INTERNATIONAL JOURNAL OF LEPROSY

Volume 48, Number 1
Printed in the U.S.A.

In vitro Cultivation and Characterization of *Mycobacterium lepraemurium*[1]

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Ogawa and Motomura ([11,12]) described the *in vitro* development of scanty, very slowly growing colonies of *Mycobacterium lepraemurium* (*M. lm*) around fragments of liver from mice infected with this organism and planted on the surface of 1% Ogawa egg yolk medium. This result was confirmed by Mori ([9]), who also obtained subcultures after incubation times of 45 to 120 days following massive inoculation and investigated some growth factors ([8]).

Saito, *et al.* ([13]) have described the *in vitro* characteristics of *M. lm* (strain Keishicho) cultured on 1% Ogawa egg yolk medium.

In this paper we present our results of the *in vitro* cultivation in successive passages of 3 different strains of *M. lm* and the characterization of these *in vitro* grown strains.

MATERIALS AND METHODS

Bacterial strains. Three strains of *M. lm* were used: strain UL, received in 1962 from H. Vande Voorde, Katholieke Universiteit Leuven (Belgium) where it had been maintained for an unknown number of years; strain CL, cat leprosy, described by Leiker and Poelma ([5]); and strain HS, the Hawaiian strain, obtained in 1978 from R. J. W. Rees, National Institute for Medical Research, London. They were maintained in female NMRI mice through successive intravenous (I.V.) injections of suspensions prepared from infected livers and containing an undefined number of acid fast bacilli (AFB). Mouse passages were performed every 4 to 5 months.

Preparation of inocula from infected mice for *in vitro* cultures. Ten percent suspensions (w/v) of infected liver and muscle (from the thigh) were prepared from aseptically harvested tissue by homogenization in sterile distilled water in a Potter-Elvehjem homogenizer cooled in ice water. Bone marrow suspensions were prepared from femurs from which the epiphyses were removed and the diaphyses rinsed with sterile distilled water through an 18 gauge needle. Marrows from 2 femurs were suspended in 1 ml and briefly but vigorously shaken with glass beads in a bijou bottle. *Bacterial counts* were performed on 5 μl drops of suspensions applied onto Wellcome plastic coated slides as used for immunofluorescence. Fixation and staining was done as described for *M. leprae* by Shepard ([16]).

In certain experiments, 2 drops of 1% NaOH were added to the suspensions before inoculation of the media since some Japanese authors had mentioned that this procedure improved the results.

In all experiments 0.1 ml of the suspensions was inoculated into at least 2 tubes.

Media. One percent Ogawa egg yolk medium (OEY) ([11]) was used as the basic medium. The composition is presented in Table 1. Modifications of this basic medium were prepared as in Table 2. Mycobactin was prepared from *M. phlei* by the method described by Francis, *et al.* ([2]) and Smith ([17]). Löwenstein-Jensen medium (L-J) was prepared by the addition of eggs to a commercial Löwenstein basis (Difco). Modifications were prepared by adding hemin in concentrations as indicated in Table 2 and by preparing the medium with egg yolk instead of whole eggs.

Conditions of incubation. Cultures were incubated at 33°C in a humidified atmosphere containing 10% CO_2. Tubes had rubber stoppers transpierced with 19 gauge disposable needles. They were examined every 1 or 2 weeks.

[1] Received for publication on 24 September 1979; accepted for publication on 7 November 1979.

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TABLE 1. *Composition of 1% Ogawa egg yolk medium (OEY).*[a]

Basal medium:	KH_2PO_4	1 g
	Na glutamate	1 g
	Glycerol	6 ml
	Water	100 ml
Autoclave: 121°C, 15 min		
Add:	egg yolk	200 ml
	Malachite green 2%	5 ml
Inspissate: on two consecutive days at 85° and 75°C for 40 min and 30 min respectively.		

[a] One percent refers to the concentration of phosphate and glutamate in the basal medium (liquid).

Animal inoculation and examination. Young adult female NMRI mice and 2 month old chickens were inoculated by the I.V. route, with suspensions prepared from *in vitro* cultures and containing respectively 10^6 and 4×10^6 AFB. Mice were killed at 4 week intervals from 4 to 36 weeks after injection, and histologic sections of liver, spleen, lung, heart, and kidney were stained by the Fite-Faracco modification of the Ziehl-Neelsen technique. Chickens were sacrificed after 2 months and histologic examination of liver and spleen performed.

