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Clinical Relevance, Diversity and Genetic Variability of Different Species within the Genus *Mycobacterium*

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Abstract

Introduction: The term non-tuberculous mycobacteria (NTM) includes different ambient species capable of sickening humans and/or animals, even by means of a potential zoonotic transmission.

Objectives: To determine: The clinical importance of several species within the genus *Mycobacterium* and the genetic diversity of the *M. avium* complex (MAC), the *in vitro* bacterial sensitivity and the success of the specific treatment.

Materials and Methods: Collection of clinical and epidemiologic data and information about isolates of the 2009-2016 period; molecular identification of the isolates; determination of the *in vitro* bacterial sensitivity and genetic diversity of the MAC; treatment evaluation.

Results: 225 mycobacteriosis cases were diagnosed, with a stable prevalence of ≈6% per year and 22 recovered species: 4 rapidly growing species isolated from 66 patients and 18 slowly growing species. The MAC was isolated in 95 cases, *M. avium hominissuis* - 40 cases, *M. intracellulare* - 51 cases, *M. chimaera* - 3 cases and *M. colombiense* - 1 case. We observed a greater probability of getting sick from *M. intracellulare* in patients previously treated for tuberculosis (TB). HIV-positive patients had a greater risk of falling ill from *M. avium hominissuis*. Aminoglycosides, fluoroquinolones and macrolides were the most active drugs against most NTM. Approximately half of the cases healed.

Conclusions: *M. intracellulare, M. aviumhominissuis* with great genetic variability and *M. abscessus* were the most commonly found pathogens. The cases of TB+NTM mixed disease were an important finding. For treating these patients, it was necessary to add second line drugs to the therapeutic regimen for TB; and most of them healed.

Key words: non-tuberculous mycobacteria, diversity and clinical importance

Introduction

The term non-tuberculous mycobacteria (NTM) includes several species capable of producing a broad spectrum of pathogens and playing a potential zoonotic role in animals and humans. Some species are strictly pathogenic, whereas other species may cause opportunistic infections, like the members of the *Mycobacterium avium* complex (MAC). However, during the last decades, there have been more cases of the disease caused by NTM, or mycobacteriosis, and more conditions causing immunosuppression, such as HIV infection, and malignant and autoimmune diseases, whose treatment generate the conditions necessary for an NTM infection to develop to a disease in infected individuals[1,2].

Rapidly growing mycobacteria (RGM) were defined as the species that need less than 7 days to produce colonies easily observed with the naked

eye in solid culture media[3]. This group includes approximately 56 ambient species. Some of them may be isolated from humans or animals. *M. fortuitum, M. abscessus, M. chelonae, M. smegmatis, M. immunogenum, M. peregrinum, M. houstonense*, *M. neworleansense* (*M. mageritense, M. septicum, M. mucogenicum* [previously *M. chelonae*-like organisms]), *M. wolinskyi* and *M. goodii* were isolated from patients[4-6]. *M. porcinum, M. farcinogenes* and *M. senegalense* are regarded as pathogens of veterinary importance, though *M. porcinum* was responsible for an outbreak in humans related to the supply of drinking water[7]. A few years ago, *M. abscessus* was subdivided into three genome species, and more recently, Totrtoli et al divided it into subspecies of the *M. abscessus* complex*: M. abscessus subspecies abscessus, M. abscessus subspecies masiliense* and *M. abscessus subspecies bolletii* [8-10]. For semantic simplicity reasons, some of the mentioned species are frequently grouped and referred to as groups or complexes: *M. abscessus*, including said species, and *M. fortuitum-chelonae,* including the species *M. chelonae, M. senegalense*, *M. smegmatis* and *M. fortuitum*[7, 10, 11].

On the other hand, slowly growing mycobacteria (SGM) are the species that need more than seven days to show visible growth in solid culture media[2,3]. The MAC includes: *M. avium* subspecies *selvaticum, M. avium* subspecies *hominissuis, M. avium* subspecies *avium, M. avium* subspecies *paratuberculosis, M. intracellulare* and the recently added species, *M. chimaera* and *M. colombiense*[12-14]. *M. avium* subspecies *hominissuis* and *M. intracellulare* are the most common and important human pathogens, whereas *M. paratuberculosis* (MAP) is a very important bovine pathogen that causes Paratuberculosis or *Johne* disease in animals. This mycobacterium has been suggested as a pathogenic agent of Chron's disease in humans[12,13].

Recently disseminated studies about the complete sequencing of a total of 120 genomes of strains considered as individual species showed that, in some cases, they could be either previously identified species or other species with a very close phylogenetic relationship with them. Thus, *M. nonchromogenicum* and *M. kumamotonense* have been included in the *M. terrae* complex; *M. chimaera* and *M. colombiense* have been included in the MAC; and *M. ulcerans* and *M. pseudozhottsii* have been included in the *M. marinum* complex[14,15].

