Memorial Lecture by the Imamura Award Winner

GENETIC RESEARCH ABOUT *MYCOBACTERIUM AVIUM* COMPLEX

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Abstract We conducted four genetic studies on the *Mycobacterium avium* complex (MAC). (1) *M.avium* genotyping : A total of 70 clinical isolates from patients with pulmonary MAC infections were typed by MATR-VNTR, IS*1245*-RFLP, and MIRU-VNTR analyses to compare discriminatory powers of these typing methods. To allow a comparison of discriminatory powers, the Hunter-Gaston discriminatory index (HGDI) was calculated, giving a HGDI of 0.960 for IS*1245*-RFLP, 0.949 for MIRU-VNTR, and 0.990 for MATR-VNTR, demonstrating that MATR-VNTR analysis is the best of the three genotyping methods. (2) Genetic characteristics of *M.avium* : Japanese clinical isolates of *M.avium* were subjected to insertion sequence (IS) analyses. First, an analysis of 81 isolates by heat shock protein 65 identified all isolates as belonging to the subspecies of *M.avium* subsp. *hominissuis*. Another analysis by IS*901* identified about 70% of the isolates as IS*901*-carriers. IS*901* had been thought to be carried by the subspecies that infect birds : *M.avium* subsp. *avium* and *M.avium* subsp. *silvaticum*. Studies have reported that most human isolates in the U.S. and Europe carry no IS*901*. The prevalence of IS*901*-carriers among Japanese clinical isolates of *M.avium* is thus a significant characteristic. A further analysis of the IS*901* showed that compared with *M.avium* subsp. *avium*, the clinical isolates shared 60 point mutations of nucleotide sequence. This novel insertion sequence was designated "IS*Mav6*". (3) The CAM-resistance gene in MAC : This study assessed the correlation between CAM-susceptibility and mutation of the gene involved in drug resistance (A DNA sequence analysis identified mutations at positions 2058 and 2059 in domain V of 23S-rRNA). Furthermore, a system was developed to rapidly detect the presence/absence of CAM resistance by ARMS-PCR, a procedure used to detect gene mutations. The utility of this new system was also evaluated. A total of 253 clinical isolates were tested for drug susceptibility, with 227 isolates identified as sensitive and 26 as resistant. Sequence analyses showed that all 28 strains randomly selected for testing from the sensitive strains were wild type, whereas 24 of the 26 resistant strains were mutant type. The rest of the 2 strains were subsequently confirmed to be mutant type after they were isolated from contaminations with sensitive strains. These results showed an association between drug susceptibility and drug-resistant gene mutation. In addition, ARMS-PCR provided a sensitivity of 84.6% (22/26) and a specificity of 100% (28/28) for the detection of gene mutations. The lower sensitive was probably attributable to the fact that every one of the 4 strains was a combination of wild type and mutant type. These results indicated that compared with drug-susceptibility tests, ARMS-PCR provides earlier results on the presence/absence of drug resistance and has the capability of rapid detection even when the specimen contains a mixture of sensitive and resistant strains. (4) Development of a VNTR analysis for *M.intracellulare* : Bioinformatics analyses were used to develop a VNTR analysis for *M.intracellulare* and to evaluate the utility of the VNTR analysis. First, the Tandem Repeat Finder (TRF) software was used to conduct a search of TR loci on the genomic data of *M.intracellulare* ATCC 13950 published in December 2007, resulting in the identification of 16 TR loci, which were used in VNTR analyses of 74 isolates from pulmonary MAC infections. The HGDI was 0.988, suggesting an excellent discriminatory power. Furthermore, a stability evaluation of the VNTR loci was conducted in isolates from patients with long-term bacilli discharge. The VNTR loci were stable without changes for up to 4 years in 14 such patients. These results indicated that this method is useful in *M.intracellulare* genotyping and in determining whether the cause of recurrence in recurred patients is endogenous from the remnant bacilli or exogenous from another infection of different bacilli, given that the VNTR loci have been confirmed to be stable.

