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Subdivision of Opportunist Mycobacteria by the Difference of Pigment Production on Lowenstein-Jensen Medium Containing Crocin

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=국문초록=

Crocin 첨가 결핵균 배지상의 색소형성에 의한 비정형 Mycobacteria 의 분류

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Mycobacteria 의 Crocin 첨가 결핵균 배지상의 보라색 색소형성과 광선조사, 호협기상태, Crocin 의 농도 및 배양시간과의 관계를 조사하고 비정형 Mycobactia 의 색소형성 특성에 따른 군분류 동정에 대하여 기술하였다.

광색정균 *M. kansasii* 는 양성으로 음성인 *M. marinum* 과 감별이 되었으며, 색정균 *M. scrofulaceum* 은 음성으로서 양성인 *M. aquae* 와, 조기발육균 *M. fortuitum* 은 3일 배양검사에서 양성으로 *M. smegmatis* 로 부터 본류 동정이 각각 가능하였다.

INTRODUCTION

The genus *Mycobacterium* contains many clearly defined species, which may be obligatory pathogens for man and animals and saprophytes, together with an illdefined groups of opportunist mycobacteria.

At present, human and animal mycobacterioses caused by opportunist mycobacteria other than typical tubercle bacilli are increasing in number of reports. Therefore, it is necessary for these infectious agents to be identified precisely in the laboratory of hospital because the prognosis and the response to drug therapy differ greatly from infections caused by each individual type of mycobacterium.

In this regard, Runyon's provisional grouping (Runyon 1955, 1959) of opportunist mycobacteria and saprophytes based on the pigmentation of mycobacteria and growth rate into photochromogens, scotochromogens, nonchromogens and rapid growers are of value. It does not relieve, however, the bacteriologist working in mycobacterioses of the task of developing and exact classification beyond such provisional grouping.

Lee and Son (1955) observed that certain strains of mycobacteria grown on Ogawa medium

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containing soluble extract of fruit of *Fructose gardeniae* produce dark violet pigment. It was a unexpected result in their works on antituberculous drug activity of various Chinese drugs. However, differentiation of various subspecies of opportunist mycobacteria by means of pigmentation on L-J medium containing crocin has not been fully investigated.

The authors were interested in this character whether it may be applied to a method for identification of mycobacteria. This paper reports the effect of aeration, exposure of light and optimal ratio of gardenia (crocin) powder to medium for the production of violet pigment, and subdivision of opportunist mycobacteria by difference in pigment production and growth rate on L-J medium containing crocin was evaluated.

MATERIALS AND METHODS

.1 Mycobacterial strains:

M. bovis AN_5, Otto, Bovine 10 and BCG; *M. tuberculosis* PN, Aoyam B, H37Rv, Huse; *M. kansasii* Janice, Swisher, P16 and 7879; *M. marinum* Hernadez andBL; *M. scrofulaceum* Bridge, Lunning and Gause; *M. avium* 16906-3380 and 14141-1395; *M. intracellulare* Borne Iowa, 141186-1424, Warsik, 3259-685, Sweatmen, Manten 157, Melnick, Findley, J2970, Lynn H. WS52, S. J. Bull No. 2, P42, 12305-406, P54 and Yandle; *M. fortuitum* DEAE 504, Stark Glidden, Taylor AT 455, Ramsey, 11997 and 16660; *M. gastri* W995; *M. aquae* 9738; *M. smegmatis* C-QV; *M. johnei* P18 were used. The sources of the strains are shown in table 1.

Table 1. Mycobacterial strains

Species	Strains	Source (Strain from the collection of)
M. bovis	AN5[a], Otto[a], Bovine 10[b], BCG[c]	a. Central Veterinary Laboratory, Weyblidge, UK.
M. tuberculosis	PN[a], Aoyama B[b], H37Rv[a], Huse[c]	b. The National Institute of Animal Health, Tokyo, Japan.
M. kansasii	Janice[d], Swisher[d], P16[d], 7879[e]	c. The Institute of Veterinary Research, Anyang, Korea.
M. marinum	Hernad[d], BL[c]	d. Dr. W. B. Schaefer, National Jewish Hospital and Research Center, Denver, Colo., USA.
M. scrofulaceum	Bridge[d], Lunning[d], Gaused[d]	e. The Tuberculosis Section, Laboratory of Pathology and Microbiology, Quensland Department of Health, Brisbane, Australia.
M. avium	16906-3380[d], 14141-1395[d]	f. The Department of Proventive Medicine, School of Veterinary Science, University of Queensland, Australia.
M. intracelluare[d]	Borne Iowa, 141186-1424, Warsik, 3259-685, Sweatman, Manten 157, Melnick, Findley, J2970, Lynn H., WS 52, S.J. Bull No.2. P42. 123 05-406, P54, Yandle	g. The Veterinary Research Laboratory, USDA, Iowa, USA.
M. fortuitum[d]	DEAE 504, Starr-Glidden, Taylor AT 455, Ramsey, 11997[e], 16660[e]	
M. gastri	W 995[b]	
M. aquae	9738[e]	
M. smegmatis	C-QV[f]	
M. johnei	P18[g]	