Unlike what happens with the microbiological diagnosis of tuberculosis (TB), and given the natural habitat of NTM that includes the soil and the water, the microbiological diagnosis of mycobacteriosis must meet certain requirements established by the American Thoracic Society (ATS), stating if it is a new colonization or if the isolated mycobacterium is the true cause for the disease[15].

The objectives of this study were: To consider the frequency and clinical importance of NTM as causative agents; determine the proportion of cases caused by SGM and RGM; explore the appearance of more than one mycobacterium isolated either simultaneously or not simultaneously in the same host; observe differences in the sensitivity of different NTM species to antibiotics; evaluate the success of the specific treatment; and analyze the genetic diversity of the MAC, the main cause for mycobacteriosis, within the period between January 2009 and December 2016.

Materials and Methods

During the study period, we gathered the following epidemiologic, demographic and clinical patient information: age, gender, district of residence; disease localization according to the images obtained; HIV infection; comorbidities causing an immunosuppressed state; previous TB or mycobacterioris treatments; and amount of analyzed clinical materials with bacteriological results.

Respiratory clinical specimens, such as sputum, bronchial and bronchoalveolar lavages and endotracheal aspirate, and also extrapulmonary specimens, such as pleural, spinal and abdominal fluids, stools, urine, tissues, blood and bone marrow were collected.

All the materials, except for the blood and bone marrow that were incubated with the Myco-F-Lytic BACTEC 9050 system (BD Argentina) were processed for direct microscopic observation by Ziehl-Neelsen staining and cultured in solid media (Löwenstein-Jensen and Stonebrink) and in the automated BACTEC MGIT 960™ [16,17] system.

Antimicrobial sensitivity was determined *in vitro* by means of a colorimetric micro-method (resazurin micro assay, REMA) that uses resazurin as a vital dye and indicates bacterial growth or the absence of it[18]. Table 1 shows which drugs were included in the antibiogram.

The identification of the different found species was carried out through the GenoType CM™ system[19]. When identification results were incomplete, we sequenced the rRNA gene in order to correctly identify the isolates[20, 21].

The differentiation of the MAC members was made through the polymerase chain reaction of insertion sequence 1311 (PCR IS1311) (21). PCR IS*901* was used to specifically differentiate subspecies *M. avium* subsp. *avium* (MAA) from *M. avium* subsp. *Hominissuis*[22].

The genetic diversity of the MAC was explored by studying the polymorphism present at 8 loci with MIRU-VNTR structure (mycobacterial interspersed repetitive units-variable number of tandem repeats), through the technique previously described by Thibault et al. 2007 (23). The allelic diversity (D) of every locus and the global discriminatory power of the MIRU-VNTR complete scheme (HGDI, Hunter Gaston Discriminatory Index) were determined with the online software edited by the Universidad del País Vasco[23-25].

http://insilico.ehu.es/mini_tools/discriminatory_power/ through 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 of every type of scheme; s is the total number of different subtypes discriminated by the typing method, and xj is the number of isolates that belong to subtype *xth*.

The patterns that were found were compared with those recorded in the international database (http://mac-inmv.tours.inra.fr/) of MIRU-VNTR for MAC and were assigned certain pattern number, INMV[22, 26, 27].

Results

During the study period, the mycobacteriology laboratory of the Hospital Dr. Antonio A. Cetrángolo, as provincial model, diagnosed 4,236 cases of mycobacterial disease. 3,965 patients (93.9%) were confirmed as cases of TB: one caused by the

TABLE 1. Concentration intervals of in vitro tested antibiotics against the different NTM species

Antibiotics	Abbreviation	Concentrations (µg/mL)	Species
Ampicillin/	AMS	0.50-1.00	*M. abscessus* complex
sulbactam		64.00-128.00	
Amikacin	AG	0.50-32.00	*M. abscessus*, MAC,
Kanamycin	KM	0.50-32.00	*M. chelonae, M. fortuitum, M. kansasii*
Amoxicillin	AMC	1.00-128.00	*M. abscessus* complex
Potassium clavulanate		0.50-16.00	
Azithromycin	AZ	2.00-128.00	MAC, *M. fortuitum*
Cefoxitin	FOX	0.13-128.00	*M. abscessus* complex
Ceftriaxone	CFX	0.50-512.00	
Cycloserine	CS	3.5-120.0	MAC, *M. kansasii*
Clarithromycin	CLA	0.03-32.00	*M. abscessus*, MAC
Clofazimine	CFZ	0.03-4.00	MAC, *M. kansasii*
Ethambutol	EMB	1.00-32.00	*M. abscessus*, MAC, *M. chelonae, M. kansasii, M. fortuitum*
Levofloxacin	FQ	0.0015-16.00	*M. abscessus*, MAC,
Moxifloxacin		0.06-8.00	*M. chelonae, M. fortuitum, M. kansasii*
Linezolid	LZ	0.06-128.00	*M. abscessus*, MAC, *M. fortuitum, M. kansasii*
Imipenem	CAR	1.00-32.00	*M. abscessus*, MAC
Meropenem		0.06-64.00	
Rifampicin	RIF	0.25-8.00	*M. abscessus*, MAC,
Rifabutin	RBT	0.06-1.00	*M. chelonae, M. fortuitum, M. kansasii*
Tigecycline	TIG	0.0015-16.00	*M. abscessus*
Trimethoprim/		2.50-80.00	*M. abscessus, M. fortuitum*
sulfamethoxazole	TMX	0.13-4.00	