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[Japanese original : Kekkaku, 2011 ; 86 : 61−68]

Key words: MAC, VNTR, HGDI, IS*901*, IS*Mav6*, Clarithromycin-resistant gene, ARMS-PCR method, MLVA method

Introduction

Let me begin with the reasons I started genetic research on the *Mycobacterium avium* complex (MAC). The number of pulmonary MAC patients started to increase from around 1992. Some of the cases progressed to respiratory failure, leading to death while the patients were still in their 50s. Although pulmonary MAC was regarded as sequelae of tuberculosis at the time, most of the patients were middle and old age women who had no apparent pre-existing disease. The disease stood out in my mind as an intractable infection with no decisive therapeutic agents available. I decided to study treatment protocols with what were available then. My study on the clinical efficacy of multidrug therapies was published in this journal in 2005.[1] In the mean time, I felt that there was a limit to what the present-state treatments can achieve. At that time, other research groups were conducting studies on host factors and human genetics, but none of the studies provided clinically applicable results. Although no precise epidemiological data are available, the number of pulmonary MAC cases in Japan seems to have increased drastically over the last 20 years, giving rise to the speculation that there may be some human infection-related mycobacterial factors present in the MAC that inhabits Japan. Thereupon I began to study the correlation between mycobacterial genotypes and clinical condition by applying variable numbers of tandem repeats (VNTR) typing, which had been used for mycobacterial identification in tuberculosis, on clinical isolates of *M.avium* and comparing VNTR typing with the then standard protocol, insertion sequence *1245*-restriction fragment length polymorphism (IS*1245*-RFLP) typing.

Mycobacterium avium Gene Typing Methods

The IS*1245*-RFLP typing reported by Guerrero et al.[2] in 1995 has been used as the standard protocol for *Mycobacterium avium* typing.[3] However, some strains have no IS*1245* sequence, and this method is unstable as the reproducibility or even the resolution declines as the number of copies increases, as with *M.tuberculosis*.[4] It is therefore unsuitable for multicenter studies, which require comparative analyses of data. Meanwhile, in Japan, Nishimori et al.[5] in 2003 published a VNTR typing method based on the *Mycobacterium avium* tandem repeat (MATR) loci, which has been used in veterinary medicine. Moriyama et al.[6] and Kasumi et al.[7] applied MATR on human clinical isolates and achieved excellent results. In 2007, Thibault et al.[8] published the *Mycobacterium* interspersed repetitive units (MIRU)-VTNR typing method and reported its utility. Our group compared 3 methods of typing to investigate which is useful in future studies. VNTRs are gene loci that consist of repeat units of 10- to 100-bp minisatellite nucleotide sequence interspersed on chromosomes. The advantage of this method lies in the facts that it uses mainly the simple and rapid PCR technique; that it facilitates the analyses of slow-growth mycobacterium as only a small quantity of culture is needed; and that it provides digital data that make it easy to compare data among study sites. Furthermore, compared with MIRU-VNTR, for which 8 VNTR loci have been identified by an exhaustive search with the Tandem Repeat Finder Program, MATR-VNTR has 16 loci identified by a homology search of tandem repeats that have been observed in *M.tuberculosis*. Although the principle of VNTR typing remains the same, these methods differ substantially in the search methods for VNTR loci. A study was conducted with 70 clinical isolates of *M.avium* to evaluate 4 patterns of analysis: these 3 methods and a combination of IS*1245*-RFLP plus MATR-VNTR. The Hunter-Gaston discriminatory index (HGDI) was used to compare discriminatory powers. The results shown in Table 1 indicated that MRTR-VNTR (HGDI ＝0.990) was the best of the 3 typing methods. The results further showed that its discriminatory power was maximized further when paired with IS*1245*-RFLP, giving an HGDI of 0.999. The utility of MRTR-VNTR alone is sufficient for the preset needs.[9]

