Original Article

# Virulence of *Mycobacterium avium* Subsp. *hominissuis* Human Isolates in an *in vitro* Macrophage Infection Model

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## Abstract

**Background:** *Mycobacterium avium* subsp. *hominissuis* (MAH) is an environmental opportunistic pathogen for humans and swine worldwide; in humans, the vast majority of MAH infections is due to strains belonging to specific genotypes, such as the internal transcribed spacer (ITS)-sequevars Mav-A and Mav-B that mostly cause pulmonary infections in elderly patients and severe disseminated infections in acquired immunodeficiency syndrome patients, respectively. To test whether the different types of infections in distinct patients' populations might reflect a different virulence of the infecting genotypes, MAH human isolates, genotyped by ITS sequencing and MIRU-VNTR minisatellite analysis, were studied for the capacity to infect and replicate in human macrophages *in vitro*. **Methods:** Cultures of human peripheral blood mononuclear cells and phagocytic human leukemic cell line THP-1 cells were infected with each MAH isolate and intracellular colony-forming units (CFU) were determined. **Results:** At 2 h after infection, i.e., immediately after cell entry, the numbers of intracellular bacteria did not differ between Mav-A and Mav-B organisms in both phagocytic cell types. At 5 days, Mav-A organisms, sharing highly related VNTR-MIRU genotypes, yielded numbers of intracellular CFUs significantly higher than Mav-B organisms in both phagocytic cell types. MIRU-VNTR-based minimum spanning tree analysis of the MAH isolates showed a divergent phylogenetic pathway of Mav-A and Mav-B organisms. **Conclusion:** Mav-A and Mav-B sequevars might have evolved different pathogenetic properties that might account for their association with different human infections.

**Keywords:** Genotyping, mycobacterial interspersed repetitive unit-variable-number tandem repeat typing, macrophage infection, *Mycobacterium avium* subsp. hominissuis, virulence

## Introduction

*Mycobacterium avium* subsp. *hominissuis* (MAH), one of the four subspecies of *M. avium*, is an opportunistic facultative intracellular pathogen for humans and swine worldwide and occasionally for other animals.[1,2] Soil and water are the natural reservoirs of the organism,[3,4] so that human infections are usually triggered by exposure to environmental clones[5,6] that seem to be endowed with a remarkable heterogeneity in virulence.[7,8]

Among the methods developed to define the molecular profiles of pathogenic clones for humans, genotyping based on minisatellite sequences of repetitive elements dispersed in the genome mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) turned out to be highly discriminative for studies on epidemiology, pathophysiology, and biodiversity of MAH infections.[9-12] Another molecular approach, based on the sequence of the 16S-23S ribosomal (rDNA) internal transcribed spacer (ITS),[13] that classifies *M. avium* into sequevars named Mav-A to Mav-H, is of practical epidemiological interest as the vast majority of MAH infections in humans are due to strains belonging to Mav-A and Mav-B sequevars.[14-16] In particular, Mav-A organisms mostly cause pulmonary infections in elderly patients and cervical lymphadenitis in children, while Mav-B organisms are almost exclusively restricted to severe disseminated infections occurring in a high proportion of severely immunocompromised acquired immunodeficiency syndrome patients.[17-19] The reason why Mav-A and Mav-B MAH organisms targets distinct patients' populations causing

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different types of infections are unknown, but it might reflect a different virulence of the infecting clones. To investigate the possible differential pathogenicity of Mav-A and Mav-B organisms, based on the assumption that macrophage infection represents a key event in MAH immunity,[20-22] we studied a set of MAH human isolates of different sequevars and MIRU-VNTR genotypes for their capacity to infect and replicate *in vitro* in human macrophages, such as blood monocytes and cells of the THP-1 cell line.