bacillus BCG (Bacillus Calmette-Guérin), three by *M. bovis* and the rest, *M. tuberculosis*. In 271 cases (6.8%), one NTM was isolated as a potential causative agent of the disease. A total of 46 cases (16.9%) were excluded from the study: one NTM was isolated only one time in 31 cases (10 isolates belonged to the *M. gordonae* complex) and was discarded as causative agent of the disease[27]. We could not obtain the adequate identification of the isolate in 6 cases, and patient information was incomplete in 9 cases. 66 of the remaining 225 cases (29.3%) had RGM, and 159 (70.7%) had SGM. Two different mycobacteria species were isolated in 15 cases (6.7%) during the same disease episode. Figure 1 and Table 2, respectively, show the frequency of appearance of mycobacteriosis caused by SGM and RGM during the study years, and the differentiation of species and complexes of the isolates that were found. Table 3 shows the characteristics of the patients included in the study. 71.1% of the cases presented pulmonary localization, 38.8% of them with bacilli in the direct examination (AARB + [acid-alcohol resistant bacilli]); 8 patients (3.6%) presented disseminated disease with bacilli isolated from serial blood cultures; 6 of those patients were coinfected with HIV. 78 patients (34.7%) showed history of previously treated TB. 151 cases (67.1%), showed, both individually or simultaneously: a) HIV infection (43 patients); b) a particular physical condition (old age, pregnancy); c) one diagnosed comorbidity (110 cases, for example, diabetes, bronchiectases with and without cystic fibrosis, a malignant disease).

Despite the gender of the patient, we observed a greater probability to develop mycobacteriosis by *M. intracellulare* in patients with previous treatment (odds ratio: 2.71487, 95% CI [confidence interval]: 1.087-6.798; X^2: 4.398, p: 0.0324), whereas the patients with HIV infection faced a greater risk to develop the disease by *M. avium* subsp. *hominissuis* (odds ratio: 7.111, 95% CI: 1.542-33.17; X^2: 7.930, p: 0.0125).

A total of 22 different species were recovered, 4 of which were RGM, and isolated from 66 patients (29.3%): 27 cases (40.9%) with the *M. abscessus* complex, where 3 isolates belonged to the subspecies *M. masiliense* and 2 belonged to *M. bolletii*; and 39 cases (59.1%) with the *M. fortuitum-chelonae* complex, where 31 (47.0%) belonged to *M. fortuitum*, 4 belonged to *M. chelonae* and 4 to *M. peregrinum*.

The SGM included the remaining 18 species. The MAC was isolated in 95 cases (42.2%), 40 of them with *M. avium* subsp. *hominissuis*, 51 with *M. intracellulare,* 3 with *M. Chimaera* and 1 with *M. colombiense.*

Table 2 shows the number and frequency of appearance of all the identified species, the cases

