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High rate of reinfection and possible transmission of *Mycobacterium avium* complex in Northeast Thailand

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ABSTRACT

The *Mycobacterium avium* complex (MAC) includes two main species of non-tuberculous mycobacteria (NTM), *M. avium* and *Mycobacterium intracellulare*. These can cause serious disease, especially in immunocompromised patients. Little information is available concerning genetic diversity of NTM. We used multilocus sequence typing (MLST) based on a highly discriminative gene set to analyze MAC serially isolated from patients to determine the rate of MAC reinfection. Genomic DNA was sequenced from 49 MAC isolates (15 cases comprised of 11 true infections and 4 instances of colonization). More than half of the MAC isolates tested were found to be multidrug resistant. The discriminatory power was assessed of 24 house-keeping genes (*fusA, atpD, pheT, glnA, topA, secA, argH, glpK, murC, cya, pta, rrl, rrs, hsp65, rpoB,* 16S-23S rRNA ITS, *recF, lipT, pepB, gnd, aspB, groEL, sodA* and *est)* previously used for genotyping of MAC and other NTM. Seven genes (*fusA, secA, rpoB, hsp65, 16S rRNA, 23S rRNA*, 16S-23S rRNA ITS) had a discriminatory power index higher than 0.9 and were included in the optimized set that we used. This set was significantly better for genotyping and diagnosis of MAC than previously used 4-gene, 5-gene and 9-gene sets. MLST using our 7-gene set indicated that the rate of reinfection was 54.55% (6/11 cases). Persistent infections ($n = 5$ cases, 45.45%) were found. A changing of clone in the same patient was found in 1/4 (25%) of the colonization cases. Two small clusters of possible MAC transmission between humans were found. Our study demonstrated that the high frequency of apparent treatment failure of MAC might be artefactual, as a consequence of a high rate of MAC reinfection in Thai population. Our useful highly discriminative gene set for MAC species and clonal strain analysis could be further applied for the diagnosis and patient management.

1. Introduction

Nontuberculous mycobacteria (NTM) are aerobic, acid-fast bacilli belonging to the family Mycobacteriaceae. The *M. avium* complex (MAC) is among the disease-causing NTM [1]. Members of the MAC are slow-growing opportunistic pathogens [2] that can cause human diseases including pulmonary disease, skin and soft-tissue infection and disseminated infections [3]. The two recognized species in the complex, *M. avium* and *Mycobacterium intracellulare*, can be found in common environments such as soil and natural waters [1,4]. *M. avium* is further subdivided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* [1]. Identification of MAC species and strains is important for definite diagnosis and patient management. MAC is associated with antibiotic resistance [5]. Treatment of MAC infections is complicated and expensive [1]. Treatment results are poor with success rates about 40% [6]. However, the factors associated with MAC treatment difficulty are still unclear.

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Table 1
Characteristics of MAC isolates used in this study.