## Methods

### Study strains and molecular typing

A set of 23 MAH strains, identified by AccuProbe and/or InnoLipa probes and by a multiplex polymerase chain reaction (PCR) designed to discriminate MAC organisms,[23] isolated in the Laboratory of Clinical Mycobacteriology of the Santa Chiara University Hospital of Pisa, Italy, from the same number of patients, were studied. Ethics approval was not needed for *in vitro* experimental research not involving humans or animals in our Institution; written informed consent was obtained from volunteer blood cell donors.

Mav subgrouping was determined by sequencing the 16S-23S rDNA ITS region as described by Frothingham and Wilson,[13] with slight modifications.[16] MIRU-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.[24]

### Human monocytes and THP-1 cells

Peripheral blood mononuclear cells (PBMC) from healthy individuals were isolated by a density gradient according to standard procedures and then plated in 24-well plates at $1 \times 10^6$ cells/well in Roswell Park Memorial Institute (RPMI) medium. After 3 h, nonadherent cells were removed with two washes of RPMI, and the cells were reincubated in RPMI with 10% human serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml fungizone (Sigma). Monocytes were then incubated at 37°C without change of medium for 3 days. Before infection, cells were washed twice with phosphate-buffered saline (PBS) and 1 ml of fresh culture medium without antibiotics was added.

The human phagocytic monocyte cell line THP-1[25] was maintained in culture medium consisting of RPMI supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotic/antimycotic solution. For infection, $1 \times 10^5$ cells per well were plated in 2 ml of culture medium in the presence of 40 nM phorbol 12-myristate 13-acetate (PMA, Sigma) in 24-well plates. Two days later, cells were washed twice to remove PMA, and 1 ml of fresh culture medium without antibiotics was added.

### Inoculum preparations and cell infection

MAH strains were grown at 37°C in Middlebrook 7H9 medium, washed twice in PBS, passed through an insulin-syringe, and resuspended in PBS to an optical density corresponding approximately to $100 \times 10^6$ colony-forming units (CFU) per ml. Cell cultures were infected at a multiplicity of infection (MOI) of approximately 50 bacteria per macrophage resuspended in 1 ml of antibiotic-free culture medium. After 2 h, cultures were washed three times to remove nonphagocytosed bacteria and further incubated at 37°C in antibiotic-free culture medium. Intracellular CFUs were counted at 2 h and 5 days after cell infection by lysing cultured cells for 15 min with 1 ml of 0.1% Tween 80 and plating appropriate aliquots onto Middlebrook 7H10 agar medium.

## Results and Discussion

Table 1 reports the characteristics of MAH clinical isolates used in this study. Mav-A organisms included a total of 10 isolates, 5 from HIV-positive, and 5 HIV-negative individuals; 4 isolates were from sputum, 2 from urine, 1 from blood, CFS, pleural fluid and stools, respectively; Mav-B organisms included a total of 13 isolates, all from HIV-positive individuals (11 from blood, 2 from respiratory specimens).

MIRU-VNTR typing of isolates detected 8 distinct patterns, 2 of which in single isolates and 6 shared by 2 or more isolates (clusters). The relationships between the MIRU-VNTR patterns were visualized by constructing the minimum spanning tree (MST) illustrated in Figure 1. This analysis shows that all but one MIRU-VNTR patterns, occurring either as clustered or unique isolates, differed from the nearest one for one allelic variation thus indicating the high relatedness of strains, as previously reported;[12] isolates of sequevars Mav-A and Mav-B segregated at the sides of the tree and were separated by a cluster including both Mav-A and Mav-B organisms.

