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Failure to Validate the Growth of *Mycobacterium leprae* on M-Y 14b Agar Medium[1]

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Since 1969, Murohashi and Yoshida ([3,4,5]) have been reporting that *Mycobacterium leprae* multiplies in their cell-free semisynthetic soft agar medium. Recently they reported colony formation of *M. leprae* on M-Y 14b agar medium ([6]), which is a modification of the soft agar medium. Ishidate (Sasakawa Memorial Health Foundation), one of the present authors, was interested in their report and organized a research group to try to confirm Murohashi and Yoshida's results. The group was composed of investigators who have had long careers in research in the cultivation of *M. leprae* and *M. lepraemurium*. The confirmation experiment was carried out by this group, who faithfully followed the directions in Murohashi and Yoshida's paper ([6]).

The present paper summarizes the results that were obtained by these individual investigators over the past two years.

MATERIALS AND METHODS

Leprosy materials. Materials for cultivation were kindly supplied by the Korean Leprosy Institute. These consisted of seven human lepromas, designated KR 9-12, KR 7-2, KR 2-18, KR 4-15, KR 8-6, KR 9-29, and KR 12-17. Bacillary suspensions were prepared in M. Kanamura's laboratory (Kurume University School of Medicine) by the following method: Each of the leprosy nodules was treated with 2% w/v NaOH for 5 min at room temperature (25°–30°C), washed three times with sterile M/30 Sörensen buffer (pH 7.0), and then homogenized in a glass homogenizer in M/30 Sörensen buffer containing 0.1% v/v bovine serum. The suspensions were kept in the cold until used. Prepared suspensions were sent to each investigator as quickly as possible.

Culture medium. The M-Y 14b agar medium developed by Murohashi and Yoshida ([6]) was used throughout. Unless otherwise stated, culture tubes of M-Y 14b agar medium were supplied to each investigator from the Suruga National Leprosarium. These culture tubes were prepared by Ishihara and Hagihara who had learned directly from Murohashi the exact and precise procedures for preparing the M-Y 14b agar medium. The composition of M-Y 14b agar medium is as follows:

KH_2PO_4	4.0 g
$CaCl_2$	0.0025 g
Asparagine	3.0 g
Sodium pantothenate	0.1 g
$Na_2HPO_4 \cdot 12H_2O$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g

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Sodium pyruvate	2.0 g
Sodium citrate	2.0 g
Sodium glutamate	3.0 g
Glucose	10.0 g
Tween 80	0.1 g
Yeast RNA	100 μg
BSA Fraction V (Armour)	5.0 g
Powdered agar	20.0 g
Water	1000 ml (pH was adjusted to 6.6–6.8)

Inoculation and cultivation of the material. Inoculation and cultivation procedures were carried out strictly according to the method of Murohashi and Yoshida. In brief, 0.2 ml of the suspension of leprosy nodules was inoculated on the slant of culture medium, and the surface of the slant was kept horizontal for ten days at 37°C. After the suspension had almost dried, the culture tubes were fitted with rubber stoppers, placed upright, and incubated at 37°C.

Evaluation of growth. Two methods were employed for evaluating the growth of *M. leprae*. One was macroscopic observation. Murohashi and Yoshida stated that visible colonies usually developed approximately 20 weeks after inoculation. Therefore, the culture tubes were examined first macroscopically, with illumination against a dark background. The other method was microscopic bacillary counting. Two ml of buffer was pipetted into the culture tube, the surface of the slant was scraped by pipetting, and then a suspension of the bacilli was made for counting. Numbers of bacilli in the suspension were counted by Hanks' pinhead method ([1]) or Shepard's method ([8]). The growth of *M. leprae* was estimated by the increase in the number of bacilli after an appropriate cultivation period at 37°C, compared to that of the inoculum or that after the same incubation period at 4°C. Morphological findings with a light and/or electron microscope were also used as parameters for the evaluation of the state of the bacilli in some cases.

Viability of *M. leprae*. In order to see whether the inoculum was viable or not, the foot pad inoculation method with the ddy strain of conventional mice ([7]) or nude (BALB/c-nu/nu) ([2]) mice was used.

Participation by each investigator. Each investigator individually received the leprosy suspension and tubes of culture medium, and carried out the culture trial in his own laboratory. Experimental procedures and evaluations of the growth of *M. leprae* carefully and faithfully followed the original instructions in Murohashi and Yoshida's reports. Names in parentheses in this paper are those of the participating investigators.

