Molecular characterization of *Mycobacterium avium* clinical isolates from Japan and development of diagnostic tools

（日本の臨床分離トリ型結核菌株の遺伝子解析と診断法開発）

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
FQ	Fluoroquinolone
FS	Frameshift
HIV	Human immunodeficiency virus
Hsp65	Heat shock protein 65
IS*1245*	Insertional sequence *1245*
IS*6110*	Insertional sequence *6110*
ITS	Internal transcribed spacer
JATA	Japan Anti-tuberculosis Association
LAMP	Loop-mediated isothermal amplification
LVX	Levofloxacin
MAC	*Mycobacterium avium* complex
MBTC	*Mycobacterium tuberculosis* complex
MIC	Minimum inhibitory concentration
MOTT	Mycobacteria other than tuberculosis
MfpA	*Mycobacterium* fluoroquinolone resistance protein A
MfpB	*Mycobacterium* fluoroquinolone resistance protein B
NTM	Nontuberculous mycobacteria
PCR	Polymerase chain reaction
PRP	Pentapeptide repeat protein
PRA	PCR restriction fragment analysis
QRDR	Quinolone resistance determining region
Qnr	Quinolone resistance protein
RFLP	Restriction fragment length polymorphism
RpoB	β subunit of RNA polymerase
TB	Tuberculosis
USA	United States of America
WGS	Whole genome sequencing
WT	Wildtype

Preface

The genus *Mycobacterium* comprises many species known to cause serious illness in humans such as *Mycobacterium tuberculosis* and *M. leprae*, the agents responsible for tuberculosis and leprosy respectively [1]. However, majority of species in the genus *mycobacterium* belong to a class of bacteria collectively known as Nontuberculous mycobacteria (NTM); also known as atypical or anonymous mycobacteria or mycobacteria other than tuberculosis (MOTT). NTM encompasses all mycobacteria other than the members of the *tuberculosis* complex (MTBC), *leprae,* and *ulcerans*. The last decades have seen a rise in the incidence and prevalence of NTM infections worldwide, particularly in the developed countries which have decreasing incidence rates of *M. tuberculosis* infection.

To date there are over 170 species of NTM distributed ubiquitously in the environment worldwide and new species are continually being discovered. NTM are opportunistic pathogens to animals and humans, including fish and poultry and are present in soil, water sources, milk, food produce as well as domestic and wild animals. In contrast with tuberculosis caused by *M. tuberculosis*, reporting of NTM infection is not mandatory; as a result the true incidence and prevalence of NTM infections are difficult to determine. However, several reports have shown a rise in the the prevalence of NTM infection in the Europe, the United States, and other countries [2] .

The memebers of the *Mycobacterium avium* complex (MAC) group are the significant NTM causing lung diseases in both immunocompromised and immune-competent individuals in most parts of the world [3,4]. The MAC group is comprised of three main species namely, *M. avium*, *M. intracellulare,* and *M. chimaera* which are all slow-growing acid-fast mycobacteria, classified under non-chromogens in group III of the Runyon classification of NTM and commonly isolated from water, house dust [5,6], and soil [7,8]. However, additional species have been identified with the availabibility of new genetic sequencing technology. Such that to date, MAC comprises about nine species of slow-growing mycobacteria: *M. avium, M. intracellulare, M. chimaera, M. colombiense, M. timonense, M. marseillense, M. vulneris, M. boucherdurhonense,* and *M. arosiense* [2].

According to Marras et al [9], approximately 80% of pulmonary diseases caused by NTM in different geographic regions, including countries such as Ireland, South Korea, and the United States of America (USA) are caused by MAC [9]. In Japan, the incidence of MAC infection was reported to have increased from 5.2/100,000 in 2007 to 13.1/100,000 in 2014. As of 2013, the prevalence of NTM in Japan was 14.7 cases per 100,000 person-years, which is over twice the incidence rate that was reported in 2007 and is one of the highest rates worldwide [10].

Mycobacterium avium is the most clinically significant organism for human infections within MAC. In Japan, up to 60% of NTM pulmonary infections were reportedly due to *M. avium* [10]. Likewise, studies in Europe, have found *M. avium* as the most frequent MAC species among pulmonary and extrapulmonary samples, as reported by a study conducted in Denmark (50.7%), Portugal (58.0%), and Italy (41.5%) [11,12]. The prevalence of MAC pulmonary and extrapulmonary infections in the Americas is not very different from that of other continents. *M. avium* was the most frequent isolate among MAC species in the USA (54%), and in Brazil, it was most frequent among NTM species (33.3%) [13]. However,variability in the dorminant species is normally observed depending on the geographical area. For instance, most pulmonary diseases in the north and east of Japan are caused by *M. avium*, while *M. intracellulare* is more prevent in the south and west of Japan [10]. Similarly, *M. intracellulare* (25.0%) is the most prevalent MAC species in Taiwan and China, while *M. avium* was the most prevalent species in Korea (88%) [14]. Nonetheless, *M. avium* undoubtedly remains the most clinically significant species in clinical practice and hence its study is warranted [3,4,15].

M. avium is a slow-growing intracellular pathogen that causes opportunistic infections with the ability to persist within the macrophages and resist the immune mechanisms of the host [16]. It can be further subdivided into four subspecies as discussed below, each with distinct pathogenic characteristics, and host preferences and all considered possible zoonoses [4,15,17–19]. *M. avium* subsp. *avium* (MAA) causes avian tuberculosis in birds which are its main reservoirs. Avian tuberculosis is a chronic wasting disease which depicts slight signs of infection and can infect a variety of mammals, especially pigs and cattle. Transmission to susceptible animals is mainly from birds through ingestion or via the fecally contaminated environment. *M. avium* subsp. *silvaticum* (MAS), the pigeon type is similar to MAA taxonomically and is a pathogen that mainly infects the wood pigeon, resulting in TB-like disease [18]. *M. avium* subsp. *paratuberculosis* (MAP) is a widely known pathogen that causes Johne's disease (paratuberculosis) in ruminants where it

causes a progressive infection in the small intestine. MAP is globally distributed and transmission is mainly by the fecal–oral route via pasture, milk, and water contaminated with feces [1,20]. In humans, MAP has been linked to Crohn's disease, however, the association remains controversial and inconclusive [21]. *M. avium* subsp. *hominissuis* (MAH) which is subspecies of interest causing pulmonary and extrapulmonary tuberculosis mostly in humans and pigs. MAH is an opportunistic environmental pathogen and infection acquisition can occur through inhalation, ingestion, and dermal contact from environmental sources or medical equipment. MAH is the most clinically relevant organism within MAC for humans and a major pathogen for individuals with T cell immunity deficiency [2].

The most common site of infection is the respiratory tract in healthy immune-competent individuals, and when it develops as a secondary complication, the typical manifestation of pulmonary disease is as a nodular/bronchiectatic form or in the fibro-cavitary form [3,17,22]. Additionally, *M. avium* can also invade the lymph nodes, bones, skin, joints, and soft tissue and can spread systemically resulting in severe disease or even death if untreated or improperly treated, especially among immunocompromised individuals such as those living with HIV and AIDS, the elderly or cancer patients [13]. From here onwards, the term *M. avium* will be used in reference to *M. avium* subsp. *hominissuis* because in Japan, *M. avium* clinical cases reported so far are restricted to subsp. *hominissuis* [23–25].

M. avium infections represent an important challenge in clinical practice because they are difficult to diagnose, treat and eliminate from contaminated surfaces due to their resistance to available antiseptics, biocides, sterilizing agents and disinfectants, and thus they are able to persist even in hostile environments [1]. The symptoms of *M. avium* infection are nonspecific and include malaise, cough, fever, weakness, dyspnea, and hemoptysis which are also seen in *Mycobacterium tuberculosis* infection and as result, *M. avium* infections are commonly misdiagnosed as *M. tuberculosis* especially where proper diagnostic facilities are lacking [13]. However, the treatment regimen for *M. tuberculosis* is different from that of *M. avium* infections, this results in case mismanagement.

Many countries, especially those with a high incidence of tuberculosis and limited resources, depend on smear microscopy for the detection of acid-fast bacilli (AFB) which is the mainstay for the diagnosis of MAC infection. Although this method is widely used due to its low

cost and rapid results, it has low sensitivity and does not identify the mycobacterial species [26]. Other traditional techniques such as culture and biochemical tests have also been for diagnosis of *M. avium* infections. Bacterial culture in solid or liquid medium is the gold standard method of identification, however, it is time consuming (3 - 6weeks) thereby making timely management of infections impossible [26–29]. Biochemical tests such as niacin production and nitrate reduction in addition to serology identification of mycobacterial isolates can be based on growth rates and colony pigmentation but these tests may be erroneous in the identification of the *Mycobacterium* species. For example, MAC species present wide variability in colony morphology, from smooth to rough colonies as well as from non-pigmented to cream-colored to bright yellow, which resembles many other mycobacterial species. In this sense, molecular-based methods are superior to the phenotypic and serological-based tests for *M. avium* identification [25,30,31].

Among the molecular methods, PCR-based assays are the technique of first choice for the identification of *M. avium* and may be performed for uncommonly encountered species or precise identification at the subspecies level. In addition, there is (partial) gene sequencing, such as for *hsp65* (heat shock protein), *rpoB* (encodes the β subunit of RNA polymerase), the 16S rRNA-23S rRNA internal transcribed spacer (ITS), and which offer high discriminatory power and can identify up to the subspecies level [32,33]. The use of such methods has revealed the vast geographic and host related genotypic diversity that exists among *M. avium* strains. Moreover, whole genome sequencing and comparative genomic analyses have unveiled some associations between certain genotypes and disease manifestation and progression [34]. Although sequencing of these genes allows for species discrimination, their practical usage is limited to laboratories that have access to sequencing facilities [32,33].

Besides the above-mentioned genes, mobile genomic elements also known as insertion sequences (IS) can be used as targets for the identification of *M. avium*. So far, there are four globally recognized IS described in *M. avium* strains isolated from humans namely; IS*1311*, IS*900*, IS*901*, and IS*1245*, which can also be used to differentiate between subspecies. For example, IS*900* is specific for *M. avium* subsp. *paratuberculosis* and IS*1245* was found mainly in *M. avium* subsp. *hominissuis*. IS*1245* is present in high copy numbers in the genome of *M. avium* and hence it has been widely used as a marker for the detection and differentiation of *M. avium*. IS*1245* restriction fragment length polymorphism (RFLP) is the standard method for the genotyping of *M. avium* strains but the high costs and expertise required for RFLP and PCR-based methods limit

their global usage [35,36]. There is therefore a sustained need for affordable, rapid, and accurate diagnostic methods for the identification of *M. avium*.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that synthesizes DNA based on auto cycling strand displacement performed by the *Bst* DNA polymerase. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. LAMP has ideal properties such as (i) isothermal amplification conditions (60 to 65°C) using Bst polymerase; (ii) rapid amplification than PCR because of no thermal cycling; (iii) high reaction specificity due to the use of four-six primers recognizing six-eight distinct regions on the target DNA; and (iv) production of extremely large amounts of amplified products which can simply be detected visually by the turbidity or fluorescence of the reaction mixture. All these characteristics make LAMP a powerful tool that facilitates point-of-care genetic testing which is very convenient in field settings [28,37].

Several LAMP-based assays have been developed targeting genes such as 16S rRNA, *rpoB*, *gyrB*, and *dnaA* for the identification of *M. avium* and other nontuberculous mycobacteria but due to the high homology of these genes across species, the authors disclosed some difficulties in accurately distinguishing *M. avium* from its closely related species [31,38,39]. Hence, to overcome the above limitations without compromising on sensitivity, specificity, and enhanced turnaround time, IS*1245* was selected as a marker for the LAMP assay due to its high specificity and high copy number [35,40–43]. The accurate identification of *M. avium* from other mycobacterial species is very important because treatments and outcomes tend to differ from species to species. It is therefore critical to rapidly detect *M. avium* to institute timely and appropriate therapy that could consequently result in better treatment outcomes for patients.

For the treatment of *M. avium* infections, a multidrug regimen consisting of macrolides such as azithromycin or clarithromycin in combination with ethambutol and rifamycin is recommended as the standard therapy. Generally, *M. avium* like with other NTM is intrinsically resistant to most anti-tuberculosis drugs, hence the treatment regimens are formulated according to established guidelines and/or drug susceptibility testing (DST) using a standardized protocol from the Clinical and Laboratory Standards Institute [44]. Given that many mycobacteria can only colonize patients without contributing to the progression of the disease, accurate disease definition must be made using the diagnostic criteria outlined by the American Thoracic Society (ATS) [7].

The treatment of *M. avium* is usually long-term (12-18 months) and patients are to remain on treatment until culture-negative on therapy for at least one year. There is still no solid effective treatment regimen for *M. avium*, therefore, the therapy continues to be a great challenge due to the long duration, toxicity, and high costs. Moreover, the treatment success rate reported for *M. avium* infections is only 50–55% [25,36]. The emergence of drug resistance to the recommended drugs (macrolides) has only compounded the existing challenges in treating *M. avium* infections.

Fluoroquinolones (FQ) are among the drugs that can be used for the treatment of macrolide-resistant cases [7,45]. FQ are broad-spectrum antimicrobials that target DNA gyrase, an essential enzyme that is involved in DNA replication, transcription, and stress responses. DNA gyrase is a type II topoisomerase composed of two GyrA and two GyrB subunits, GyrA binds DNA, while GyrB is an ATPase [46]. Structural and biochemical studies have described the mode of action of DNA gyrase to involve a two-gate mechanism. The DNA topology is altered by promoting the passage of one DNA duplex (the transported or 'T' segment) in a second double-stranded DNA segment (the gate or 'G' segment) through a transient break in an ATP-dependent manner (figure 3) [47].

Many structural studies have demonstrated that mutations in *gyrA* and *gyrB* conferring quinolone resistance play important roles in drug-protein interactions, however, the correlation between antibiotic resistance and particular mutation sites in *M. avium* clinical isolates is not strong [48–50]. This phenomenon suggests that there are other as yet unknown mechanisms that may also be important contributing factors to resistance and that the DNA gyrase mechanisms may not be the only factor determining drug resistance.