To test the capacity of MAH isolates to infect and replicate in human phagocytes, cultures of PBMC and THP-1 cells were infected at a MOI of 50:1 with each isolate and intracellular CFUs were determined at 2 h and 5 days postinfection. At 2 h, i.e., immediately after cell entry, the numbers of intracellular bacteria did not differ between Mav-A and Mav-B organisms in both phagocytic cell types (data not shown). At 5 days, Mav-A organisms yielded mean numbers of intracellular CFUs significantly higher than Mav-B organisms in both phagocytic cell types ($P = 0.003$ for PBMC; $P = 0.015$ for THP-1 cells, by Student's *t*-test) [Figure 2]. Notably, in preliminary experiments *M. avium* Mav-C isolates of environmental (stream water) source, not identified at the subspecies level, tested in the macrophage infection assay, turned out to be much less efficient in macrophage infection as they were rapidly phagocytosed and killed by PBMC and THP-1 cells (data not shown). It is, therefore, reasonable to assume that Mav-A and Mav-B ITS sequevars represent distinct MAH subgroups that have evolved different capabilities to infect and replicates in human macrophages. Actually, divergent phylogenetic pathways of sequevar Mav-A and Mav-B isolates are also suggested by the MIRU-VNTR-based MST analysis [Figure 1]; indeed,

**Table 1: Characteristics of *Mycobacterium avium* subspp. *hominissuis* clinical isolates[a]**

| Isolate number | Clinical specimen | Patient'sHIV serology[b] | ITS sequevar[c] | MIRU-VNTR pattern[d] |
|---|---|---|---|---|
| 1 | Sputum | + | Mav-A | 82521222 |
| 2 | Blood | + | Mav-A | 80531223 |
| 3 | Sputum | - | Mav-A | 80531223 |
| 4 | Sputum | - | Mav-A | 80531222 |
| 5 | Urine | + | Mav-A | 80531222 |
| 6 | CSF | + | Mav-A | 80531222 |
| 7 | Pleural fluid | - | Mav-A | 80531222 |
| 8 | Urine | - | Mav-A | 80531222 |
| 9 | Sputum | - | Mav-A | 80531223 |
| 10 | Stools | + | Mav-A | 80531222 |
| 11 | Blood | + | Mav-B | 82221222 |
| 12 | Blood | + | Mav-B | 82421222 |
| 13 | Blood | + | Mav-B | 82221222 |
| 14 | Blood | + | Mav-B | 8-221222 |
| 15 | Blood | + | Mav-B | 52421222 |
| 16 | Blood | + | Mav-B | 52421222 |
| 17 | Bronchial wash | + | Mav-B | 82421222 |
| 18 | Blood | + | Mav-B | 82521222 |
| 19 | Blood | + | Mav-B | 82521222 |
| 20 | Blood | + | Mav-B | 82421222 |
| 21 | Blood | + | Mav-B | 82421222 |
| 22 | Sputum | + | Mav-B | 92421222 |
| 23 | Blood | + | Mav-B | 82421222 |

[a]Isolates were identified by AccuProbe and/or InnoLipa probes and by a multiplex PCR designed to discriminate MAC organisms. Abbreviations: ITS, 16S-23S rDNA internal transcribed spacer; CSF, cerebro-spinal fluid, b+, HIV-positive; -, HIV-negative, [c]ITS sub-grouping was determined by sequencing the 16S-23S rDNA ITS region as previously reported, dMIRU-VNTR typing was performed by PCR using specific primers for 8 polymorphic loci in *M. avium subsp. paratuberculosis* K10. 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 and 47