Patient No.	Locations	Dates of collection	Sample sites	MAC species (LPA)	True infection / colonization	Disease types
Patient1.1	Nong Khai	19/5/2014	Knee fluid	*Mycobacterium intracellulare*	True infection	Disseminated
Patient1.2		25/8/2014	Knee fluid	*M. intracellulare*		
Patient1.3		23/9/2014	Synovial fluid	*M. intracellulare*		
Patient2.1	Buriram	6/12/2013	Sputum	*Mycobacterium avium*	True infection	Pulmonary
Patient2.2		19/12/2013	Sputum	*M. avium*		
Patient3.1	Kalasin	12/9/2012	Neck (Pus)	*M. intracellulare*	True infection	Skin
Patient3.2		20/9/2012	Tissue	*M. intracellulare*		
Patient3.3		20/9/2012	Pus	*M. intracellulare*		
Patient3.4		9/10/2012	Arm	*M. intracellulare*		
Patient4.1	Khon Kaen	2/3/2016	Sputum	*M. intracellulare*	Pulmonary colonization	
Patient4.2		26/4/2016	Sputum	*M. intracellulare*		
Patient5.1	Mahasarakham	25/5/2016	Skin (Tissue/Biopsy)	*M. avium*	True infection	Skin
Patient5.2		24/6/2016	Tissue	*M. intracellulare*		
Patient6.1	Khon Kaen	15/2/2013	Sputum	*M. intracellulare*	Pulmonary colonization	
Patient6.2		11/3/2013	Sputum	*M. intracellulare*		
Patient7.1	Khon Kaen	31/5/2016	Tracheal suction	*M. intracellulare*	Pulmonary colonization	
Patient7.2		22/6/2016	Sputum	*M. intracellulare*		
Patient8.1		18/9/2014	Fluid	*M. intracellulare*		
Patient8.2	Yasothon	18/11/2014	Cheeks	*M. intracellulare*	True infection	Disseminated
Patient8.3		20/3/2015	Pus from wound swab	*M. intracellulare*		
Patient9.1		10/6/2015	Sputum	*M. intracellulare*		
Patient9.2	Khon Kaen	8/7/2015	Sputum	*M. intracellulare*	True infection	Pulmonary
Patient9.3		5/8/2015	Sputum	*M. intracellulare*		
Patient10.1		19/12/2012	Sputum	*M. intracellulare*		
Patient10.2		20/3/2013	Sputum	*M. intracellulare*		
Patient10.3		24/2/2014	Sputum	*M. intracellulare*		
Patient10.4		14/5/2014	Sputum	*M. intracellulare*		
Patient10.5	Khon Kaen	29/7/2014	Sputum	*M. intracellulare*	True infection	Pulmonary
Patient10.6		21/11/2014	Sputum	*M. intracellulare*		
Patient10.7		10/2/2015	Sputum	*M. intracellulare*		
Patient10.8		22/7/2015	Sputum	*M. intracellulare*		
Patient10.9		14/1/2016	Sputum	*M. intracellulare*		
Patient11.1		4/7/2014	Sputum	*M. avium*		
Patient11.2	Khon Kaen	15/12/2014	Sputum	*M. avium*	True infection	Pulmonary
Patient11.3		20/2/2015	Sputum	*M. avium*		
Patient12.1	Khon Kaen	5/10/2015	Sputum	*M. avium*	Pulmonary colonization	
Patient12.2		18/4/2016	Sputum	*M. avium*		
Patient13.1		2/2/2016	Fluid	*M. intracellulare*		
Patient13.2		8/3/2016	Knee fluid	*M. intracellulare*		
Patient13.3	Khon Kaen	23/5/2016	Knee fluid	*M. intracellulare*	True infection	Disseminated
Patient13.4		26/5/2016	Elbow (Pus)	*M. intracellulare*		
Patient13.5		28/6/2016	Knee fluid	*M. intracellulare*		
Patient14.1		7/6/2014	Chest (Pus)	*M. avium*		
Patient14.2		9/6/2014	Inguinal abscess	*M. avium*		
Patient14.3	Nakhon Phanom	10/3/2015	Pus from wound swab	*M. avium*	True	Disseminated
Patient14.4		5/5/2016	Elbow (Pus)	*M. avium*		
Patient14.5		13/5/2016	Synovial fluid	*M. avium*		
Patient15.1	Nongbua Lamphu	21/8/2012	Stool	*M. avium*	True	Disseminated
Patient15.2		12/6/2013	Bone Marrow (Pus)	*M. avium*		

Multilocus sequence typing (MLST) is a common tool used to genotype MAC isolates. Various combinations of genes have been used in the past for this purpose. These include a four-gene set (*rpoB, hsp65, 16S rRNA,*16S–23S rRNA ITS) [7], five-gene set (*recF, lipT, pepB, gnd1, est*) [8] and a nine-gene set (*recF, lipT, pepB, gnd1, est, aspB, sodA, groEL1, hsp65*) [9]. However, these gene sets have never been assessed for their discriminatory power or evaluated using samples serially isolated from the same patient. One previous study investigated serial isolates of MAC from 49 patients in Korea based on the four-gene set and reported a high reinfection rate (73%) [7]. Such a high reinfection rate needs to be confirmed in a different population.

Distinguishing between the two species of MAC is generally based on differences in the rRNA genes. A commercial line-probe assay kit is usually used in the clinical laboratory for MAC identification [10]. One such kit is the GenoType *Mycobacterium* Assay, which is based on 23S rRNA gene sequences. Sequences of a set of genes including *rpoB*, *16S rRNA*, *23S rRNA*, *hsp65* and 16S-23S rRNA ITS also showed a high utility for MAC species identification [7,11]. Since MAC can show genetic differences in different geographical regions [12], it is necessary to evaluate these approaches in Southeast Asia.

Exposure to environmental sources has been suggested as the main route of MAC infection [13]. Transmission of other NTM between humans may be possible [14,15], but has not yet been reported for MAC.

We aimed to optimize MLST, testing the utility of different sets of genes to identify MAC. We then wished to use the optimized set to analyze the genetic diversity of MAC in Thailand and to characterize serial isolates of MAC from the same patient. We also aimed to evaluate the performance of MLST based on various gene sets for species identification relative to results from the GenoType *Mycobacterium* Assay.