TABLE 1. *Lack of multiplication of* M. leprae *sample Nos. KR 9-12 and KR 7-2 on M-Y 14b agar medium.*

Sample	Inoculum size	Incubation period (months)	Incubation temperature		Reporter
			4°C	37°C	
KR 9-12	2.0×10^6	3	6.2×10^5	6.7×10^5	Mori
		6		1.1×10^6	
		9	5.0×10^5	2.4×10^5	
	1.7×10^7	6	1.4×10^7	1.7×10^7	Matsuo
				1.6×10^7	
				9.3×10^{6a}	
		10	1.5×10^7	8.7×10^6	
				7.9×10^{6a}	
KR 7-2	1.5×10^7	3	8.5×10^6	8.3×10^{6a}	Matsuo
				7.7×10^{6a}	
		6	7.1×10^{6a}	7.4×10^{6a}	
				6.7×10^{6a}	

[a] MY-H medium was used. MY-H medium was prepared by Matsuo, Department of Bacteriology, Hiroshima University School of Medicine.

TABLE 2. *Lack of multiplication of* M. leprae *sample No. KR 2-18 on M-Y 14b agar medium.*

Inoculum size	Incubation period (months)	Incubation temperature		Reporter
		4°C	37°C	
2.0×10^7	3	2.4×10^7	2.1×10^7 1.6×10^7	Matsuo
	6	2.0×10^7	1.8×10^7 1.5×10^7	
6.7×10^7	2	2.7×10^7	1.3×10^7	Mori
	6		1.6×10^7	
	5	4.1×10^6	1.9×10^6	M. Nakamura
	10		3.3×10^6	
	3		2.4×10^7	Nakayama
	6		1.4×10^7	
	10		2.8×10^7	
8.8×10^6	3	3.4×10^5	3.4×10^5	Saito
	6	4.8×10^4	5.2×10^4	

RESULTS

Problem concerning colony formation of *M. leprae* on M-Y 14b agar medium. The tubes were examined for the formation of colonies of *M. leprae* after three months and six months of cultivation at 37°C. The results reported by all participants indicated that small, yellow-white, colony-like spots were fequently seen on the slants, but that these spots were not bacterial colonies but tissue debris as determined by microscopic observation. Therefore, it can be concluded that no colony of *M. leprae* was ever obtained in any of the culture tubes examined.

Change in the number of bacterial cells of *M. leprae* during cultivation. In parallel with the macroscopic observations of the culture tubes, the bacilli on the surface of the solid medium in the culture tube were counted by the methods described. The results from the individual investigators were collected and are summarized in Tables 1 through 6. None of the results showed any data indicating an increase in the number of bacterial cells during cultivation. Accordingly, it can be stated that no growth of *M. leprae* took place on M-Y 14b agar medium.

Morphological changes in bacterial cells of *M. leprae* during cultivation. Light and elec-

TABLE 3. *Lack of multiplication of* M. leprae *sample No. KR 4-15 on M-Y 14b agar medium.*

Inoculum size	Incubation period (months)	Incubation temperature		Reporter
		4°C	37°C	
9.0×10^7	3	2.7×10^7	9.9×10^{7a}	Mori
3.4×10^8	3	3.4×10^8	3.3×10^{8a} 3.5×10^{8a}	Matsuo
	6	1.6×10^8	2.7×10^{8a} 1.4×10^{8a}	
1.6×10^8	3	1.5×10^8	7.6×10^7	Saito
	6	6.4×10^7	3.4×10^7	
G-2[b]	3	7.5×10^6	4.5×10^6	Mori

[a] MY-H medium was used.
[b] G-2 represents the second generation from the first column in this table which was cultivated at 37°C.

