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Mycobacterium malmoense in Italy: The modern Norman invasion?

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Abstract. The isolation of *Mycobacterium malmoense* has for a long time been restricted to few countries of Northern Europe; reports from countries other than Sweden, Great Britain and Finland are rare and the first Italian case report has been published in 1995. Since 1988, however, fifteen strains of *M. malmoense* have been isolated in Italy, eleven of which in the last two years; of these, ten appeared clinically significant on the basis of medical records. The susceptibility of the strains and the role of high performance liquid chromatography of cell wall mycolic acids for a reliable identification are discussed.

Key words: Antimicrobial susceptibility, Mycobacteriosis, *Mycobacterium malmoense*

Abbreviations: GI = growth index; HPLC = High performance liquid chromatography; MAC = *Mycobacterium avium* complex

Introduction

Mycobacterium malmoense, first described in 1977 [1], is a slowly growing nonchromogenic mycobacterium, which may cause pulmonary infections in adults and lymphadenitis in children [2]. Single cases and extensive reviews have been reported mainly in Sweden [1, 3, 4], Great Britain [5–7] and Finland [8]. In other countries *M. malmoense* isolations are on the contrary rare [9–13]; in Southern Europe in particular they have been quite exceptional. In Italy the first report of a *M. malmoense* infection was published in 1995 [14], and it concerned a case observed in 1991; to our knowledge only another strain of *M. malmoense* had been isolated before in this country. In the last 25 months however we identified as *M. malmoense* a number of strains, mostly received for speciation from other centers. We review here these Italian isolates of *M. malmoense*, placing emphasis on the microbiological procedures for their identification and susceptibility testing.

Material and methods

The isolation of the strains was achieved on various media in different laboratories. Lowenstein-Jensen, which was inoculated in all centers, was used alone in two labs; in all the remaining but one (which employed Middlebrook 7H11) it was flanked by radiometric broths (Bactec, Becton Dickinson, USA). In one instance the liquid medium MGIT (Becton Dickinson) was also used. Acidified media and incubation temperatures below 36 °C, which have been proven to enhance the recovery of *M. malmoense* [15, 16] were never implemented.

While the first three strains were sent from isolating laboratories to foreign reference centers (the Pasteur Institute of Paris in two cases, and the Mycobacterium Reference Unit of Cardiff in the third) the identification of all other isolates was performed in Florence using both high performance liquid chromatography (HPLC) of cell wall mycolic acids and conventional tests.

Biochemical and cultural tests used for conventional identification are reported in Table 1 and were performed according to standard procedures [17].

HPLC analysis was carried out on mycolic acids after saponification, extraction, and derivatization, as reported previously [18]. Standardized HPLC conditions were followed for the elution of various fractions of mycolic acid blends. The HPLC profiles were compared with the ones of the reference strain ATCC 29751 and with those of the three strains

Table 1. Results of conventional biochemical, cultural and inhibition tests on 15 isolates of *Mycobacterium malmoense*

Test	Result	(% of positive)
Niacin	–	(0)
Nitrate reduction	–	(0)
Thermostable catalase	+	(87)
β-glucosidase	–	(0)
Tween 80 hydrolysis (10 days)	+	(100)
Tellurite reduction	–	(7)
Arylsulfatase (3 days)	–	(0)
Urease	+	(100)
Catalase (over 45 mm of foam)	–	(7)
Photochromogenicity	–	(0)
Scotochromogenicity	–	(0)
Growth at 25 °C	+	(100)
Growth at 37 °C	v	(47)
Growth at 45 °C	–	(0)
MacConkey	–	(0)
Tolerance to:		
p-nitrobenzoate (500 μg/ml)	+	(100)
NaCl (5%)	–	(0)
Thiophene-2-carboxylic hydrazide (5 μg/ml)	+	(100)
Thiacetazone (10 μg/ml)	+	(100)
Hydroxylamine (500 μg/ml)	+	(80)
Isoniazid (1 μg/ml)	+	(100)
Oleate (250 μg/ml)	–	(20)
Growth rate	> 30 days	(100)
Colonial morphology	Smooth	(100)

whose identification had been validated by reliable foreign reference centers.