Other alternative mechanisms of FQ resistance include the regulation of efflux pumps in which the bacteria extrudes the drug by its stimulated cell efflux system. Multidrug efflux pumps are thus capable of mediating resistance to FQ [30]. Another interesting mechanism recently described is the DNA mimicry by pentapeptide repeat proteins such as *Mycobacterium* fluoroquinolone resistance protein A (MfpA) which induced intrinsic FQ resistance exclusively in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* [51,52]. In *M. smegmatis*, *mfpA* was shown to play a role in determining the innate MICs of FQ in the *mfpA* mutant strain which had a two to four-fold decrease in the level of FQ resistance. MfpA reportedly limits the efficacy of FQ by decreasing the FQ-induced DNA cleavage, and thereby protects the enzyme from the inhibitory

activity of these antibiotics [53]. Therefore, mutations that alter or increase the expression of MfpA could lead to the development of FQ resistance [51,54]. However, the role of MfpA in FQ resistance in *M. avium* clinical strains has not yet been investigated.

In light of the above, in Chapter I, an IS*1245* LAMP assay for the rapid detection of *M. avium* was developed. Secondly, due to the high geographic and host-related genetic diversity known to exist among *M. avium* strains, the influence of this diversity on the applicability of the LAMP assay was assessed using human and pig samples from Japan. In Chapter II, the role of MfpA in levofloxacin resistance among *M. avium* clinical isolates was investigated by minimum inhibitory concentration (MIC) determination and sequencing FQ resistance associating genes namely, *gyrA*, *gyrB*, *mfpA*, and *mfpB*.

Tables and Figures

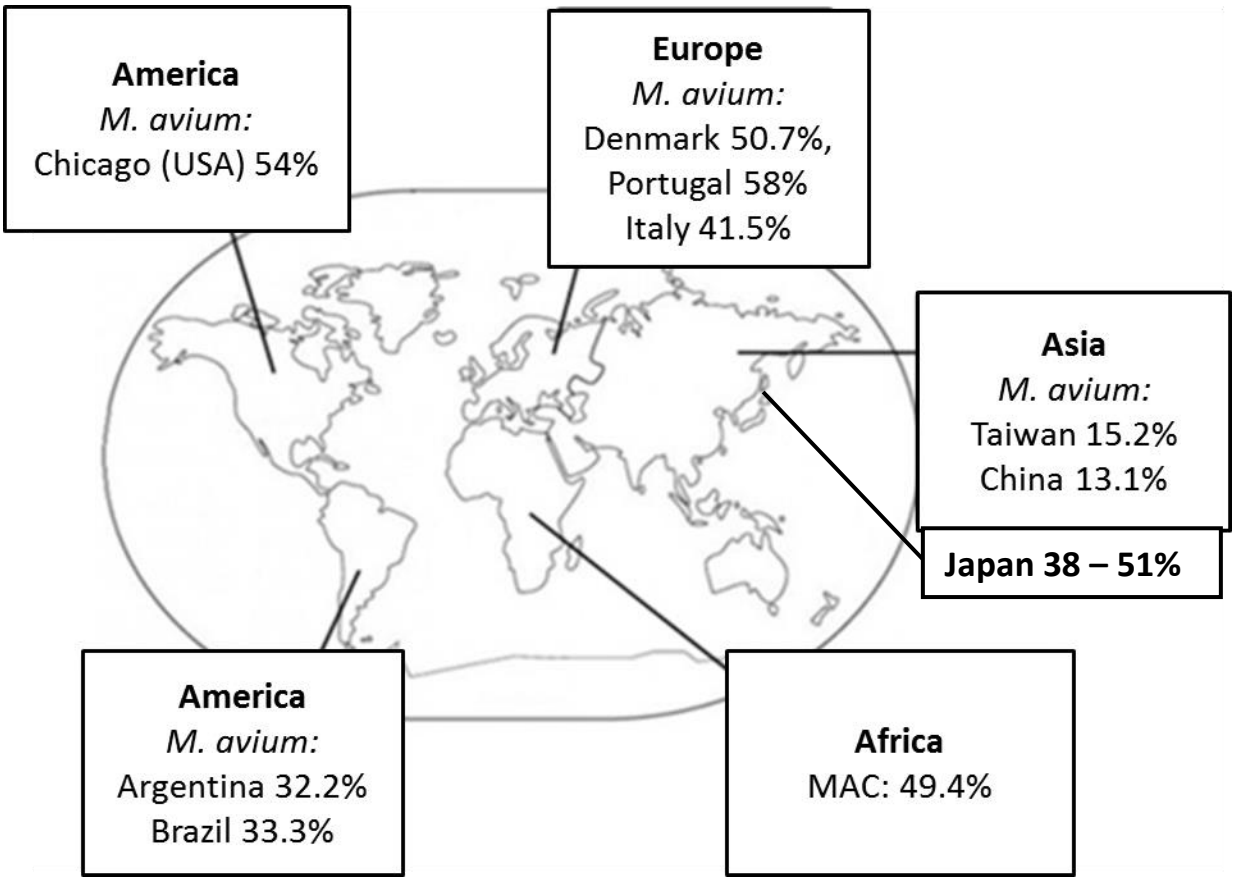

Figure 1. Geographic representation of the prevalence of *M. avium* in NTM infections across the world [39,55]**.**

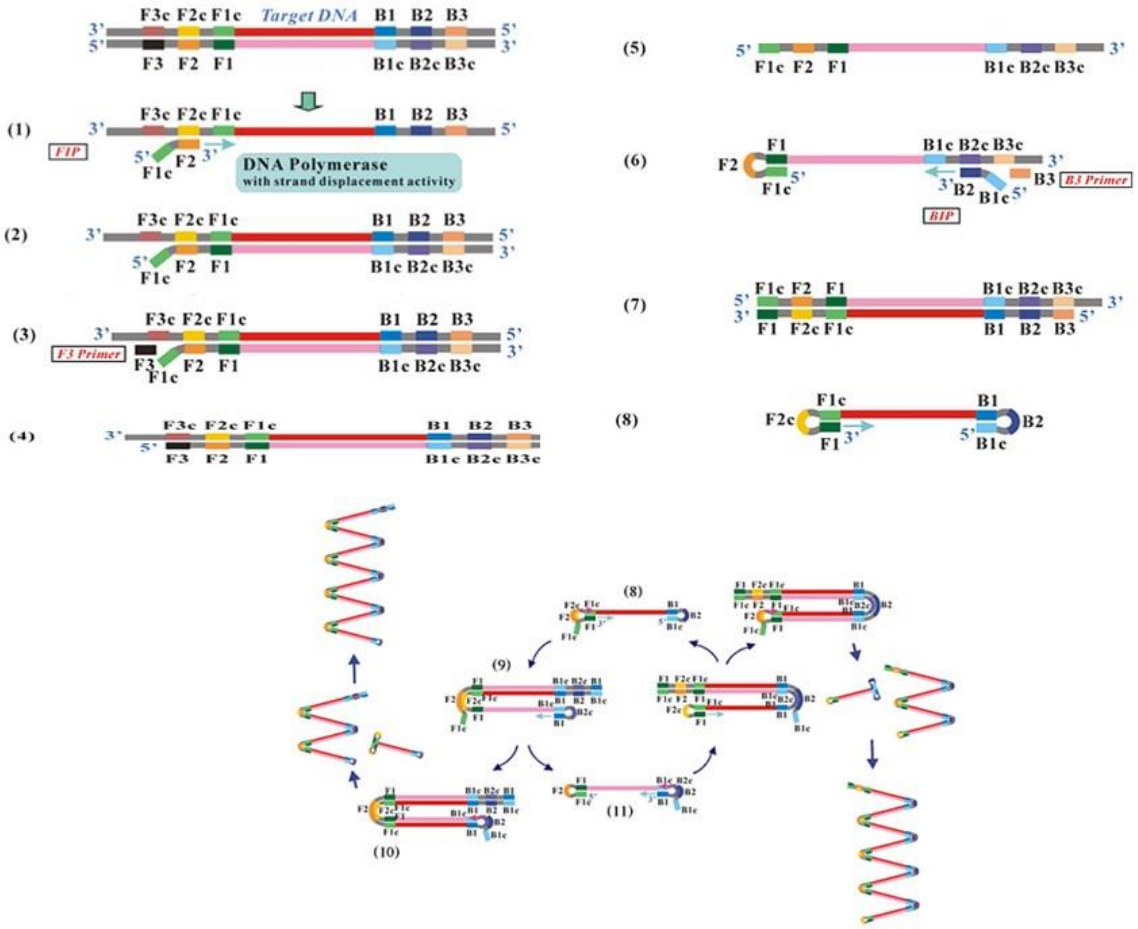

Figure 2. Principles of LAMP amplification. Adapted from Eiken Chemical Co. Ltd., Japan (2015). Primers are designed based on the Forward (F3c, F2c and F1c) and the Backward (B1, B2 and B3) that represent 6 distinct regions of the target gene at the 3' side and regions at the 5' side respectively.

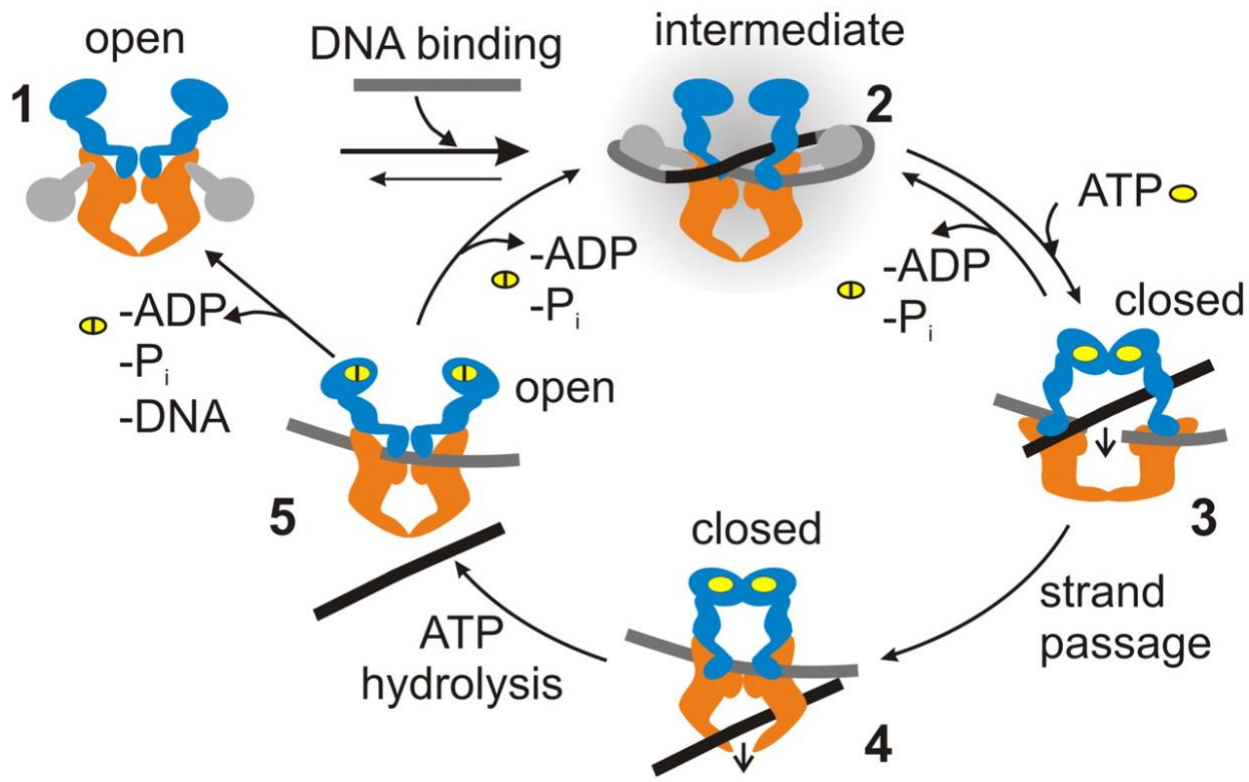

Figure 3. Strand DNA passage mechanism of negative supercoiling with DNA gyrase and conformational changes. Modified from Gubaev et.al (2011) [56].

