Full paper

Molecular characterization of *Mycobacterium abscessus* subspecies isolated from patients attending an Italian Cystic Fibrosis Centre

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Running title: Mycobacterium abscessus subspecies isolated from cystic fibrosis patients

SUMMARY

Mycobacterium abscessus (MABS) infection represents significant management challenge in cystic fibrosis (CF) patients. This retrospective study (2005-2016) aims to determine the prevalence of the subspecies of MABS isolated from CF patients, to evaluate the persistence over the years of a single subspecies of MABS and to correlate mutations responsible for macrolides and amikacin resistance with MIC values.

We investigated 314 strains (1 isolate/patient/year) isolated from the lower respiratory tract of 51 chronically infected CF patients. Sequencing of rpoB gene was performed to identify the MABS subspecies. The erm(41) gene was sequenced to differentiate the strains with and without inducible macrolide resistance. Regions of 23S and 16S rRNA were sequenced to investigate mutations responsible for constitutive resistance to macrolides and aminoglycosides, respectively. Antibiotic susceptibility, using commercial microdilution plates, was evaluated according to CLSI.

M. abscessus subsp. abscessus accounted for 64% of the isolates, bolletii subspecies for 16% and massiliense subspecies for 20%. All the massiliense strains presented truncated erm(41) gene while 12 abscessus strains presented the mutation T28->C in the erm(41) gene, which makes it inactive. The 23S rRNA analysis did not show constitutive resistance to macrolides in any strain. Mutation of the 16S rRNA gene was highlighted in 2 strains out of 314, in agreement with high MIC values.

The correct identification at the subspecies level and the molecular analysis of 23S rRNA, 16S rRNA and erm gene is useful to guide the treatment strategy in patients with M. abscessus lung infection.

Keywords: Cystic Fibrosis, Mycobacterium abscessus (MABS), Non-Tuberculous Mycobacteria (NTMs), Antibiotic susceptibility

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INTRODUCTION

Mycobacterium abscessus (MABS) and the species belonging to *Mycobacterium avium* complex are the Non-Tuberculous Mycobacteria (NTMs) isolated with increasing frequency from patients with cystic fibrosis (CF), with a prevalence ranging from 3.8% to 22.6% (Torrens *et al.*, 1998). In a recent study a significantly more severe decline of lung functionality has been reported in CF patients with chronic MABS infection than in those infected by other NTM species (Esther *et al.*, 2010).

Given the ubiquitous nature of NTMs, isolation of an NTM from respiratory specimen is not synonymous with disease, nor it is necessarily an indication to initiate treatment. Current American Thoracic Society and Infectious Diseases Society of America (ATS/IDSA) criteria for the diagnosis of NTM lung disease call for the presence of 2 or more positive cultures, in the setting of compatible symptoms and radiographic findings and the exclusion of other diseases (Torrens *et al.*, 1998).

MABS, whose involvement in lung infections is increasing worldwide (Lai *et al.*, 2010; Thomson and NTM working group at Queensland TB Control Centre and Queensland Mycobacterial Reference Laboratory, 2010), in particular in CF patients (Griffith, 2003; Olivier *et al.*, 2003), is characterized by resistance to most of the antibiotics potentially active on rapid growing mycobacteria (RGM). MABS can cause severe lung infections and has proven to be a serious threat to patients with CF and a challenge for clinicians due to difficulties in timely diagnosis and complex multidrug treatment regimens (Nessar *et al.*, 2012)[8].

In recent years, whole genome sequencing has produced substantial evidence of the presence, within the MABS, of three subspecies: *M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* differing for the spectrum of resistance to antibiotics (Bryant *et al.*, 2013; Tortoli *et al.*, 2016).

The isolation of MABS from the sputum of CF patients is technically challenging as it is often hampered by the concurrent overgrowth of *Pseudomonas aeruginosa* or by the heavy decontamination procedure required by samples from CF patients; it follows that the prevalence of MABS is probably underestimated.

Although to date its role in the decline in lung functionality in CF patients is unclear, some CF patients have chronic MABS infection while in others it occurs intermittently. At times patients with apparent chronic infection are instead infected, at different times, by different strains even belonging to different subspecies.