Susceptibility testing was performed in radiometric liquid medium using the method developed for *Mycobacterium avium* complex (MAC) [19]. In short, a pre-inoculum broth culture was prepared by seeding a Bactec 12B vial with 100 μl of a No. 1 McFarland mycobacterial suspension; it was then incubated and read daily with a Bactec 460TB instrument until the achievement of a growth index (GI) ⩾ 999. An 1:100 dilution in Diluting fluid (Becton Dickinson) of such broth culture was used for the inoculum of radiometric vials added with twofold dilutions of the drugs to be tested. According to the standard procedure two drug-free control vials were inoculated with 100 μl of the above mentioned diluted culture and with the same volume of its further 1:100 dilution. The internal control conditions proposed for testing strains of MAC were applied without changes except for the time of final reading which was considered valid if achieved within 10 days (instead of 8 days of original technique). The alteration was adopted because of the slightly slower growth rate of *M. malmoense*.

Clinical and microbiological reports of all patients who grew in culture *M. malmoense* were carefully reviewed to ascertain the clinical significance of the finding according to the criteria proposed by the American Thoracic Society [20].

Table 2. Minimal inhibitory concentrations (MICs) of seven drugs against 15 isolates of *Mycobacterium malmoense*

Drug	MIC_{50}	MIC_{90}	Range
Amikacin	4	8	⩽ 2–8
Ciprofloxacin	4	4	⩽ 1–4
Clarithromycin	⩽ 2	⩽ 2	⩽ 2
Ethambutol	4	8	⩽ 2–8
Rifabutin	⩽ 0.5	⩽ 0.5	⩽ 0.5
Rifampin	2	8	⩽ 0.5–8
Streptomycin	4	8	⩽ 2–> 8

Results

Clinical and microbiological features are reported in Table 3. The finding appeared clinically significant for ten patients out of 15. Of these, six were adults, mean age 55, and five of them presented pulmonary infections; four were children with lymphonodal localization of *M. malmoense* infection.

The 15 isolates were obtained in eight different cities, five of them contributing one isolate each. Excluding a center in which three strains were isolated within a one month period (only one of them being clinically significant), for all other cases spatial and temporal relation are excluded.

Only in five cases a clinical significance could not be proven; in one instance (case 1) the responsibility

Table 3. Clinical and microbiological data on patients with *Mycobacterium malmoense*

Case (sex, age)	Disease	Treatment	Outcome	Specimen	Microscopy[a]	Histology[a]	Isolations	Media[b]	Clinical significance	Isolation site	Year
1[c] (M, 68)	cavitary tuberculosis	antituberculosis regimen	relapse	sputum	POS	n.d.	single	LJ	NO	Florence	1988
2 (F, 5)	cervical lymphadenopathy	surgical resection	recovery	lymph node	NEG	typical	single	Bactec 12B LJ	YES	Ancona	1991
3 (M, 32)	pneumonia	antituberculosis regimen	recovery	sputum	NEG	n.d.	single	Bactec 12B	NO	Bergamo	1992
4 (M, 6)	cervical lymphadenopathy	surgical resection	recovery	lymph node	NEG	typical	single	7H11 LJ	YES	Pisa	1993
5 (M, 57)	tenosynovitis	surgical resection	relapse	pus	NEG	typical	repeated	Bactec 12B	YES	Bergamo	1994
6 (F, 51)	none	none	unchanged	urine	NEG	n.d.	single	Bactec 12B	NO	Bergamo	1994
7 (M, 65)	pleurisy	non-antituberculosis drugs	recovery	pleural fluid	NEG	n.d.	single	Bactec 12B	NO	Bergamo	1994
8 (M, 64)	pulmonary cavities	antituberculosis regimen	chronicization	sputum	POS	n.d.	repeated	LJ	YES	Castelnuovo Garfagnana	1994
9 (M, 30)	AIDS; pulmonary infiltrate	none	death	blood	n.d.	n.d.	single	Bactec 13A	YES	Brescia	1994
10 (M, 6)	cervical lymphadenopathy	surgical resection	recovery	lymph node	NEG	typical	repeated	Bactec 12B LJ	YES	Pordenone	1995
11 (M, 74)	pulmonary cavities	antituberculosis regimen	improvement	sputum	POS	n.d.	repeated	Bactec 12B LJ, MGIT	YES	Florence	1995
12 (M, 9)	cervical lymphadenopathy	surgical resection	recovery	lymph node	NEG	typical	single	7H11	YES	Pisa	1995
13 (F, 75)	pulmonary cavities	antituberculosis regimen	improvement	sputum	NEG	n.d.	single	Bactec 12B	YES	Bergamo	1995
14 (F, 81)	chronic bronchiectasia	none	unchanged	sputum	POS	n.d.	repeated	Bactec 12B LJ	Uncertain	Bergamo	1995
15 (M, 33)	AIDS: pulmonary interstitial infiltrate	antituberculosis regimen	improvement	sputum	POS	n.d.	repeated	Bactec 12B	YES	Belluno	1996