Present Status of the Clinical Application of MATR-VNTR Typing

Pulmonary *M.avium* infections are air-borne infections, while disseminated *M.avium* infections associated with HIV are primarily enteric infections. In porcines, infections are transmitted by the enteric route, just as in HIV patients. VNTR

Table 1 Discriminatory index of VNTR and IS*1245*-RFLP typing of *M.avium* clinical isolates

Typing method	IS*1245*-RFLP	MATR-VNTR	MIRU-VNTR	IS*1245*-RFLP plus MATR-VNTR
No. of different patterns	53	56	27	68
No. of clusters	4	8	16	2
No. of clustered isolates	21	22	59	4
No. of unique isolates	49	48	11	66
HGDI	0.960	0.990	0.949	0.999

HGDI: Hunter-Gaston discriminatory index

typing was performed on isolates from patients with pulmonary *M.avium* infections, HIV patients, and porcines. The results showed that isolates from patients with pulmonary *M.avium* infections and porcines, which came from different routes of transmission, formed different clusters. Further, some isolates from HIV patients were found to cluster with isolates from porcines, indicating a homology. These results suggested that similarity among mycobacterial strains may be highly depending on their routes of transmission. A retrospective study on clinical isolates, clinical data, and clinical progression in 104 patients with a pulmonary *M.avium* infection who were under the care of our hospital has yet to yield any significant findings. These study results described above have been presented in an academic lecture in the 47th Meeting of the Japanese Respiratory Society and the 84th Annual Meeting of the Japanese Society for Tuberculosis. Further studies from different perspectives are needed to find out the strength of virulence and treatment responses, among other things.

Gene Characteristics of Clinical Isolates of *Mycobacterium avium* from Patients with Pulmonary MAC Infections

Presently, *M.avium* is classified into 4 subspecies: *M.avium* subsp. *avium* and *M.avium* subsp. *silvaticum*, which infect birds; *M.avium* subsp. *paratuberculosis*, which infects caws and sheep; and *M.avium* subsp. *hominissuis*, which infects humans and porcines. Subspecies can be determined by analyses such as a sequence analysis of the heat shock protein 65 gene (*hsp65* analysis)[10] and an analysis of gene insertion sequences, which are present on bacterial genomes and reported to be involved in bacterial evolution, drug resistance, and virulence (IS analysis).[11)~17)] Both *hsp65* analysis and IS analysis were performed on 81 isolates of *M.avium* from the sputum of patients with pulmonary MAC infections, 33 isolates of *M.avium* from the blood of HIV-positive patients, and 23 isolates of *M.avium* from porcines. First, the *hsp65* analysis identified all isolates as subsp. *hominissuis*. Next, the IS analysis by PCR detected IS*901*, which had been thought to be carried exclusively by the subspecies that infect birds, in 75% of the isolates from patients with pulmonary MAC infections and 45% of the isolates from HIV patients. Overseas studies have reported a minimal prevalence of 0% to 8% in the U.S. and Europe.[14)18)] In other words, the subsp. *hominissuis* isolated in Japan appeared to have a characteristic not seen in the U.S. and European isolates. Given that the detection of IS*901* was performed by the PCR technique, amplification of closely homologous sequences was a concern. A sequence analysis thus was performed to investigate details of the nucleotide sequence. The analysis identified base mutations at 60 sites compared with the IS*901* of the type that infects birds, showing a sequence homology of 95%. Given these results, this sequence was considered different from the IS*901* in the type that infects birds. It was registered as a novel IS and designated "IS*Mav6*."[19)] With a report implicating the involvement of IS*901* in the virulence for birds,[18)] IS*Mav6* was suspected of a potential involvement in the virulence for humans, and a study was performed on its insertion point. The study revealed that upon infection *in vivo*, IS*Mav6* is inserted into the Shine-Dalgarno sequence of the *cfp29* gene, which codes the protein that induces interferon-γ in human immune cells.[20)] In other words, insertion into the transcription initiation site of this gene may inhibit its expression and thus may suppress attacks by human immune cells. Further *in vivo* and *in vitro* studies on these mechanisms or actions are ongoing.