2. Preparation of crude gardenia(crocin) powder:

Dried fruit of gardenia was collected from the market and its rind was peeled off and yellow fruit was obtained. It was grounded in mortar and sieved through two layers of gauze. The fine powder was dried at 80°C for 60 minutes and kept in desicator until used. Crocin was extracted by boiling the crude gardenia in distilled water at 80°C for 30 minutes. With the aid of the Bausch and Lomb "Spectronic 20" absorption tubes to give an optical density A 415=0, 31± 0. 005 (water in blank) when 0. 1 ml of the extract (mg/ml) was diluted in 20 ml of distilled water.

3. Culture media:

Bacto-Dubos oleic agar and L-J coagulated egg medium bases containing crocin were prepared. Different volume of gardenia powder was boiled in distilled water or mineral salt solution of medium to be prepared. Dubos agar base was distributed in 180 ml amounts in 300 ml flasks, sterilized in autoclave for 15 minutes at 15 pounds pressure, cooled to 50°C and 20 ml of sterile normal bovine serum and 10. 000 units of penicillin were added. The medium was distributed in 5ml amounts in sterile 1 Oz McCartney bottles and slanted. L-J medium was distributed in 5 ml amounts in steile 1 oz McCartney bottles and inspissated by holding at 80°C in an electric oven for one hour.

Stock cultures of mycobacteria maintained on L-J medium and subcultured at approximately 6 months intervals were employed. For observation of pigmentation of culture, one loopful of a heavy growth of mycobacteria to be tested was plated on the slant of medium and cultured at 37°C for 20 days.

EXPERIMENT AND RESULTS

1. Optimal ratio of gardenia powder:

Experiments were designed to determine the optimal ratio of gardenia powder to culture medium. One loopful of a heavy growth of *M. fortuitum.* (DEAE 504) was plated each on L-J medium containing crocin extraction of 0. 35, 0. 70, 1. 40 2. 80 and 5. 0 mg/ml of gardenia powder and cultivated at 37°C for 7 days.

Table 2. Pigmentation of *M. fortuitum* on Löwenstein-Jensen medium Containing various amount of "gardenia powder.

Crocin powder (mg/ml)	Intensity of Pigmentation*							
	1	2	3	4	5	6	7	8 days
0. 00	−	−	−	−	−	−	−	−
0. 35	−	±	1+	1+	1+	2+	2+	2+
0. 70	±	±	1+	1+	1+	2+	2+	2+
1. 40	±	1+	1+	1+	1+	2+	2+	2+
2. 80	±	2+	3+	3+	4+	4+	4+	4+
5. 00	±	2+	3+	3+	4+	4+	4+	4+

* 4+strongly positive, 3+moderate positive, 2+positive, 1+weak positive, ± partial or doulitful, − negative.

Table 2 illustrates that the optimal ratio of gardenia powder for the most intensive pigmentation of culture was 2. 8 mg/ml. Moderate positive (3+) reaction was observed on this medium in 3 days.

2. Effect of aeration and exposure of light:

Effect of aeration and exposure of light on the production of pigment was observed. To determine the effect of aeration and light, four subcultures of each strain of *M. kansasii, M. aquae M. fortuitum* were slanted on L-J medium in one oz McCartney bottles. One of the subcultures was incubated in the dark with sufficient oxygen at 37°C for 24 days and others

Table 3. Effects of aeration and exposrue of light on pigmentation of mycobacteria.