2. Methods

2.1. Study population and classification

Forty-nine serial isolates of *M. avium* complex (MAC) came from 15 patients at Srinagarind Hospital, Khon Kaen Province, Northeast Thailand during the period 2012 to 2016. Age, gender, locations (provinces) and other details for all 15 patients are summarized in Table 1. Ages of patient ranged from 27 to 91 years (with an average of 55 years). Seven were men (four <60 and three ≥60 years of age) and

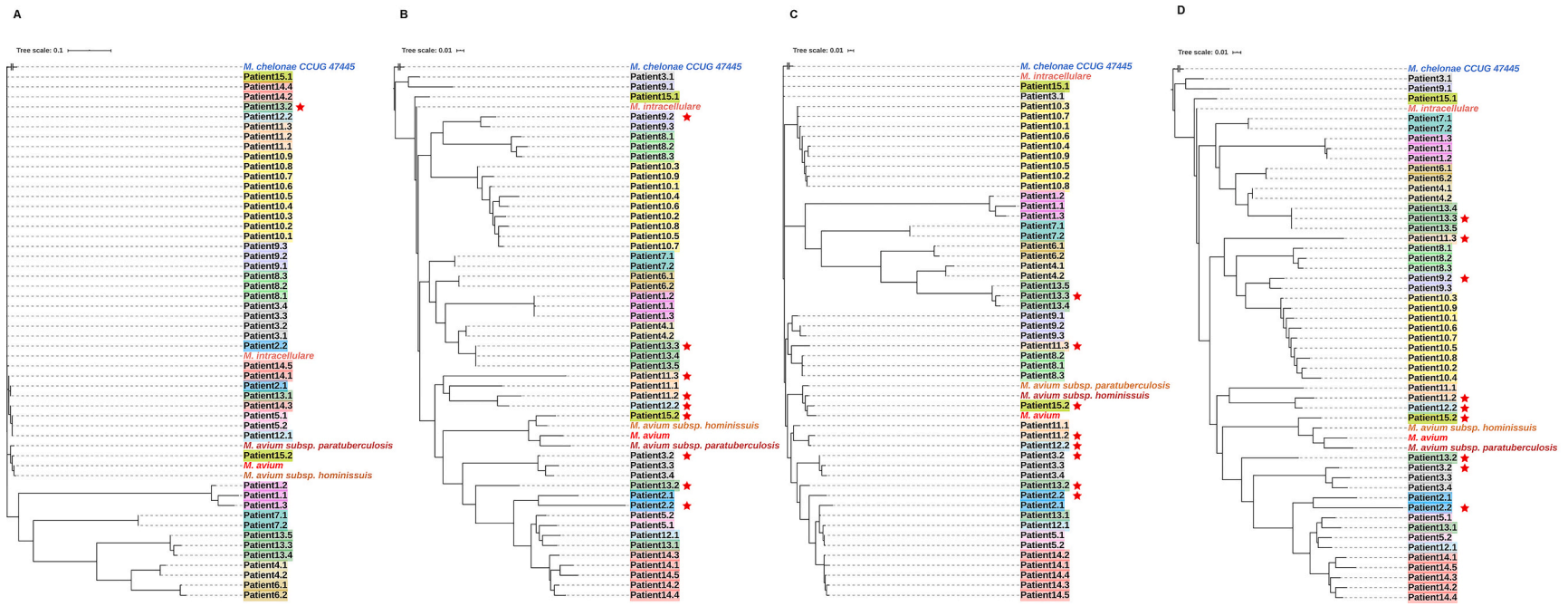

Fig. 1. Phylogenetic trees based on 5-gene set (248 SNPs) (**A**), 4-gene set (457 SNPs) (**B**), 9-gene set (476 SNPs) (**C**) and optimized 7-gene set (925 SNPs) (**D**). These bootstrap consensus trees were inferred from 1000 replicates. Different highlight colors represent the isolates from each patient. One isolate, 9 isolates, 8 isolates and 9 isolates were identified as examples of reinfection (red stars) based on the trees inferred from the 5-gene, 4-gene, 9-gene and the optimized 7-gene sets. Reinfection was identified when serial isolates collected from the same patient fell on different branches in the tree (refer to SNP distances shown in Fig. 2**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

Table 2
Pairwise comparisons of the phylogenetic interpretations based on 4-gene, 5-gene and 9-gene sets, and the optimized 7-gene set.

Characteristics	% Concordance					
	4 genes vs. 5 genes	4 genes vs. 7 genes	4 genes vs. 9 genes	5 genes vs. 7 genes	5 genes vs. 9 genes	7 genes vs. 9 genes
Species identification	58.62% (17/29 isolates)	96.67% (29/30 isolates)	96.67% (29/30 isolates)	60% (18/30 isolates)	60% (18/30 isolates)	100% (30/30isolates)
Mycobacterium intracellulare	45% (9/20 isolates)	100% (19/19 isolates)	100% (19/19 isolates)	47.37% (9/19 isolates)	47.37% (9/19 isolates)	100% (19/19 isolates)
Mycobacterium avium	53.06% (26/49 isolates)	97.96% (48/49 isolates)	97.96% (48/49 isolates)	55.10% (27/49 isolates)	55.10% (27/49 isolates)	100% (49/49 isolates)
Total						
Reinfection*	11.11% (1/9 isolates)	100% (9/9 isolates)	88.89% (8/9 isolates)	11.11% (1/9 isolates)	11.11% (1/9 isolates)	88.89% (8/9 isolates)