For ultramicroscopy, colonies were fixed in glutaraldehyde and ultrathin sections prepared and stained by the usual methods.

RESULTS

Primary cultures from mouse suspensions. The earliest evidence for *in vitro* growth was obtained after 2 to 3 weeks when very small but distinctive colonies appeared in some tubes. The incubation time varied between 3 weeks and 3 months, but cultures from different organs of the same animal had a very similar incubation time.

TABLE 2. *Modifications of the OEY medium used.*

1. OEY prepared with whole eggs
2. OEY without glutamate
3. OEY + *M. phlei* mycobactin
4. OEY + glycyl-L-histidine-L-lysine acetate
   + isovitalex
   + vitamin K
5. OEY + $FeNH_4$ citrate
   (10, 100, 1000, 5000 μg/ml)
6. OEY + hemin
   (0.5, 5, 15, 30, 50, 100, 150, 200 μg/ml)
7. OEY without glycerol + hemin
   (0.5, 5, 15, 50, 100 μg/ml)
8. OEY without glycerol + Na pyruvate
   (0.03, 0.3, 1%)
   + hemin (as in 7)

TABLE 3. *Primary* in vitro *cultivation of* M. lm *from mouse tissues.*

M. lm strains	Liver		Muscle		Bone marrow	
	pos.	neg.	pos.	neg.	pos.	neg.
UL	6	0	2	2[a]	16	3[a]
CL	1	0	–	–	5	1
HS	1	0	–	–	2	0
Total	8	0	2	2	23	4

[a] Inoculum $<10^4$ AFB.

Table 3 shows that from a total of 39 experiments 33 were successful. More cultures were started from bone marrow because these had produced the earliest results, and it was thought at first that bone marrow constituted a privileged source for *M. lm*. However, results with liver suspensions were also consistently positive, and results with muscle suspensions illustrate that *in vitro* cultures of *M. lm* can be readily obtained from probably any infected organ.

Five of the 6 failures were related to low inocula containing less than 10^4 AFB. The relationship between the number of AFB in the bone marrow inocula, and the *in vitro* growth is presented in Table 4 together with the results obtained after the addition of two drops of 1% NaOH to the inoculum. Positive results were obtained when 10^5 AFB were inoculated; confluent cultures could be observed with inocula of 5×10^6 AFB but more readily with 10^7 and more. The addition of NaOH to the inoculum did not significantly alter the results. There is also a direct relationship between the number of organisms in the inoculum and the incubation time, as shown in Fig. 1, the richest inocula having the shortest incubation times.

In all experiments, tubes with L-J were inoculated and incubated in parallel with OEY. No growth was ever obtained on L-J.

Subcultures. Transfers performed with an inoculating loop gave rise to good growth after 2 months, which might be-

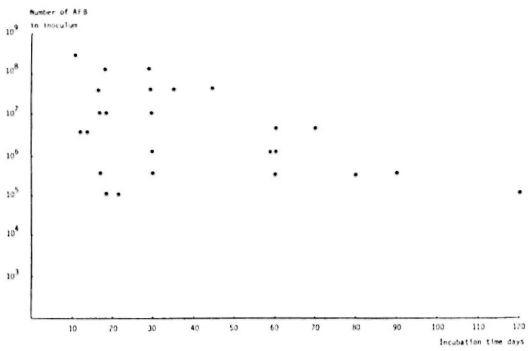

FIG. 1. *In vitro* cultures of *M. lm* from bone marrow. Relationship between the number of *M. lm* inoculated and the incubation time of the *in vitro* cultures.

TABLE 5. *Growth of* M. lepraemurium *(all strains) on OEY + additives and L-J.*

Medium	Growth[a]
O E Y	+
+ whole egg	−
− (without) glutamate	+
+ G L H L L A[b]	+
+ isovitalex	+
+ vitamin K	+
+ iron-ammonium citrate	+[c]
+ *M. phlei* mycobactin	+
Löwenstein	−
+ hemin	−
− (without) egg white	−

[a] Results given as all or none phenomena.
[b] G L H L L A = glycyl-L-histidine-L-acetate.
[c] To a maximum of 1.000 μg/ml.

come confluent after 3 months. Transfers from cultures of different ages were positive, provided the starting cultures were less than 5 months old. All data presented for the primary isolations concerning the quantitative relationships between the number of AFB in the inocula and the degree of positivity and length of incubation time were equally valid for subcultures.