The present retrospective study investigates MABS strains isolated from pulmonary specimens of CF patients attending the CF Regional Reference Centre (CRRFC) of Milan between 2005

and 2016. Objectives of the study, restricted to CF patients with chronic MABS infection, include: determination of the prevalence of the different subspecies, evaluation of the persistence over the years of a single subspecies of MABS or the alternation of different subspecies, and the evaluation of the correlation between mutations responsible for macrolides and amikacin resistance and the minimum inhibitory concentrations (MIC) detected by broth microdilution.

MATERIALS AND METHODS

Study population

A total of 314 MABS strains, collected between 2005-2016 (1 isolate/patient/year stored at -80°C) were obtained from respiratory specimens (bronchial aspirates, sputum or nasopharyngeal aspirates) of 51 chronically infected CF patients (31 males). The specimens were collected during the quarterly routine check-up provided by the protocol used in the Milan CF center, in agreement with national and international guidelines (Griffith *et al.*, 2007; Floto *et al.*, 2016; Haworth *et al.*, 2017).

Microbiological analysis

Our laboratory, in agreement with literature data, has introduced in its routine a culture system for rapid growing of mycobacteria based on the use of *Burkholderia cepacia* selective agar (BCSA) (Esther *et al.*, 2011). Colony morphology was investigated and the colonies were differentiated as rough or smooth. An initial presumptive identification of species was performed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF, VITEK-MS Mycobacterium/Nocardia kit, BioMérieux). The strains of MABS were frozen at -80 °C for subsequent molecular analysis.

Molecular characterization of MABS strains

The identification of the MABS subspecies was performed by partial amplification and sequencing of the housekeeping gene rpoB using the primers rpoB-F: GGCAAGGTCACCCCGAAGGG and rpoB-R: AGCGGCTGCTGGGTGATCATC that allowed us to sequence a tract of about 700 pb (Adékambi et al., 2006). The sequences obtained were compared with those of type strains of the three subspecies of MABS available in GenBank.

Detection of mutations responsible for resistance

The *erm*(41) gene, responsible for inducible resistance to macrolides, was sequenced by using the primers: erm-F: GACCGGGGCCTTCTTCGTGAT and erm-R:

GACTTCCCCGCACCGATTCC to differentiate wild type strains from the ones with *erm*(41) gene either truncated or harboring the T28C mutation (Kim *et al.*, 2010).

The 19-F: GTAGCGAAATTCCTTGTCGG and 21-R: TTCCCGCTTAGATGCTTTCAG primers were used to amplify the peptidyltransferase region of the 23S rRNA gene, to search the mutations (A2058C, A2058G, A2059C, A2059G) responsible for constitutive resistance to macrolides (Meier *et al.*, 1994).

The 16S rRNA gene was sequenced with the 16S-f2: CAGCAGCCGCGGTAATAC and 1541-r: CACCTTCCGGTACGGCTA primers (Prammananan *et al.*, 1998), targeting the variable region around nucleotide 1000 involved in mutations responsible for amikacin-resistance (A1408G and C1409T) (Nessar *et al.*, 2011). The sequences obtained were compared to the wild type gene sequence of MABS deposited in GenBank database.

Antimicrobial susceptibility testing

MIC values were measured following the recommendations of Clinical Laboratory Standards Institute (Woods *et al.*, 2011; Clinical & Laboratory Standards Institute, 2019) using a broth (cation-adjusted Mueller-Hinton) microdilution (RAPMYCO SENSITITRE®). The following 9 antibiotics were tested: Amikacin (AMI), Cefoxitin (FOX), Ciprofloxacin (CIP), Clarithromycin (CLA), Doxycycline (DOX), Imipenem (IMI), Linezolid (LZD), Moxifloxacin (MXF), Sulfamethoxazole-trimethoprim (SXT). The plates were read after five days of incubation and the MIC of clarithromycin was read again after an incubation period of 14 days. For quality control purposes, *M. peregrinum* ATCC 700686 and *Staphylococcus aureus* ATCC 29213 were used. Additionally, MIC_{50} and MIC_{90} values were derived from the MIC distribution for each antibiotic.