* Agreement between the two methods in recognition of reinfection cases.

eight were women (five <60 and three ≥60 years of age). The patients were from many provinces in the region: Khon Kaen, Kalasin, Nong Khai, Nong Bua Lamphu, Yasothon, Mahasarakham, Buriram and Nakhon Phanom. The isolates were taken from specimens such as sputum, tracheal suction, neck (pus), stool, synovial fluid, skin, cheek (pus) and other tissues. Cases of true infection were identified on the basis of isolation of NTM from sterile sites (i.e., bone joint samples and blood: 13 isolates) and/or the criteria in ATS/IDSA guidelines [16]. Briefly, these criteria included availability of radiological data, exclusion of tuberculosis and isolation of three or more sputum specimens for acid-fast bacilli analysis. Additionally, the relevant antibiotic treatment history was also available for the cases of true infection. The study protocol was approved by KKU Human Ethics committee (No. HE591454).

2.2. Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) was determined using a SLOMYCOI Sensititre 96-well plate (TREK Diagnostic Systems, Ohio, USA) following the manufacturer's protocol. The plates were incubated at 37 °C for 7–14 days. The MIC was defined as the lowest concentration of antibiotic that inhibits the growth of the tested isolate. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [17].

2.3. DNA extraction and sequencing

All of the MAC isolates (n = 49) were subcultured on Lowenstein-Jensen medium. DNA extraction was done using the cetyltrimethylammonium bromide-sodium chloride method [18]. Illumina sequencing was performed by a sequencing service company (Novogene Corporation Inc., Singapore) using the Illumina HiSeq platform generating 150-bp paired-end reads.

2.4. Identification of MAC based on the line-probe assay (LPA)

Line probe assay (GenoType *Mycobacterium* CM VER 2.0, Hain Life Science GmbH, Nehren, Germany) was used. This test uses probes for species identification based on the 23S rRNA gene region. The DNA samples were prepared, the target genes were amplified and the amplified products were detected by hybridization to the species-specific probes immobilized on the membrane strips, according to standard protocol of the manufacturer [11].

2.5. Bioinformatics analysis of sequence data

The quality of sequence reads was checked using FastQC version 0.11.7 [19]. Reads below 75 bp were trimmed using Trimmomatic version 0.38 [20]. Reads from each isolate were mapped to the reference genome of *M. intracellulare* ATCC 13950 (SRA accession no. CP003322) using BWA-MEM version 0.7.17 [21]. SAMtools version 0.1.19 [22] was used for sorting and indexing of mapped sequences. Local realignment of the mapped reads was performed using GATK version 3.4.0 [23].

2.6. Multilocus sequence typing (MLST)

Sequences from 24 housekeeping genes (*fusA, atpD, pheT, glnA, topA, secA, argH, glpK, murC, cya, pta, rrl, rrs, hsp65, rpoB,* 16S-23S rRNA ITS*, recF, lipT, pepB, gnd, aspB, groEL, sodA* and *est*) were used for genetic analysis of MAC and other NTM based on findings from previous studies [7–9,11,24,25]. Characteristics of these genes and the primers used to amplify them are described in Supplementary Table 1. Each gene sequence from the 49 MAC isolates was extracted from the aligned mapped sequences. The gene sequences of reference strains *M. avium* subsp. *avium* (SRA accession number CP028731), *M. avium* subsp. *hominissuis* (CP018363), *M. avium* subsp. *paratuberculosis* (NC_002944), *M. intracellulare* ATCC 13950 (CP003322) and *Mycobacterium chelonae* CCUG 47445 (NZ_CP007220) were used for comparisons.

2.7. Phylogenetic analysis

Phylogenetic analysis was done using MEGA-7 [26] based on a four-gene set (*16 s rRNA, hsp65, rpoB,*16S–23S rRNA ITS) [7], a five-gene set (*recF, lipT, pepB, gnd1, est*) [8], a seven-gene set (*fusA, secA,* 16S-23S rRNA ITS*, rpoB, hsp65, 16S rRNA, 23S rRNA*) and a nine-gene set (*hsp65, recF, lipT, pepB, gnd1, aspB, sodA, groEL1, est*) [9]. The maximum-likelihood method was employed using the most suitable model of sequence evolution (GTR) and 1000 bootstrap replications. *M. chelonae* CCUG 47445 was used as an outgroup and *M. intracellulare* ATCC 13950*, M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis* were used as reference strains.

2.8. Data analysis

The discriminatory power (D) of each gene for classification of MAC strain was calculated based on the number of unrelated strains tested (N), the number of different types identified (S) and x_j the number of strains belonging to the j^{th} type using the formula $D = 1 - \frac{1}{N(N-1)}\sum_{j=1}^{S} x_j(x_j - 1) \times 100$ [27].