A suspension of strain CL prepared from an *in vitro* culture was enumerated and inoculated in dilutions on OEY tubes. Tubes inoculated with 10^5 AFB and less remained negative.

Some variables in incubation conditions were tried. All routine observations were done at 35°C in a humidified 10% CO_2 atmosphere; growth at 33°C was slower; 37°C was not tested. Aeration and humid atmosphere are essential. There was no growth in microaerophilic (Gaspack jar with

TABLE 4. *Relation of number of AFB in inoculum and results of* in vitro *growth of* M. lm *from bone marrow.*[a]

Number of AFB inoculated	Strain UL		Strain CL		Strain HS	Three strains combined	
	−[b]	+NaOH[d]	−	+NaOH	−	positive/ negative	% positive
$<10^4$	−					0/2	0
10^4	−						
10^5	−, +			+	+	7/11	64
5×10^5	−, −	+, +, +	−	+			
10^6	−	+	+	c	+	11/12	92
5×10^6	c, +, +	+, c	+	+			
10^7		+				6/7	86
5×10^7	+, +, c	+, −		+			
10^8	+, c					4/4	100
$>10^8$	c	c					
Number positive cultures	10	9	2	5	2	28/36	77.8
Number negative cultures	6	1	1	0	0		
% positive	63%	90%	67%	100%	100%		

[a] − = no growth. + = isolated colonies. c = confluent growth.
[b] − = no NaOH added.
[d] +NaOH = two drops of 1% NaOH added to inoculum before inoculation of media.

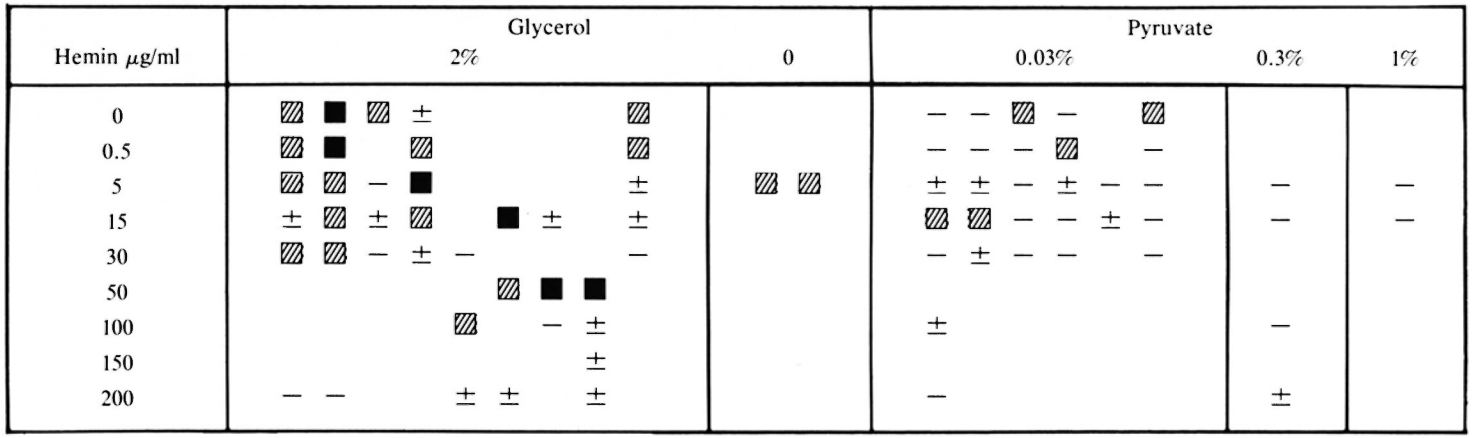

FIG. 2. Effect of different carbon sources and concentrations of hemin on the growth of *M. lm* strain UL.