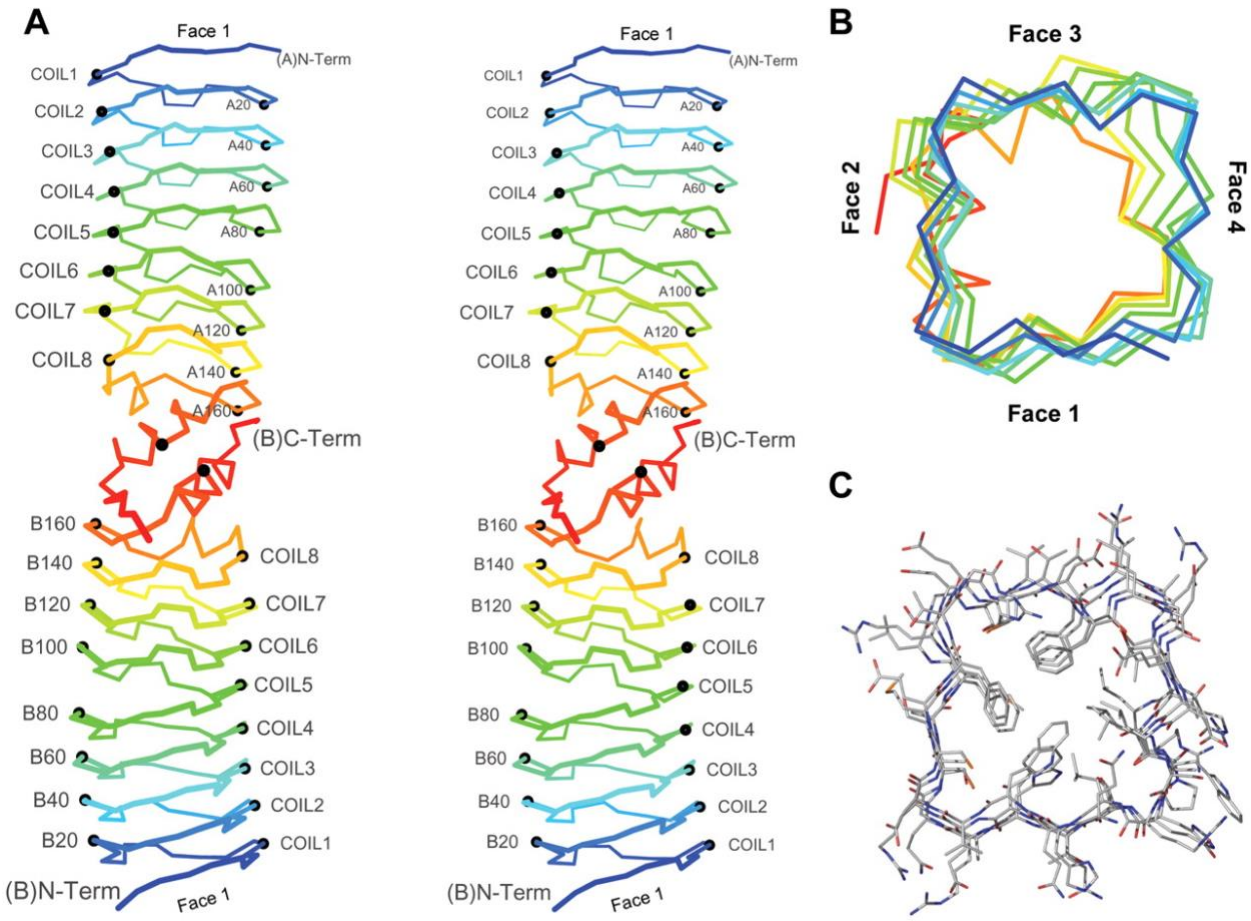

Figure 4. *MfpA* structural fold illustrations. Adapted from Hegde et. al (2015) [51].

Chapter I: Evaluation of IS*1245* LAMP in *Mycobacterium avium* and the influence of host-related genetic diversity on its application

1.1. Introduction

Mycobacterium avium is a slow-growing pathogen that is increasingly causing pulmonary and extrapulmonary infections in both immune-compromised individuals and healthy people worldwide. *M. avium* is ubiquitous in the environment and especially thrives in human-engineered environments thus making transmission very easy especially for the vulnerable groups with predisposed health conditions. Because the clinical presentation of *M. avium* infections is nonspecific, there is a need for accurate diagnostic tools that can correctly and rapidly identify *M. avium* infections. Correct identification of *M. avium* from other mycobacterial species is critical because treatment is variable depending on the species involved due to different drug susceptibility patterns.

In this chapter, I focused on LAMP targeting an *M. avium*-specific insertional sequence, IS*1245*, for its detection. IS*1245* has been widely used for the detection and typing of *M. avium* and has been proposed as the standard marker for strain typing of *M. avium* clinical isolates [35,57–59]. Due to its presence in multiple copies, systems targeting IS*1245* have exhibited high sensitivity and the ability to distinguish *M. avium* from other species. Despite being widely used in PCR-based assays, there are limited studies that have evaluated the utility of IS*1245* LAMP for the detection of *M. avium* in clinical isolates. Hence, I describe the development of the IS*1245* LAMP assay which was achieved through a series of experiments described in the methods section.

Previous studies have shown that there are significant genetic differences among *M. avium* strains circulating in humans, pigs, and the environment. So as a second objective, I sought to assess how much influence the infamous genetic diversity among *M. avium* strains would have on the applicability of IS*1245* LAMP for the detection of *M. avium* isolates in human and pig samples from Japan.

1.2. Materials and Methods

A total of 228 *M. avium* strains were used in this study including 137 clinical isolates obtained from patients diagnosed with *M. avium* infections from National Hospital Organization

Osaka Toneyama Medical Center (n=50), Keio University Hospital (Tokyo, n=27), Fukujuji Hospital, Japan Anti-Tuberculosis Association (Tokyo, n=29) and Hokkaido University Hospital (n=31); and 84 isolates from pigs collected in a slaughterhouse in Osaka and 7 pig isolates from Hokkaido (Table 3). Isolates were grown on 2% Ogawa medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) at 37ºC for 3 to 4 weeks. Genomic DNA was extracted from the colonies by mechanical disruption according to the modified procedure of Suzuki et al. (1995) [60].

Briefly, colonies were scraped from Ogawa medium and suspended in 0.5 ml of 0.1M Tris-EDTA (TE) buffer in a vial which contained 0.1mm Zirconia/Silica Beads (BioSpec Products, Inc., Bartlesville, OK, USA) and 0.5 ml of chloroform. Mycobacterial cells were disrupted by shaking vigorously by Micro Smash (TOMY SEIKO Co. Ltd., Tokyo, Japan) at 3,000 rpm for 1 minute. This was followed by centrifugation, then the aqueous layer was recovered, and DNA concentrated by ethanol precipitation, dissolved in 100 µl of TE buffer and kept at -30ºC until use.

For the specificity study, extracted DNA from 23 nontuberculous mycobacterial type strains including *M. avium* JATA 51-01 (ATCC 25291) [61], two *Mycobacterium tuberculosis* complex (MTBC) reference strains, four *M. bovis* clinical isolates from Zambia, 10 *M. tuberculosis* clinical isolates from Osaka and an additional five non-mycobacterial species, that are common pathogens of pneumonia, were used (Table 1).

1.2.1. Primer design

From the conserved sequence of the mycobacterial IS*1245* element as a target (GenBank Accession no: L33879) [35], I designed several sets of primers to select the best combination. The set consisted of two inner primers, namely the forward inner primer (FIP) and backward inner primers (BIP), two outer primers (F3 and B3), and two loop primers (FL and BL) for recognizing 8 distinct regions of the IS*1245* gene. The LAMP primers were designed by using Primer Explorer V5 software (https:// primerexplorer.jp; Eiken Chemical Co. Ltd, Tokyo, Japan). Furthermore, by using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), multiple sequence alignment of the IS*1245* sequences available from NCBI GenBank databases (https://www.ncbi.nlm.nih.gov/nucleotide/) was performed to ensure compatibility. The primers were commercially synthesized by Life Technologies Japan Ltd. The list of the selected primers and their respective locations on the IS*1245* gene are listed in Figure 1.

1.2.2. LAMP reactions and Optimization

The LAMP reaction was performed in 25µl reaction volumes as described by Pandey et al, 2008 [62]. Each reaction contained 2µl of the DNA template, 1.6µM each of FIP and BIP, 0.2 µM each of F3 and B3, 2.2 µM each of LF and LB loop primers, 1.25 mM deoxynucleoside triphosphate mix, 0.8 M betaine (Sigma-Aldrich, St Louis, MO, USA), 20 mM Tris-HCl (pH 8.8) (Wako Pure Chemical Industries, Osaka, Japan), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20 (Sigma-Aldrich), 6 mM $MgSO_4$ and 8 U of Bst DNA polymerase (Nippon Gene Co., Ltd., Tokyo, Japan). These reagents were mixed in one tube and the volume was adjusted to 25µl by addition of double distilled water (DDW). Subsequently, the mixture was incubated at 66°C in a Loopamp real-time turbidimeter (LA-200; Teramecs Co., Kyoto, Japan) for 120 minutes. The results were considered positive based on the rise in the curves of turbidity greater than a threshold of 0.1 according to the manufacture's instruction. Extracted DNA from *M. avium* JATA 51-01 and DDW was used in each run as a positive and a negative control, respectively. These above conditions were adopted following a series of preliminary optimization LAMP reactions in which some parameters were tested in varying concentrations, e.g. a range of temperatures (64°C - 68°C), primer volumes as well as different volumes of betaine and magnesium.

1.2.3. Sensitivity of IS*1245* LAMP assay

For the sensitivity evaluation, the concentration of *M. avium* ssp. *hominissuis* HP-22 genomic DNA was first measured using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, it was diluted with TE buffer to prepare DNA solution at a final concentration of 3ng/µl. This DNA solution was used to make 10-fold serial dilutions of the target DNA to concentrations ranging from 3pg/µl, 300fg/µl, 30fg/µl, 3fg/µl and an additional 1fg/µl (6pg~2fg/reaction). Two microliters of each dilution were used as a DNA template for each LAMP reaction. To examine reproducibility, all reactions were performed in duplicates and repeated three times (6 reactions) at 66°C in a Loopamp real-time turbidimeter (LA-200; Teramecs Co.) for 120 minutes. Additionally, the effect of the number of copies of IS*1245* on the sensitivity of the developed LAMP was evaluated by analyzing *M. avium* type strain JATA 51-01 with only one copy of IS*1245* [59]. The sensitivity determination was carried out as outlined above.

The sensitivity of the assay was further evaluated using a total of 228 *M. avium* isolates including 137 clinical isolates obtained in Osaka (n=50), Tokyo (n=56) and Hokkaido (n=31), and

84 isolates obtained from pigs in Osaka and 7 pig isolates from Hokkaido (Table 3). To ensure reproducibility, all reactions were performed in duplicates while *M. avium* JATA 51-01 strain and DDW were used as positive and negative controls respectively.

1.2.4. Specificity of IS*1245* LAMP assay

To confirm the specificity of the assay, the designed primers were first subjected to a BLAST search against all available sequences in the database to screen for any possible cross-reaction with other non-targeted heterologous bacteria using the BLASTn software algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Thereafter, the LAMP assay was assessed against 22 nontuberculous mycobacterial type strains, 2 MTBC reference strains, 14 MTBC clinical isolates, and an additional 5 non-mycobacterial species listed in (Table 1). *M. avium* JATA 51-01 strain was used as positive control while DDW was a negative control.

1.2.5. IS*1245* PCR reaction

To investigate the validity of the LAMP assay, IS*1245* PCR was also performed using forward (5'-CCGGATCTGCAAAGACCTC-3') and reverse (5'-CGACACCACCCGATGATTC-3') oligonucleotides as reported by Slana et al, 2010 [63]. The following PCR reaction conditions were used; 1 µl of template DNA, 4µl 5x GoTaq buffer (Promega Corporation, Madison, WI, USA), 0.2µl 25mM dNTP, 0.8µl 25mM $MgCl_2$, 2µl 5M betaine, 0.5µl each primer (0.25µM), 0.2µl GoTaq DNA polymerase (5 units/µl) and DDW to make up a total volume of 20µl. The amplification protocol consisted of an initial denaturation at 96°C for 1 minute, followed by 30 cycles of denaturation at 94°C (1 minute), annealing at 60°C (1 minute), and extension at 72°C (1 minute) and a post-run elongation of 10 minutes at 72°C. The PCR amplicons were analyzed by using 2% agarose gel electrophoresis with GelRed (Biotium, Inc., CA, USA) in 1× TAE buffer at 100v for 15 minutes and visualized by ultraviolet trans-illumination. The expected band size was 130bp.

1.2.6. 16S-23S ribosomal DNA internal transcribed spacer sequence (ITS) sequencing

To confirm the bacterial species and the genotype of *M. avium* isolates, ITS sequencing was performed. The region was amplified with a pair of primers, 16S-univ-4 (5'-AGTCCCGCAACGAGCGCAACCC-3') and myco ITS-23s-Rv (5'-

CGGTTGACAGCTCCCCGAGGC-3') with the same PCR reagent conditions shown in section 1.2.5. The amplification protocol consisted of an initial denaturation at 96°C for 1 minute, followed by 35 cycles of denaturation at 96°C (10 sec), annealing at 62°C (10 sec), and extension at 72°C (50 sec) and a post-run elongation of 5 minutes at 72°C. The PCR amplicons were analyzed by 1.5% agarose gel electrophoresis. The ITS sequence was read on a 3500xL Genetic Analyzer (Thermo Fisher Scientific) using a primer, 16S-univ-ITS (5'-CTTGTACACACCGCCCGTCA-3') and using Big Dye Terminator v3.1 chemistry (Thermo Fisher Scientific) following the manufacturer's protocol. The resulting sequences were compared with the ITS sequence of *M. avium* strain 104 (NC_008595.1) and classified [18,25].

1.2.7. Whole-genome sequencing (WGS)

The DNA concentration was verified by Qubit 3.0 (Thermo Fisher Scientific). WGS was performed on an Illumina MiSeq 2500 for selected Hokkaido isolates (Table 3). Library preparation and sequencing were performed according to the Illumina MiSeq manual. The quality of sequences was evaluated, trimmed, and mapped to the reference genome TH135 (NZ_AP012555.1) and IS*1245* (accession No. L33879) using CLC Genomics Workbench 10 (QIAGEN, Hilden, Germany).

1.3. Results

1.3.1. Sensitivity of IS*1245* LAMP assay

As shown in Figure 2, the LAMP reaction was able to detect as low as 6fg, which is the equivalent of 1 genome copy of *M. avium* HP-22 DNA per reaction within 30 minutes. I further tested its sensitivity in detecting *M. avium* strains with low IS*1245* copy numbers by evaluating the type strain JATA 51-01 with only one copy of IS*1245*. The IS*1245* LAMP was able to detect 60fg (10 copies) of the *M. avium* JATA 51-01 genomic DNA in 30 minutes as indicated in Figure 7. Among the 137 human clinical isolates evaluated, the IS*1245*LAMP assay was able to detect 96/137 (70.1%) isolates, while 41/137 (29.9%) were negative. Specifically, 37/50 (74.0%) isolates from Osaka were positive whereas 13/50 (26.0%) were LAMP negative, 33/56 (58.9%) isolates from Tokyo were positive while 23/56 (41.1%) were negative and 26/31 (83.9%) isolates from Hokkaido were positive and 5/31 (16.1%) were negative. The assay was able to detect all pig-

derived *M. avium* isolates (91/91, 100%) from both Osaka and Hokkaido Prefectures (Table 2, Table 3).