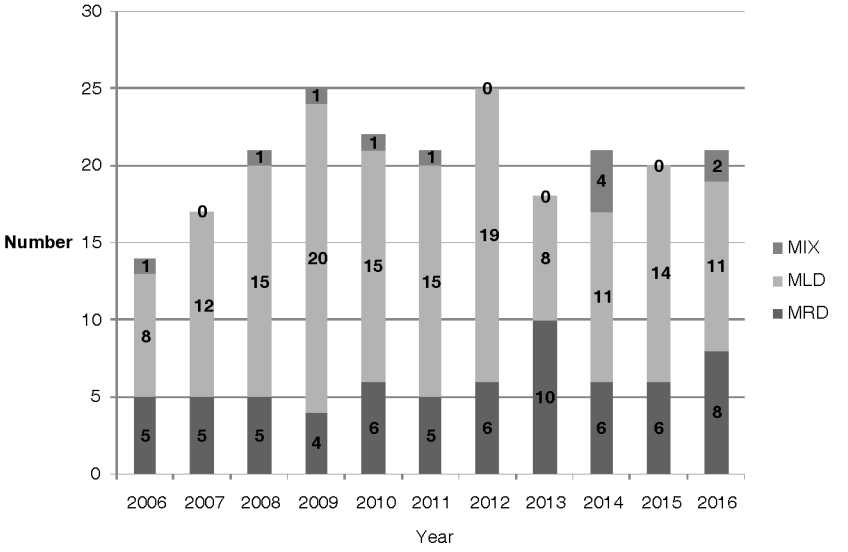

Figure 1. Number of patients with MB caused by SGM and RGM during the study years MIX: mixed infection by *M. tuberculosis* and *M. avium* (4 cases), *M. intracellulare* (2 cases), *M. fortuitum* (2 cases), *M. cheonae* and *M. scrofulaceum* (1 case each); RGM and SGB: mycobacteria of rapid and slow growth.

TABLE 2. Characteristics of patients included in the study

Characteristic		Males N (%) 126 (56.0%)	Females N (%) 99 (44.0%)	Total N (%) 225 (100)
Age (years), mean, (interval)[1] (95% CI)		44.58 (15-87) (39.79-49.38)	41.73 (1-81)[1] (36.56-46.89)	43.23 (1-87)[1] (39.76-46.69)
HIV	P	28 (22.2)	15 (15.2)	43 (19.1)
	N	39 (31.0)	38 (38.4)	77 (34.2)
	NI	59 (46.8)	46 (46.4)	105 (46.7)
T	Yes	49 (38.9)	29 (29.3)	78 (34.7)
	No	64 (50.8)	60 (60.6)	124 (55.1)
	NS	13 (10.3)	10 (10.1)	23 (10.2)
LOC	PUL	92 (73.0)	68 (68.7)	160 (71.1)
	EXP	25 (19.8)	28 (28.3)	53 (23.6)
	PUL+EXP	4 (3.2)	0	4 (1.7)
	DISS	5 (4.0)	3 (3.0)	8 (3.6)
Condition or comorbidity	≥65 years	9 (7.1)	10 (10.1)	21 (9.3)
	Malignant disease	6 (4.8)	10 (10.1)	16 (7.2)
	Diabetes	25 (19.8)	15 (15.2)	40 (17.8)
	Pregnancy	-	3 (3.0)	3 (1.3)
	Bronchiectases	10 (7.9)	6 (6.0)	12 (5.3)
	Cystic fibrosis	6 (4.8)	8 (8.1)	18 (8.0)
	No/ND	70 (55.5)	45 (45.5)	115 (51.1)
AARB	PUL-P	40 (31.7)	22 (22.2)	62 (27.6)
	PUL-N	36 (28.6)	47 (47.5)	83 (36.9)
	EXP-P	2 (15.9)	2 (2.0)	4 (1.8)
	EXP-N	21 (16.7)	11 (11.1)	32 (14.2)
	EXP-ND	18 (14.3)	11 (11.1)	29 (12.9)
	Isolates	9 (7.1)	6 (6.1)	15 (6.6)

P: positive; N: negative; NS: not specified; LOC: disease localization; AARB: result of direct examination of the material; PUL: pulmonary; EXP: extrapulmonary; PUL+EXP: pulmonary plus extrapulmonary; DISS: disseminated; ND: not determined; T: history of previous treatment for tuberculosis or mycobacteriosis.

attributed to mixed infection and the localization of the disease that produced the isolates of the different species.

11 of a total of 27 *M. abscessus* cases (40.7%) were related to pulmonary disease, whereas the remaining 16 were obtained from skin lesions and/or injuries caused by surgical or cosmetic procedures.

The *M. avium* subsp. *hominissuis* strains presented pulmonary, extrapulmonary and disseminated localization. *M. intracellulare* did not cause a disseminated disease in the study cases, but cases of disseminated disease were proven with *M. kansasii*. The species *M. flavescens, M. kubicae, M. lentiflavum, M. haemophilum, M. sherrisi*, *M. branderi, M. celatum, M. rhodesiae* and *M. xenopi* were capable of producing pulmonary, extrapulmonary and disseminated disease according to the origin of the samples from which the bacteria were isolated and the clinical data of the patients.

A total of 15 cases presented mixed infection. In 11 patients, *M. tuberculosis* associated with other species was isolated. In 4 HIV-positive cases, the second germ that was found was *M. avium* subsp. *hominissuis*; in 2 HIV-positive cases, the second germ was *M. intracellulare* and in HIV-negative patients we found the following associations: 1 case with *M. abscessus*, 2 cases with *M. fortuitum*, 1 case with *M. chelonae* and 1 case with *M. kubicae*. The last case was an old patient at a nursing home.