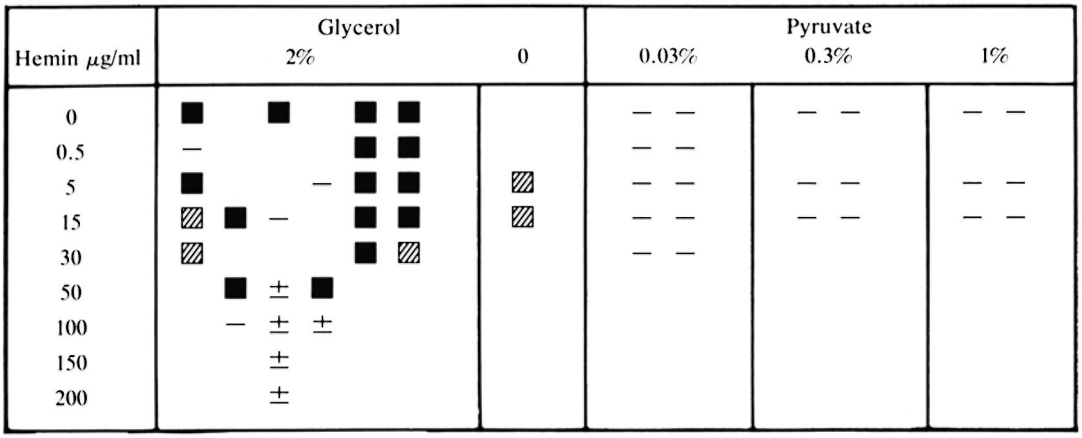

FIG. 3. Effect of different carbon sources and concentrations of hemin on the growth of *M. lm* (strain CL).

anaerobic reagents but without catalyst) or anaerobic conditions. Because of lack of incubators, it was impossible to test whether the 10% CO_2 atmosphere is essential and what occurs at 37°C.

Growth factors. Table 5 shows the results obtained with the different medium compositions. Addition of egg white to the Ogawa medium prevents all growth. Addition of different compounds known to be important for other organisms and/or mycobacteria did not influence the results. Glutamate may be omitted. L-J prepared without egg white or with added hemin did not support growth.

Since Mori ([6]) had mentioned improved subcultures by the addition of hemin and since glycerol and sodium pyruvate stimulate some mycobacterial species and inhibit others, the influence of these compounds was studied in different combinations on two *M. lm* strains. Figures 2 and 3 show the results. Pyruvate was not beneficial and was inhibitory in a 1% concentration. Hemin at concentrations higher than 50 μg/ml inhibited growth. Hemin is not a growth factor. There was growth in the absence of glycerol, but it was less than in the presence of 2% glycerol.

Characterization and identification of the *in vitro* cultures. When first visible, colonies of *M. lm* were 1 mm in diameter, white, glistening, and heaped up. On longer incubation, they became pale yellow and attained 1–2 mm, showing sometimes a central crater. Growth was eugonic and appeared quite rough although it was possible to prepare suspensions that remained stable for many hours. When colonies were removed from the medium, brown rust-like spots could be seen on the sites where the colonies were situated. These spots were also observed by Mori and Kohsaka ([10]). This has not been observed with *M. avium*, *M. ulcerans*, and *M. paratuberculosis* grown on the same medium.

The microscopic appearance was rather unique. The AFB were pleomorphic with very long filamentous, beaded forms and definite branching. The microscopic appearance of *M. lm* in culture reminds one of that of *M. lm* observed in suspensions and tissue sections of infected organs of mice. Some AFB, frequently the very long but also some shorter rods, showed swollen ends. These were interpreted as degenerative forms. Ultrathin sections confirmed that the organisms were mycobacteria with the presence of degenerative forms. Extremely long organisms or shorter swollen

TABLE 6. In vitro *characteristics of* M. lepraemurium *(M. lm).*

	Growth Löwenstein	Pigmentation	Growth at 33°C	Growth at 37°C	Growth at 42°C	Resistance to INH	Resistance to TCH	Resistance to HA	Resistance to PNB	Catalase >45 mm	Niacin production	Nitrate reduction	Acid phosphatase	Tween hydrolysis	β-galactosidase	Urease	Nicotinamidase	Pyrazinamidase	Putrescin	Growth in 5% NaCl	OAA colonial morphology
M. lm strain UL	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	R
M. lm strain CL	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	R
M. lm Keishicho[a]	−	−	+	+	−				+	−						−	+	+	−	−	R
M. haemophilum[b]	−[c]	−	+	−	−	+				−	−	−		−	−	−	+	+		−	Sm
M. paratuberculosis	−[d]	−	+	+	+	+	+	+	−	−	−	−	−	−	−				−	−	R
M. bovis	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	R
M. ulcerans	+	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	R
M. avium	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−	Sm