1.3.2. Specificity of the IS*1245* LAMP assay

The IS*1245* LAMP correctly identified the *M. avium* type strain JATA 51-01, with no amplification seen with any of the 43 non-*M. avium* and non-mycobacterial species included in the specificity assessment, as shown in Table 1.

1.3.3. IS*1245* PCR reaction

To validate the results of the IS*1245* LAMP, conventional PCR targeting IS*1245* for the detection of *M. avium* was performed using primers described by Slana et al. (2010) [63]. The IS*1245* positivity and negativity results in IS*1245* PCR were 100% consistent with those of the IS*1245* LAMP (Table 2, Table 3).

1.3.4. ITS sequencing

ITS sequence types were determined and shown in Table 3. Human isolates consisted of two major ITS types, MavF (73/137, 53.3%) and MavA (61/137, 44.5%), and a minor ITS type MavB (5/137, 3.6%). On the other hand, the vast majority of the pig isolates were MavB (80/91, 87.9%) while MavA was minor (11/91, 12.1%). No MavF types were observed among pig isolates. All the MavB isolates and pig-derived MavA isolates were IS*1245* positive. Among human isolates, 32.4% (23/71) of MavF and 30.5% (18/59) of MavA isolates were IS*1245* negative.

1.3.5. Whole-genome sequencing

Some LAMP positive and LAMP negative Hokkaido isolates were subjected to whole-genome sequencing. The results of WGS revealed that all the IS*1245* LAMP positive isolates carried the insertion element IS*1245* in their genome whereas all IS*1245* LAMP negative isolates lacked the insertional sequence. These findings, therefore, explain why some isolates could be detected by neither the IS*1245* LAMP nor the IS*1245* PCR (Table 3).

1.4. Discussion

The rise in the global incidence of *M. avium* infections constitutes a threat to public health and hence the quest to continue developing sensitive, low-cost diagnostic tools remains of

paramount importance. In the current study, I designed a sensitive LAMP system to rapidly detect *M. avium* by targeting IS*1245*, a multi-copy element believed to be exclusive to *M. avium* clinically important subspecies strains [35,59]. LAMP has become a very useful method for pathogen detection targeting a variety of sequences including insertional sequences and many housekeeping genes [28,29,37,39,62,64–66].

The IS*1245*-LAMP described in this study was highly sensitive, able to detect as low as one copy of *M. avium* genomic DNA (6fg) in 30 minutes. This sensitivity was higher than two previously reported LAMP-based systems targeting the 16S rRNA and *gyrB* which had detection limits of 100fg and 1pg of *M. avium* DNA respectively within 60 minutes [28]. Most *M. avium* isolates from humans have been described to contain 3-27 copies of IS*1245* [35,40,67] and this multi-copy characteristic is a possible reason for the enhanced sensitivity of assays targeting IS*1245*, especially when contrasted with single-copy genes. Equally, in *M. tuberculosis* diagnosis, LAMP assays targeting insertion sequence IS*6110* have been reported to have higher sensitivity when compared to other assays that targeted single-copy genes such as *gyrB* or *rrs* [28,29,68]. However, even when I evaluated the IS*1245* LAMP assay on an *M. avium* strain with a single copy of IS*1245*, the sensitivity (60fg of *M. avium* DNA/reaction) was still higher, and the assay time shorter (30 minutes), than previously reported LAMP-based assays for *M. avium* detection [28,39].

Recently, during the preparation of my manuscript, another IS*1245* LAMP-based system for the detection of *M. avium* was reported by Yashiki et al. (2019) [69]. Although their LAMP target was the same as my target, their primary focus was to distinguish *M. avium subsp. hominissuis* from *M. avium subsp. avium* in pigs in Japan. As a result, all the 31 *M. avium* isolates analyzed in that study were pig isolates and their assay was not validated on human isolates. The authors also reported a detection limit of 0.4pg while the limit of detection of my LAMP assay was improved by almost 100-fold (0.006pg). Besides that, Yashiki et al. (2019) [69] reported that at the highest concentration of 4151pg, *M. avium* could be detected in 21 minutes; however, with the assay, a lower concentration (6pg) could detect *M. avium* quicker than 20 minutes (Fig 2). Therefore, in terms of sensitivity and speed, the IS*1245* LAMP assay had comparably improved sensitivity, shorter turnaround time, and was evaluated on human isolates as well as pig isolates.

In addition to having enhanced sensitivity, the LAMP assay demonstrated high specificity for *M. avium*, namely for IS*1245*, thus eliminating any possibility of false-positive results. The

LAMP results were also in agreement with parallel validation by PCR of the same panel of isolates using *M. avium* IS*1245* specific published primers [63], and therefore the assay itself is accurate and precise (Table 2, Table 3).

As well as reporting the performance of the newly developed IS*1245* LAMP as a detection tool, the study further highlights the unique epidemiological aspects of the IS*1245* gene in *M. avium* strains and the caution with which the assay results should be interpreted. The newly established IS*1245* LAMP was able to correctly detect all *M. avium* isolates carrying IS*1245* from both humans and pigs across Japan. However, during the evaluation, I encountered a proportion of the *M. avium* strains from the Japanese human population that lacked IS*1245*. The lack of IS*1245* in some isolates was confirmed by comprehensive whole-genome sequencing of twelve Hokkaido clinical isolates which were all LAMP and PCR negative (Table 3). A previous Japanese study [24] conducted in Nagoya, a city located between Osaka and Tokyo, found IS*1245* in 84% (96/114) of the examined *M. avium* isolates. In the study, the IS*1245* carriage rates in clinical isolates from Osaka, Tokyo, and Hokkaido were determined to be 74% (37/50), 59% (33/56), and 84% (26/31) respectively, with an overall total of 70% (96/137). This rate is significantly lower than the 96% (88/92) reported from neighboring South Korea [70] and 98% (90/92) reported from Belgium (Fisher's exact test, $p<0.05$) [71].

In contrast to the observation made with *M. avium* clinical isolates, all pig-derived *M. avium* isolates maintained the IS*1245* in their genome (91/91, 100%). This difference underscores one of the possibly many molecular aspects whereby *M. avium* strains in the Japanese human population may be genetically distinct from the pig strains, as previously suggested [23, 25,72].

The study by Iwamoto et al. (2012) [72] reported that the *M. avium* clinical isolates in Japan had high relatedness with the genotypic profiles of environmental strains, whereas pig-derived *M. avium* strains in Japan were similar genetically to strains isolated from the European human population. This could be because livestock pigs in Japan have a Euro-American origin and were introduced to Japan several decades ago, with hundreds of breeding pigs being continuously imported from these countries every year (https://www.maff.go.jp/aqs/tokei/toukeinen.html). Additionally, the feed or sawdust given to pigs in Japan is usually imported from other countries and so this could be a plausible route of infection [73].

MavA and MavF were the two major ITS types in Japanese clinical isolates, whereas MavB was predominant in pig isolates, which was consistent with the result of a previous study in Japan by Adachi et al. (2016) [25]. Mijs et al. (2002) [18] observed multi-banded IS*1245* RFLP patterns in MavB isolates from pigs and humans in the Netherlands, Belgium, and the United States. They confirmed the presence of IS*1245* in all samples, even in MavA isolates, which was also the case for pig isolates in Japan. Therefore, the lack of IS*1245* may be a unique event that occurred in a particular population of Japanese *M. avium* ssp. *hominissuis* strains.

In light of the findings, the newly developed IS*1245* LAMP system may not benefit the Japanese human population due to low IS*1245* carriage rates in the circulating *M. avium* strains. However, the IS*1245* LAMP assay is highly suitable for application in Euro-American countries and other regions that have a high IS*1245* prevalence and routinely use IS*1245* as a marker of *M. avium* for both detection and differentiation [36,57,59,71,74–77]. In such settings, this LAMP assay may be considered as an additional approach for *M. avium* detection, particularly in point-of-care or resource-limited settings.

However, in regions where IS*1245* is not routinely used as a marker, this IS*1245* LAMP may only be used to complement other methods for *M. avium* detection. The lack of IS*1245* in some *M. avium* strains remains the main limitation of the IS*1245* LAMP assay, particularly in regions with low or unknown IS*1245* prevalence. Hence, in the next study, I plan to employ the use of IS*1245* with a combination of an essential gene to ensure complete detection of all *M. avium* strains by a LAMP. The current study only investigated *M. avium* clinical isolates from northern and central Japan, hence the IS*1245* carriage rates may not be representative of Japan as a whole. For a more representative view, isolates from all major regions in Japan will need to be analyzed.

1.5. Summary

The incidences of *M. avium* infections have been increasing especially in industrialized countries causing higher burdens than *M. tuberculosis*. Unlike, *M. tuberculosis*, *M. avium* is especially harder to treat due to the lack of effective treatment regimens and high drug resistance rates. Therefore, early detection before full disease progression is paramount for the timely control of *M. avium* infections. This, therefore, places an urgent need for accurate and rapid diagnostic tools that are cost-friendly to accommodate regions with limited resources.

In this chapter, a LAMP assay targeting IS*1245* for the rapid detection of *M. avium* using 228 *M. avium* strains, 137 clinical isolates and 91 isolates from pigs from Hokkaido, Osaka and Tokyo in Japan. The effects of genotypic diversity on the applicability of this tool in Japanese *M. avium* was also explored.

The developed assay could detect as low as 1 genome copy of *M. avium* DNA within 30 minutes. All 91 (100%) *M. avium* isolates from pigs were detected positive while all other tested bacterial species were negative. Interestingly, among the 137 clinical *M. avium* isolates, 41 (30%) were undetectable with this LAMP assay as they lacked IS*1245*, the absence of which was revealed by PCR and whole-genome sequencing. These findings highlighted genotypic differences in *M. avium* strains from humans and pigs in Japan and how this diversity can influence the applicability of a detection tool across different geographical areas and hosts.

The IS*1245* marker is known as a gold standard for the detection of *M. avium*, however, the evaluation showed that *M. avium* genetic diversity has a major influence on the applicability of the diagnostic tool across different geographical areas. Hence, it is very critical to understand the local genetic make-up of a pathogen population before adopting descriptions formed at the global level (which are often focussed on European or US findings).

Pathogen populations should be tested for the presence or absence of IS elements (or other markers) used in diagnostics tests when designing and evaluating a new detection method. Nevertheless, the newly developed IS*1245* LAMP assay detailed in this chapter, exhibited high sensitivity and specificity, thereby making it a fast, cost-friendly alternative method of *M. avium* detection especially in the Euro Americas which have reported very high IS*1245* carriage rates.

Table 1. Bacterial strains used to determine the specificity of IS*1245*-LAMP assay

	Bacterial species	Sample ID	LAMP Results
NTM reference strains	*M. avium* (ssp. *avium*)	JATA 51-01	P
	M. chelonae	JATA 62-01	N
	M. fortuitum	JATA 61-01	N
	M. gastri	KK 44-01	N
	M. gordonae	JATA 33-01	N
	M. intermedium	JATA 9H-01	N
	M. intracellulare	JATA 52-01	N
	M. kansasaii	KK 21-01	N
	M. lentiflavum	JATA 9N-01	N
	M. abscessus	JATA 63-01	N
	M. asiaticum	KK 24-01	N
	M. malmoense	JATA 47-01	N
	M. mucogenicum	JATA 9P-01	N
	M. nonchromogenicum	JATA 45-01	N
	M. peregrinum	JATA 61-01	N
	M. shimodei	JATA 54-01	N
	M. smegmatis	JATA 64-01	N
	M. ulcerans	JATA 43-03	N
	M. celatum	JATA 9L-01	N
	M. triviale	JATA 50-01	N
	M. xenopi	JATA 42-01	N
	M. simiae	JATA 32-01	N
	M. scrofulaceum	JATA 46-01	N
MTBC reference strains	*Mycobacterium bovis* BCG	BCG Tokyo 172	N
	Mycobacterium tuberculosis	H37Rv	N
MTBC clinical isolates	*M. bovis*	L090-1	N
	M. bovis	1199	N
	M. bovis	24189 (55)	N
	M. bovis	24208 (56)	N
	M. tuberculosis	06-13	N
	M. tuberculosis	06-82	N
	M. tuberculosis	07-21	N
	M. tuberculosis	07-49	N
	M. tuberculosis	07-61	N
	M. tuberculosis	07-81	N
	M. tuberculosis	08-10	N
	M. tuberculosis	08-14	N
Non-mycobacteria	*Streptococcus pneumoniae*	NBRC 102642	N
	Klebsiella pneumoniae	NBRC 3318	N

	Pseudomonas aeruginosa	NBRC 12689	N
	Staphylococcus aureus	NBRC 100910	N
	Mycoplasma pneumoniae	NBRC 14401	N

P: result of assay positive, N: negative
JATA: Japan Anti-Tuberculosis Association (= KK: Kekkaku Kenkyusyo in Japanese)
NBRC: Biological Resource Center, NITE
* JATA 51-01 is the type strain of *M. avium* that shares its origin with ATCC 25291.