Statistical analysis

Descriptive statistics including MIC median, MIC_{50} and MIC_{90}, were performed for each drug. Pearson's chi-squared test and Fisher's exact test statistics were used to assess proportion of resistant profiles of *M. abscessus, M. Bolletii and M.* massiliense. P-values were two-sided and $P<0.05$ was considered statistically significant. All analyses were performed using the open source software R version 3.5.1 (http://www.R-project.org)

RESULTS

Distribution of the MABS subspecies in the CRRFC of Milan

Among 51 chronically infected patients, 314 isolates were collected during the period 2005-2016. According to *rpo*B gene sequencing, *M. abscessus* subsp. *abscessus* accounted for 64%, *M. abscessus* subsp. *bolletii* for 16% and *M. abscessus* subsp. *massiliense* for 20%.

Evaluation over time of the MABS subspecies isolated from CF patients

As shown in Figure 1, a large majority of patients (90%) were persistently infected by the same subspecies of MABS. Only 5 patients (10%) had either a co-infection or alternation of infections by different subspecies (*abscessus* and *bolletii* or *abscessus* and *massiliense*).

Analysis of the erm gene

Out of 202 *M. abscessus* subsp. *abscessus*, 190 (94%) presented wild-type (WT) *erm*(41) gene which was able to confer inducible resistance to macrolides. The 12 remaining (6%) showed the mutation T28C responsible for inactivation of *erm* gene, which was therefore unable to induce resistance (Table 1, 2).

All the 52 *M. abscessus* subsp. *bolletii* isolates had WT *erm* gene, therefore responsible for inducible resistance (Table 1, 2).

All the isolates of *M. abscessus* subsp. *massiliense* (60 strains) showed a truncated *erm* gene, thus excluding the possibility of inducible macrolide resistance (Table 1, 2).

Analysis of the 16S and 23S rRNA genes

A->G mutation at positions 1408 of the 16S rRNA gene, responsible for amikacin resistance, was detected in two strains of the subspecies *abscessus*.

None of the clinical isolates showed in 23S rRNA gene any of the mutations (A2058C/A2058G/A2059C/A2059G) responsible for constitutive macrolide resistance (Table 1, 2).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing confirmed the multidrug-resistance of MABS; in fact, the strains showed resistant phenotype to most antibiotics (Nie *et al.*, 2014; Mougari *et al.*, 2017; Kusuki *et al.*, 2018).

The drug susceptibility patterns of MABS, including the MIC50/MIC90 values, are presented in Table 3. Amikacin was the most active; the resistance rate to amikacin was 1% (2/202) for the subspecies *abscessus* while no resistance was detected among the subspecies *massiliense* and *bolletii*. The difference between the subspecies (P=1) was not significant.

Resistance rates to trimethoprim/sulfamethoxazole and imipenem (IMI P=0.0729) were significantly higher (SXT P<0.001; IMI P=0.0729) in the subspecies *abscessus* (SXT R=86%, IMI R=39%) than in *massiliense* (SXT R=52%, IMI R=25%) and *bolleti* (SXT R=63%, IMI R=27%).

Almost all isolates were resistant (R>88%) to doxycycline and no significant difference was detected between the three subspecies (P=0.6488).

Resistance rates to moxifloxacin and ciprofloxacin were equally high in the subspecies *abscessus* (CIP R=55%; MXF R=60%), *massiliense* (CIP R=62%; MXF R=68%) and *bolletii* (CIP R=52%; MXF R=50%). Almost all the isolates of the three subspecies were susceptible to linezolid (S>93%).

Resistance rates to cefoxitin were low (R<3%) in the three subspecies with no significant differences (*P=0.2636).*

For clarithromycin, 6% (12/202) of the isolates of the subsp. *abscessus* were still susceptible at 14 days. The remaining 94% (190/202) were susceptible at 5 days but resistant at day 14, in agreement with the WT *erm* gene. Similarly, 100% (52/52) of the strains of the subsp. *bolletii,* all presenting WT *erm gene,* were susceptible at day 5, but resistant at day 14. On the other hand, all the strains of the subsp. *massiliense* remained susceptible at day 14, as expected, because of the truncated *erm* gene (100% of the strains showed MIC equal to 0.5 µg/mL) (Table 1, 2).