In other 4 cases, microbiological findings were produced generally during the treatment for the first isolated germ. These 4 cases were:

a) Old patient: in 2010, *M. fortuitum* was isolated and in 2011, before ending the corresponding treatment, *M. intracellulare* was isolated.
b) HIV-positive patient: *M. avium* subsp. *hominissuis* was isolated and during the specific treatment in the same year, *M. intracellulare* was isolated.
c) HIV-positive patient: *M. chelonae* and *M. avium* subsp. *hominissuis* were simultaneously isolated in the same disease episode.
d) HIV-positive patient: *M. intracellulare* was isolated from a sputum sample in 2008. In 2009, during the treatment for mycobacteriosis, *M. scrofulaceum* was isolated from a blood culture sample.

TABLE 3. Mycobacterial species isolated from different disease sites

Growth rate	Complex	Species (N°)	Origin of isolate	Localization
		M. abscessus subsp. *boletti* (n: 2)	TBX	EXP
	M. abscessus	M. *abscessus* subsp.	TBX	EXP
RGM	(n:27)	*masiliense* (n: 3)		
(N: 66)		*M. abscessus* (n: 22)	S, TBX, SBX	P, EXP
	M. fortuitum-chelonae	*M. chelonae* (n: 5)	TBX, S	P, EXP
	(n: 39)	*M. fortuitum* (n: 29)	S, AL, TBX	P, EXP
		M. peregrinum (n: 5)	S	P
		M. avium (n: 4)	S, U, LNBX	P, EXP
		M. fortuitum (n: 2)	S, TBX	P, EXP
Mixed TB	***M. tuberculosis***	*M. intracellulare* (n: 2)	S	P
(N: 11)		*M. scrofulaceum* (n: 1)	TBX	EXP
		M. chelonae (n: 1)	S	P
		M. abscessus (n: 1)	S	P
	M. avium	*M. intracellulare* (n: 1)	S	P
		M. chelonae (n: 1)	ST, S	EXP, P
Mixed NTM	***M. intracellulare***	*M. fortuitum*+ (n: 1)	S	P
(N: 4)	MAC (n: 95)	*M. scrofulaceum* (n: 1)	BC	P, DISS
		M. avium (n: 40)	ST, S, BAL, BC, LNBX, IBX	P
		M. chimaera (n: 3)	S	P
		M. colombiense (n: 1)	S	P, DISS
		M. intracellulare (n: 51)	S, U, BC	P, EXP
SGM	***M. terrae***	*M. nonchromogenicum* (n: 1)	S	P
(N: 144)	(n: 6)	*M. kumamotonense* (n: 2)	BAL	P
		M. terrae (n: 3)	S	P
		M. kansasii (n:17)	S, BC	P, DISS
		M. scrofulaceum (n: 8)	S, TBX	P
		M. flavescens (n: 5)	S	P
Other		*M. kubicae* (n: 2)	BAL, BC	P, DISS
(N: 43)		*M. lentiflavum* (n: 3)	BC, CERBX	P, EXP
		M. haemophilum (n: 2)	BC	DISS
		M. sherrisi (n: 2)	S, LNBX	P, EXP
		M. branderi; M. celatum; M. rhodesiae; M. xenopi (n: 1)	S, BC	P, DISS

BAL: bronchoalveolar lavage; S: sputum; BC: blood culture; ST: stools; U: urine; BX: biopsies; LNBX: lymph node biopsy; IBX: intestine biopsy; PLBX: pleural biopsy; TBX: tissue biopsy; SBX: skin biopsy; P: pulmonary; EXP: extrapulmonary; DISS: disseminated.

In these 15 cases, mixed infection was associated with the history of previous and/or current treatment in the male group (odds ratio: 1.5933 (95% CI: 0.6096-3.8217).

Table 4 shows the number and percentage of isolates of every species sensitive to each one of the drugs detailed in Table 1. EMB and RIF were the only first line drugs used in TB treatment to be included in the NTM antibiograms. RIF was tested in a total of 165 isolates (75.7%). 109 cases (66.0%) were sensitive to the drug. The EMB showed 84.8% global inhibitory activity (140/165 isolates), especially toward *M. kansasii* and some strains of MAC and *M. chelonae*. *M. abscesus*, MAC and *M. kansasii* were mostly susceptible to CLA, and *M. foruitum* and MAC to AZ. The CFZ showed activity in over one third of the MAC strains, whereas *M. kansasii* proved to be very sensitive to this antibiotic. The FQs (levofloxacin and moxifloxacin) under evaluation inhibited more than 70% of the species. From the beta-lactamic antibiotics, AMS, AMC and FOX, only FOX was active against the *M. abscessus* complex. Two thirds of these strains were inhibited by AG, FOX, CLA, FQ, RIF and TMS, whereas a lower percentage (around 60%) were also inhibited by EMB and TG. Most of the MAC strains were inhibited by AG, AZ, CLA, EMB, FQ, RIF and RBT. The activity of LZ was higher on *M. intracellulare* isolates than on *M. avium* isolates.