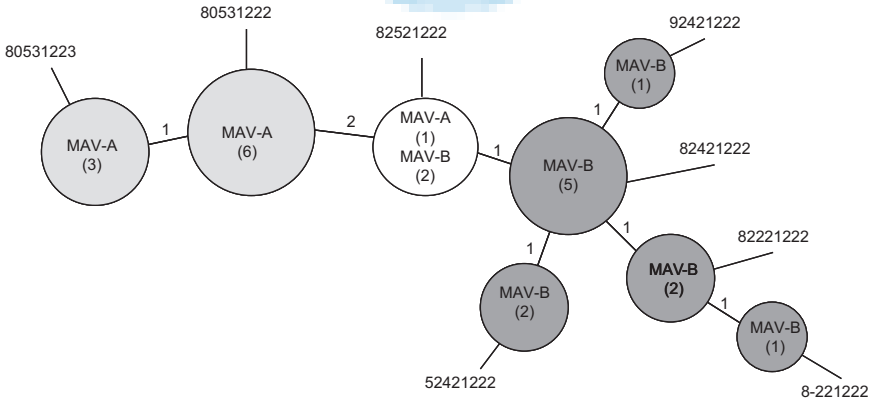


**Figure 1:** Minimum spanning tree based on variable-number tandem repeat patterns of a set of 8 mycobacterial interspersed repetitive unit loci of 23 *Mycobacterium avium* subsp. *hominissuis* clinical isolates. Circles include isolates sharing a given mycobacterial interspersed repetitive unit-variable-number tandem repeat pattern (the number is given in parenthesis); circle callouts report the mycobacterial interspersed repetitive unit-variable-number tandem repeat pattern expressed as a string of 8 numbers, each representing the number of tandem repeats at the variable-number tandem repeat loci 32, 292, X3, 25, 3, 7, 10 and 47. Light grey and dark grey circles include isolates exclusively of genotype Mav-A and Mav-B, respectively; white circle includes isolates of Mav-A and Mav-B. Numbers next to the branches connecting circles indicate the level of changes induced by loss or gain of variable-number tandem repeat copies at a given locus, yielding a change from one allele to another

clustered isolates with MIRU-VNTR profiles 80531222 and 80531223 (all of Mav-A) yielded numbers of intracellular CFUs >10-fold higher than 82421222 isolates (Mav-B) in both phagocytic cell types, while clustered isolates with

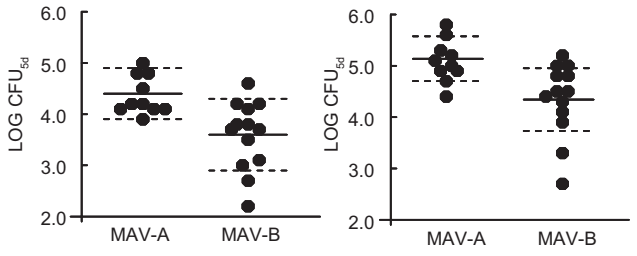


**Figure 2:** Phagocytosis of *Mycobacterium avium* subsp. *hominissuis* human isolates of Mav-A, Mav-B genotype by peripheral blood mononuclear cells (left panel) and THP-1 cells (right panel). Cultures of Peripheral blood mononuclear cells and THP-1 cells were infected at a multiplicity of infection of 50:1 and intracellular colony forming units were counted in cell lysates at 5 days after infection. Each circle represents the mean $Log_{10}$ of intracellular colony forming units determined in triplicate cultures for each isolate. Horizontal continuous and dashed lines represent mean ± standard deviation of intracellular colony-forming units of the tested isolates for each Mav genotype

MIRU-VNTR profile 82521222, including both Mav-A and Mav-B isolates, yielded intermediate numbers of intracellular CFUs.

Although our results indicate that different MAH genotypes differ in the capacity of infection and replication in human macrophages, the demonstration of a straightforward link with virulence for humans of the MAH subgroups will require the comparative investigation of specific virulence factors and immunogenicity of the distinct genotypes. Some studies have described that *M. avium* isolates of different subspecies possess genetically distinct features that may be associated with pathogenetic characteristics[26] and show different behaviors during human macrophage infection in terms of cytokine responses and ability to replicate intracellularly.[27,28] However, only a few studies have reported the association of specific MAH genotypes to certain virulence factors, for example, we showed that *M. avium* Mav-A and Mav-B isolates of human origin were strong producers of a magnesium-dependent, cell-wall associated haemolysin, a potential virulence factor, as compared to environmental Mav-C organisms;[29] more recently, specific virulence factors of the mammalian cell entry family have been reported in highly virulent MAH isolates sharing the MST22 genotype which also displayed suppression of the interferon-γ and interleukin (IL)-17 responses, and increased IL-10 production.[8] Whole-genome sequencing-based analysis of representative human, animal, and environmental MAH isolates will likely clarify at the genome level the possible link of virulence with the isolate genotype and the association of MAH genotypes with different human infections.

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## Conflicts of interest

There are no conflicts of interest.

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