Questions have been raised on whether it is appropriate to classify strains that infect humans and porcines under the same subspecies of *M.avium* subsp. *hominissuis*, one of the 4 subspecies under the present classification system. Pulmonary MAC infections are air-borne infections, while the infections in porcines are digestive tract infections. They differ in the route of transmission. The 2008 report by Turenne et al. on a phylogenetic analysis of *M.avium* subspecies in multilocus sequence typing (MLST) with 10 different housekeeping genes, including *hsp65*, indicates that of the 4 subspecies, only subsp. *hominissuis* is widely distributed.[21)] As shown in Fig., *M.avium* 104, the standard strain in HIV infections for which a whole-genome analysis has been performed, and *M.avium* HN135, the pulmonary MAC infection strain that we studied, are located in the other side. The whole-genome analyses of the 2 strains showed a concordance rate of 98.9% (unpublished data), which also supports the view that they should not be grouped under the same subspecies. In addition, a phylogenetic diagram from MATR-VNTR typing showed that these 2 strains are located furthest apart, and that isolates from porcines with gastrointestinal infection and *M.avium* 104 are in the same cluster. These data suggested that it may be more appropriate to classify the type from air-borne infection and the type from digestive tract infection, currently under subsp. *hominissuis*, into separate subspecies.

Study on Methods for Rapid Detection of Clarithromycin (CAM)-resistant Gene in MAC

The basic treatment for pulmonary MAC infections is multidrug therapy, including clarithromycin (CAM). Of these, CAM is the only key drug for which reliable MIC data are avilable.[22)] CAM resistance is an important prognostic factor in predicting clinical progression in pulmonary MAC infections, and early detection of the presence/absence of resistance is clinically useful.[23)] DNA sequence analyses were performed on gene mutations in domain V of 23S-rRNA, which has been implicated to be involved in CAM resistance, in clinical isolates of MAC from patients with pulmonary MAC infections. The goals were to evaluate correlation with CAM-susceptibility tests using BrothMIC NTM and to assess the amplification refractory mutation system (ARMS)-PCR technique used for rapid detection of the presence/absence of CAM resistance. First, a CAM-susceptibility test was performed on a total of 253 isolates: 33 clinical isolates of *M. avium* from HIV-