Species of Mycobacteria	Aeration	Light*							Dark						
		3	5	7	9	11	22	24	3	5	7	9	11	22	24 days
Photochromogens															
M. kansasii (J2970)	Yes	−	1+	Y	Y	DG	DG	DG	−	1+	2+	G	G	G	DG
	No	−	1+	2+	3+	3+	3+	4+	−	1+	2+	4+	4+	4+	4+
Scotochromogen															
M. aquae (9738)	Yes	−	−	1+	BY	Y	Y	Y	−	−	BY	BY	BY	BY	BY
	No	−	−	1+	2+	3+	4+	4+	−	−	1+	3+	4+	4+	4+
Rapid grower															
M. fortuitum (DEAE 504)	Yes	2+	4+	4+	4+	G	DG	M	2+	4+	4+	4+	BG	BG	BG
	No	2+	4+	4+	4+	4+	4+	4+	2+	4+	4+	4+	4+	4+	4+

±; partial pigmentation, + to 4+; intensityof pigmenation.
Y; yellow, DG; dark green BY; bluish yellow, BG; bluish grey.
* Cultures were exposured to light (30 Wlamp) at a distance of 30 cm for one hour.

were tightened as control. Two subcultures were incubated for 6 days in the dark. When good growth develops, the bottle was exposed for one hour to light from a 30-W bulb at a distance of approximately 30 cm. The Cultures were reincubated for additional 18 days.

Table 3 illustrates that both aeration and exposure to light affect producing both yellow or orange pigment and violet pigment from cultures. When the strains were cultivated with sufficient oxygen the conventional yellow or orange pigment of photochromogens and sotochromogens were observed with a little change in color. On the contrary, cultures kept in tightened botttles produced a little more intensive violet pigment while conventional pigmentation was weak or not observed. Photochromogens produced a yellowish or orange -violet pigment only in the light, wherase scotochromogens produced it also in the dark although often to a lesser degree.

3. Characters of pigmentation on different medium base:

The formation of pigment of various mycobacteria on Dubos agar base and L-J medium base was compared.

It was characterized by producing mucilaginous cultures of all strains of *M. bavis*, *M. tuberculosis*, *M. kansasii*, *M. aquae*, *M. scrofulaceum*, *M. gastri* and *M. smegmatis* subcultured on Dubos medium base. All strains of *M. fortuitum* and *M. kansasii* showed positive rea-

Table 4. Comparison of pigmentation of mycobacteria on Dubos-oleic agar base and Löwenstein-Jensen egg medium base containing "Gardenia" powder(2.8 mg/ml).

Species	No. of strain tested	Dubos-oleicagar		L-J egg medium	
		Medium	Bacilli	Bacilli	Medium
M. bovis	4	−	−M	−	1/4P (BCG)
M. tuberculosis	4	−	−M	−	1/4P (Aoyama B)
M. kansasii	4	1/4	1/4M	4/4	4/4
M. marinum	2	—	—	—	—
M. aquae	1	—	−M	—	1
M.scrofulaceum	3	—	−M	—	—
M. gastri	1	—	−M	—	1
M. terrae	1	—	—	—	—
M. fortuitum	6	4/6	5/6	6/6	6/6
M. smegmatis	1	—	−M	—	1

—; no pigmentation, Number of pigmentation/number of tested strains.
M; Muc id type of cultures.
* cultured in the dark with sufficient oxygen at 37°C for 14 days.

Table 5. Pigmentation of opportunist mycobacteria on L-J medium with 2.8 mg/ml crocin powder.

Group	Number of strains tested	Pigmentation						
		3	5	7	9	11	15	20days
Group I								
M. kansasii	4	—	—	1+(3)	1+(4)	3+(4)	4+(4)	4+(4)
M. marinum	2	—	—	—	—	—	—	—
Group II								
M. scrofulaceium	3	—	—	—	—	±(1)	±(1)	±(1)
M. aquae	2	—	—	1+(2)	4+(2)	4+(2)	4+(2)	4+(2)
Group III								
M. avium	2	—	—	—	1+(1)	2+(1)	2+(1)	2+(1)
M. intracellulare	18	—	—	1+(5)	1+(8)	2+(9)	2+(13)	3+(13)
M. gastri	2	—	—	—	—	1+(1)	1+(1)	1+(1)
M. terrae	2	—	—	—	—	—	—	—
Group IV								
M. fortuitum	6	2+(5)	3+(6)	4+(6)	4+(6)	4+(6)	4+(6)	4+(6)
M. smegmatis	1	—	±	3+	4+	4+	4+	4+
Mammalian type								
M. bovis	4	—	—	—	—	—	1+(1)	1+(1)
M. tuberculosis	4	—	—	—	—	1+(1)	3+(1)	3+(1)
M. johnei	1	—	—	2+	3+	3+	3+	4+

—; negative; ±; partial reaction, 1+to 4+; intensity of pigmentation.
Number in the parenthesis; number of postive strains.

ction on L-J medium base while only 5 of 6 strains of *M. fortuitum* and one of 4 strains of *M. kansasii* produced violet pigment on Dubos medium base. It was observed that positive reaction on L-J egg medium base was superior to Dubos agar base medium.

