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GENETIC DIVERSITY OF *MYCOBACTERIUM AVIUM* subsp. *HOMINISSUIS* STRAINS ISOLATED IN ITALY BASED ON VNTR LOCI ANALYSIS

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Abstract. *Background. Mycobacterium avium* subsp. *hominissuis* (MAH) is an important pathogen responsible for most of the human-associated nontuberculous mycobacteria infections. Over the past few decades the incidence of MAH infections is increasing in Italy, as in many countries worldwide. The present study is aimed to elucidate the genetic characteristics of MAH strains isolated from human patients using VNTR typing and to show the genetic relatedness among them. *Methods.* The genetic diversity of 108 human isolates of MAH was determined by VNTR analysis targeting 8 loci, coded 32, 292, X3, 25, 3, 7, 10 and 47. *Results.* The VNTR analysis revealed 25 distinct VNTR patterns; of these, 13 patterns were unique, while 12 patterns were shared by 2 or more isolates, thus yielding 12 clusters including a total of 95 isolates. The discriminatory power of our VNTR analysis yielded an HGDI of 0.990, indicating that VNTR typing has an excellent discriminatory power. No association of a particular VNTR pattern with a particular clinical feature, such as the disseminated, pulmonary or extrapulmonary type of infection, was observed. Minimum spanning tree analysis showed that 21 VNTR patterns, occurring either as clustered or unique isolates, differed from the nearest one for one allelic variation. *Conclusions.* The results obtained through the VNTR analysis showed that most MAH strains displayed a close genetic relationship. This high phylogenetic proximity of the VNTR loci over a long time period supports the concept that the MAH genotype is highly homogeneous in our geographical area, suggesting the hypothesis of the presence of possible sources of infection and transmission pathways at the local level.

Key words: *Mycobacterium avium, population structure, Italy, VNTR loci, mycobacteriosis.*

ГЕНЕТИЧЕСКОЕ РАЗНООБРАЗИЕ ШТАММОВ *MYCOBACTERIUM AVIUM* subsp. *HOMINISSUIS*, ВЫДЕЛЕННЫХ В ИТАЛИИ, НА ОСНОВЕ АНАЛИЗА ЛОКУСОВ VNTR

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Резюме. *Mycobacterium avium* subsp. *hominissuis* является наиболее актуальным возбудителем микобактериоза человека. За последние несколько десятилетий в Италии заболеваемость микобактериозом *M. avium* subsp. *hominissuis* растет, как и во многих странах мира. Целью исследования была молекулярно-генетическая характеристика и оценка генетического родства штаммов *M. avium* subsp. *hominissuis*, выделенных от больных микобактериозом в Италии, с использованием VNTR (variable number of tandem repeats)-типирования. Аллельный полиморфизм 108 штаммов *M. avium* subsp. *hominissuis* оценивали методом VNTR-типирования по 8 локусам — 32, 292, X3, 25, 3, 7, 10 и 47. С помощью VNTR-типирования было выявлено 25 вариантов VNTR-типов; из них 13 профилей были уникальными, а 12 профилей представлены кластерами (включающими

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2 и более изолятов), в состав которых входило 95 изолятов. Дискриминирующая способность VNTR-типирования (индекс Хантера–Гастона, Hunter Gaston discriminatory index) составила 0.990, что указывает на высокую дискриминирующую способность использованной схемы VNTR. Связи между профилем VNTR и клинической формой микобактериоза (генерализованная, легочная или внелегочная) не обнаружено. Анализ минимального связывающего дерева профилей VNTR показал, что 21 VNTR-тип (как уникальные изоляты, так и кластеры двух и более изолятов) входили в единый клональный комплекс в котором соседние узлы различались по одному локусу. Полученные результаты VNTR-типирования выявили близкое родство изученных штаммов *M. avium* subsp. *hominissuis*. Высокий уровень филогенетического родства по локусам VNTR для штаммов, выделенных в течение длительного периода, подтверждает концепцию о том, что *M. avium* subsp. *hominissuis* очень гомогенен в нашей географической области в Италии, что, в свою очередь, подкрепляет гипотезу о наличии возможных источников инфекции и путей ее передачи на местном уровне.