TABLE 4. *Lack of multiplication of* M. leprae *sample No. KR 8-6 on M-Y 14b agar medium.*

Inoculum size	Incubation period (months)	Incubation temperature		Reporter
		4°C	37°C	
1.1×10^8	3	8.7×10^7	6.9×10^7	Itoh
	6	7.8×10^7	4.1×10^7	
2.0×10^7	3	1.5×10^6	2.4×10^6	Kozeki
	6	6.9×10^6	1.7×10^6	
	3	1.5×10^7	1.3×10^7	Matsuo
			1.3×10^7	
	6	1.5×10^7	1.2×10^7	
		1.4×10^7	1.1×10^7	
1.6×10^7	4	2.3×10^7	2.4×10^6	M. Nakamura
	6		2.8×10^6	
	3		4.6×10^7	Nakayama
	6		3.7×10^7	
	3	1.1×10^7	6.9×10^6	
			8.2×10^6	
	6	7.7×10^6	6.2×10^6	Okada
			7.0×10^6	
9.8×10^5	6	1.1×10^7	5.4×10^6	Saito

tron microscopic observation of *M. leprae* during cultivation was carried out. The results indicate that there was no definite increase in the clumps of *M. leprae* compared with the initial inoculum and that an increase in non-solid forms was regularly seen (Ogawa and Saito). On the other hand, electron microscopic observations showed that the number of ghost cells increased with the time of cultivation (Okada).

TABLE 5. *Lack of multiplication of* M. leprae *sample No. KR 9-29*[a] *on M-Y 14b agar medium.*

Inoculum size	Incubation period (months)	Incubation temperature		Reporter
		4°C	37°C	
4.3×10^7	6	7.9×10^6	1.5×10^7	Itoh
1.4×10^7	3	2.6×10^6	4.5×10^6	Koseki
	6	3.3×10^6	2.2×10^6	
2.5×10^7	6		1.8×10^7	Matsuo
	6		1.8×10^7	Mori
5.5×10^6	3	8.6×10^6	1.1×10^7	M. Nakamura
	6		1.1×10^7	
9.0×10^7	3		7.4×10^7	Nakayama
	6		7.7×10^7	
	10		2.4×10^7	
3.5×10^7	3	3.0×10^7	1.8×10^7	Okada
			2.0×10^7	
	6	2.8×10^7	1.2×10^7	
			1.9×10^7	
4.5×10^6	6	4.1×10^6	6.0×10^6	Saito

[a] This sample was contaminated with *Staphylococcus aureus*.

TABLE 6. *Lack of multiplication of* M. leprae *sample No. KR 12-17 on M-Y 14b agar medium.*

Inoculum size	Incubation period (months)	Incubation temperature		Reporter
		4°C	37°C	
2.7×10^7	3	9.1×10^6	8.6×10^6	Itoh
	6	8.7×10^6	9.7×10^6	
2.9×10^6	3	1.9×10^6	1.1×10^6	Kohsaka
	6	1.5×10^6	4.7×10^6	
1.1×10^7	3	7.7×10^6	5.8×10^6 7.9×10^6	Matsuo
	6	6.4×10^6	4.2×10^6 6.8×10^6	
	3	1.4×10^6	2.9×10^6	Mori
	6	1.6×10^6	1.3×10^6	
	3	3.1×10^6	2.1×10^6	M. Nakamura
	6	3.0×10^6	2.0×10^6	
	3		1.4×10^7	Nakayama
	6		1.4×10^7	
7.2×10^6	3	5.2×10^6	1.0×10^7 5.3×10^6	Okada
	6	1.7×10^6	2.6×10^6 5.1×10^6	
7.9×10^6	6	7.6×10^6	1.7×10^7	Saito

Viability of inoculum of *M. leprae*. At the time of inoculation of *M. leprae* onto M-Y 14b agar medium, the viability of the *M. leprae* contained in the inoculum was tested by foot pad inoculation of ddy mice and nude mice, in order to validate the results of cultivation trials (Matsuo, Kohsaka, and K. Nakamura). The results obtained are summarized in Table 7 (Kohsaka). The number of *M. leprae* increased in the mouse foot pads in all samples except KR 9-12 and KR 8-6. Although not stated in the table, KR 7-2 (Table 1) was determined to be viable by Matsuo, who used ddy mice. Therefore, it can be seen from the results that all samples used for the cultivation experiment, except KR 9-12 and KR 8-6, contained *M. leprae* which had the potential for multiplication in the mouse foot pad. In other words, the inoculum definitely contained viable cells of *M. leprae*.

