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Research paper

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

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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

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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

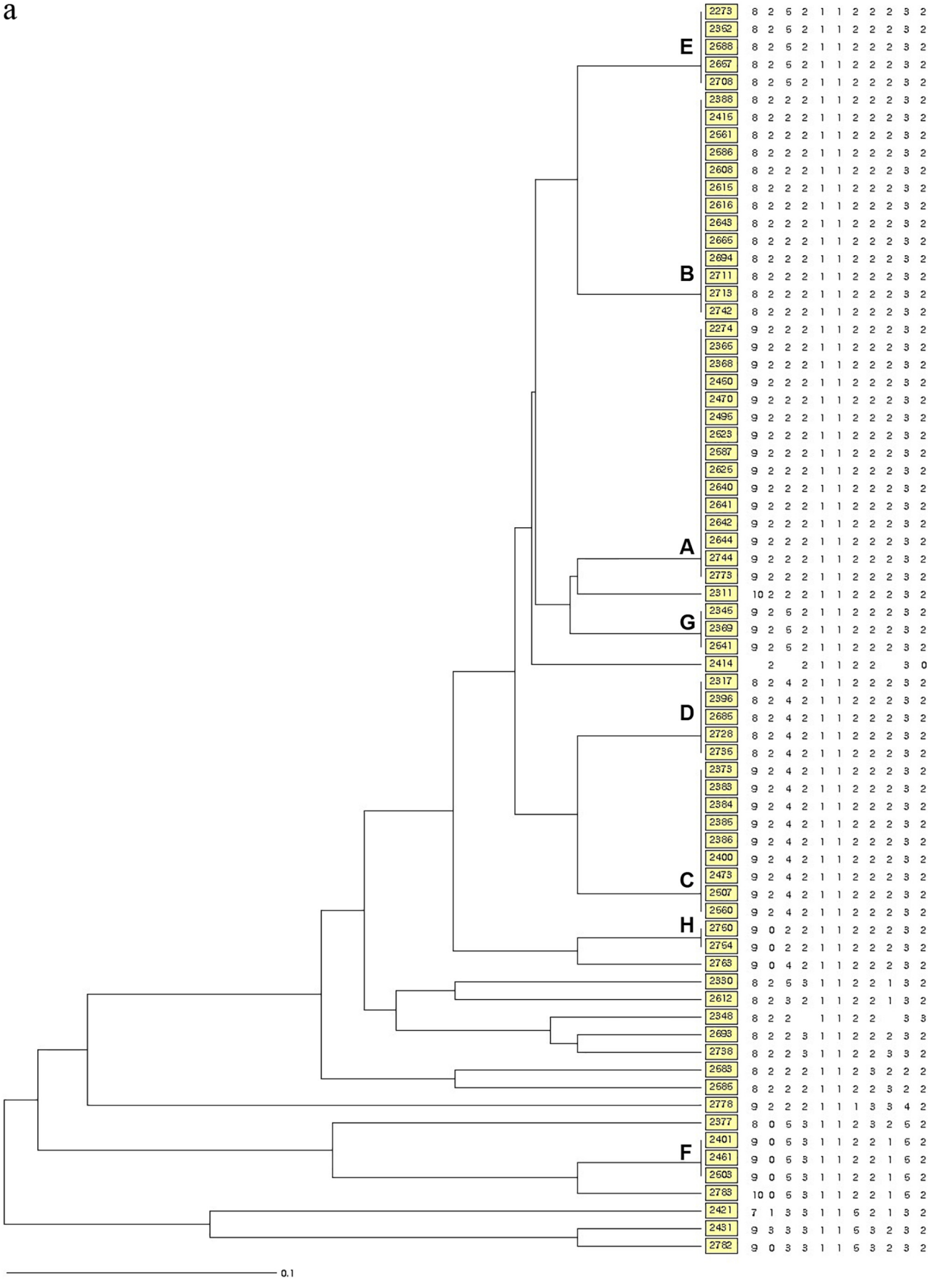
3.1. Epidemiology

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).