Table 2. Results of the detection by IS*1245*-LAMP assay and by IS*1245*-PCR assay using *M. avium* isolates in Japan

a) Osaka patients

		IS*1245* PCR*		
		Positive	Negative	Total
IS*1245* LAMP	Positive	37	0	37
	Negative	0	13	13
	Total	37	13	50

b) Tokyo patients

		IS1245 PCR*		
		Positive	Negative	Total
IS*1245* LAMP	Positive	33	0	33
	Negative	0	23	23
	Total	33	23	56

c) Hokkaido patients

		IS1245 PCR*		
		Positive	Negative	Total
IS*1245* LAMP	Positive	26	0	26
	Negative	0	5	5
	Total	26	5	31

d) Osaka pigs

		IS1245* PCR		
		Positive	Negative	Total
IS*1245* LAMP	Positive	84	0	84
	Negative	0	0	0
	Total	84	0	84

e) Hokkaido pigs

		IS1245* PCR		
		Positive	Negative	Total
IS*1245* LAMP	Positive	7	0	7
	Negative	0	0	0
	Total	7	0	7

*Primers according to Slana et al. (2012) [63]

Table 3A. Osaka MAH clinical isolates

No.	Sample ID	IS*1245* LAMP Run 1	IS*1245* LAMP Run 2	IS*1245* PCR	ITS code
1	Cl-A-1	N	N	N	MavF
2	Cl-A-2	P	P	P	MavF
3	Cl-A-3	P	P	P	MavA
4	Cl-A-4	P	P	P	MavF
5	Cl-A-5	P	P	P	MavA
6	Cl-A-6	P	P	P	MavA
7	Cl-A-7	P	P	P	MavA+MavF
8	Cl-A-8	P	P	P	MavF
9	Cl-A-9	N	N	N	MavF
10	Cl-A-10	P	P	P	MavF
11	Cl-A-11	P	P	P	MavA
12	Cl-A-12	P	P	P	MavA
13	Cl-A-13	P	P	P	MavF
14	Cl-A-14	P	P	P	MavF
15	Cl-A-15	P	P	P	MavA
16	Cl-A-16	P	P	P	MavF
17	Cl-A-17	P	P	P	MavF
18	Cl-A-18	P	P	P	MavF
19	Cl-A-19	P	P	P	MavF
20	Cl-A-20	P	P	P	MavF
21	Cl-A-21	P	P	P	MavF
22	Cl-A-22	P	P	P	MavF
23	Cl-A-23	P	P	P	MavA
24	Cl-A-24	N	N	N	MavA
25	Cl-A-25	P	P	P	MavA
26	Cl-A-26	P	P	P	MavA
27	Cl-A-27	P	P	P	MavA
28	Cl-A-28	P	P	P	MavF
29	Cl-A-29	P	P	P	MavF
30	Cl-A-30	N	N	N	MavF
31	Cl-A-31	P	P	P	MavF
32	Cl-A-32	N	N	N	MavF
33	Cl-A-33	N	N	N	MavF
34	Cl-A-34	P	P	P	MavA
35	Cl-A-35	N	N	N	MavF
36	Cl-A-36	N	N	N	MavA
37	Cl-A-37	P	P	P	MavA
38	Cl-A-38	P	P	P	MavF
39	Cl-A-39	P	P	P	MavA
40	Cl-A-40	N	N	N	MavA
41	Cl-A-41	N	N	N	MavF
42	Cl-A-42	P	P	P	MavF
43	Cl-A-43	P	P	P	MavA
44	Cl-A-44	N	N	N	MavF
45	Cl-A-45	P	P	P	MavA
46	Cl-A-46	P	P	P	MavF
47	Cl-A-47	P	P	P	MavF
48	Cl-A-48	N	N	N	MavA

49	Cl-A-49	P	P	P	MavF
50	Cl-A-50	N	N	N	MavF

B. Tokyo MAH clinical samples

No.	Sample ID	IS*1245* LAMP		IS*1245* PCR	ITS code
		Run 1	Run 2		
1	Koav-01	N	N	N	MavF
2	Koav-02	N	N	N	MavF
3	Koav-03	P	P	P	MavF
4	Koav-04	N	N	N	MavF
5	Koav-05	N	N	N	MavA
6	Koav-06-1	P	P	P	MavF
7	Koav-06-2	P	P	P	MavF
8	Koav-07	N	N	N	MavF
9	Koav-08	N	N	N	MavF
10	Koav-09	P	P	P	MavA
11	Koav-10-1	P	P	P	MavA
12	Koav-10-2	N	N	N	MavA
13	Koav-11	N	N	N	MavA
14	Koav-12	P	P	P	MavF
15	Koav-13	P	P	P	MavF
16	Koav-14	P	P	P	MavA
17	Koav-15	N	N	N	MavA
18	Koav-16	P	P	P	MavA
19	Koav-17	P	P	P	MavF
20	Koav-18	N	N	N	MavA
21	Koav-19	P	P	P	MavF
22	Koav-20	P	P	P	MavA
23	Koav-21	N	N	N	MavF
24	Koav-22	P	P	P	MavA
25	Koav-23	P	P	P	MavA
26	Koav-24	P	P	P	MavF
27	Koav-25	N	N	N	MavA
28	Fuku-av-1	N	N	N	MavA
29	Fuku-av-2	P	P	P	MavF
30	Fuku-av-3	P	P	P	MavF
31	Fuku-av-4	N	N	N	MavF
32	Fuku-av-5	P	P	P	MavF
33	Fuku-av-6	N	N	N	MavA
34	Fuku-av-7	P	P	P	MavF
35	Fuku-av-8	P	P	P	MavF
36	Fuku-av-9	P	P	P	MavA
37	Fuku-av-10	N	N	N	MavA
38	Fuku-av-11	P	P	P	MavF
39	Fuku-av-12	N	N	N	MavF
40	Fuku-av-14	N	N	N	MavF
41	Fuku-av-15	P	P	P	MavF
42	Fuku-av-16	N	N	N	MavA
43	Fuku-av-17	P	P	P	MavA
44	Fuku-av-18	P	P	P	MavA
45	Fuku-av-19	N	N	N	MavA
46	Fuku-av-20	P	P	P	MavA

47	Fuku-av-21	P	P	P	MavB
48	Fuku-av-22	N	N	N	MavA
49	Fuku-av-23	P	P	P	MavA
50	Fuku-av-24	N	N	N	MavF
51	Fuku-av-25	P	P	P	MavF
52	Fuku-av-26	P	P	P	MavA+MavF
53	Fuku-av-27	P	P	P	MavF
54	Fuku-av-28	P	P	P	MavF
55	Fuku-av-29	P	P	P	MavF
56	Fuku-av-30	N	N	N	MavF

C. Hokkaido MAH clinical isolates

No.	Sample ID	IS*1245* LAMP		IS*1245* PCR	WGS IS*1245*	ITS code
		Run 1	Run 2			
1	Hokudai HP 1	P	P	P	P	MavA
2	Hokudai HP 6	P	P	P	P	MavA
3	Hokudai HP 7	N	N	N	N	MavA
4	Hokudai HP 9	P	P	P	-	MavA
5	Hokudai HP 12	P	P	P	P	MavA
6	Hokudai HP 17	P	P	P	P	MavA
7	Hokudai HP 18	P	P	P	-	MavA
8	Hokudai HP 20	P	P	P	-	MavF
9	Hokudai HP 22	P	P	P	P	MavF
10	Hokudai HP 29	N	N	N	N	MavF
11	Hokudai HP 30	P	P	P	-	MavF
12	Hokudai HP 31	P	P	P	-	MavF
13	Hokudai HP 34	P	P	P	-	MavB
14	Hokudai HP 39	N	N	N	N	MavF
15	Hokudai HP 41	P	P	P	-	MavF
16	Hokudai HP 45	P	P	P	-	MavA
17	Hokudai HP 49	P	P	P	-	MavA
18	Hokudai HP 51	P	P	P	P	MavB
19	Hokudai HP 52	P	P	P	-	MavA
20	Hokudai HP 53	P	P	P	P	MavB
21	Hokudai HP 54	N	N	N	-	MavF
22	Hokudai HP 55	P	P	P	-	MavA
23	Hokudai HP 56	P	P	P	-	MavB
24	Hokudai HP 58	N	N	N	N	MavA
25	Hokudai HP 59	P	P	P	P	MavF
26	Hokudai HP 60	P	P	P	-	MavA
27	Hokudai HP 61	P	P	P	-	MavA
28	Hokudai HP 64	P	P	P	-	MavF
29	Hokudai HP 67	P	P	P	-	MavA
30	Hokudai HP 68	P	P	P	-	MavA
31	Hokudai HP 70	P	P	P	-	MavF

D. Osaka MAH pig isolates

No.	Sample ID	IS*1245* LAMP		IS*1245* PCR	ITS code
		Run 1	Run 2		
1	OP 1-1	P	P	P	MavB
2	OP 2-1	P	P	P	MavA
3	OP 2-2	P	P	P	MavA
4	OP 3-1	P	P	P	MavB
5	OP 3-2	P	P	P	MavB
6	OP 3-3	P	P	P	MavB
7	OP 3-4	P	P	P	MavB
8	OP 3-5	P	P	P	MavB
9	OP 3-6	P	P	P	MavB
10	OP 4-2	P	P	P	MavB
11	OP 4-3	P	P	P	MavB
12	OP 5-1	P	P	P	MavA
13	OP 6-1	P	P	P	MavB
14	OP 6-2	P	P	P	MavB
15	OP 6-3	P	P	P	MavB
16	OP 6-4	P	P	P	MavB
17	OP 7-1	P	P	P	MavB
18	OP 7-2	P	P	P	MavB
19	OP 8	P	P	P	MavB
20	OP 9-1	P	P	P	MavB
21	OP 9-2	P	P	P	MavB
22	OP 11	P	P	P	MavB
23	OP 12	P	P	P	MavB
24	OP 13	P	P	P	MavB
25	OP 14	P	P	P	MavB
26	OP 15	P	P	P	MavB
27	OP 16	P	P	P	MavB
28	OP 17	P	P	P	MavA
29	OP 18	P	P	P	MavB
30	OP 19	P	P	P	MavB
31	OP 21	P	P	P	MavB
32	OP 22	P	P	P	MavB
33	OP 23	P	P	P	MavB
34	OP 24	P	P	P	MavB
35	OP 25	P	P	P	MavB
36	OP 26	P	P	P	MavB
37	OP 27	P	P	P	MavB
38	OP 28	P	P	P	MavB
39	OP 29	P	P	P	MavB
40	OP 30	P	P	P	MavA
41	OP 31	P	P	P	MavB
42	OP 32	P	P	P	MavA
43	OP 33	P	P	P	MavB
44	OP 34	P	P	P	MavB

45	OP 35	P	P	P	MavB
46	OP 36	P	P	P	MavB
47	OP 37	P	P	P	MavB
48	OP 38	P	P	P	MavB
49	OP 39	P	P	P	MavB
50	OP 40	P	P	P	MavB
51	OP 41	P	P	P	MavB
52	OP 42	P	P	P	MavB
53	OP 43	P	P	P	MavA
54	OP 44	P	P	P	MavB
55	OP 46	P	P	P	MavB
56	OP 47	P	P	P	MavB
57	OP 48	P	P	P	MavB
58	OP 49	P	P	P	MavB
59	OP 50	P	P	P	MavB
60	OP 51	P	P	P	MavB
61	OP 52	P	P	P	MavB
62	OP 53	P	P	P	MavB
63	OP 54	P	P	P	MavB
64	OP 55	P	P	P	MavB
65	OP 56	P	P	P	MavB
66	OP 56-2	P	P	P	MavB
67	OP 58	P	P	P	MavB
68	OP 59	P	P	P	MavB
69	OP 60	P	P	P	MavB
70	OP 61	P	P	P	MavB
71	OP 62	P	P	P	MavB
72	OP 63	P	P	P	MavB
73	OP 64	P	P	P	MavB
74	OP 65	P	P	P	MavB
75	OP 66	P	P	P	MavA
76	OP 67	P	P	P	MavB
77	OP 68	P	P	P	MavA
78	OP 69	P	P	P	MavA
79	OP 70	P	P	P	MavB
80	OP 71	P	P	P	MavB
81	OP 72	P	P	P	MavB
82	OP 73	P	P	P	MavA
83	OP 74	P	P	P	MavB
84	OP 75	P	P	P	MavB

E. Hokkaido MAH pig isolates

No.	Sample ID	IS*1245* LAMP		IS*1245* PCR	ITS code
		Run 1	Run 2		
1	Rakuno pig 30	P	P	P	MavB
2	Rakuno pig 31	P	P	P	MavB
3	Rakuno pig 32-1	P	P	P	MavB
4	Rakuno pig 32-2	P	P	P	MavB
5	Rakuno pig 34	P	P	P	MavB
6	Rakuno pig 38-1	P	P	P	MavB
7	Rakuno pig 38-2	P	P	P	MavB

MAH: *M. avium* ssp. *hominissuis*
P: detection positive
N: detection negative
-: not done

ITS sequence type* (accession number of reference sequence)
MavA (L07855)
MavB (L07856)
MavF (AF315838)

* Mijis et al. (2002) [18], Adachi et al. (2016) [25]

A

329 GCAGGGGACC TGGAACTGCG GATTCCCAAG CTGCGCACC G GGTCATTTTT
F3 F2

379 CCCGGCGTTG TTGGAGCGGC GTCGCCGGGT CGATCAGTGC TTGTTCGCGG
LF F1

429 TGGTGATGGA GGCCTACCTG CACGGCACCT CCACCCGCAA GGTCGACGAT
B1

479 C TGGTCAAGG CACTGGGTAC CGATACCGGG ATCTCCAAAA GCGAGGTCAG
LB B2

529 CCGGATCTGC AAAGACCTCG ACACCGAGGT CGCGGCCTTC C 569
B3

B

Primer	Length	Sequence 5' to 3'
F3	18	ATTCCCAAGCTGCGCACC
B3	20	GGTCTTTGCAGATCCGGCTG
FIP	40	CACCGCGAACAAGCACTGAT-GGGTCATTTTTCCCGGCGTT
BIP	40	CACCCGCAAGGTCGACGATC-CCTCGCTTTTGGAGATCCCG
LF	17	GACCCGGCGACGCCGCT
LB	19	TGGTCAAGGCACTGGGTAC

Figure 5. Primer sets used for the amplification of *M. avium* IS*1245* by the loop-mediated isothermal amplification (LAMP) technique. (A) Locations of the primers on the target sequence IS*1245*. (B) Names, length, and sequences of six primers. F3 and B3 represent forward and backward external primers, respectively; FIP and BIP represent forward and backward internal primers, respectively; and LF and LB represent forward and backward loop primer. Primer FIP consists of F1 complementary sequence and F2 direct sequence. Primer BIP consists of B1 direct sequence and B2 complementary sequence.

