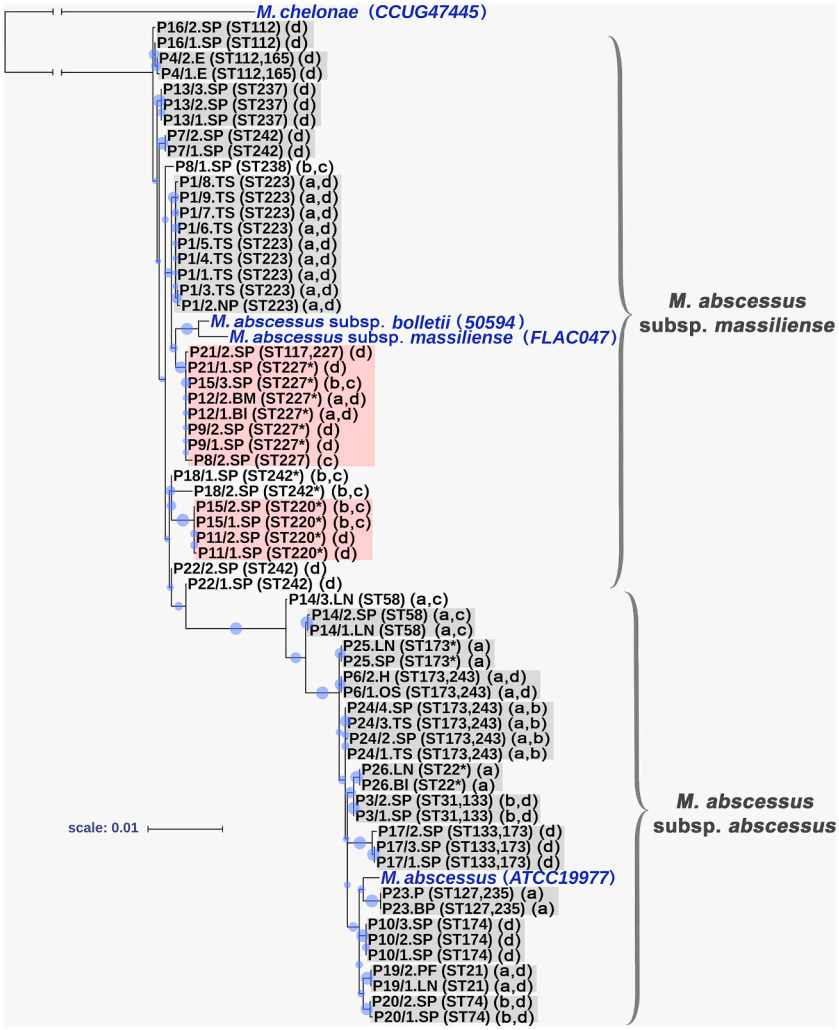

Fig. 3. Distribution of sequence types (STs) based on sequences from 7 genes (4123 bp). The phylogenetic tree was inferred from sequences of 62 *M. abscessus* isolates using the maximum likelihood method. All 62 strains were classified into either *M. abscessus* subsp. *massiliense* or subsp. *abscessus*. A bootstrap consensus tree was inferred from 1000 replicates. Blue circles refer to bootstrap values and the size of each circle is proportional to its value (the largest blue circle indicates a value of 100%). Sequences of *M. chelonae* were used as the outgroup. Reference sequences of various subspecies from GenBank are indicated by their accession numbers. * Exact match of the sequences of 7 loci to numbered STs from the MLST database. In some cases, our sequences were equally close to two STs in the MLST database. In such cases, both STs are listed in the tree. Grey boxes enclose clonal strains isolated at different times from the same patient. Red boxes enclose clonal strains possibly transferred among patients. "a", "b", "c" and "d" in brackets refer to strains causing multi-organ infection (a), colonization (b), strains that split from cluster of the same patient (c) and strains causing persistent infection (d), respectively. TS (tracheal suction), E (eye), SP (sputum), H (humerus tissue), Bl (blood), BM (bone marrow), LN (lymph nodes), PF (pleural fluid), BP (back pus) and P (pus from other tissues). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individuals in which genetically very similar strains were present but there was no apparent epidemiological link between the patients (Harris et al., 2015). A previous study investigating the genetics of *M. abscessus* serially isolated from individual infected/colonized patients found that strains isolated at different time points were generally unique (Jonsson et al., 2007). This implies re-acquisition from the environment rather than human-to-human transmission (Jonsson et al., 2007). In our study, we found that most *M. abscessus* strains serially isolated from individual infected patients formed a cluster indicating persistent infection or colonization. We found 2 clonal groups of very closely related strains (≤1 SNP difference) from different patients; clonal group I (5 patients, 8 isolates, ST227) and clonal group II (2 cases, 4 isolates, ST220). However, there was no apparent epidemiological like between individuals: most of the patients lived in different provinces and time intervals of isolation among patients (2 pairs of clonal strains were isolated 1–3 years apart) seemed too great. In addition, the clonal strains were isolated from sputum of 5 cases supporting the inhalation route, except one case from blood. Therefore, human-to-human transmission among the patients was hardly supported by our results. Previous studies, including ours, did not sample environmental strains from the vicinity of patients. Therefore, acquisition from the same environmental source such as fomite (Malcolm et al., 2017) cannot be excluded. At this stage, we cannot discriminate between a common environmental source or human-to-human transmission. Further study using whole-genome sequencing techniques, which provide the highest possible discriminatory power, could help to clarify these points.