3. Results

3.1. Study population and characteristics

All 49 MAC isolates were from 15 patients from Srinagarind Hospital, a super-tertiary hospital located in Northeast Thailand. Eleven cases (41 isolates) were defined as true NTM infections and 4 cases (8 isolates) were regarded as examples of colonization (Table 1). Almost half of the isolates from patients with true infections (41.46%, n = 17/41) caused pulmonary disease. The remainder of such isolates (58.54%, n = 24/41) had caused extra-pulmonary infection including disseminated infection

(caption on next page)

Fig. 2. SNP distances and interval times of MAC serial isolates from individual patients (n = 15, P#1-P#15). MLST analysis based on 5-gene (**A**), 4-gene (**B**) 9-gene (**C**) and optimized 7-gene (**D**) sets. Numbers in grey boxes refer to the SNP distances separating sequential isolates. Red circles refer to identified reinfection (based on SNP distance and the presence of isolates from the same patient falling on different branches in the phylogenetic tree). Sp = sputum, Ts = tracheal suction, EP = elbow pus, KF = knee fluid, Np = neck pus, T = tissue, A = arm, Sk = skin, F = fluid, Ck = cheeks, PW = pus from wound, CP = chest pus, IA = inguimal abscess, SF = synovial fluid, St = stool, BM = Bone marrow, P=Pus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(43.90%, n = 18/41) and skin infection (14.63%, n = 6/41). All eight isolates from four colonized patients were from pulmonary sites (100%, n = 8/8) (Table 1).

According to the compiled clinical breakpoints from the CLSI [17], the MIC values of the 13 tested antibiotics for the 49 MAC isolates were determined and are shown in Supplementary Table 2. The most common resistance phenotypes observed were those to moxifloxacin (MIC 4 to ≥8 μg/mL) and linezolid (MIC 32 to ≥64 μg/mL). Among the MAC isolates, seven showed an MIC of amikacin equal to or higher than the breakpoint (MIC ≥64 μg/mL). Twenty-one MAC isolates were considered resistant to clarithromycin. Although ethambutol, rifampin, rifabutin and streptomycin are useful clinically, breakpoints for determining susceptibility and resistance have not been established. Interestingly, 27 of the 49 MAC isolates in our study were multidrug resistant (three or more antibiotic categories) and six MAC isolates were highly resistant to all four antibiotics tested (amikacin, clarithromycin, linezolid and moxifloxacin).

All 49 MAC isolates were identified to the species level using GenoType *Mycobacterium* line-probe assay (LPA): 34/49 were *M. intracellulare* and 15/49 were *M. avium*.

3.2. Analysis of discriminatory power of house-keeping genes and comparisons among 4-gene-, 5-gene-, 7-gene- and 9-gene-based MLST

The discriminatory power of MLST for classification of MAC strain using various combinations of 24 house-keeping genes was analyzed. Seven genes individually had a discriminatory power index higher than 0.9 (Supplementary Table 1) and were combined into a set (the optimized 7-gene set) that was then compared with other previously reported gene sets (4-gene, 5-gene and 9-gene). The phylogenetic trees constructed based on the various gene sets are shown in Fig. 1.

The 4-gene, 7-gene and 9-gene sets agreed equally well (83.67% of cases; *M. intracellulare* = 28/49, *M. avium* = 13/49) with the GenoType *Mycobacterium* line-probe assay (LPA) for species identification (data not shown). The 5-gene set had lowest agreement with the LPA (55.10%, 27/49, *M. intracellulare* = 20/49, *M. avium* = 7/49). There was 83.67% (41/49 isolates) concordance between LPA and the 7-gene set (Fig. 1). Among the isolates with discordant results, 2 isolates (patients#15.1 and #11.3) were identified by LPA as *M. avium*, but by the 7-gene set as *M. intracellulare*. Further, 6 isolates (patients#3.2, #3.3, #3.4, #13.1, #13.2, #5.2) were identified as *M. intracellulare* by LPA but as *M. avium* by the 7-gene set.

In pairwise comparisons among the four different gene sets, the 5-gene set agreed least well with the others in terms of species identification and recognition of reinfection (Table 2). One isolate was identified as resulting from reinfection based on the 5-gene tree whereas 9, 8 and 9 isolates were identified as due to reinfection based on the 4-gene, 9-gene and the optimized 7-gene sets, respectively (Fig. 1).