TABLE 7. *Infectivity of* M. leprae *used for inoculation onto M-Y 14b agar medium.*[a]

Sample	Inoculum/ 0.05 ml	Harvest/ foot (after inoculation)	Judgment
KR 9-12	1.6×10^6	$\leqq 5.3 \times 10^3$ (9 mo.)	No growth
KR 2-18	6.7×10^6	2.2×10^8 (10 mo.)	Growth
KR 4-15	3.0×10^7	8.3×10^8 (7.5 mo.)	Growth
KR 8-6	1.3×10^6	4.3×10^5 (8 mo.)	No growth
KR 9-12	2.7×10^5	1.1×10^7 (6 mo.)	Growth
KR 12-17	—	3.0×10^5 (3.3 mo.) 4.8×10^6 (7 mo.)	Growth

[a] Kohsaka; foot pad inoculation of nude mice (BALB/c-nu/nu).

Maintenance of viability of *M. leprae* on M-Y 14b agar medium. The survival period of *M. leprae* on M-Y 14b agar medium during cultivation was tested by washing off the inoculum after a definite incubation period and inoculating it into mouse foot pads. The results obtained indicate, as shown in Table 8 (Kohsaka), that the viability of *M. leprae* was completely lost within approximately seven weeks of cultivation on M-Y 14b agar medium at 37°C.

TABLE 8. *Lack of survival of* M. leprae *on M-Y 14b agar medium during cultivation.*[a]

Sample	No. of cells at 0 time/0.05 ml	No. of cells/foot (after inoculation)	Judgment
KR 12-18: 7 weeks' cultivation	6.5×10^5	2.8×10^4 (8.5 mo.)	No growth
KR 4-14: 3 months' cultivation	1.4×10^6	1.9×10^5 (8 mo.)	No growth
KR 12-17: 3 months' cultivation	7.0×10^5	4.8×10^4 (10 mo.)	No growth

[a] Kohsaka; foot pad inoculation of nude mice (BALB/c-nu/nu).

DISCUSSION

Since 1969, Murohashi and Yoshida have published a series of reports on the development of a culture medium for the growth of *M. leprae*. In this series they initially developed a semisolid soft agar medium ([3,4]), next a liquid culture medium ([5]), and finally a solid medium ([6]), which is claimed to be able to produce macroscopic colonies of *M. leprae*.

Some of us were interested in these reports, and discussed confirmation of the results. Consequently, a research group for confirmation consisting of expert investigators who have rich empirical experience in attempts to cultivate *M. leprae* and *M. lepraemurium* was organized. At the beginning of the confirmation study, guidance in the procedures for confirmation was set up as a chart, according to the papers of Murohashi and Yoshida, and the chart was distributed to each investigator.

Items in the chart are as follows:

a) Culture medium: M-Y 14b agar medium prepared by Ishihara and Hagihara of the National Leprosarium of Suruga.
b) Inoculum: 0.2 ml of bacillary suspension per tube. The number of bacilli is to be determined by the method selected by the individual investigator.
c) Culture conditions: After inoculation, tubes are maintained in the horizontal state with loose stoppers for ten days at 37°C, in order to dry. After ten days, the tubes are tightly fitted with rubber stoppers and also sealed with vinyl tape. Four tubes are incubated at 37°C, and two tubes are kept in the cold.
d) Assessment of growth:
   1) Observation periods: three months and six months after inoculation.
   2) Macroscopic observation: Colony formation.
   3) Microscopic observation: A sample is taken from the center part (5 mm wide) of the slant with a loop, and a smear is made by spreading the sample with one drop of water previously poured on the slide glass. The smear is stained by the Ziehl-Neelsen method.
   4) Bacillary count: One ml of water is pipetted into a test tube and the surface is scraped with a pipette. One more ml of water is added. The bacilli in 2 ml are counted by the method selected by the individual investigator.
   5) Electron microscopic observation is carried out.
   6) Viability of *M. leprae*: Viability of *M. leprae* in the inoculum and the cultured samples is determined by mouse foot pad inoculation.
e) Report:

Cultivation period	Incubation temperature	Macroscopic observation	Smear BI MI	Bacillary count
3 month	37°C 4°C			
6 month	37°C 4°C			

BI = Bacillary index
MI = Morphological index (solid or non-solid)

According to this instruction, each investigator carried out the experiments in his own laboratory. In spite of some data indicating multiplication of *M. leprae* report-

ed by Murohashi and Yoshida, our results from both *in vitro* and *in vivo* experiments strongly indicate that no propagation of *M. leprae* was obtained and also that M-Y 14b agar medium is not suitable for maintaining the viability of *M. leprae*. From the results in these confirmation experiments, it can be presumed that the false growth-like findings observed by Murohashi and Yoshida are due to autolysis or destruction of subcutaneous tissues containing a large number of *M. leprae* during a long cultivation period. To avoid this false interpretation, a bacillary counting method should be useful and productive. It should be pointed out that in the papers of Murohashi and Yoshida there were no quantitative aspects of their evaluation of the growth of the fastidious microorganism, *M. leprae*. Indeed, quantitative assessment like bacillary counting would be unnecessary if colonies of *M. leprae* were visible and would grow with time. Unfortunately, however, none of the investigators in our research group were ever able to find any colony of acid-fast bacilli on M-Y 14b agar medium.