DISCUSSION

To date, the CRRFC and the CF Adult Section of Milan (IRCCS Cà Granda Foundation, Ospedale Maggiore Policlinico), comprises 892 patients, aged between less than one month to 74 years.

In our twelve-year surveillance (2005-2016) the number of CF patients in the center, as well as the prevalence of MABS infection, has progressively increased from 3.5% (2005) to 5.2% (2016). The factors that may have contributed to such increase include: 1) the use, since 2010, of Burkholderia cepacia Selective Agar, which makes it much easier to isolate RGM, including MABS; 2) the implementation of genetic sequencing for identification; 3) the lengthening of life expectancy of patients, and 4) the acquisition, from other Italian CF Centers, of patients previously infected by NTM including MABS.

Several studies reported the proportion of the three subspecies among MABS isolates. Out of 40 patients monitored at the National Institutes of Health (Bethesda, MD), the prevalence of *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* was 28% and 5%, respectively (Zelazny *et al.*, 2009). In the Netherlands, 21% of clinical isolates of *M. abscessus* were identified as *M. abscessus* subsp. *massiliense* and 15% as *M. abscessus* subsp. *Bolletii* (van Ingen *et al.*, 2009). In France, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* accounted for 22% and 18%, respectively, of 50 CF patients infected by *MABS* (Roux *et al.*, 2009). In Korea, half (47%) of *M. abscessus* clinical isolates were identified as *M. abscessus* subsp. *massiliense* while the prevalence of *M. abscessus* subsp. *bolletii* was low (2%)

(Kim *et al.*, 2008). The present study included 51 patients with chronic MABS lung disease, and molecular identification revealed that in CF patients attending our center the infection was more frequently due to *M. abscessus* subsp. *abscessus* (64%), followed by *M. abscessus* subsp. *massiliense* (20%) and *M. abscessus* subsp. *bolletii* (16%). The alternation of the subspecies responsible for infection was observed in only five patients (10%), while in the other patients the subspecies remained unchanged over time (Figure 1). The prevalence of the three *M. abscessus* subspecies did not significantly differ from that reported in other case studies (Zelazny *et al.*, 2009; O'Driscoll *et al.*, 2016).

Lee et al. (Lee *et al.*, 2014), reported a low frequency of 23S rRNA and 16S rRNA mutants, only 3.8% of strains possessed *rrl* mutation (Nessar *et al.*, 2011) while the mutation A1408G of the 16S rRNA gene was present in 1% of MABS strains. Prammananan et al. (Prammananan *et al.*, 1998) reported that M. *abscessus* subsp. *abscessus* strains mutated in the 16S rRNA gene had high MIC values (>64 µg/ml) of amikacin and were isolated from the patients who had received aminoglycoside therapy.

In our Center, we detected the A->G mutation at positions 1408 of the 16S rRNA gene, responsible for amikacin resistance, in 2 strains of the subspecies *abscessus* (MIC values > 64 µg/ml). None of our clinical isolates showed, in 23S rRNA gene, any of the mutations responsible for constitutive macrolide resistance.

In this study, amikacin was the most active antimicrobial agent against MABS species, showing a 99% overall susceptibility rate. After amikacin, linezolid was the second most effective antimicrobial agent, with 93% susceptibility rate.

Although prior studies have reported a variable susceptibility rate for linezolid ranging from 32.0% to 97.0% (Yang *et al.*, 2003; Chua *et al.*, 2015), the majority of the strains of MABS species are susceptible to linezolid.

Linezolid represents the best choice in association with amikacin. Resistance rates to trimethoprim/sulfamethoxazole (SXT P<0.001) and imipenem (IMI P=0.0729) were higher in the subsp. *abscessus* than in *massiliense* and *bolletii.*

Resistance rates to cefoxitin was low (R<3%) in MABS and there was no significant difference in resistance rates among the three subspecies *(P=0.2636).*

Contrary to our results, one Australian study reported that the subspecies massiliense (27.8%) was more resistant to cefoxitin than abscessus (10.0%) (Chua *et al.*, 2015).