[a] Results from Saito, *et al.* (15)
[b] Results from Sompolinsky, *et al.* (18)
[c] Needs hemin
[d] Needs mycobactin

INH = isoniazid 10 μg/ml
TCH = thiophene-2-carboxylic acid hydrazide 1 μg/ml
HA = hydroxylamine hydrochloride 250 μg/ml
PNB = p-nitrobenzoic acid 500 μg/ml
OAA = oleic acid albumin agar

ones might be filled with granules, presumably coagulated cytoplasm, sometimes extruding from ruptured bacilli.

Identification of the strains as *M. lm* is based on the following characteristics:

a) An identical growth pattern was observed for all cultures obtained from 3 different strains of *M. lm* as to the visual appearance of the colonies, incubation time, conditions of incubation, and absence of growth on L-J.

b) Three months after being inoculated I.V. with suspensions containing 10^6 AFB/ml prepared from primary cultures and from subcultures as well, mice developed lesions in the liver and the spleen which were histologically identical to those observed in mice inoculated with mouse passage material. Beaded and often ramified AFB were found in large cells without evidence of inflammatory reaction. After 5 to 6 months, AFB were found in the bone marrow of these mice in numbers from 10^6 to 10^8, large accumulations of acid-fast organisms were found in the liver and the spleen, and AFB also appeared in the lungs, the myocardium, and sometimes in the kidney. Cultures on OEY from the liver and bone marrow of mice injected with primary cultures or *in vitro* passages gave rise to the same type of *in vitro* growth as described above. Chickens inoculated with an *in vitro* grown *M. lm* remained well, and no AFB or histopathologic lesion could be found in their livers and spleens.

c) The strains were identified by the tests described in the identification scheme of Pattyn and Portaels (14) in its more recent revision of 1979) (4) but with OEY replacing L-J. The results, available for strains UL and CL, appear in Table 6. For comparison, characteristics of 3 other slow growing mycobacteria, *M. bovis*, *M. ulcerans*, and *M. avium*, and two other species not growing on L-J, *M. paratuberculosis* (Portaels, unpublished) and *M. haemophilium* (18) have been included as well as the results on the *M. lm* strain Keishicho by Saito, *et al.* (15). The two strains UL and CL have identical characteristics and differ from the mycobacteria listed and all other known mycobacteria (4). *M. lm* is particularly nonreactive. Although catalase production as detected in the standard test for the identification of mycobacteria remained negative, catalase is present in small amounts in *M. lm* since bubbles were produced when

M. lm was suspended in hydrogen peroxide on a microscope slide. That *M. lm* is not hemin dependent was confirmed by the porphyrin test [10], which became positive after 2 to 3 days of incubation.

Tube agglutination tests performed with suspensions of *in vitro* grown *M. lm* and 18 *M. avium* agglutinating sera [13] were negative.

DISCUSSION

Ogawa and Motomura [11,12] and Mori [6,7,9] obtained limited *in vitro* growth of *M. lm* from fragments of liver tissue very rich in AFB and planted on the surface of OEY. We have regularly and consistently obtained *in vitro* growth of *M. lm* from suspensions of liver and bone marrow. Whereas Mori [8] obtained subcultures with difficulty after 6 to 9 months of incubation, we obtained them within 2 to 3 months. As with other very slow growing mycobacteria such as *M. ulcerans*, transfers are preferably made with a bacteriological loop rather than from suspensions. This points to the necessity for rather heavy inocula and is illustrated by our quantitative data, which indicate that the lowest number of AFB giving rise to macroscopically visible growth is 10^5 both for primary isolation and for transfer of suspensions prepared from *in vitro* grown organisms. This points to either an important fraction of the bacteria being nonviable or a deficiency in the medium. Ultramicroscopy reveals degenerating bacteria even in young colonies; however, quantitative data are not available. Several additives important for other bacteria did not enhance growth. Pyruvate as a carbon source inhibited growth. The influence of different concentrations of glycerol should be further investigated. Absence of growth on medium containing egg white points to iron as an important growth factor since egg white is an iron chelator. The brown, rust-like spots under the colonies might be accumulations of iron or an iron compound. Mori and Kohsaka [10] also observed these spots and suspected them to be accumulations of coproporphyrin. The addition of iron-ammonium citrate did not enhance growth. The beneficial effect of the addition of a small amount of NaOH to the inocula, as mentioned by Ogawa and Motomura [12], could not be confirmed. Neither is there a need for added hemin. Our results confirm our previous findings that *M. lm* is an aerobic organism [3].

