Infection, Genetics and Evolution 65 (2018) 144–149

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Genotyping and clarithromycin susceptibility testing of *Mycobacterium avium* subsp. *hominissuis* isolated in Tuscany, Italy

Flavia Genua, Melissa Menichini, Nicoletta Lari, Laura Rindi*

Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie, in Medicina e Chirurgia, Università di Pisa, I-56127 Pisa, Italy

ARTICLE INFO

Keywords:
Mycobacterium avium subsp. *hominissuis*
Molecular typing
VNTR analysis
Clarithromycin

ABSTRACT

Mycobacterium avium subsp. *hominissuis* (MAH) is a major cause of nontuberculous mycobacteria infection and the incidence of MAH infections is increasing in many countries. This study aimed at determining the VNTR-based genetic diversity and the susceptibility to clarithromycin of a collection of 71 MAH human strains isolated in the last seven years. The VNTR analysis, revealing 16 unique patterns and 8 clusters including a total of 55 isolates, showed that most MAH isolates displayed a close genetic relationship, indicating that the MAH genotypes are quite homogeneous in our geographical area. Clarithromycin showed strong antimicrobial activity against MAH isolates, as indicated by the high proportion (94.4%) of susceptible strains. No association between specific VNTR patterns and the clinical features or the MIC of clarithromycin was found.

1. Introduction

Mycobacterium avium complex is responsible for most of the human-associated nontuberculous mycobacteria infections in many countries (Griffith et al., 2007). *Mycobacterium avium*, one of the members of the *M. avium* complex, is ubiquitous in the environment, including soil, water, aerosols, dust (Nishiuchi et al., 2017). *M. avium* is classified into 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 (Mijs et al., 2002; Turenne et al., 2007). MAH is an important pathogen that causes infections in the respiratory tract, lymph node, and, occasionally, soft tissue of immunocompetent patients; moreover, it causes disseminated diseases in patients with human immunodeficiency virus infection (Karakousis et al., 2004). MAH infection is hard to be treated and the antimicrobial susceptibility of MAH is essential for appropriate patient management (Griffith et al., 2007). In Italy, as in many other countries worldwide, MAH is the most common cause of nontuberculous mycobacteria infection and the incidence of MAH infections is increasing (Rindi and Garzelli, 2016). 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 rapid and highly discriminatory genotyping method that has been successfully applied for MAH isolates (Ichikawa et al., 2015; Inagaki et al., 2009; Iwamoto et al., 2012; Radomski et al., 2010; Tirkkonen et al., 2010; Thibault et al., 2007).

Our group previously demonstrated a close genetic relationship of MAH isolates over the period from 1990 to 2011, suggesting that the MAH genotype is conserved (Rindi et al., 2013). In the present study, in light of the significant increase in MAH infections occurred in recent years (Rindi and Garzelli, 2016), we determined the VNTR-based genetic diversity of a collection of MAH human strains isolated from 2010 to 2016 in order to estimate the genetic relationships among MAH isolates in our setting and to confirm the homogeneity of MAH isolates at our local level in more recent years. Moreover, we performed the clarithromycin susceptibility test in order to investigate whether there was any association between the VNTR pattern and the minimal inhibitory concentration (MIC) of clarithromycin.

2. Materials and methods

2.1. Clinical isolates

A set of 71 MAH strains, identified by InnoLipa probes and by a multiplex PCR designed to discriminate MAC organisms (Shin et al., 2010), isolated from 2010 to 2016 in the Laboratory of Clinical Mycobacteriology of the University Hospital of Pisa, Italy, from the same number of patients, were studied. Thirty-six isolates were from respiratory specimens, 12 from lymph nodes, 3 from specimens other

* Corresponding author at: Dipartimento di Ricerca Traslazionale, e delle Nuove Tecnologie in Medicina e Chirurgia, Via San Zeno, 35/39, 56127 Pisa, Italy.
E-mail address: laura.rindi@med.unipi.it (L. Rindi).

https://doi.org/10.1016/j.meegid.2018.07.032
Received 13 July 2018; Received in revised form 24 July 2018; Accepted 24 July 2018
Available online 25 July 2018

Table 1
Epidemiological features of MAH patients.[a]

Male/ Female	Age group				Localization		
	0–14	15–39	40–64	≥65	Respiratory tract	Lymph node	Other[b]
33/37	10	2	8	36	36	12	3

[a] Gender, age and localization were unknown for 1, 15 and 20 patients, respectively.
[b] Include isolates from blood, stool and synovial fluid.

than respiratory specimens and lymph nodes, and 20 from an unknown source.

2.2. 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 (Thibault et al., 2007) and for three additional VNTR loci, *i.e.,* MATR-1, −7 and − 13, according to Inagaki et al. (2009). 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 (Thibault et al., 2007; Inagaki et al., 2009). VNTR profile is expressed as a string of 11 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) (Hunter and Gaston, 1988). 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 xth subtype.

2.3. Genetic relationships analysis

VNTR data were analyzed by the MIRU-VNTR*plus* 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.

2.4. Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) for clarithromycin (Sigma-Aldrich, USA) was determined by the resazurin microtiter assay plate method as described by Palomino et al. (2002). Briefly, the mycobacterial inoculum was prepared in 7H9 medium (Middlebrook 7H9 broth containing 0.5% glycerol and 10% oleic acid, albumin, glucose and catalase supplement), adjusted to a McFarland tube No. 1 standard and diluted 1:20; 100 µl of this suspension was used as inoculum. Clarithromycin stock solution was diluted in 7H9 medium and serial dilution were prepared in a 96-well microtitre plate using 100 µl of 7H9 medium; the range of concentrations tested was 0.5–64.0 µg/ml. A growth control containing no clarithromycin and a sterility control without inoculum were included in each plate. The plates were covered and incubated at 37 °C. After 7 days incubation, 30 ml of 0.01% resazurin solution was added to each well and the plates were re-incubated for 24 h. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was defined as the lowest concentration of clarithromycin that prevented this change in colour. According to the Clinical and Laboratory Standards Institute (2011), the MIC breakpoint of clarithromycin indicating resistance was ≥ 32 µg/ml.

3. Results and discussion

3.1. Epidemiology

A total of 71 MAH strains isolated in the years 2010–2016 from the same number of patients resident in Tuscany, Italy, were studied. As shown in Table 1, no association between MAH infection and patient sex was observed, similarly to what previously reported (Moore et al., 2010; Jankovic et al., 2013). MAH strains were prevalently isolated from subjects older than 65 years (51%), in whom the infection occurred at the pulmonary level; these results confirm data described in epidemiological studies carried out in different geographic regions

Table 2
VNTR allelic distribution in 71 MAH clinical isolates.

No. of tandem repeat copies	No. of isolates at the VNTR locus										
	32	292	X3	25	3	7	10	47	MATR-1	MATR-7	MATR-13
0		9									1
1		1			71	71	1		7	2	
2		60	37	59			67	66	59	65	69
3		1	4	11				5	3	1	1
4			15							3	
5			14				3				
6											
7	1										
8	31										
9	36										
10	2										
nd[a]	1		1	1					2		
h[b]	0.52	0.26	0.63	0.25	0	0	0.09	0.12	0.25	0.15	0.04

[a] Not determined (no PCR product was obtained).
[b] Allelic diversity (h) was calculated as described by Selander et al. (1986).