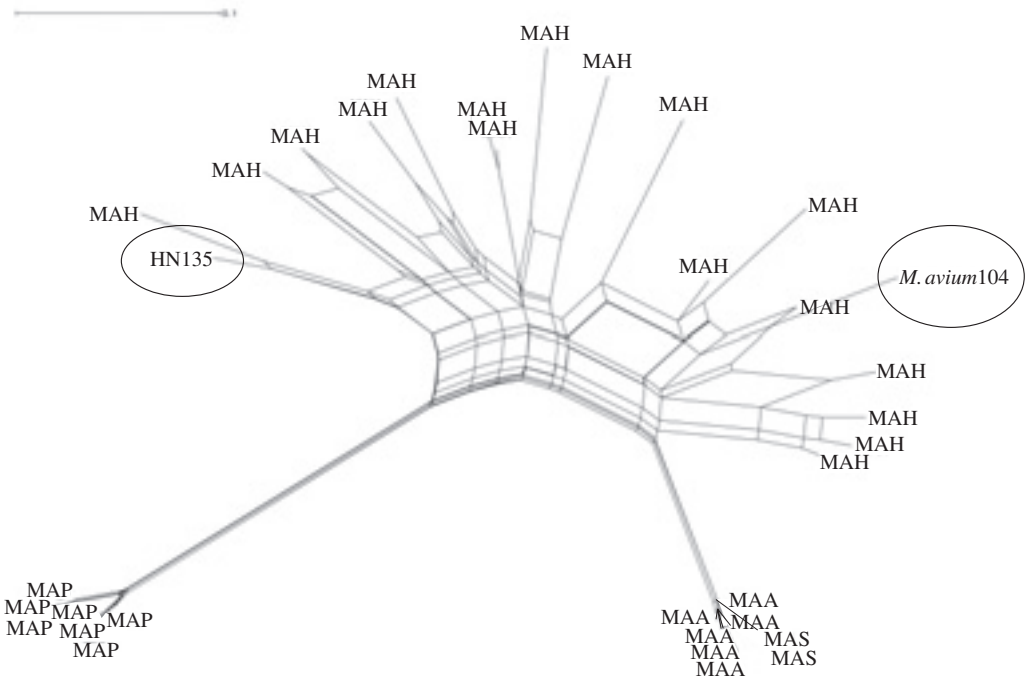

Fig. Splits graph of the phylogenetic relationship among the *M.avium* subspecies genotypes. The vertices are labeled with each subspecies. The graph was generated from the concatenated sequences of the ten gene loci (8064 bp). The scale bar represents a 10% difference in nucleotide sequences.

MAP: *M.avium* subsp. *paratuberculosis*
MAA: *M.avium* subsp. *avium*
MAS: *M.avium* subsp. *silvaticum*
MAH: *M.avium* subsp. *hominissuis*
HN135: isolated from HIV negative patient with pulmonary infection
M.avium 104: isolated from HIV positive patient with disseminated infection

Table 2 Comparison of the results from sequence analysis and ARMS-PCR with an *in vitro* susceptibility test

in vitro susceptibility test		CAM-sensitive n=28	CAM-resistant n=26
Sequence analysis	WT	28	1
	MT	0	24
	WT+MT	0	1
ARMS-PCR method	WT	28	1
	MT	0	22
	WT+MT	0	3

CAM: Clarithromycin WT: CAM wild type MT: CAM mutant type

positive humans; 134 clinical isolates of *M.avium* from HIV-negative patients with pulmonary MAC infections; and 86 clinical isolates of *M.intracellulare* from HIV-negative patients with pulmonary MAC infections. The test detected 227 susceptible strains with a MIC of $<8\,\mu$g/mL and 26 resistant strains with a MIC of $\geqq 8\,\mu$g/mL. Next, sequence analysis of domain V of 23S-rRNA and ARMS-PCR were performed on a total of 54 strains: the 26 CAM-resistant strains and 28 strains randomly selected from the 227 CAM-sensitive strains. The results are presented in Table 2. The sensitivity of sequence analysis in detecting CAM resistance was 24/26 or 92.3%. Furthermore, with strains containing a combination of wild and mutant types counted as mutants, the sensitivity increased to 25/26 or 96.2%. The specificity was 28/28 or 100%. The sensitivity of ARMS-PCR in detecting CAM resistance was 22/26 or 84.6%. With the combination-type strains counted as mutants, the sensitivity of ARMS-PCR was 25/26 or 96.2%, which is equivalent to that of sequence analysis. The specificity of ARMS-PCR was 28/28 or 100%. These results indicated a strong correlation between CAM-resistance gene mutation and CAM susceptibility in clinical isolates of MAC from pulmonary MAC infections and that the mechanism of resistance in most CAM-resistant MAC can be explained by this gene mutation.