Ключевые слова: *Mycobacterium avium, структура популяции, Италия, локусы VNTR, микобактериоз.*

Introduction

In many countries worldwide the incidence of nontuberculous mycobacteria (NTM) infections is increasing over the past few decades [11]. *Mycobacterium avium* complex is responsible for most of the human-associated nontuberculous mycobacteria infections [1]. *Mycobacterium avium*, one of the members of the *M. avium* complex, includes 4 subspecies, each endowed with specific pathogenetic and host range characteristics: *M. avium* subsp. *paratuberculosis*, that causes the Johne's disease in ruminants; *M. avium* subsp. *avium*, that infects birds; *M. avium* subsp. *silvaticum*, that infects wood pigeons; and *M. avium* subsp. *hominissuis* (MAH), that is usually isolated from human and swine sources [14, 20]. MAH is an important pathogen that causes not only disseminated diseases in patients with human immunodeficiency virus infection but also pulmonary disease, even in immunocompetent patients [19], and the incidence of pulmonary MAH infection is increasing in Italy [14].

Control of MAH infections in humans requires knowledge of its epidemiology and biodiversity of the strains. The variable numbers of tandem repeats (VNTR) analysis is a genotyping method that has been proven to be a rapid and reliable method with a high discriminatory power for MAH isolates [5, 17]. The present study is aimed to elucidate the genetic characteristics of MAH strains isolated from human patients using VNTR typing and to show the genetic relatedness among them.

Materials and Methods

Clinical isolates. A set of 108 MAH strains, identified by InnoLipa probes and by a multiplex PCR designed to discriminate MAC organisms [16], isolated from 1990 to 2016 in the Laboratory of Clinical Mycobacteriology of the University Hospital of Pisa, Italy, from the same number of patients, were studied. Fifty isolates were from respiratory specimens, 19 from blood, 15 from lymph nodes, 7 from specimens other than respiratory specimens, blood and lymph nodes, and 17 from an unknown source.

VNTR analysis. Genomic DNA was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method. VNTR typing was performed by PCR using specific primers for the eight loci identified as polymorphic for *M. avium* subsp. *paratuberculosis* K10 and coded 32, 292, X3, 25, 3, 7, 10 and 47, as described previously [17]. The PCR fragments were analyzed by gel electrophoresis using 2% NuSieve agarose (Cambrex Bio Science Rockland). For each locus, sizes of amplicons were estimated by comparison with 20 bp and 100 bp markers (Superladder-low; GenSura, CA, USA) and the numbers of repetitive units were determined according with a previously described allele-calling table [17]. VNTR profile is expressed as a string of 8 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the order given above. The allelic diversity (h) of the VNTR loci was calculated using the equation $h = 1 - \Sigma\, x_i^2\, \{n/(n-1)\}$ where n is the number of isolates and x_i the frequency of the i^{th} allele at the locus (Selander et al., 1986). The global discriminatory power of complete VNTR scheme (HGDI) was determined using the Hunter and Gaston discriminatory index (HGDI) [2]. The HGDI was calculated using the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{s} xj\,(xj-1),$$

where N is the total number of isolates in the typing scheme, s is the total number of distinct subtypes discriminated by the typing method, and xj is the number of isolates belonging to the x^{th} subtype.

Genetic relationships analysis. VNTR data were analyzed by the MIRU-VNTRplus web application available at www.miru-vntrplus.org; VNTR profile similarities were visualized by generating a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA); the genetic relationships among the isolates were analyzed by constructing a minimum spanning tree (MST), an undirected network in which all the VNTR profiles are linked together with the smallest possible linkages between nearest neighbours, by the UPGMA method.

Table 1. VNTR allelic distribution in 108 MAH clinical isolates

No. of tandem repeat copies	No. of isolates at the VNTR locus							
	32	**292**	**X3**	**25**	**3**	**7**	**10**	**47**
0		19						
1		1		1	108	108	1	
2		85	48	82			104	96
3		1	4	23				12
4			25	1				
5	2		30				3	
6								
7	2							
8	62							
9	39							
10	2							
nd*	1	2	1	1				
*h***	0.57	0.32	0.66	0.36	0	0	0.06	0.19

* not determined (no PCR product was obtained). ** allelic diversity (h) was calculated as described by Selander et al. (1986).

Results

The genetic diversity of 108 MAH human strains, isolated over a two 25 year-period in the Laboratory of Clinical Mycobacteriology of the University Hospital of Pisa, Italy, was investigated by determining the polymorphism of a set of eight MIRU-VNTR loci as previously described by Thibault et al. [17]. We first quantified the resolution provided by each VNTR locus by calculating its allelic diversity, which depends upon both the number and the distribution of the alleles, according to Selander et al. [15]. As shown in Table 1, the allelic diversity *(h)* of the VNTR loci of our collection varied widely, from 0 to 0.66. The VNTR loci 32 and X3 had a high diversity index ($h \geq 0.5$); three loci (292, 25, 47) showed medium diversity index ($0.1 \leq h \leq 0.5$); the locus 10 achieved a low diversity index ($h \leq 0.1$); the last two loci (3, 7) did not show any allelic diversity.