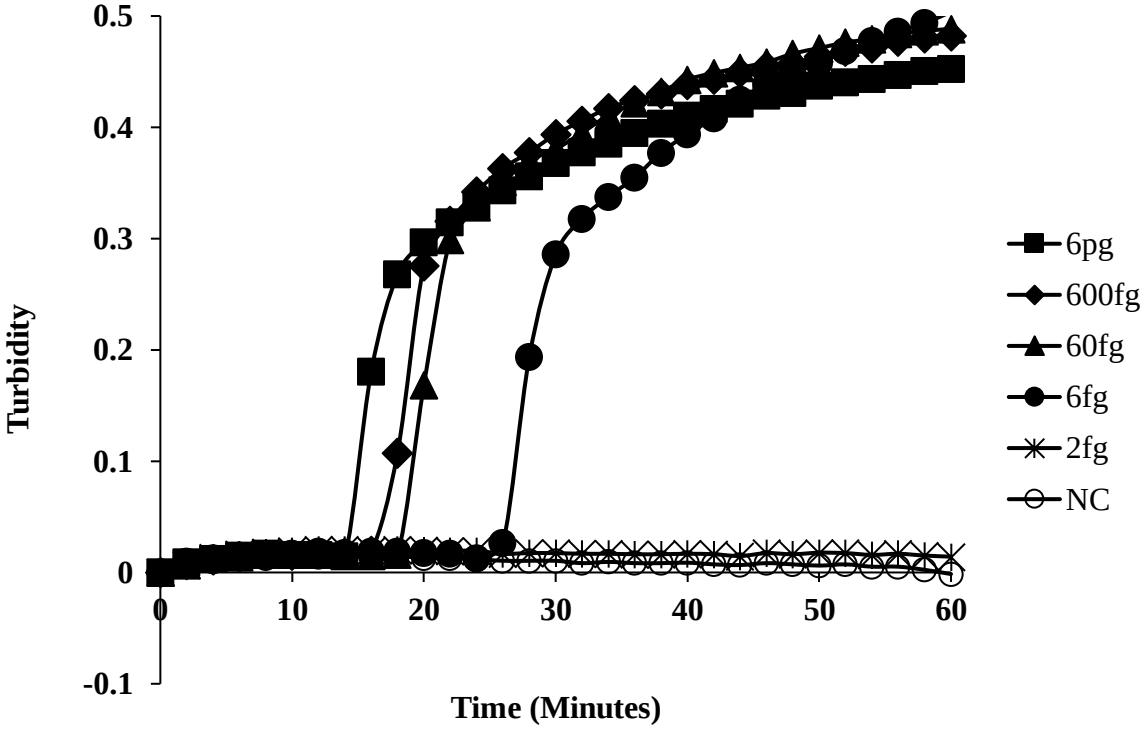

Figure 6 . Sensitivity of established LAMP assay to detect *M. avium* ssp. *hominissuis* clinical strain (HP-22) observed by rising curves on turbidimeter LA-200 (Teramecs Co.)

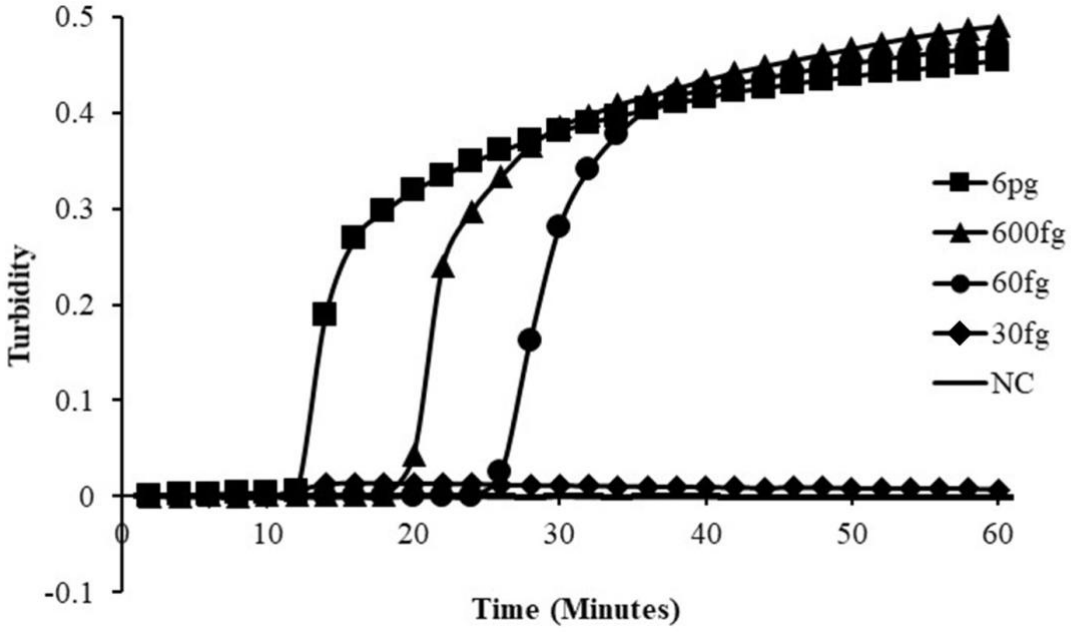

Figure 7. Sensitivity of established LAMP assay to detect *M. avium* ssp. *avium* (JATA 51-01) strain possessing a single copy of IS*1245* as observed by rising curves on the turbidimeter

Chapter II: The role of pentapeptide protein MfpA in conferring fluoroquinolone resistance in *Mycobacterium avium*

2.1. Introduction

Fluoroquinolones (FQs) are broad-spectrum antibiotics that form a critical component of antituberculous drug regimens for multidrug-resistant (MDR) *M. avium* as well as tuberculosis (TB) [78,79]. FQ inhibit the activity of DNA gyrase, a biologically essential type II DNA topoisomerase enzyme that regulates DNA topology leading to efficient transcription in bacteria. FQ resistance in mycobacteria has been increasingly reported worldwide. It is generally caused by mutations at the codons, 88, 90, or 94 in DNA gyrase A subunit-coding gene and/or DNA gyrase B subunit [30].

In a previous study, several *M. avium* isolates were found to be FQ resistant despite not having any mutations in DNA gyrase genes. After performing WGS of these isolates, no mutations were found in DNA gyrase genes and my attention was shifted to *mfpA*. Interestingly, WGS results revealed that *mfpA* was intact in FQ resistant strains while susceptible strains had a frameshift in *mfpA*. Due to the poor correlation between DNA gyrase mutations and drug resistance, the contribution of *mfpA* was investigated.

MfpA is a member of the pentapeptide repeat proteins which is present in the chromosomes of all known mycobacterial genomes and has been suggested to compete with DNA for binding to the DNA gyrase. FQs sorely bind to the DNA-gyrase complex hence when MfpA binds to the gyrase, it either replaces DNA in a FQ-inhibited complex which prevents the formation of the gyrase DNA complex, or releases the gyrase from FQ inhibition thereby conferring resistance [52,80]. However, the contribution of MfpA to FQ resistance in *M. avium* clinical strains has not yet been elucidated.

Therefore, in this chapter, I investigated the role of *mfpA* to resistance in levofloxacin, a representative FQ, by checking the association of the MfpA genotypes and the level of phenotypic susceptibility to FQ through MIC determination and sequencing of FQ associating genes.

2.2. Materials and Methods

2.2.1. Bacterial strains and cultures conditions

A total of 88 *M. avium* clinical strains obtained from Keio University Hospital in Tokyo (n=23), the National Hospital Organization Osaka Toneyama Medical Center (n=40), and Hokkaido University Hospital (n=25), were used in this study. Using the modified procedure of Suzuki et al. (1995), genomic DNA was extracted from the colonies by mechanical disruption [60], whereas speciation was done by the sequencing of the 16S rRNA gene and results of ITS sequencing confirmed all of the isolates to belong to *M. avium* following an early publication [18]. The samples were grown in Ogawa medium for confirmation and bacterial colonies were then transferred to Difco Middlebrook 7H11 agar plates (Becton Dickinson & Co, Sparks, NV) containing 10% oleic acid-albumin-dextrose complex (OADC) (Becton Dickinson & Co) and 0.2% glycerol and incubated for 7 days.This was followed by a subculture of selected colonies on Ogawa medium and after a week they were inoculated into Difco Middlebrook 7H9 broth medium (Becton Dickinson & Co) supplemented with 10% OADC and 0.2% glycerol and incubated at 37ºC for 5-7 days. Bacterial suspensions were kept at -80ºC until required whereas extracted DNA samples were kept at -30ºC until they were used.

2.2.2. Levofloxacin susceptibility test for *M. avium* isolates

M. avium isolates were cultured on Ogawa media for 10-14 days at 37ºC and then sub-cultured into Difco Middlebrook 7H9 broth medium supplemented with 10% OADC and incubated at 37°C. The broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) of levofloxacin (LVX) (LKT Laboratories Inc, Santa Paul, MN) as per CLSI recommendations (Clinical and Laboratory Standards Institute, 2011) [82]. A bacterial culture (OD_{600} = 0.15) was diluted 40-fold with Middlebrook 7H9 broth medium and 100 µl of the dilution was added to each well of a sterile round bottom microtitre plate containing the 2-fold serially diluted LVX (100 µl/well).

After the test plate was incubated for 14 days at 37°C, bacterial growth was observed and MICs were defined as the lowest concentrations of LVX that inhibited visible bacterial growth. *M. avium* isolates with MIC of < 2 µg/ml were considered susceptible, MIC of 2 - 4 µg/ml as intermediate, and MIC of ≥ 8 µg/ml as resistant according to CLSI guidelines [82].

2.2.3. Sequencing of FQ resistance associating genes

All primers used for the amplification of *gyrA* and *gyrB* DNA fragments are listed in Table 1. The following reaction mixture was used; 1 µl (20ng) of template DNA, 4µl of 5x Go Taq buffer (Promega Co, Fitchburg, WI), 0.8µl of 25mM MgCl2, 2µl of 5M Betaine, 0.2µl of 25mM dNTP, 0.5µl each of 10 µM primer, 0.1µl of GoTaq DNA polymerase (0.4units/µl) and DDW to make up a total volume of 20µl. The amplification protocol consisted of an initial denaturation at 96°C for 1 minute, followed by 35 cycles of denaturation at 96°C for 60sec, annealing at 55°C for 10sec, extension at 72°C for 30sec, and final post-run elongation at 72°C for 1 minute.

The *mfpA* was amplified using the following reaction mixture; 1 µl (10-30ng) of template DNA, 10µl of 2x GCI buffer (Takara Bio, Co, Shiga, Japan), 0.5µl of 25mM dNTP, 0.5µl each of 10µM primer, 0.2µl of GoTaq DNA polymerase (0.8units/µl), and DDW to make up the final volume of 20µl. The amplification reaction protocol consisted of an initial denaturation at 96°C for 10sec, followed by 35 cycles of denaturation at 96°C for 60sec, annealing at 60°C for 10sec, and extension at 72°C for 80sec, and thereafter, a final post-run elongation step at 72°C for 1 minute. On the other hand, the reaction mixture for the amplification of the *mfpB* gene consisted of 1 µl (10-30ng) of template DNA, 4µl of 5x Go Taq buffer (Promega), 0.8µl of 25mM $MgCl_2$, 2µl of 5M Betaine, 0.2µl of 25mM dNTP, 0.5µl each of 10µM primer, 0.1µl GoTaq DNA polymerase (0.4units/µl), and DDW to make up a total volume of 20µl. The reaction was amplified as indicated above for *gyrA* and *gyrB*. PCR amplicons were analyzed by using 2% agarose gel electrophoresis with gel red (Biotium, Inc, Fremont, CA) in 1× TAE and the bands were visualized by ultraviolet transillumination.

Thereafter, 2ul of 10x diluted ExoSAP-IT™ Express (Thermo Fisher Scientific, Walthum, MA) was directly added to the PCR product for product clean-up before sequencing. This treatment was carried out for 15 minutes at 37°C, followed by 3-minute incubation at 80°C. Sequencing reactions were performed in the ABI PRISM 3500 Genetic Analyzer (Thermo Fisher Scientific) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer's protocol using corresponding primers as used in the PCR reactions. The sequences were assembled by Sanger sequencing and were compared to the reference strain sequence (*M. avium* 104; GenBank accession number NC_008595.1) using BioEdit software.

2.2.4. Differentiation of two genotypes of *mfpA* by multiplex PCR

Sequencing of the *mfpA* revealed two distinct *mfpA* genotypes among the *M. avium* isolates. Some isolates harbored *mfpA* with a C170frameshift deletion which will be referred to as "C170deleted *mfpA*" while other isolates had an "Intact *mfpA*", where the frameshift mutation was absent. The significance of this frameshift mutation is discussed in detail later. Owing to this finding, a multiplex PCR for rapid differentiation of the above described *mfpA* genotypes in *M. avium* was developed to simplify the work. All primers used in the multiplex PCR are outlined in Table 8.

The following PCR reaction mixture was used; 1 µl (20ng) of template DNA, 1.5µl of 10x PCR buffer (Takara Bio), 0.2 µl 25 mM dNTP, 0.8 µl of 25mM $MgCl_2$, 2µl of 5M Betaine, 0.5 µl of 10 µM of mfpA-F and mfpA-R1' and 0.2 µl of 10 µM mfpA-R2', 2 µl Taq DNA polymerase (8units/µl) and DDW to make up a total volume of 15 µl. The amplification protocol consisted of an initial denaturation at 96°C for 1 minute, followed by 30 cycles of denaturation at 96°C for 10sec, annealing at 60°C for 10sec, extension at 72°C for 10sec, and a post-run elongation at 72°C for 60sec. The PCR amplicons were analyzed by using 2% agarose gel electrophoresis with gel red in 1× TAE and the bands were visualized by ultraviolet trans-illumination. The expected band sizes for the Intact *mfpA* and C170deleted *mfpA* were 64bp and 126bp respectively.