[a] NEG = negative; POS = positive; n.d. = not done.
[b] LJ = Lowenstein-Jensen.
[c] *M. tuberculosis* was isolated from other specimens of this patient.

of pulmonary cavitations was attributed to the concomitant presence of *Mycobacterium tuberculosis*, while in another (case 7), despite the isolation from a 'sterile site' (pleural fluid), the recovery following a non-antituberculosis antibiotic treatment seems to suggest an etiology due to a different microbial agent and the contamination of the sample seems likely.

At least in one of the instances in which clinical significance was not acknowledged (case 14), some doubt exist because of the disagreement between microbiological (three positive smears and cultures) and clinical data (lack of suggestive chest abnormalities in an elderly bronchiectasis patient); no therapeutic regimen was undertaken and the patient remained stationary. On the contrary a treatment was given to patient 3, pending the identification of the isolate; the success of the therapy has not been sufficient to establish the clinical significance of the isolate, owing to the stringent criteria adopted.

M. malmoense was isolated twice from heavily immunocompromised AIDS patients (cases 9 and 15) suffering from cavitary pulmonary disease; the promptly undertaken antimycobacterial regimen resulted in improvement in one of them (case 15) while in the other (case 9), characterized also by disseminated *M. malmoense* infection, only a post mortem diagnosis was made because of the immediate death of the patient.

All children with cervical lymphadenopathy were surgically cured with complete recovery. Histology investigated in all such cases (patients 2, 4, 10 and 12) revealed the typical picture of this disease with granulomatous infiltration and caseating necrosis; acid fast bacilli were not seen.

Antimycobacterial regimen brought about amelioration in all patients with pulmonary lesions attributed to *M. malmoense* with evident improvement of general conditions and sterilization of cultures in two of them.

M. malmoense grew on Lowenstein Jensen in seven cases out of 15 (47%) and on Bactec 12B in all the ten instances in which this medium was inoculated. Rarely used media (Middlebrook 7H11, Bactec 13A and MGIT) were positive whenever were used, twice the first one and once each of the others.

Growth on solid media became evident only after six weeks or more while broth cultures scored positive at least two weeks early. The extent of primary growth on solid media was not regularly recorded, the presence of almost confluent colonies was however the prevailing occurrence.

The identification performed using conventional tests revealed a very homogeneous pattern for all the isolates (Table 1) which were all characterized by the scanty growth at 36 °C, the positivity of Tween 80 hydrolysis and urease, and the negativity of nitrate reductase.

The reference strain and all clinical isolates presented an identical mycolic acid pattern in HPLC characterized by 13 major peaks arranged in an uninterrupted late sequence (Figure 1); this is a unique pattern, clearly different from that of any other previously studied mycobacterial species.

The procedure used for susceptibility testing appeared quite suitable for the purpose, as it fulfilled all the requirements established in order to avoid errors due to excess or defect of inoculum.

The antimicrobial pattern of our *M. malmoense* strains is characterized by susceptibility to rifabutin and clarithromycin and by variable behavior of all other drugs; the less active molecules appeared to be streptomycin, ethambutol and ciprofloxacin (Table 2).

Discussion

In countries other than Sweden, Finland and Great Britain the isolation of *M. malmoense* is not frequent and very few cases have been reported in areas of Southern Europe [9, 14, 21–23]. Since Italy has been one of the last countries to report its first isolation of *M. malmoense*, the number of isolations obtained recently was unexpected. Only time will tell whether