(caption on next page)

Fig. 1. VNTR genotyping based on a set of 11 loci (32, 292, X3, 25, 3, 7, 10, 47, MATR-1, MATR-7 and MATR-13) of 71 MAH strains.
a. Dendrogram and allelic profiles. The dendrogram was generated using the UPGMA method by the MIRU-VNTR*plus* web application available at www.miru-vntrplus.org. The columns 1 to 12 on the right of the dendrogram represent respectively: 1) isolate ID code (boxed); 2–12) isolate MIRU-VNTR profiles expressed as a string of 11 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the order stated above. The clusters are labelled A to H. b. Minimum spanning tree. Each small-size circle represents a single isolate; larger circles represent clusters of 2–15 isolates, depending on the circle size, with identical MIRU-VNTR profiles. For each cluster the number of the isolates and the VNTR profile are given in the callouts. Circles without callouts represent isolates with unique VNTR patterns. Numbers next to the branches indicate the level of changes induced by loss or gain of VNTR copies at a given locus, yielding a change from one allele to another. Black, dark grey and light grey circles indicate VNTR profiles belonging to clonal complexes CC1, CC2 and CC3, 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.

(Moore et al., 2010; van Ingen et al., 2010). The other most represented age group (14%) consisted of pediatric patients who had a lymph node infection; in fact, nontuberculous mycobacteria lymphadenitis are typically childhood diseases caused mainly by mycobacterial species belonging to MAC (Eriksson et al., 2001). Finally, the 3 strains isolated from extrapulmonary sites other than lymph nodes (blood, stool and synovial fluid) reflect the ability of MAH to colonize and infect different body districts (Tortoli, 2009).

3.2. Genotyping and clustering analysis of MAH isolates

The genetic diversity of 71 MAH human strains, isolated over a 7 year-period in the Laboratory of Clinical Mycobacteriology of the University Hospital of Pisa, Tuscany, Italy, was investigated by determining the polymorphism of a set of eleven VNTR loci (Thibault et al., 2007; Inagaki et al., 2009). 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. (1986). As shown in Table 2, the allelic diversity (h) of the VNTR loci of our collection varied widely, from 0 to 0.63. The VNTR loci 32 and X3 had a high diversity index ($h \geq 0.5$); five loci (292, 25, 47, MATR-1, MATR-7) showed medium diversity index ($0.1 \leq h \leq 0.5$); two loci (10, MATR-13) achieved a low diversity index ($h \leq 0.1$); the last two loci (3, 7) did not show any allelic diversity. In agreement with previous reports (Radomski et al., 2010; Tirkkonen et al., 2010; Thibault et al., 2007; Pate et al., 2011; Imperiale et al., 2017), 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. The discriminatory power of VNTR typing yielded an HGDI of 0.901, very similar to that obtained with VNTR schemes used by other authors (Imperiale et al., 2017; Inagaki et al., 2009).

The VNTR analysis was used to construct a dendrogram, reported in Fig. 1a, in which the VNTR patterns were ordered by similarity. Our VNTR analysis revealed 24 distinct VNTR patterns; of these, 16 patterns were unique, while 8 patterns were shared by 2 or more isolates, thus yielding 8 clusters including a total of 55 isolates, labelled A to H in the figure. In particular, 1 cluster consisting of 15 isolates, 1 cluster of 13 isolates, 1 cluster of 9 strains, 2 clusters of 5 strains, 2 clusters of 3 strains and finally 1 cluster of 2 strains were identified.