3.3. Ability to distinguish between reinfection and persistent infection of MAC

Identification of reinfection and persistent infection was based on different cut-off levels according to the optimized 7-gene set (≥87 SNPs for reinfection and ≤ 41 SNPs for persistent infection) (Fig. 3) and concordance of species identification based on LPA (Table 2). Reinfection rate was estimated to be 54.55% (6/11 true infection cases) based on both the optimized 7-gene set and the 4-gene set (Table 2). Different strains were recovered from one colonization case (patient#12), a situation analogous to reinfection. The interval time between samples during which reinfection occurred ranged from 8 to 296 days with an average of 97.9 days (Fig. 3). Reinfection in one patient (patient#9) was not identified by the 9-gene set. The 5-gene set identified only one reinfection case.

3.4. Cluster analysis for possible transmission between patients

Cluster analysis of MAC infections showed two possible clonal transmission clusters (cluster 1 = P#11, P#12 and cluster 2 = P#5, P#12 and P#13), based on distances ≤41 SNPs (Fig. 2). Cluster 1 was supported by a geographical link (the same province) and collection time (16 months apart). Cluster 2 was supported by their occurrence in adjacent provinces (Khon Kaen and Mahasarakham) and collection time (7 months apart) (Fig. 3 and Table 1).

4. Discussion

MAC infection is a public health problem worldwide and an important cause of morbidity and mortality. The two recognized species with the MAC are *M. avium* and *M. intracellulare*: both can infect humans. MAC infection is usually chronic and is highly associated with drug resistance and treatment failure [1]. More than half of the MAC isolates (55%) in this study were multidrug resistant, which is a major public health concern [28,29]. *M. avium* and *M. intracellulare* cannot be differentiated through conventional microbiological tests and their clinical features are often considered indistinguishable [1]. However, a study in Korea found that patients with *M. intracellulare* lung disease exhibited a more severe illnesses and worse prognosis than patients with *M. avium* lung disease [30]. A previous study using an animal model also suggested that *M. intracellulare* was the more virulent species [31]. Therefore, identification of the exact species involved is of clinical importance. Some serious MAC infections and treatment failures are associated with antibiotic resistance. In addition, during the course of antibiotic treatment, the MAC isolates sampled might be derived from the same clone that initially infected the patient (persistent infection) or may represent a new clone (reinfection). The extent to which reinfection by MAC can influence the apparent treatment failure rate is still unclear. Here, we optimized a gene set that can be used for species and strain classification. Use of this gene set demonstrated a high reinfection rate of MAC in the Thai population. Our optimized gene set also allowed us to explore relationships among serial isolates, making it possible to infer instances of human-to-human transmission of MAC.

Molecular typing is a useful tool to discriminate between reinfection and persistent infection cases, allowing us to investigate below the species level [15]. A high-resolution gene set for MLST is necessary to distinguish whether MAC isolates are from the same clone or different clones. Such information can be used for molecular epidemiology and diagnosis. Here, we analyzed the discriminatory power of 24 house-keeping genes selected from the various genotyping sets used in previous studies on MAC [7–9,11,25] and other NTM [24,25]. We showed that our 7-gene set has the highest discriminatory power, best resolution to differentiate reinfection from persistent infection and the highest concordance with LPA for species identification. We also used this gene set to demonstrate possible human-human transmission of MAC.

MLST is the genetic analysis tool most commonly used to genotype MAC [9,32]. Many house-keeping genes have been used for