We investigated subspecies of *M. abscessus* associated with drug susceptibility pattern. A study from Brazil reported 43 isolates *M. abscessus* subsp. *bolletii* 100% resistant to ciprofloxacin, doxycycline, moxifloxacin, sulfamethoxazole and tobramycin, but fully susceptible to amikacin and partially (14%) resistant to clarithromycin (Nunes Lde et al., 2014). Inducible resistance to clarithromycin of *M. abscessus* subsp. *abscessus,* which was not found in subsp. *massiliense*, can be detected after 14-days incubation (Jeong et al., 2017; Kim et al., 2016; Kim et al., 2015). We found that subsp. *abscessus* was significantly associated with resistance to clarithromycin compared to subsp. *massiliense*. We also compared the antibiotic resistance pattern between persistent vs reinfection, true infection vs colonization, but no statistical differences were found from the comparisons.

Limitations of our studies must be noted. MLST has lower discriminatory power than PFGE (Machado et al., 2014). Nonetheless, we tried to maximize the performance of this method by adding sequences from an additional gene. This allowed us to discriminate between reinfection and re-colonization. The studied population and *M. abscessus* isolates in our study were from a single hospital (Srinagarind Hospital). However, this hospital is a super-tertiary hospital serving all provinces in Northeast Thailand and our study can represent molecular epidemiological data for *M. abscessus*.

5. Conclusion

In conclusion, we discriminated between reinfection and persistent infection, and re-colonization vs persistent colonization by *M. abscessus*