To visualize the genetic relationships between the study isolates, a minimum spanning tree (MST) was constructed. The MST, illustrated in Fig. 1b, is based on variations from one allele to another due to the loss or gain of one tandem repeat sequence at a single VNTR locus. By this analysis, the 24 VNTR profiles described above yielded three clonal complexes, termed CC1, CC2 and CC3, including 15, 4 and 2 unique profiles, respectively. CC1 (black in Fig. 1b) included a total of 60 isolates, 52 of which clustered in the 7 clusters A, B, C, D, E, G and H of 15, 13, 9, 5, 5, and 2 isolates, respectively. CC2 (dark grey in Fig. 1b), that differed from CC1 for three allelic variations, included exclusively the 3 isolates that were grouped in the cluster F and 1 isolate with unique VNTR profile. Finally, CC3 (light grey in Fig. 1b), that differed from CC1 for four allelic variations, included 2 isolates with unique VNTR profile. Overall, the results obtained through the VNTR analysis showed that most MAH strains, isolated in recent years, displayed a close genetic relationship, indicating that the MAH genotypes are quite homogeneous in our geographical area, confirming our previous results for the years prior to 2010. This finding is consistent with those of other studies demonstrating that strains of MAH exhibit geographical differences in genetic diversity (Dirac et al., 2013; Ichikawa et al., 2015; Iwamoto et al., 2012; Kalvisa et al., 2016). Such genotypic stability of the MAH strain population circulating in our region supports the hypothesis of the presence of possible local sources of infection and transmission pathways at the local level, as suggested in recent studies demonstrating different degrees of genetic correlation between human, swine and environmental MAH strains depending on the geographical area (Iwamoto et al., 2012; Lahiri et al., 2014; Muwonge et al., 2014; Nishiuchi et al., 2017).

3.3. Clarithromycin susceptibility of MAH isolates

The results of the clarithromycin susceptibility test for the 71 MAH clinical isolates are shown in Fig. 2. We found that most MAH isolates (94.4%) were susceptible to clarithromycin; only 3 isolates out of 71 (4.2%) were resistant to clarithromycin, as indicated by MIC values ≥ 64. These results, according to previously reported findings (Brown-Elliot et al., 2012; Cowman et al., 2016; Schon and Chryssanthou, 2017), confirm the strong *in vitro* antimicrobial activity of clarithromycin against MAH. The three resistant strains were isolated exclusively from elderly female patients, suggesting cases of Lady Windermere Syndrome (Dhillon and Watanakunakorn, 2000).

3.4. Association between VNTR genotypes and characteristics of MAH isolates

Table 3 shows the characteristics of MAH isolates occurring in clusters. No association of a specific VNTR pattern with a particular clinical feature, such as the pulmonary or lymph node infection, was observed. Further studies will provide information about possible association between MAH genotypes and other clinical characteristics in our geographical region; about this, other studies report that in Japan *M. avium* genotypes were associated with disease progression or therapeutic response (Kikuchi et al., 2009; Kikuchi et al., 2014), or that there was no significant association between MAH genotypes isolated from Korean patients and clinical manifestation or treatment response (Kim et al., 2016; Kim et al., 2012; Tatano et al., 2012).

No significant association between VNTR genotype and MIC of clarithromycin was observed; moreover, due to the small number of resistant isolates, it was not easy to evaluate the correlation between VNTR genotypes and clarithromycin susceptibility. In this context, a study done in China failed to demonstrate an association between MAH genotypes and clarithomycin resistance (Wei et al., 2015); interestingly on this subject, a recent study has shown significant differences in susceptibility to several drugs among MAH clusters (Uchiya et al.,