In addition, the results also suggested that an analysis of the drug-resistance gene may be a simple detection method for specimens with a mixture of susceptible and resistant strains, for which detection is difficult with drug-susceptibility tests. This study further showed that more combination-type strains can be detected by using ARMS-PCR instead of sequence analysis. The combination-type strains were confirmed to be

Table 3 VNTR allelic distribution in *M. intracellulare* clinical isolates

Locus	No. of isolates with the specified TR allele								Allelic diversity
	0	1	2	3	4	5	6	Total	
VNTR-1		5	52	14		3		74	0.46
VNTR-2	3	43	18	8	2			74	0.58
VNTR-3	7	62	5					74	0.27
VNTR-4		32	9	13	20			74	0.69
VNTR-5	14	2	55	3				74	0.40
VNTR-6		20	35	16	3			74	0.65
VNTR-7		20	38	15	1			74	0.62
VNTR-8	35		37	2				74	0.52
VNTR-9	2	1	71					74	0.07
VNTR-10	1	44	3	1	20	5		74	0.56
VNTR-11		1	34		36	2	1	74	0.54
VNTR-12			60	14				74	0.30
VNTR-13	3	1	33	37				74	0.54
VNTR-14		1	65	8				74	0.21
VNTR-15		38	31	5				74	0.55
VNTR-16		4	70					74	0.09

identical strains by MATR-VNTR typing.[24)]

Development and Evaluation of the Utility of Multi Locus VNTR Analysis (MLVA) in *Mycobacterium intracellulare*

Mycobacterium intracellulare is one of the pathogens of pulmonary MAC infections, but, compared with *M. avium*, it has rarely been the subject of molecular epidemiological studies.[25)] We developed a VNTR typing method for *M. intracellulare* with bioinformatics analysis and evaluated its utility. First, the Tandem Repeat Finder (TRF) software was used to conduct a search of VNTR loci on the genomic data of *M. intracellulare* ATCC 13950 published in December 2007 under the following search conditions: (1) possessing of repeat sequences of 2 or more; (2) minimum repeat-size of 50 to 65 bp so as to facilitate agarose electrophoretic analyses of the diversity of VNTR loci; and (3) a homology of ≧90% among tandem repeats. Among loci identified in the search, those that cannot be analyzed or amplified were excluded, leaving the 16 loci shown in Table 3. Next, the discriminatory power of these 16 loci was evaluated in 74 isolates from patients with pulmonary MAC infections. A phylogenetic analysis of the results separated the 74 isolates into 49 genotypes in 17 clusters, demonstrating an excellent strain discriminatory power with a HGDI of 0.988. In contrast, the conventional heat shock protein 65-PCR restriction enzyme analysis (PRA) classified all 74 isolates as *M. intracellulare* type I, showing no discriminatory power. In addition, the stability of these 16 loci was investigated in 33 isolates from 14 patients with long-term bacilli discharge. The 16 loci in the 14 patients were stable without changes for up to 4 years. In a single patient, the bacilli discharged stopped after treatment was given for *M. intracellulare* infection but resumed about 1 year later. The isolates collected from this patient prior to and after the resumption of discharge have been confirmed to be identical. These results demonstrated that MLVA can provide highly reproducible results rapidly in a single day and that its discriminatory power is superior to that of PRA. In addition, with the confirmed stability of the repeat loci, this method is useful to determine whether the cause of recurrence in recurred patients is endogenous from the remnant bacilli or exogenous from another infection of different bacilli.[26)]

Conclusions

Mycobacterial gene research has taken a great leap forward with whole-genome analyses of *M. avium* isolates from pulmonary MAC infections. The nationwide study conducted by the National Hospital Organization hospitals has begun to yield evidence showing an association between IS*Mav6* in *cfp29*-carrying strains and clinical progression. Clinical applications of these findings are likely in the near future. Our ultimate goal is to cure pulmonary MAC infections by developing new treatments based on both host and mycobacterial factors.

Acknowledgment

I would like to take this opportunity to express my gratitude to Doctors Kei Nishimori, Tetsuya Yagi, Kazuya Ichikawa, Takayuki Inagaki, Taku Nakagawa, Makoto Moriyama, Keiichi Uchiya, and Toshiaki Nikai and many other collaborators, without whose help this research would not have been possible.

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