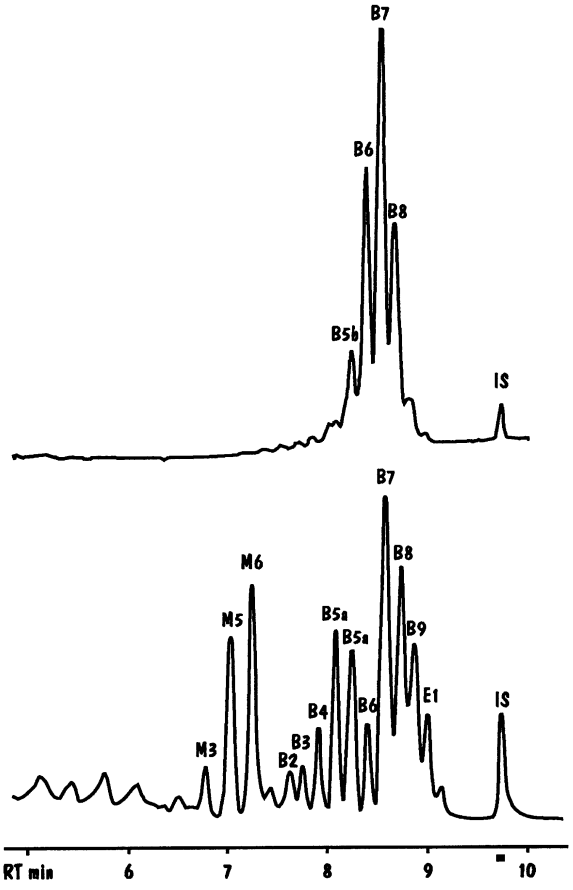

Figure 1. HPLC profile of *M. malmoense* compared with the one of *M. tuberculosis*; peaks labelled according to CDC convention (RT = retention time, IS = internal standard).

this is due to chance or it is the beginning of a rising trend.

The strains reported here, six of which were isolated in liquid medium and failed to grow in the parallel cultures on Lowenstein Jensen, confirm the well known cultural difficulties of *M. malmoense* which grows best at 30 °C on acid medium [15, 16] and presents its optimal cultural results in broth. A firm conclusion on the ability of liquid nonradiometric media to enhance the growth of *M. malmoense* cannot be reached here; the prompt (2–4 weeks) growth on MGIT of several isolates from different samples of the same patient, achieved during an occasional trial of this novel medium, although promising, is in fact not probative.

The fastidious nature of *M. malmoense* suggests that the organism has a wider diffusion than estimated from the reported isolations; this is particularly true in countries like Italy where there is little awareness of this organism, and therefore no effort to use acid media and lower incubation temperatures. If this is correct, the trend towards an increased use of liquid media should contribute to raise the frequency of isolation of *M. malmoense*. The unusual recovery rate achieved in Italy in the last months cannot however be attributed to technical improvements, as none of the isolating centers had concomitantly modified its cultural procedures.

Opportunistic organisms are often isolated from clinical specimens in absence of disease, clinical relevance of our *M. malmoense* isolations is however extraordinarily high (67%), much more than the rate of most frequently encountered not tuberculous mycobacteria. A similar high percentage has however already been reported [8]; on the other hand, *M. malmoense* appears to have a very low prevalence in the environment, from which it has been isolated on very rare occasions [24, 25]; this suggests that this species has a higher virulence for man than most other nontuberculous mycobacteria, which are encountered more frequently, but have a lower chance to infect and cause disease. In this connection, the small proportion of cases with underlying immunodeficiency [26–30] (two of 15 our cases, five others in literature) demonstrate the ability of *M. malmoense* to overcome the normal host defenses.

The identification of *M. malmoense* using conventional tests is often problematic and reference centers always support it with other tests, mainly thin layer chromatography of cell wall mycolates [31]. The uniqueness of HPLC profile [32] allows unambiguous identification of *M. malmoense* without resorting to other tests.

Little is known about the antimicrobial susceptibility of *M. malmoense*. The full susceptibility of our strains only to clarithromycin and rifabutin somehow confirms the high level of resistance put in evidence by the only exhaustive susceptibility study available in literature [33]. Although performed with different procedures, both studies were carried out in liquid medium, which is known to provide the most reliable results [34].

The isolation of two strains from extremely immunocompromised AIDS patients emphasizes the preferential connection between disseminated mycobacterioses and HIV infection. In this connection the predominant role of *M. avium* complex and *M. tuberculosis* should not induce to underestimate the relevance of the steadily increasing group of other involved species.

Despite the slightly sensationalist title, whose aim is only to arouse reader's curiosity, no importation of *M. malmoense* from abroad is hypothesized here. At present no documentation exists concerning such mycobacterial flow and other hypotheses, first of all ecological modifications, appear more likely.

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