80% of the MAH (n: 32) were properly genotyped, exhibiting great genetic diversity. With the MIRU-VNTR technique, we found 5 known patterns and other 6 patterns recently published by our group, not previously reported in the literature[28]. X3 and 292 loci showed the highest discrimination index (D) among the MAH isolates (0.7424 and 0.6515), obtaining a high HGDI (0.9697).

TABLE 4. Number and percentage of NTM inhibited by antimicrobials

Drug	Total number and percentage (%) of sensitive isolates according to the species					
	Mab (n: 27)	MAH (n:37)	Mint (n: 51)	Mche (n: 5)	Mfor (n: 28)	Mkan (n: 17)
AG	27 (100)	29 (78.4)	31 (60.8)	3/5	15 (53.6)	17/17
AZ	ND	34 (92.0)	50 (98.0)	ND	23 (82.1)	ND
AMS/C	2 (7.4)	ND	ND	ND	ND	ND
CAR	11 (36.7)	38 (43.2)		ND	ND	ND
FOX	15 (56.0)	16 (43.2)	ND	ND	ND	ND
CS	ND	15 (40.5)	31 (60.8)	3/5	ND	13/17
CLA	24 (80.0)	33 (89.2)	46 (90.2)	ND	ND	ND
CFZ	ND	50 (35.1)		0	ND	15/17
EMB	17 (62.9)	26 (70.3)	36 (71.4)	4/5	6 (21.4)	15/17
ETH	ND	18 (48.6)	13 (25.0)	ND	ND	4/17
FQ	21 (70.0)	32 (86.5)	34 (66.6)	3/5	25 (89.3)	16/17
LZ	11 (40.7)	21 (56.7)	47 (92.1)	ND	11 (39.3)	11/17
RBT	10 (37.0)	27 (72.9)	42 (82.4)	4/5	6 (21.4)	16/17
RIF	19 (70.4)	26 (70.3)	39 (76.5)	1/5	10 (35.7)	14/17
TG	17 (62.9)	ND	ND	ND	ND	ND
TMS	21 (77.8)	ND	ND	ND	57 (92.8)	ND

Mab: Mycobacterium abscessus; MAH: M. avium hominissuis; Mint: M. intracellulare; Mfor: M. fortuitum; Mkan: M. kansasii. AG: aminoglycosides (kanamycin, amikacin); AZ: azithromycin; AMS/C: ampicillin-sulbactam/amoxicillin-potassium clavulanate; CAR: carbapenems (imipenem, meropenem); CS: cycloserine; CLA: clarithromycin; CFZ: clofazimine; EMB: ethambutol; ETH: ethionamide; FOX: cefoxitin; FQ: fluoroquinolones (levofloxacin, moxifloxacin); LZ: linezolid; RBT: rifabutin; RIF: rifampicin; TG: tigecycline; TMS: trimethoprim-sulfamethoxazole; ND: not determined.

Figures 2 and 3 show the genetic patterns (INMV) of the MAH that were found and the existent phylogenetic relationship among them. Relationships among the different INMV patterns are shown in a dendrogram in Figure 2. Figures 3 and 4 show clonal relationships among the different patterns, represented by the minimum spanning tree (MST) created from the goeBURST algorithm. It is generally assumed that the genetic distance between two INMV patterns is proportional to the difference in the number of repetitions of each locus. These relationships were established using the *Phyloviz 2* software (http://goeburst.phyloviz.net)[26].

Regarding the success of the specific treatment, we evaluated a total of 109 (63.7%) out of the 171 patients (76.0%) who began the treatment in the 2009-2014 period. In 71 cases (65.1%), the cohort showed "ended treatment" or "healed" (ET/H). Therapy suspension and deaths represented 12.8%, with 8 and 6 patients, respectively. There were 6 relapses (5.5%) and 16 cases (14.7%) were labeled as "continuing treatment" at the moment of the evaluation. No information was obtained about the remaining 62 patients (36.3%).

Of the 54 patients (24.0%) who initiated the treatment in 2015 and 2016, it was possible to evaluate treatment success in 41 patients (76.0%). 3 of them died (7.3%), 4 (9.8%) were registered as ET/H and 34 (83.0%) were still under treatment during the data collection phase. Of the 11 cases with mixed infection, 7 were registered as ET/H. The treatment global success was estimated at 50.0% according to the relationship between the number of cases evaluated in the 2009-2015 period (n: 150) and the ET/H cases (n: 75).