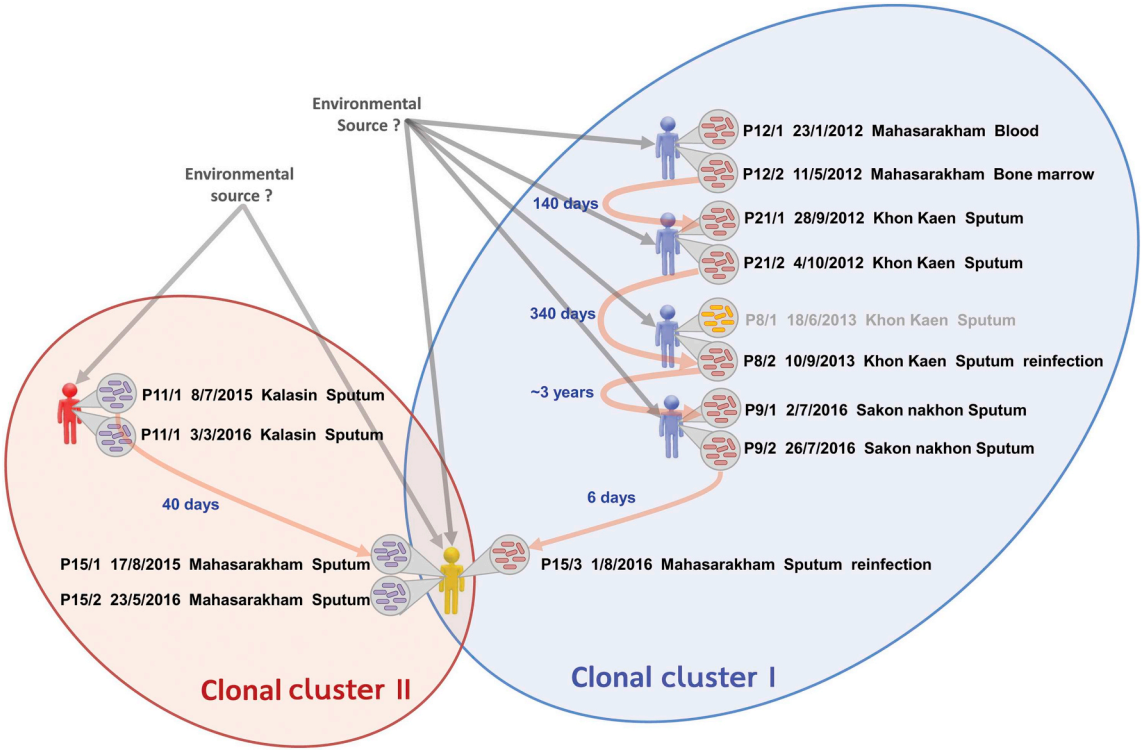

Fig. 4. Clonal isolates from *M. abscessus*-infected cases and possible relationship among strains. Clonal cluster I contained 5 patients and clonal cluster II contained 2 patients. Isolates from both clonal clusters were obtained from a single patient. The dates of isolation, province where each patient resided and specimen type are provided. Possible common environmental sources of infection are indicated.

Table 2
Comparison of antibiotic resistance patterns between clusters/subspecies of *M. abscessus* based on MLST (7-gene-based tree).

DST	Number (%) of resistant isolates			Average MIC level (ug/ml)		
	Subsp. *massiliense* (*n* = 35)	Subsp. *abscessus* (*n* = 27)	*p*-Values	Subsp. *massiliense* (n = 35)	Subsp. *abscessus* (n = 27)	p-Values
Amikacin	1 (2.86)[a]	2 (7.41)	0.575	12.11	17.19	0.431
Cefoxitin	31 (88.57)	21 (77.78)	0.308	120.69	112.59	0.238
Ciprofloxacin	35 (100)	27 (100)	–	4	4	1.000
Clarithromycin	8 (22.86)	19 (70.37)	< **0.001**	4.24	9.62	< **0.001**
Doxycycline	33 (94.29)	24 (88.89)	0.645	15.2	14.41	0.425
Imipenem	35 (100)	27 (100)	–	60.34	62.81	0.272
Linezolid	23 (65.71)	17 (62.96)	0.822	25.71	24.59	0.7
Minocycline	33 (94.29)	25 (92.59)	1.000	7.94	7.52	0.508
Moxifloxacin	35 (100)	25 (92.59)	0.186	7.89	7.56	0.39
Tobramycin	35 (100)	26 (96.3)	0.435	15.09	14.07	0.238
SXT	35 (100)	27 (100)	–	8	8	1.000

Bold letters refer to significant p-Values.
[a] Trimethoprim/sulfamethoxazole.

based on the MLST method. We demonstrated the presence of 2 major subspecies (subsp. *abscessus* and subsp. *massilense*) and the predominance of ST227 in our region. *Mycobacterium abscessus* subsp. *abscessus* was associated with multi-organ infection and clarithromycin resistance but no association between drug resistance and re-infection or colonization was found. No association between subspecies of *M. abscessus* and reinfection, colonization was found.

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Competing interests

The authors declare there are no competing interests.

Authors' contribution

KF designed the study, acquired funding, supervised the research assistant (IK) and interpreted the results. IK, PrC and PA collected microbiological data. KF and IK analyzed the data and wrote the manuscript. PlC, WN and WR provided overall guidance, assisted the result interpretation and co-supervised IK. KF prepared the text of the paper. All authors approved the final manuscript.

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