2.2.5. Statistical analysis

The proportions of resistant and susceptible isolates harboring or not harboring the specific mutation in *mfpA* were compared using the chi-square test or Fisher's exact test as appropriate. A *p*-value of <0.05 was considered significant and all tests were two-tailed. All statistical analyses were performed with the SPSS v.26 statistical package from IBM.

2.3. Results

2.3.1. LVX susceptibility of *M. avium* subsp. *hominissuis* isolates

The LVX susceptibility patterns are shown in Table 10. Of the total 88 *M. avium* isolates, 25% (19/88) were susceptible to Levofloxacin, whereas (69/88) were resistant. High-level resistance (≥8 μg/ml) was observed in 45% (31/69) of the isolates while the rest (38/69) exhibited intermediate resistance (2 - 4 μg/ml). The LVX resistance rates per sampling location were 75%, 73.9%, and 88% in isolates from Osaka, Tokyo, and Hokkaido respectively.

2.3.2. Sequencing of fluoroquinolone resistance associating genes

The *gyrA* and *gyrB* QRDR, as well as *mfpA* and *mfpB*, were sequenced for all 88 isolates and the sequences were analyzed and compared with MIC data to determine the correlation between specific mutations within these genes and FQ resistance. Of 69 LVX-resistant isolates, four harbored resistance-conferring mutations (D94Yor D94G) were found within the *QRDR* of *gyrA* (Table 9). The MIC values of these four isolates ranged from 2 μg/ml (1/4, 25%), 8 μg/ml (1/4, 25%) and 32 μg/ml (2/4, 50%) (Table 10). In *mfpA*, five substitutions independent of the C170delwere found in some isolates from Tokyo (14/23, 60.9%). No mutations were found in *gyrB* and *mfpB* of all the isolates (Table 9).

2.3.3. Correlation between *mfpA* genotypes and MICs of LVX

The *mfpA* of all *M. avium* isolates was sequenced and analyzed for correlation with FQ resistance. All the LVX MICs and the distribution of the *mfpA* genotypes are outlined in Table 10. Out of the total 88 isolates analyzed in this study, 58 had the intact *mfpA* genotype while 30 had C170deleted *mfpA* type (Table 10). Among the 58 isolates with intact *mfpA*, 53/58 (91.4%) were resistant to LVX, with the majority of these isolates having MICs of 4 μg/mL and 8 μg/mL (56.6%, 30/53). Overall, more than two-thirds (53/65, 81.5%) of the LVX resistant isolates with WT-*gyrA* and -*gyrB* (four isolates with GyrAB mutations excluded), were of the intact *mfpA* genotype. From these results, a statistically significant (p-value <0.001) association was observed between *mfpA* (intact *mfpA* genotype) and resistance to LVX (Table 10D). Moreover, five discordant isolates were observed that displayed increased susceptibility to LVX despite having an intact *mfpA* type. On the other hand, among isolates with C170deleted *mfpA*, 14 out of 30 (46.7%) were susceptible to LVX while the remaining 16 (53.3%) were resistant. Two of the resistant isolates each harbored a LVX resistance-conferring mutation in codon 94 of *gyrA* namely; (Y94D, Asp-Tyr) and (D94G,

Asp-Gly). Furthermore, fourteen other isolates with the C170deleted *mfpA* and WT- *gyrA* and -*gyrB* were observed to exhibit low to high-level LVX resistance (MIC values range: 2-32 µg/mL). MIC determinations were repeated and consistent values were obtained.

2.3.4. Evaluation of multiplex PCR for differentiation of *mfpA* genotypes

A simple multiplex PCR targeting the deletion of C at the nucleotide position 170 was developed and evaluated for rapid differentiation of the *mfpA* genotypes. The multiplex was initially designed using *M. avium* isolates from Osaka and then applied for the differentiation of all the *M. avium* isolates that were analyzed in this study. The multiplex had good diagnostic accuracy since all results were concordant with Sanger sequencing results (Table 11). The developed multiplex PCR had a sensitivity of 91.4% but only had a specificity of 50% to predict LVX resistance in the *M. avium* clinical specimens (Table 11).

2.4. Discussion

Fluoroquinolones are considered important alternative therapy for *M. avium* pulmonary infections when the recommended regimen is insufficient [83]. However, the resistance to fluoroquinolones is increasing and this has impacted the effective management of *M. avium* infections. In this present study, the involvement of *gyrA*, *gyrB*, *mfpA*, and *mfpB* to LVX resistance in *M. avium* clinical isolates from Japan was investigated through MIC determination and sequence analysis of the FQ resistance associating genes. All analyzed genes have previously been reported to play a contributory role in resistance to FQ in mycobacteria [48,51,84–86].

From the results of this study, only two amino acid substitutions occurring at codon 94 of *gyrA*, were independently found in four LVX resistant isolates. Both of these amino acid substitutions, D94Y (Aspartic acid – Tyrosine) and D94G (Aspartic acid – Glycine); have been associated with high-level FQ resistance in *M. tuberculosis* [85,87,88]. Therefore, the contribution of mutations in the QRDR of *gyrA* to FQ resistance in this study was 4.5% (4/69).

The percentage although low was higher than that reported by Kim et al, 2018 [48], who reportedly detected no amino acid substitutions associated with FQ resistance in GyrA or GyrB in any of their 105 MAC and MABC isolates, including 96 moxifloxacin-resistant isolates. Yamaba et al, 2019 [49], also reported moxifloxacin resistance in one-fourth of *M. avium* and *M. intracellulare* isolates from Japan with no association with mutations in *gyrA* and *gyrB* and similar findings have also been described elsewhere involving different classes of FQs [89]. However, a

separate study by Pang et al, 2018 [50] described several mutations in *gyrA* and *gyrB* in *M. avium* isolates, although, it was not disclosed whether these mutations occurred in the QRDR. No mutation associated with FQ resistance was observed in *gyrB*, consistent with data reported by earlier studies in *M. avium* and *M. tuberculosis* [48,87,90,91]. From these previous studies and the findings, it is apparent that in contrast to *M. tuberculosis*, there is no strong association between mutations of the DNA gyrase genes (*gyrA* and *gyrB*) and FQ resistance in *M. avium* [85,92].

I, therefore, explored the role of *mfpA* and *mfpB* using the same procedure as for the *gyrA* and *gyrB*. The contribution of *mfpA* has been previously evaluated in a some studies, however, these studies were exclusively conducted in *M. tuberculosis* and *M. smegmatis* using recombinant proteins. Nonetheless, in both organisms, MfpA reportedly increased the FQ MICs but when it was disrupted, increased FQ susceptibility was observed [51,53,84].

According to the sequencing results, in *M. avium* clinical isolates, MfpA naturally occurred in two states. In some *M. avium* isolates (34%, 30/88), MfpA existed with a natural polymorphism (C170deletion) while in 66% (58/88) of the isolates, it existed as a functional/intact *mfpA*, without the deletion. The MIC data showed distinct patterns of LVX susceptibility between the two classes (C170deleted *mfpA* and Intact *mfpA*) (Table 10). Isolates with intact type *mfpA* generally showed a higher correlation with resistance to LVX than those with C170 deleted type with statistical significance (p-value < 0.001).

This observation is thought to be because the functional/intact *mfpA* did not have the C170 deletion and/or any other disruptive mutations and therefore, *mfpA* could be fully transcribed into a functional protein with the potential to influence the activity of FQ. However, the occurrence of the C170 deletion caused a frameshift at codon 57 of MfpA resulting in the attachment of an unnatural (defective) amino acid sequence and subsequently early termination. This could ultimately disrupt the function of MfpA protein to induce LVX resistance as elucidated by earlier studies in *M. tuberculosis* and *M. smegmatis* [51,84,86].

Surprisingly, two isolates with intact type *mfpA* were found to be LVX-susceptible. This observation is hypothethized to be due to enhanced cell permeability which can cause high drug concentrations inside the cell and has previously been shown to suppress the effects of inhibitory mechanisms thereby inducing resistance to drugs [85,93]. Other missense mutations in *mfpA* were observed among FQ resistant isolates. None of these mutations co-occurred with the C170 deletion hence their contribution to FQ resistance has yet to be ascertained (Table 12). A previous study by

Tao et al. 2013 [54], proposed that *mfpA* activity required the presence of the GTPase, *Mycobacterium* fluoroquinolone resistance protein B (MfpB), hence mutation analysis of *mfpB* nuceleotide sequences was also performed in this current study. However, no mutation associated to FQ resistance was observed, a result that has been recently supported by findings from Feng et al. 2021[53], which suggested that MfpB played no role in assisting MfpA in protecting gyrase from FQ activity [53].

There were several isolates with no resistance-conferring mutations observed in all the FQ resistance associating genes that were analyzed including MfpA, suggesting that some other mechanism of FQ resistance is at play in these isolates. Reports have shown that efflux pumps also contribute to FQ resistance in some bacteria, such as *M. tuberculosis* [44,94]. The overexpression of efflux pumps results in drug extrusion out of the membrane and thereby prevents the drug from reaching bactericidal concentrations [95]. The effects of efflux pumps have been described to account for most of the low-level resistance in *Mycobacterium tuberculosis*, and similar efflux pumps are also present in *M. avium* and other NTMs [96]. Put together, the findings in this study emphasize the need to consider DNA mimicry (exhibited by MfpA) as a potential contributor to FQ resistance especially where there is minimal or no involvement of *gyrA* or *gyrB* mutations in *M. avium*. In future studies, I plan to utilize recombinant proteins and a wider range of FQs to verify the role of *mfpA* in FQ resistance. The collective knowledge obtained from all these investigations will be beneficial in providing more insights into FQ resistance mechanisms in *M. avium*.

2.5. Summary

In this chapter, the basis for Levofloxacin resistance in 88 *M. avium* isolates from Japan was investigated through MIC determination and sequencing of *gyrA*, *gyrB*, *mfpA*, and *mfpB.* Due to the limited association observed between mutations in *gyrA* and *gyrB* (5/69, 7.2%) and LVX resistance, the role of *mfpA* in resistance was explored. To simplify the *mfpA* analysis, a multiplex PCR was developed to detect the two *mfpA* genotypes (C170deleted *mfpA* and Intact/Non-C170deleted *mfpA*). From the total 88 isolates analyzed in this study, 58 had the intact *mfpA* genotype while 30 had C170deleted *mfpA* type. Among the 58 isolates with intact *mfpA,* 53/55 (96.4%) were resistant to LVX. Totally, more than two-thirds (56/65, 86.2%) of the LVX resistant isolates with WT-*gyrA* and -*gyrB* (four isolates with GyrAB mutations excluded), were of the

intact *mfpA* genotype. From these results, a statistically significant (*p*-value <0.001) association was observed between *mfpA* (intact *mfpA* genotype) and resistance to LVX. These results although preliminary, suggest that *mfpA* has some contributory role in either inducing and/or enhancing LVX resistance in *M. avium* and therefore warrants more attention.

Tables and Figures

Table 8. Oligonucleotide sequences of primers used for sequencing of *gyrA*, *gyrB*, *mfpA*, and *mfpB* genes and multiplex-MfpA PCR

Gene	Primer name	Sequence 5'- 3'	Amplicon length (bp)
gyrA	gyrA-F	GACTGACACCACGCCTGCCACC	500
	gyrA-R	CACCCGGCCGTCGTAGTTGG	
gyrB	gyrB-F	CAGCTCACCCACTGGTTCGAAG	500
	gyrB-R	CGCATGAACCGGAACAGCAG	
mfpA	mfpA-F	GACAGCCCGTCGGTCGCCGAG	1403
	mfpA-R	CCCGCGGGACAGCCACTTCC	
mfpB	mfpB-F	GACAGCCCGTCGGTCGCCGAG	588
	mfpB-R	CGGAAGGCCGACGCGCGGTG	
Multiplex Primers			
mfpA	mfpA-mF	CACCGCGCGTCGGCCTTCC	
	mfpA-mR[1]	CGAAAGTGCTGTGCCACAACGATG	64
	mfpA-mR[2]	ATCGAACGTCACCGGCCGCA	126

Table 9. Profile of *gyrA* , *gyrB, mfpA and mfpB* mutations among 88 *M. avium* subsp. *hominissuis* isolates

Gene	Nucleotide substitions	No. of isolates/Frequency (%)
gyrA	G283T (Asp94Tyr)	2 (2.3)
	A284G (Asp94Gly)	2 (2.3)
	WT	84 (95.5)
gyrB	None	-
mfpA	T156C, T225C	2 (2.3)
	C321T, C450A, C524G	12 (13.6)
	C170del	30 (34.1)
	WT	44 (50)
mfpB	None	-

Reference sequence: *M. avium* TH135

Table 10. Correlation between *mfpA* genotypes and MICs of LVX

***mfpA* genotype**	**Total**	**No. of isolates**									***p*-value**
		MIC (μg/mL) of Levofloxacin									
		<0.25	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	
Osaka isolates											
Intact	24	-	-	2	-	3	9	6	2	2	0.007*
C170 deleted	16	2	-	-	6	3	2	2	-	1	
Tokyo isolates											
Intact	14	-	-	-	1	5	4	1	1	2	0.02*
C170 deleted	9	-	2	2	1	3	1	-	-	-	
Hokkaido isolates											
Intact	20	-	-	-	2	1	6	4	4	3	0.5
C170 deleted	5	-	-	-	1	1	-	2	-	1	
Osaka, Tokyo, Hokkaido isolates											
Intact	58	-	-	2	3	9	19	11	7	7	**<0.001***
C170 deleted	30	2	2	2	8	7	3	4	-	2	
Total	88	2	2	4	11	16	22	15	7	9	

Table 11: Differentiation of *mfpA* genotype by multiplex PCR

		Sanger sequencing		
		C170 deleted	Intact	Total
Multiplex PCR	C170 deleted	30	0	30
	Intact	0	58	58

*Sensitivity and specificity were 100%, respectively.