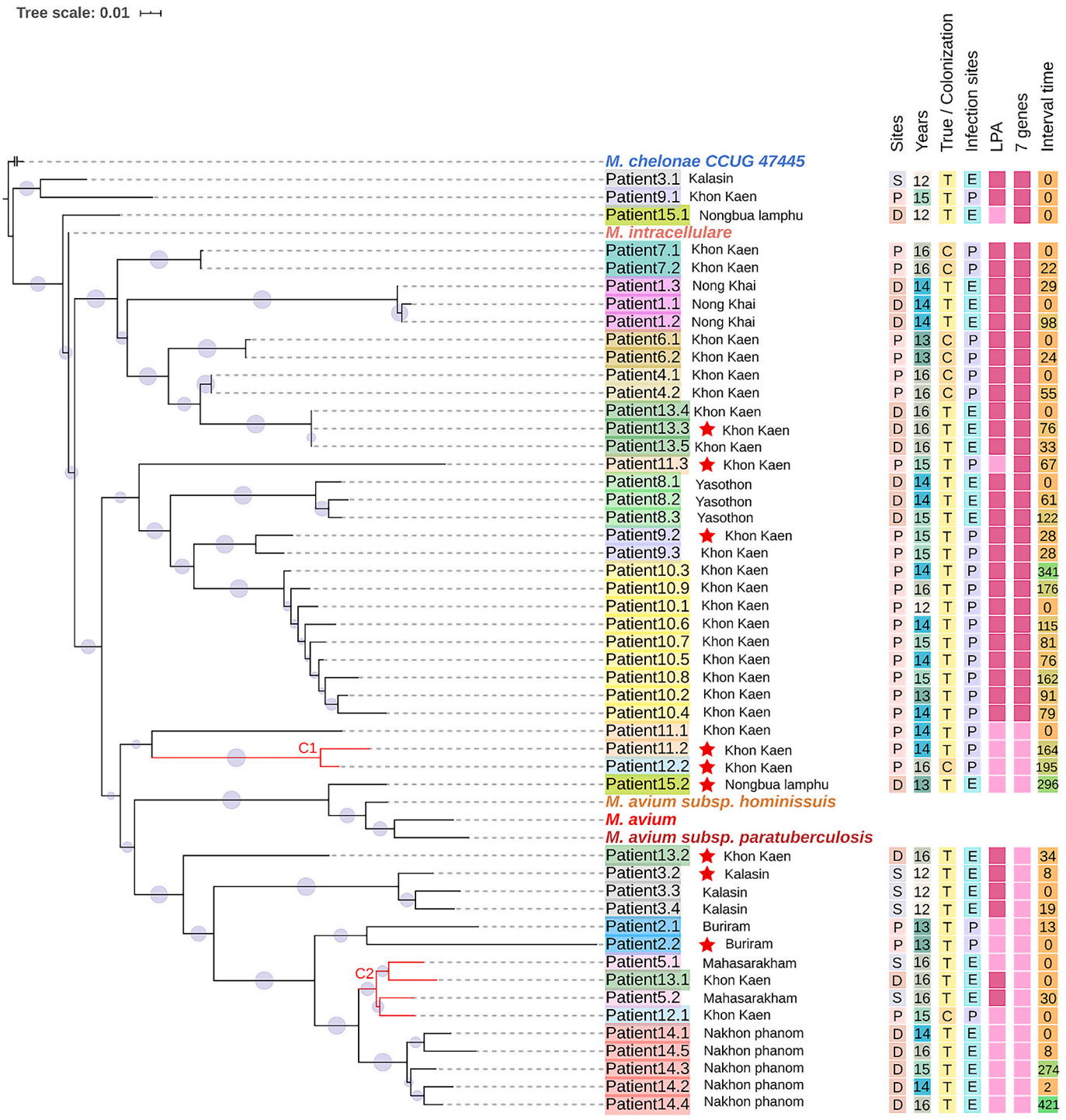

Fig. 3. Phylogenetic tree of 925 SNPs from the optimized 7-gene set (*fusA, secA,* 16S-23S rRNA ITS*, rpoB, hsp65, 16S rRNA, 23S rRNA*) of MAC isolates using the maximum likelihood method. All 49 strains were identified as either *Mycobacterium intracellulare* or *Mycobacterium avium* subsp. *avium.* This bootstrap consensus tree was inferred from 1000 replicates. Blue circles represent bootstrap values and the size of each circle is proportional to its value (the largest blue circle indicates a value of 100%). *M. avium* subsp. *avium, M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis* and *M. intracellulare* ATCC 13950 (accession numbers CP028731, CP018363, NC_002944 and CP003322, respectively) were used as reference strains. (D = Disseminated, P = Pulmonary, S = Skin, T = True, C = Colonization, E = Extra-pulmonary, Pink colour = *M. avium*, Dark pink = *M. intracellulare*). C1 = cluster 1 (Patient 11.2 and 12.2), C2 = cluster 2 (Patient 5.1–5.2, 13.1 and 12.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mycobacterial identification, such as the 16S and 23S rRNA genes, *hsp*65, *rpoB*, superoxide dismutase gene, and internal transcribed spacer (ITS) region [33]. Previous studies have simply adopted a convenient gene set reported by others or have failed to optimize for the most suitable gene set for genetic analysis of MAC [7–9,11,25]. We determined the discriminatory power of 24 house-keeping genes using serial isolates of MAC from the same patients, in whom treatment had not apparently been successful. Such isolates could include examples of persistent infection (infection by the same clone across different time points) and/or reinfection (infection due to the acquisition of a new clone of bacteria). Here, we showed that 7 of the 24 genes (*fusA, secA,* 16S-23S rRNA ITS*, rpoB, hsp65, 16S rRNA, 23S rRNA*) had a

discriminatory power index higher than 0.9 for differentiating serial isolates of MAC. We compared this optimized 7-gene set with previously used gene sets. The discriminatory power of each was proportional to the number of SNPs in each. The 7-gene set (925 SNPs) is better than the 4-gene (457 SNPs), 9-gene (476 SNPs) and 5-gene (248 SNPs) sets. The optimized 7-gene set had the highest concordance (but comparable to the 4-gene and 9-gene sets) for species identification of MAC compared to LPA.