The VNTR analysis revealed 25 distinct VNTR patterns; of these, 13 patterns were unique, while 12 patterns were shared by 2 or more isolates, thus yielding 12 clusters including a total of 95 isolates. In particular, 1 cluster consisting of 24 strains, 1 cluster of 15 strains, 2 clusters of 11 strains, 1 cluster of 9 strains, 1 cluster of 7 strains, 1 cluster of 5 strains, 1 cluster of 4 strains, 1 cluster of 3 strains and finally 3 clusters of 2 strains were identified. The discriminatory power of our VNTR analysis yielded an HGDI of 0.990, indicating that VNTR typing has an excellent discriminatory power. Table 2 shows VNTR profiles and localization of infection of clustered and unique MAH strains; no association of a particular VNTR pattern with a particular clinical feature, such as the disseminated, pulmonary or extrapulmonary type of infection, was observed.

The genetic relationships between the study isolates were then visualized by constructing a minimum spanning tree (MST) based on the VNTR profiles. The MST reflects the variations from one

Table 2. Characteristics of MAH strains

VNTR pattern[a]	No. of Isolates	No. of isolates with specific localization[b]			
		Respiratory tract	Blood	Lymphnode	Other
82221122	24	12	5	5	1
92221122	15	10	–	3	–
82421122	11	2	6	1	1
92421122	11	4	–	–	–
82521122	9	3	1	3	–
80531122	7	2	1	–	4
80531123	5	3	2	–	–
92521122	4	1	1	1	–
90531122	3	–	–	1	–
82231122	2	2	–	–	–
52421122	2	–	2	–	–
90221122	2	2	–	–	–
82321122	1	–	–	–	1
10 2221122	1	1	–	–	–
90421122	1	1	–	–	–
82231123	1	1	–	–	–
82241123	1	1	–	–	–
82221123	1	1	–	–	–
92221113	1	1	–	–	–
82531122	1	–	–	1	–
10 0531122	1	1	–	–	–
72511123	1	–	1	–	–
71331152	1	–	–	–	–
93331153	1	1	–	–	–
90331153	1	1	–	–	–

[a] VNTR patterns are expressed as strings of 8 numbers, each representing the number of tandem repeats at a given VNTR position, in the following order: locus 32, 292, X3, 25, 3, 7, 10, 47.
[b] Localization was unknown for 17 patients.

allele to another due to the loss or gain of one tandem repeat sequence at a single VNTR locus. The MST, illustrated in Figure, shows that most (21 out of 25) VNTR patterns, occurring either as clustered or unique isolates, differed from the nearest one for one allelic variation; one VNTR pattern differed for 2 allelic variations; three VNTR patterns differed for 3 allelic variations. By this analysis, the 25 VNTR profiles described above yielded two clonal complexes, termed CC1 and CC2, including 21 and 2 unique profiles, respectively. CC1 (white in Fig.) included a total of 104 isolates, 95 of which clustered in the 12 clusters. CC2 (grey in Fig.), that differed from CC1 for three allelic variations, included 2 isolates with unique VNTR profile.

Discussion

The aim of the present study was to determine the genetic diversity of MAH strains isolated in a region of Italy by analyzing a set of eight VNTR loci. The VNTR typing assay employed in the present study showed that 5 VNTR loci of our MAH isolates (i.e., loci 32, 292, X3, 25 and 47) were enough polymorphic to yield an acceptable allelic diversity. Indeed, in agreement with previous reports [4, 10, 12, 17, 18], locus VNTR X3 turn out to be the most polymorphic, while loci VNTR 3, VNTR 7 and VNTR 10 were the least suitable for VNTR typing of MAH isolates. Our VNTR analysis, that yielded 25 unique VNTR patterns and identified 12 clusters including a total 95 isolates, showed an excellent discriminatory power (HGDI = 0.990), similar to that obtained with VNTR schemes used by other authors [4, 5]. The results obtained through the VNTR analysis showed that most MAH strains displayed a close genetic relationship, as indicated by the minimum spanning tree analysis; in fact, 21 out of 25 VNTR patterns of the MAH isolates, occurring either as clustered or unique isolates, differed from the nearest one only for one allelic variation. This high phylogenetic proximity of the VNTR loci over a long time period supports the concept that the MAH genotype is highly homogeneous in our geographical area. Other studies demonstrated geographical differences in genetic diversity of MAH, suggesting the hypo-