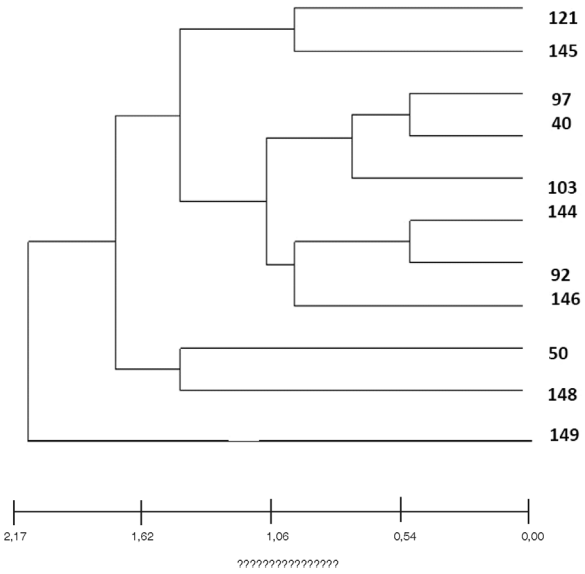

Figure 2. Dendrogram with the genetic *M. avium hominissuis* patterns found
Numbers 121 to 149 belong to the different genetic patterns (INMV) according to the MAC-INMV database. The dendrogram shows the distance between the different patterns that were found, where 2.17 is the value for the most genetically remote isolates.

Conclusions

NTM are ubiquitous, naturally free-living organisms, considered as opportunistic infectious agents, especially in hosts with cellular immunity disorders, where they produce a disease similar to TB, generically called mycobacteriosis[28].

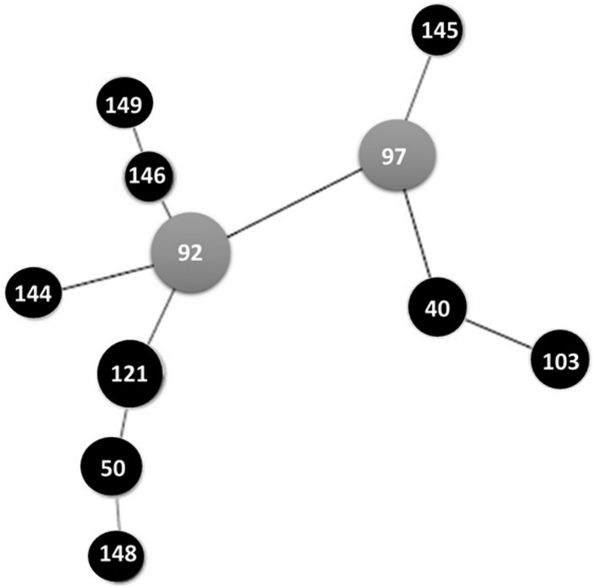

Figure 3. Minimum spanning tree with the genetic *M. avium hominissuis* patterns found in the study
The minimum spanning tree represents the clonal relationship among the different genetic patterns (INMV). The numbers represent the different INMV patterns found for MAH. The grey circles are the clones that gave origin to the rest of the MAH patterns. The circle size is related to the number of isolates of every pattern.

The incidence of NTM was about 6.0% taking into account all the cases initially considered as TB, with a kind of stable tendency during the period under evaluation. These results, in comparison with the ones previously published show a stable tendency in the appearance of MB in our environment[20].

In this study, patients mostly presented lesions with a respiratory localization, though the disease can also be extrapulmonary, mixed or disseminated; over one third of patients had previously received treatment for TB and almost half of the patients presented comorbidities as base disease. This information agrees with previously published data[29-31]. However, previous TB was the most important risk factor for mycobacteriosis, especially for the cases caused by *M. intracellulare* which, together with *M. avium* subsp. *hominissuis* were the most commonly found pathogens.

In total, 22 different species of NTM were recovered, where SGM predominate.

RGM, mainly the members of the *M. abscessus* complex, are considered emerging opportunistic pathogens that may cause chronic pulmonary infections and diseases of the skin, soft tissues, bones and joints. There are numerous publications where