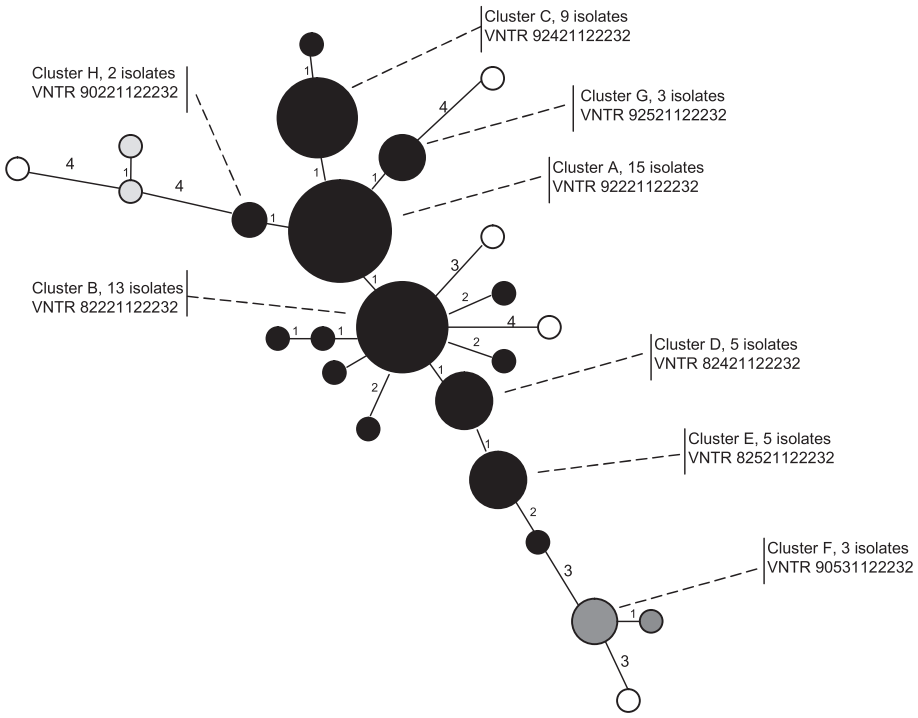


Fig. 1. (*continued*)

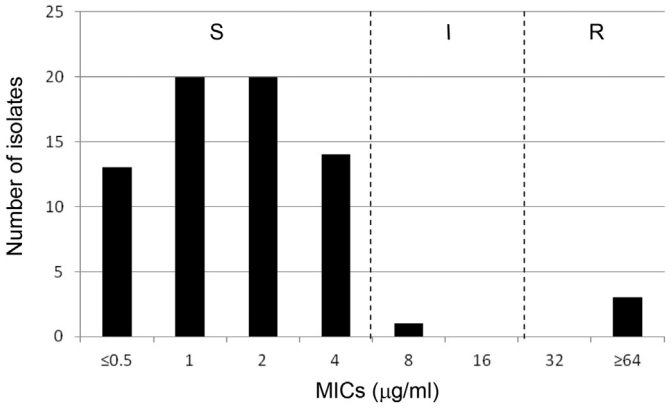


Fig. 2. Clarithromycin MICs for all 71 MAH clinical isolates. S, susceptible; I, intermediate; R, resistant.

2018) and a further study has reported a significant association between VNTR genotypes and susceptibility to quinolones (Tatano et al., 2012).

4. Conclusions

The evaluation of the genetic diversity of MAH strains isolated from humans in a 7-year period, carried out using the VNTR analysis, provided the identification of circulating genotypes in Tuscany, Italy. Our study confirmed, even for the most recent time period, the close genetic relationship of MAH isolates, indicating that the MAH genotypes are homogeneous in our geographical region. As expected, clarithromycin showed strong antimicrobial activity against MAH isolates. No association between the specific VNTR patterns and the localization of infection or the MIC of clarithromycin was found. Further investigations on larger collections of MAH strains of human, animal and environmental origin, are needed both to define the correlation between genotypes and clinical features and to clarify the sources of infection and

Table 3
Characteristics of MAH clinical isolates occurring in clusters.

Cluster code	No. of Isolates	VNTR pattern[a]	No. of isolates with specific localization[b]			No. of isolates with specific MIC (μg/ml) of clarithromycin							
			Respiratory tract	Lymph node	Other	≥64	32	16	8	4	2	1	≤0.5
A	15	92221122232	8	2	–	1	–	–	–	–	5	6	3
B	13	82221122232	8	3	1	–	–	–	–	3	6	3	1
C	9	92421122232	2	–	–	1	–	–	–	1	2	4	1
D	5	82421122232	1	1	2	–	–	–	–	2	2	–	1
E	5	82521122232	1	3	–	–	–	–	–	2	1	–	1
F	3	90531122132	–	1	–	–	–	–	–	1	1	1	–
G	3	92521122232	1	1	–	–	–	–	–	–	1	1	1
H	2	90221122232	2	–	–	–	–	–	1	–	–	–	1

[a] VNTR patterns are expressed as strings of 11 numbers, each representing the number of tandem repeats (TR) at a given VNTR position, in the following order: locus 32, 292, X3, 25, 3, 7, 10, 47, MATR-1, MATR-7 and MATR-13.
[b] Localization was unknown for 18 patients.

the specific transmission pathways of our region, in order to achieve a better control of MAH infection.

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