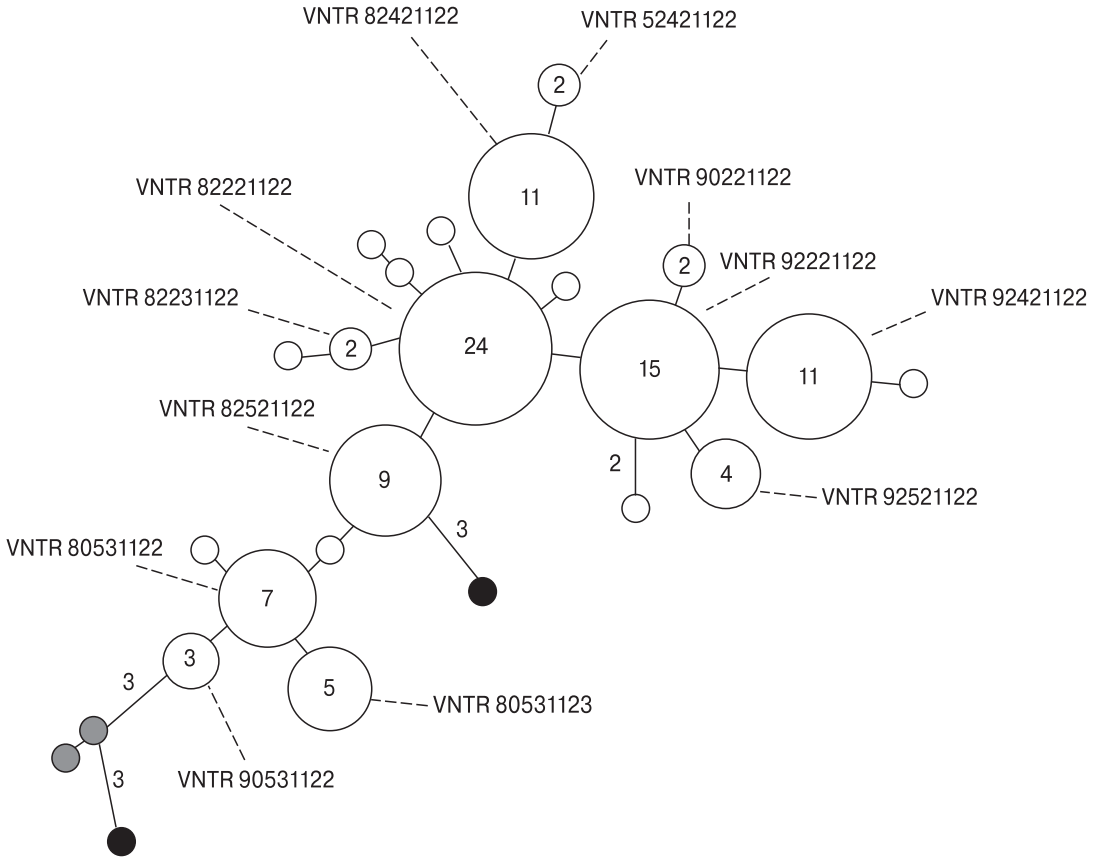

Figure. Minimum spanning tree based on VNTR profiles of a set of 8 loci of 108 MAH clinical isolates
Each small-size circle represents a single isolate; larger circles represent clusters of 2–24 isolates, depending on the circle size, with identical VNTR profiles. For each cluster the number of the isolates is given in the circle and the VNTR profile in the callouts. Numbers next to the branches indicate the level of changes more than 1 induced by loss or gain of VNTR copies at a given locus, yielding a change from one allele to another. White and grey circles indicate VNTR profiles belonging to clonal complexes CC1 and CC2, respectively, detected by the analysis at single locus variance. The tree was generated using the UPGMA method by the MIRU-VNTRplus web application available at www.miru-vntrplus.org.

thesis of the presence of possible sources of infection and transmission pathways at the local level [3, 6, 7, 8, 9]. Interestingly on this subject, a recent population structure study postulated the emergence of human-adapted MAH lineages on local scale, and suggested that recombination facilitates local adaptation of MAH [21].

In order to achieve a better control of MAH infection, further investigations on larger collections of MAH strains of human, animal and environmental origin are needed to clarify the sources of infection, the specific transmission pathway and the local adaptation mechanisms of MAH.

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