The vast majority of MABS isolates were resistant to moxifloxacin and ciprofloxacin, as previously reported (Yang *et al.*, 2003; Chua *et al.*, 2015). Studies investigating the activities in vitro of different generations of quinolones against clinical and reference strains of rapidly

growing mycobacteria found high levels of resistance, confirming our results (de Moura *et al.*, 2012; Maurer *et al.*, 2014; Ravnholt *et al.*, 2018).

In South Korea, prior research reported that almost all MABS isolates were resistant to ciprofloxacin and moxifloxacin (Lee *et al.*, 2015; Jeong *et al.*, 2017). Based on these findings, the treatment effects of moxifloxacin and ciprofloxacin seem to be limited.

Susceptibility to macrolides varied widely depending on the subspecies. One drawback pertinent to *M. abscessus* is the possible occurrence of inducible resistance to clarithromycin (Jayasingam *et al.*, 2017). This is caused by the presence of an active erythromycin resistance methylase (*erm*) gene as is the case in most strains of subsp. *abscessus* and *bolletii* (Nash *et al.*, 2009). In the subsp. *massiliense*, the *erm*(41) gene is inactive (truncated) and consequently this subspecies is not affected by inducible resistance. As expected, all *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* that presented WT *erm*(41) gene were associated, in the microdilution test, with the transition from complete susceptibility to clarithromycin, at first reading (5 days), to resistance, at day 14.

We found that the subsp. abscessus had a 94% inducible resistance rate. Contrary to the low susceptibility to clarithromycin observed in subsp. abscessus and bolletii, 100% of massiliense isolates were susceptible to clarithromycin. These findings highlight the importance of using different treatment strategies for the three subspecies and the consequent need for precise differentiation between the three subspecies.

In agreement with others (Yang *et al.*, 2003; Park *et al.*, 2008; Kim *et al.*, 2010; Bastian *et al.*, 2011; Koh *et al.*, 2011) our *M. abscessus* isolates were characterized by high resistance rates to the majority of antibiotics. The use of macrolides is therefore suitable only for the treatment of infections due to *M. abscessus* subsp. *massiliense* and for those *M. abscessus* that present the T28C mutation. These data are in agreement with the more favorable clinical outcome reported for patients infected by *M. abscessus* subsp. *massiliense* in comparison with other *M. abscessus* subspecies (Koh *et al.*, 2011).

The treatment of MABS pulmonary infections is an emerging challenge in patients with cystic fibrosis. Multidrug therapy for prolonged durations is required and carries the significant burden of drug-related toxicity, cost and selective pressure for multidrug resistant bacteria. International guidelines (British Thoracic Society (BTS), United States Cystic Fibrosis Foundation (US CFF) and the European Cystic Fibrosis Society (ECFS)) (Floto *et al.*, 2016; Haworth *et al.*, 2017) acknowledge that clinical and in vitro data to support treatment regimens are limited, particularly in children, and the presence of differences in susceptibility of the *Mycobacterium abscessus* subspecies confirms the need for accurate identification and the

usefulness of susceptibility testing performed in microdilution with MIC determination (Ravnholt *et al.*, 2018; Shaw *et al.*, 2019).

In conclusion, differences in drug susceptibility patterns of MABS could explain the different treatment outcomes among the three subspecies. The goal of antimicrobial susceptibility testing is to predict whether patients treated with specific antibiotics are likely to be cured for their infections. Since antibiotic susceptibilities differ substantially among strains in different geographical locations and clinical settings, local antibiotic susceptibility data are very helpful in choosing the treatment for infections, especially in critical patients. Prospective studies of MABS species are needed to understand the association between different antimicrobial susceptibility testing results and clinical outcome.

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Table 1. Drug susceptibility profiles of the subspecies *abscessus*, *bolletii* and *massiliense*.