The results of Brown [1], who concluded that the primary multiplication site of *M. lm* in the mouse is in the bone marrow, the bone marrow containing larger numbers of viable organisms when compared with other organs, were not confirmed by the present observations. Suspensions of liver, muscle, and bone marrow, prepared from one animal and adjusted for comparable numbers of bacteria, all gave rise to comparable numbers of colonies *in vitro*.

In contrast to the results published by Saito, *et al.* [15], the strains of *M. lm* tested in this study did not grow in the presence of 500 μg/ml of paranitrobenzoic acid. Because it has been said that there is a relationship between *M. lm* and *M. avium*, our strains were also inoculated into chickens and agglutination tests performed with all available known antisera against *M. avium*. These investigations did not reveal any relationship between *M. lm* and *M. avium* by these criteria.

1) Identical *in vitro* grown strains obtained from 3 different mouse strains of *M. lm*, 2) requiring a special medium, 3) requiring particular incubation circumstances (although the role of the CO_2 enriched atmosphere could not be defined), 4) having identical, distinctive *in vitro* characteristics, 5) producing lesions in mice identical to those produced by mouse passaged strains, and 6) from which mice the same *in vitro* strains can be obtained, produce proof that the *in vitro* grown organisms are *M. lm*.

M. lm is a very slow growing acid fast organism developing within 2 months on OEY and not L-J and requiring particular conditions of incubation. It differs from all presently known mycobacteria both in its microscopic morphology and in its *in vitro* characteristics.

SUMMARY

Three different strains of *M. lm* were regularly grown *in vitro* from suspensions of mouse organs if at least 10^5 organisms were inoculated on Ogawa egg yolk medium and incubated at 35°C in a humidified, CO_2 enriched atmosphere. Growth is slow and requires 2–3 months. Colonies are 1–2 mm in diameter, white to pale yellow. Microscop-

ically the bacteria are acid-alcohol-fast pleomorphic rods with branchings and beaded filamentous forms. Mice inoculated with *in vitro* grown subcultures develop an infection indistinguishable from the one observed after injection with mouse passage strains of *M. lm*. The *in vitro* characteristics of the strains are identical and different from all other known mycobacteria.

RESÚMEN

Se logró el crecimiento *in vitro* de 3 diferentes cepas de *M. lm* obtenidas de ratones infectados. El crecimiento fue consistente cuando el medio de Ogawa con yema de huevo se inoculó con no menos de 10^5 bacterias. Los cultivos se incubaron a 35°C, en una atmósfera húmeda enrriquecida con CO_2. El crecimiento fue lento y requirió de 2 a 3 meses. Las colonias fueron pequeñas, con un diámetro de 1 a 2 mm, de color blanco o amarillo pálido. Al microscópio, las bacterias fueron ácido-resistentes, pleomórficas, filamentosas y con ramificaciones. Los ratones inoculados con los subcultivos crecidos *in vitro* desarrollaron una infección indistinguible de aquella que presentaron los animales inoculados con las cepas de *M. lm* obtenidas de ratones infectados. Las características *in vitro* de las 3 cepas son idénticas entre si, pero diferentes de todas las otras micobacterias conocidas.

RÉSUMÉ

Trois souches de *M. lm* furent régulièrement cultivées *in vitro* à partir de suspensions d'organes de souris, pour autant qu'un minimum de 10^5 germes soient inoculés sur du milieu au jaune d'oeuf de Ogawa incubé à 35°C en atmosphère humidifiée et enrichie de CO_2. La croissance est lente et demande 2 à 3 mois. Les colonies font 1–2 mm de diamètre et sont blanches à jaune pâle. Les germes sont acido-alcoolo-résistants, pléomorphes avec beaucoup de ramifications et des éléments granuleux et filamenteux. Des souris inoculées avec des subcultures *in vitro* développent une infection identique à celle observée après injection de souches passées de souris à souris. Les souches possèdent des caractères *in vitro* identiques entr'elles mais différents de toutes les autres mycobactéries connues.

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