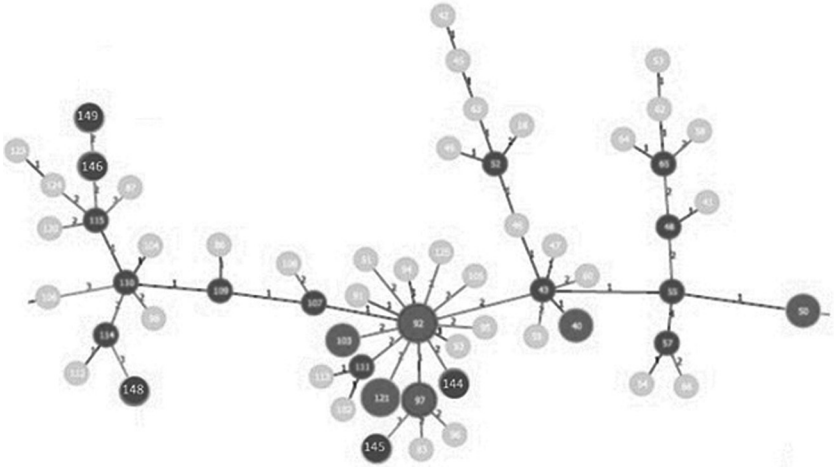

Figure 4. *Minimum spanning tree* with the *M. avium hominissuis* patterns found and the ones reported in the MAC-INMV database.
The numbers represent the different INMV patterns. Dark grey circles represent the INMV found in the study, whereas the light grey circles are the MAH patterns reported in the database which were not found in the isolates under evaluation. The circle size is related to the number of isolates of every pattern. In this study, INMV 92 is shown as the main clone that gave origin to the rest of the isolates. The number between each line joining the different clones represents the genetic distance between two patterns, proportional to the difference in the number of repetitions of each locus.

these RGM are involved in infections associated with traumatic injuries, surgeries and various procedures: acupuncture, liposuction, mesotherapy, and breast implant surgery [29-31].

In this study, the number of cases with pulmonary localization of the disease caused by the *M. abscessus* complex isolated from tissue and sputum samples was similar to the number of cases with extrapulmonary localization. *M. nonchromogenicum* and *M. peregrinum* were also found, but with low frequency and with pulmonary localization exclusively.

The species of the *M. abscessus* and *M. fortuitum-M. chelonae* complexes were the most frequently isolated among the RGM. The species of the *M. fortuitum-M. chelonae* complex were mostly related to pleuropulmonary diseases.

Within the MAC, the most commonly found pathogens were *M. intracellulare* and *M. avium* subsp. *hominissuis,* though *M. chimaera* and *M. colombiense* were also isolated. The members of the MAC, together with *M. kubicae* and *M. haemophilum* caused the disseminated disease. *M. avium* subsp. *hominissuis* presented great genetic variability, suggesting a diversity of infection sources. Also, previous TB was the most important risk factor, especially for the cases where the disease had been caused by *M. intracellulare.*

Other species of SGM such as *M. kumamotonense* and *M. rhodesiae* were also found, though in small proportion.

Some NTM, such as the members of the MAC, are able to infect not only birds but also mammals, for example, pigs. In a study recently published in Belgium, more than 98% of pig isolates belonged to *M. avium* subsp. *hominissuis*. This finding could suggest the zoonotic transmission of these mycobacteria[25]. This makes us think about the need to plan genetic and epidemiologic studies to corroborate or discard potential zoonosis in mycobacteria and also to evaluate possible direct transmission between hosts.

Although the results of NTM antibiograms are informative and must be carefully evaluated by attending physicians, the results of this study showed concordance with the information previously published by several authors[31-33]. In general terms, of all the drugs under evaluation, aminoglycosides, fluoroquinolones and macrolides were the most active against a greatest variety of NMT, but there wasn´t any species homogenously sensitive to said drugs. The other drugs showed a more evident species-dependent relationship. In this regard, the identification of the germ at the species level is an important predictor of its sensitivity to drugs and a guide to aid the physician in the implementation of a suitable therapy. A review has been recently published about new antimicrobials active against NTM at several growth phases. In this study, the evaluation of cohorts showed that around half of the patients with mycobacteriosis healed, though an important loss of information was registered and the cohorts were incomplete.

A remarkable fact was to find that both *M. tuberculosis* and NTM were causing the disease. The simultaneous isolation of *M. tuberculosis* together with NTM was considered as cause of joint disease. The patients were treated with regularized regimens for TB using, in the early phase, isoniazid, rifampicin, etambutol and pyrazimide, to which *M. tuberculosis* isolates were originally sensitive. They continued with conventional treatment until detection of NTM and/or treatment failure (positive smear tests and cultures) resulted in the customization of therapeutic regimens, by adding aminoglycosides, fluoroquinolones and any other drug depending on the identified NTM species (Tártara, personal communication), the recommended regimens and the *in vitro* sensitivity results obtained from the isolates[33]. Most of these patients healed.

Regarding the variability of the *M. avium* subsp. *hominissuis* isolates obtained, the molecular technique we used allowed us to show the existence of great genetic diversity among the isolates. Also, the INMV patterns obtained in this study could be compared and analyzed with the ones reported by other authors in the international database (MAC-INMV)[24]. The creation of the MST allowed us to establish clonal relationships among the different isolates. INMV 92 turned out to be a native clone, both when the MST included only the study isolates and also when it included the isolates globally reported in the database. This confirms its role as a clone from which other *M. avium* subsp. *hominissuis* isolates derive.

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BRI, MPS and MIR are researchers at CONICET; DM is a postdoctoral fellow at CONICET.

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