A study from Korea using the 4-gene set reported a high reinfection rate of MAC (73%) and suggested that this might be a factor contributing to chronic infection that creates treatment difficulties [7]. In our study, the 4-gene set identified the same proportion (54.55%) of reinfection cases as did the optimized 7-gene set. We confirmed a high rate of reinfection due to MAC in Thailand, in agreement with the previous report from Korea. We also found one out of four cases of MAC colonization included a change of clone through time, a situation analogous to reinfection and possibly due to independent acquisitions from the environment. As MAC infection usually occurs in immunocompromised hosts [34], the reacquisition of MAC from environmental exposure during treatment might explain the high reinfection rate observed, leading to chronic infection and treatment difficulties.

Using the number of SNPs differing between serial isolates of MAC from individual patients, we identified cut-off values to distinguish between reinfection and persistent infection. For the high-resolution 7-gene set, reinfection was identified based on $\geq$87 SNP differences between sequential isolates and persistent infection was identified based on $\leq$41 SNPs. The high average interval time (98 days), and high number of SNPs, separating the reinfection isolates also supported the identification of reinfection. The results of the same 4-gene set compared to the previous study [7] also support the validity of both the optimized 7-gene set and the reinfection rate identified from this study.

We used the cut-off values based on the optimized 7-gene set to identify possible clonal transmission clusters of MAC. There were 2 clusters found. Cluster 1 comprised two cases from Khon Kaen occurring two years apart. However, the social data and exposure history from cluster 1 are not available for analysis. Cluster 2 comprised 4 isolates from 3 cases from the adjacent provinces of Mahasarakham and Khon Kaen within the same time period in February–June 2016. Such clusters could be a result of exposure to the same environmental source, such as soil [35]. There is increasing speculation that human-to-human transmission of some NTM infections can occur [36,37]. Such transmission has never been reported for MAC but cannot be excluded. Additional study that includes the social links and exposure history is needed to confirm human transmission. This should also include analysis of MAC environmental isolates, such as from soil and the household environment.

Many molecular methods can be used to identify NTM. The line-probe assay is the most widely used. This method enables simultaneous detection and identification of different mycobacterial species using house-keeping genes and DNA sequences such as the 16S-23S rRNA gene spacer, 23S rRNA gene and *rpoB* gene [7]. The LPA assay we used (GenoType *Mycobacterium* CM) has 97% and 92.4% sensitivity and specificity, respectively, compared to biochemical methods, HPLC, INNO-LiPA MYCOBACTERIA (Innogenetics NV) and *16S rRNA* gene sequencing [10]. The LPA has 98.23% and 50% sensitivity and specificity compared to HPLC [38]. MLST using our 7-gene set achieved a high concordance with LPA (83.67%). As no bacterial taxon other than MAC was analyzed, specificity cannot be calculated. Since the LPA uses only a single gene (23S rRNA), the higher discriminatory power of the 7-gene set might lead to some discordance. However, biochemical tests were not available to us for comparison. Therefore, we cannot conclude whether the 7-gene set has a higher performance for MAC species identification. At least, the 7-gene set was comparable with the previous 4-gene and 9-gene sets for MAC species identification. Given its higher overall discriminatory power, the 7-gene set is the optimal set for genetic analysis of MAC.

MAC can infect many organs, especially in HIV patients, and is considered the most common cause of chronic lung infection [39]. A previous study reported that MAC causes pulmonary infection far more frequently than extrapulmonary [34]. In our study, *M. intracellulare* was the major species isolated from both pulmonary and extrapulmonary sites. However, no significant difference was seen between the two species comparing pulmonary and extrapulmonary sites. Also, we found no evidence to support an association between the number of mutations and the time interval between serial isolates, nor any association between the number of mutations and site of infection. Our sample size limited the power of statistical analysis. A correlation analysis between site of infection and sub-specific strains was not done due to the limited range of sample sites and because serial isolates from the same patient could not be regarded as independent samples. [36,37]

5. Conclusion

We evaluated a 7-gene set for MLST analysis that provided high discriminatory power and diagnostic performance for the genetic study of MAC. MLST analysis using this gene set can be used for MAC species identification. The results we obtained indicated that the rate of reinfection was 54.55%. Two small clusters of possible transmission of MAC between humans were found.

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CRediT authorship contribution statement

Wicharajit Boonjetsadaruhk: Data curation, Formal analysis, Investigation, Supervision, Validation, Visualization, Writing – original draft. **Orawee Kaewprasert:** Formal analysis, Investigation. **Arnone Nithichanon:** Supervision. **Pimjai Ananta:** Resources. **Prajuab Chaimanee:** Resources. **Kanin Salao:** Supervision. **Wisitsak Phoksawat:** Methodology. **Marut Laohaviroj:** Supervision. **Auttawit Sirichoat:** Data curation, Formal analysis, Supervision, Writing – review & editing. **Yang Fong:** Supervision. **Suwin Wongwajana:** Supervision. **Wises Namwat:** Supervision. **Viraphong Lulitanond:** Supervision. **Ploenchan Chetchotisakd:** Resources, Supervision. **Kiatichai Faksri:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare there are no competing interests.

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