	Susceptible			Intermediate			Resistant			
	***abscessus* (202)**	***bolletii* (52)**	***massiliense* (60)**	***abscessus* (202)**	***bolletii* (52)**	***massiliense* (60)**	***abscessus* (202)**	***bolletii* (52)**	***massiliense* (60)**	**P-value**
Antibiotics*	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	**No. of isolates (%)**	
SXT	29 (14%)	19 (37%)	29 (48%)	0	0	0	173 (86%)	33 (63%)	31 (52%)	<0.001
LZD	190 (94%)	52 (100%)	56 (93%)	12 (6%)	0	4 (7%)	0	0	0	1
CIP	46 (13%)	6 (11,5%)	0	45 (22%)	19 (36,5%)	23 (38%)	111 (55%)	27 (52%)	37 (62%)	0.5454
IMI	53 (26%)	12 (23%)	10 (17%)	71 (35%)	26 (50%)	35 (58%)	78 (39%)	14 (27%)	15 (25%)	0.0729
MXF	39 (20%)	14 (27%)	19 (32%)	40 (20%)	12 (23%)	0	123 (60%)	26 (50%)	41 (68%)	0.1386
FOX	80 (40%)	18 (35%)	35 (75%)	115 (57%)	34 (65%)	25 (25%)	7 (3%)	0	0	0.2636
AMI	200 (99%)	52 (100%)	60 (100%)	0	0	0	2 (1%)	0	0	1
DOX	1 (1%)	0	2 (3,5%)	16 (8%)	6 (11,5%)	2 (3,5%)	185 (91%)	46 (88.5%)	56 (93%)	0.6488
CLA on day 5	202 (100%)	52 (100%)	60 (100%)	0	0	0	0	0	0	1
CLA on day 14	12 (6%)	0	60 (100%)	0	0	0	190 (94%)	52(100%)	0	<0.001

***SXT: trimethoprim/sulfamethoxazole, LZD: linezolid, CIP: ciprofloxacin, IMI: imipenem, MXF: moxifloxacin, FOX: cefoxitin, AMI: amikacin, DOX: doxycyclin

Table 2. Correlation of clarithromycin MICs with the presence of mutations in *erm*(41) and *rrl*. *****truncated

	Clarithromycin MIC (µg/mL)										***erm (41)***	***rrl***
	≤ 0,06	***0,12***	***0,25***	***0,5***	***1***	***2***	***4***	***8***	***16***	***32***		
M. abscessus* subsp. *abscessus (202)	/	/	/	/	/	/		**83**	**107**	/	**WT**	**WT**
	1	**/**	**1**	**10**	/	/	/	/	/	/	**mut**	**WT**
M. abscessus* subsp. *bolletii (52)	**/**	**/**	**/**	**/**	**/**	**/**	**/**	**6**	**46**	**/**	**WT**	**WT**
M. abscessus* subsp. *massiliense (60)	**18**	**6**	**19**	**17**	**/**	**/**		**/**	**/**	**/**	***trunc.**	**WT**

Table 3. MIC 50/90

	abscessus		***bolletii***		***massiliense***	
Antibiotics*	MIC 50 (μg/ml)	MIC 90 (μg/ml)	MIC 50 (μg/ml)	MIC 90 (μg/ml)	MIC 50 (μg/ml)	MIC 90 (μg/ml)
SXT	**8**	**8**	**8**	**8**	**4**	**8**
LZD	**8**	**8**	**8**	**8**	**1**	**8**
CIP	**4**	**4**	**4**	**4**	**4**	**4**
IMI	**8**	**32**	**8**	**16**	**8**	**16**
MXF	**4**	**8**	**2**	**8**	**4**	**8**
FOX	**32**	**64**	**32**	**32**	**16**	**32**
AMI	**2**	**4**	**2**	**4**	**4**	**8**
DOX	**16**	**16**	**16**	**16**	**16**	**16**
CLA on day 5	**0,25**	**1**	**0,5**	**1**	**0,12**	**0,5**
CLA on day 14	**16**	**16**	**16**	**16**	**0,25**	**0,5**

*SXT: trimethoprim/sulfamethoxazole, LZD: linezolid, CIP: ciprofloxacin, IMI: imipenem, MXF: moxifloxacin, FOX: cefoxitin, AMI: amikacin, DOX: doxycyclin

Figure 1: MABS subspecies and duration of infection in chronically infected patients