Table 12: Mutations and MIC results of MAH clinical isolates from Osaka, Hokkaido and Tokyo

A. Osaka MAH isolates

No.	Sample ID	M-PCR *mfpA* genotype	LVX MIC (µg/mL)	Mutations			
				gyrA	*gyrB*	*mfpA*	*mfpB*
1	Cl-A-2	C170del	8	G283T	None	C170del	None
2	Cl-A-3	Intact	8	None	None	None	None
3	Cl-A-4	C170del	1	None	None	C170del	None
4	Cl-A-5	Intact	4	None	None	None	None
5	Cl-A-6	Intact	2	None	None	None	None
6	Cl-A-8	C170del	1	None	None	C170del	None
7	Cl-A-9	C170del	1	None	None	C170del	None
8	Cl-A-10	Intact	8	None	None	None	None
9	Cl-A-11	Intact	8	None	None	None	None
10	Cl-A-13	Intact	4	None	None	None	None
11	Cl-A-14	Intact	4	None	None	None	None

12	Cl-A-16	C170del	1	None	None	C170del	None
13	Cl-A-17	Intact	4	None	None	None	None
14	Cl-A-18	C170del	8	None	None	C170del	None
15	Cl-A-20	C170del	2	None	None	C170del	None
16	Cl-A-22	Intact	2	None	None	None	None
17	Cl-A-24	C170del	2	None	None	C170del	None
18	Cl-A-26	Intact	4	None	None	None	None
19	Cl-A-27	Intact	8	None	None	None	None
20	Cl-A-28	Intact	4	None	None	None	None
21	Cl-A-29	C170del	4	None	None	C170del	None
22	Cl-A-30	Intact	4	None	None	None	None
23	Cl-A-31	C170del	4	None	None	C170del	None
24	Cl-A-32	C170del	1	None	None	C170del	None
25	Cl-A-33	C170del	32	None	None	C170del	None

26	Cl-A-34	Intact	4	None	None	None	None
27	Cl-A-36	Intact	16	None	None	None	None
28	Cl-A-37	Intact	32	None	None	None	None
29	Cl-A-38	Intact	2	None	None	None	None
30	Cl-A-39	Intact	8	None	None	None	None
31	Cl-A-41	C170del	2	None	None	C170del	None
32	Cl-A-42	WT	0.5	None	None	None	None
33	Cl-A-43	WT	8	None	None	None	None
34	Cl-A-44	WT	0.5	None	None	None	None
35	Cl-A-45	C170del	1	None	None	C170del	None
36	Cl-A-46	C170del	<0.25	None	None	C170del	None
37	Cl-A-47	Intact	16	None	None	None	None

38	Cl-A-48	Intact	4	None	None	None	None
39	Cl-A-49	Intact	32	None	None	None	None
40	Cl-A-50	C170del	<0.25	None	None	C170del	None

B. Hokkaido MAH isolates

No.	Sample ID	M-PCR *mfpA* genotype	LVX MIC (µg/mL)	Mutations			
				gyrA	*gyrB*	*mfpA*	*mfpB*
1	Hokudai HP 1	C170del	1	None	None	C170del	None
2	Hokudai HP 6	C170del	2	None	None	C170del	None
3	Hokudai HP 7	Intact	16	None	None	None	None
4	Hokudai HP 9	C170del	8	None	None	C170del	None
5	Hokudai HP 18	Intact	8	None	None	None	None
6	Hokudai HP 20	C170del	32	None	None	C170del	None
7	Hokudai HP 29	Intact	16	None	None	None	None
8	Hokudai HP 30	Intact	8	None	None	None	None
9	Hokudai HP 31	C170del	8	None	None	C170del	None
10	Hokudai HP 34	Intact	2	None	None	None	None
11	Hokudai HP 39	Intact	4	None	None	None	None
12	Hokudai HP 45	Intact	4	None	None	None	None
13	Hokudai HP 49	Intact	4	None	None	None	None
14	Hokudai HP 51	Intact	16	None	None	None	None
15	Hokudai HP 52	Intact	1	None	None	None	None
16	Hokudai HP 54	Intact	1	None	None	None	None
17	Hokudai HP 55	Intact	4	None	None	None	None
18	Hokudai HP 56	Intact	8	None	None	None	None
19	Hokudai HP 58	Intact	32	None	None	None	None
20	Hokudai HP 59	Intact	32	None	None	None	None
21	Hokudai HP 60	Intact	8	None	None	None	None
22	Hokudai HP 61	Intact	4	None	None	None	None
23	Hokudai HP 67	Intact	32	None	None	None	None
24	Hokudai HP 68	Intact	4	None	None	None	None
25	Hokudai HP 70	Intact	16	None	None	None	None

C. Tokyo MAH isolates

No.	Sample ID	M-PCR *mfpA* genotype	LVX MIC (µg/mL)	Mutations			
				gyrA	***gyrB***	***mfpA***	***mfpB***
1	Koav-01	C170del	0.25	None	None	C170del	None
2	Koav-02	C170del	4	None	None	C170del	None
3	Koav-03	C170del	2	A284G (D94G)	None	C170del	None
4	Koav-05	Intact	4	None	None	C321T, C450A, C524G	None
5	Koav-06-1	C170del	0.5	None	None	C170del	None
6	Koav-07	Intact	2	None	None	C321T, C450A, C524G	None
7	Koav-08	C170del	1	None	None	C170del	None
8	Koav-09	Intact	2	None	None	C321T, C450A, C524G	None
9	Koav-10-1	Intact	4	None	None	C321T, C450A, C524G	None
10	Koav-11	Intact	32	G283T (D94Y)	None	C321T, C450A, C524G	None
11	Koav-12	Intact	2	None	None	C321T, C450A, C524G	None
12	Koav-13	Intact	32	A284G (D94G)	None	C321T, C450A, C524G	None
13	Koav-14	Intact	8	None	None	T156C, T225C	None
14	Koav-15	Intact	4	None	None	C321T, C450A, C524G	None
15	Koav-16	Intact	4	None	None	C321T, C450A, C524G	None
16	Koav-17	C170del	2	None	None	C170del	None
17	Koav-18	Intact	2	None	None	C321T, C450A, C524G	None
18	Koav-19	C170del	2	None	None	C170del	None
19	Koav-20	C170del	0.5	None	None	C170del	None
20	Koav-21	Intact	1	None	None	C321T, C450A, C524G	None
21	Koav-22	Intact	16	None	None	T156C, T225C	None
22	Koav-24	C170del	0.25	None	None	C170del	None
23	Koav-25	Intact	2	None	None	C321T, C450A, C524G	None

Figures

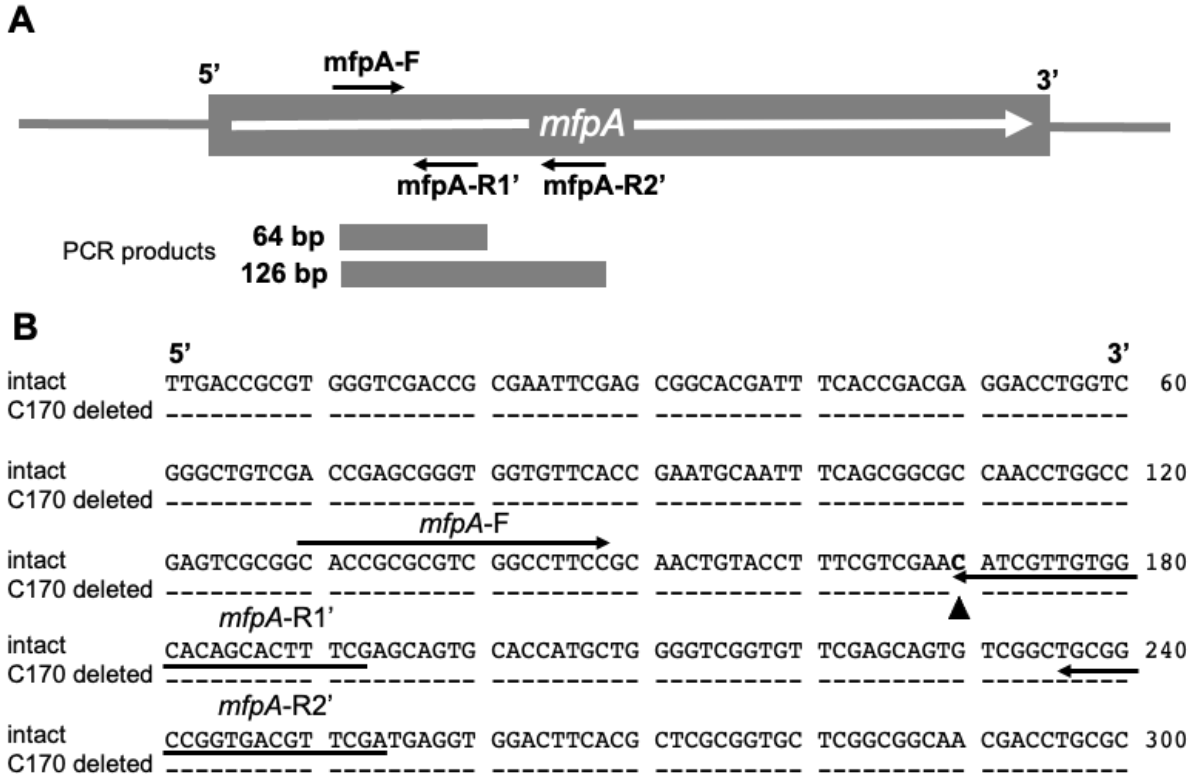

Figure 7. Differentiation of Intact and C170 deleted *mfpA* by multiplex PCR
A: Schematic representation of the position of PCR primers and expected amplicons. **B**: nucleotide sequence of 5' half of *M. avium* subsp. *hominissuis mfpA* and the position and orientation of PCR primers. -: same nucleotides. F, R1' and R2' denotes forward primer, reverse primer 1 and 2, respectively. Arrowhead denotes the nucleotide deletion at the position of 170.

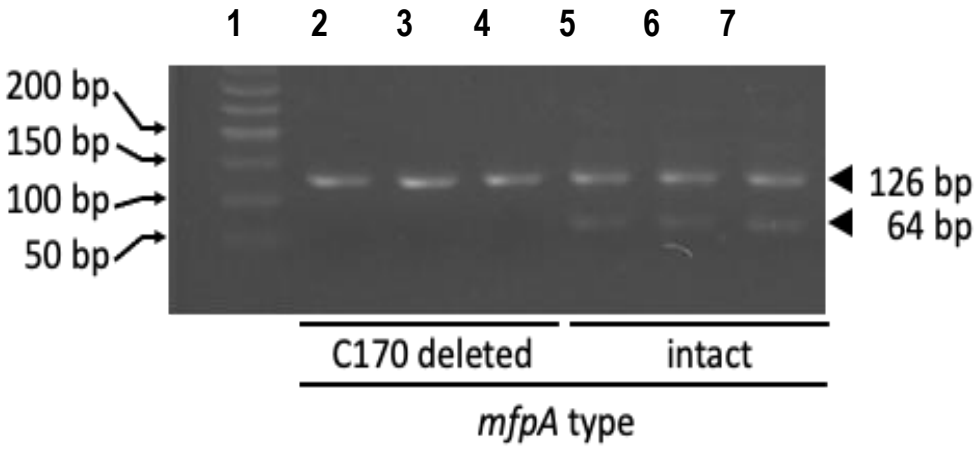

Figure 8: Multiplex PCR for differentiation of the intact and C170 deleted *mfpA* types in *M. avium* subsp. *hominissuis* isolates. Lane 1: 50bp ladder DNA size marker; Lane 2-4: PCR amplicon of C170 deleted type *mfpA*; Lane 5-7: PCR amplicon of Intact type *mfpA*.

Conclusion

The prevalence of infectious diseases caused by NTM has increased worldwide, and *M. avium* is one of the most frequent species involved. However, there are still very limited studies on *M. avium*, the scarcity of scientific knowledge about many aspects of *M. avium* has resulted in continued mismanagement of these diseases and consequently loss of lives. It is generally accepted that early disease detection and initiation of therapy are cardinal for good prognoses. However, the sustained need for suitable cost-effective diagnostic tools for *M. avium* remains. On the other hand, treatment presents its challenges due to the emergence of drug resistance which has rendered the drugs of choice ineffective.

This present study contributes to the better management of *M. avium* by adding a highly sensitive tool to the already existing diagnostic kits and reveals alternative resistance mechanisms that *M. avium* is utilizing to evade the actions of the limited second-line *M. avium* drugs in the name of FQ through molecular approaches.

In Chapter I of this thesis, a new rapid and affordable method for the detection of *M. avium* was established. Secondly, the effects of the infamous genetic diversity in *M. avium* was investigated and found to negatively influence the applicability of new detection tools in some regions thereby limiting their usefulness and impact as observed in the case of IS*1245*-targeting diagnostic tools. Although IS*1245* is the widely used marker for *M. avium* identification and detection, this marker was lacking in over a third of the *M. avium* strains circulating in the Japanese human population thereby limiting its usage.

In Chapter II, mechanisms of FQ resistance were investigated and the mutations in the QRDR had a low contribution to the apparent resistance. Moreover, MfpA was observed to decrease *M. avium* susceptibility to levofloxacin, a type of FQ. This finding could explain why some *M. avium* isolates in literature have been found resistant despite not having any known resistance-conferring mutations in the QRDR. Therefore, it is recommended to consider MfpA as potential contributor to FQ resistance in *M. avium*.

Overall, this study provided some contribution to the control of *M. avium* infections by developing a rapid diagnostic tool for timely diagnosis and highlighted the impact of geographical and host-related diversity in *M. avium* strains on the application of diagnostic tools. Furthermore, the study also provided insights into the alternative mechanisms of FQ resistance in *M .avium* by highlighting MfpA. These findings form the basis upon which more in-depth studies can be undertaken to further explore the relationship between *mfpA* and FQ resistance in *M. avium*. Taken together, the discussions in this research will contribute to the management of *M. avium* infections.

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