SUMMARY

Confirmation experiments on colony formation of *M. leprae* on the M-Y 14b agar medium developed by Murohashi and Yoshida were carried out for two years independently by individual members of an organized research group, according to the method described by Murohashi and Yoshida. The results obtained can be summarized as follows:

a) No colony production by *M. leprae* on M-Y 14b agar medium was seen.
b) No increase in the number of cells of *M. leprae* on M-Y 14b agar medium during cultivation was seen.
c) Light and electron microscopic observation indicated that there was an increase in the number of non-solid bacterial cells and ghost cells with time of cultivation.
d) It was found by mouse foot pad inoculation that four of six samples of *M. leprae* used as inocula were definitely viable.
e) By means of mouse foot pad inoculation, it was shown that viability of *M. leprae* inoculated onto M-Y 14b agar medium was lost within approximately seven weeks of cultivation.

From these results, it can be definitely concluded that there is no evidence indicating that multiplication of *M. leprae* took place on M-Y 14b agar medium.

RESUMEN

Durante 2 años, un grupo de investigadores realizó experimentos para confirmar la formación de colonias del *M. leprae* en el medio de agar M-Y 14b desarrollado por Murohashi y Yoshida, de acuerdo a las indicaciones de los autores. Los resultados se pueden resumir así:

a). No se observó la producción de colonias de *M. leprae* sobre el medio de agar M-Y 14b.
b). Durante el cultivo, no se observó incremento en el número de *M. leprae* en el medio de agar M-Y 14b.
c). Las observaciones con los microscópios de luz y electrónico indicáron que hubo un incremento en el número de formas no sólidas y de células vacías conforme avanzó el tiempo de cultivo.
d). Se encontró, por inoculación en el cojinete plantar del ratón, que cuatro de las seis muestras de *M. leprae* que se usaron como inóculo fueron definitivamente viables.
e). También se encontró, por inoculación en el cojinete plantar del ratón, que la viabilidad del *M. leprae* inoculado en el medio de agar M-Y 14b se perdió dentro de las 7 semanas siguientes al inicio del cultivo.

De estos resultados se puede concluir definitivamente que no hay evidencias de la multiplicación del *M. leprae* en el medio de agar M-Y 14b.

RÉSUMÉ

Des études expérimentales en vue de confirmer des résultats antérieurs ont été menées concernant la formation de colonies de *M. leprae* sur le milieu à l'agar M-Y 14b développé par Murohashi et Yoshida. Ces études ont été menées indépendamment, pendant deux ans, par les membres individuels d'un groupe de recherche organisé. Le protocole à suivi la méthode décrite par Murohashi et Yoshida. Les résultats obtenus peuvent être résumés comme suit:

a) Aucune production de colonies par *M. leprae* n'a été observée sur le milieu à l'agar M-Y 14b;
b) Aucune augmentation du nombre de cellules de *M. leprae* n'a été observée sur le milieu à l'agar M-Y 14b durant l'essai de culture;
c) Les observations menées par microscopie optique et par microscopie électronique ont permis de mettre en évidence une augmentation du nombre des cellules bactériennes non-solides, et des silhouettes de cellules en association avec le moment de la culture;
d) L'inoculation au coussinet plantaire de la souris

a montré que quatre des six échantillons de *M. leprae* utilisés comme inoculats étaient indubitablement viables;

e) L'inoculation au coussinet plantaire de la souris a également permis de démontrer que la viabilité de *M. leprae* est perdue dans les cultures dans approximativement les 7 semaines qui suivent l'inoculation sur le milieu à l'agar M-Y 14b.

Ces résultats permettent catégoriquement de conclure qu'il n'existe aucune évidence indiquant une multiplication de *M. leprae* sur le milieu à l'agar M-Y 14b.

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