4. Pigmentation of various mycobacteria:

To facillitate the Runyon's classsification of opportunist mycobacteria and saprophytes together with other important mammalian pathogens, *M. bovis*, *M.tuberculosis* and *M. johnei*, subcultures of mycobacteria were made by inoculating a loopful of culture (one match head) on L-J medium slant with extraction of 2.8 mg/ml crude gardenia powder and cultivated in the dark with sufficient oxygen at 37°C for 20 days.

Positive reaction was observed in subcultures of *M. kansasii*, *M. aquae*, *M. fortuitum*, *M. smegmatis* and *M. johnei*. Subcultures of *M. marinum*, *M. scrofulaceum* and *M. terrae* were differentiated from other species in the Runyon's groups with negative in 9 days culture. Strains of *M. fortuitum* was easily differentiated from other mycobacteria by crocin 3+ in 3 days culture. Pigmentation of *M. avium*, *M. intracellulare*, and *M. gastri* was indefinite by strains tested, but the number of positive strains mostly partiral reaction was increased by the extension of cultivation period. Three strains of *M. bovis* was negative, but BCG strain produced positive (1+) in 15 days culture. Of 4 strains of *M. tuberculosis*, 3 strains were negative, but one strains (Aoyama B) was positive (1+) in 11 days culture. The biological strain of johnin, M. johnei, P18 produced typical dark

brownish violet pigment in 7 days culture.

DISCUSSION

On the basis of growth rate on enriched medium at various temperature and determination of difference in pigment production, opportunist mycobacteria in relation to clinical significance are subdivided provisionally into four groups (Runyon 1959); Among these, photochromogens Group I consists of well defined species, *M. kansasii* (Hauduroy 1955) and *M. marinum* (Aronson 1926). *M. scrofulaceum* (Prissick and Masson 1956) is pathogen of scotochromogen Group Ⅱ. *M. intracellulare* (Runyon 1967), *M. avium* (Rivolta 1889) and M. xenopi (Schwabacher 1959) are the major pathogens of nonchromogen Group Ⅲ and Group Ⅵ rapid grower *M. fortuitum* (Cruz 1938) is known as pathogen.

Although Runyon's classification is not considered valid from the taxonomic point of view, this provisional subdivision of opportunist mycobacteria serves a valuable purpose of providing both clinians and bacteriologists to study epidemiology of opportunist mycobacterioses.

Lee and Son (1955) observed that some strains of mycobacteria grown on Ogawa medium containing gardenia extract produced violet pigment. They prepared crocin extract by boiling one gram of fruit of Fructose gardenia in 4 ml of distilled water at 100°C for 20 min. The extract was filtered and filtrate was added to Ogawa-Katakura medium at the ratio of 1 in 500 to 1,000 dilution. However, in this study, to save the time various amounst of fine gardenia powder was added to hot mineral salt solution of the medium in proportion to produce the desired concentration and shaken to be freely solved. This ext act was added to agar medium or egg medium or egg medium base without filtration.

The maximum volume of gardenia powder producing intensire crocin reaction was 2.8 mg/ml. Of the various factors, that play a role in the production of pigment, aeration was not so critical. When the strains of photochromogens and scotochromogens were culticated with suffucient oxygen on L-J medium containing crocin powder (2.8 mg/ml), conventional yellow or orange pigmentation of bacili was still observed with a little change in color. On the contrary, cultures kept in tightened bottle-cap produced an intensive violet pigment. However, conventional pigmentation of bacilli kept in tightened bottle was rather more obscure than that in loosened bottle-cap. The result indicates that oxygen is quite important in production of yellow or orage pigment of mycobacteria whereas the production of violet pigment is not affected as in others.

When production of violet pigmentation of mycobacteria on Dubos agar base and L-J egg medium base was compared, L-J medium base was superior to Dubos agar base in the sensitivity of pigmeptation. An interesting finding was that all strains of *M. bovis*, *M. tuberculosis*, *M. kansasii*, *M. aquae*, *M. scrofulaceum*, *M. gastri* and *M. smegmatis* cultivated on Dubos agar containing crocin produced smooth mucilaginous cultures whereas it was not observed on L-J egg base medium.

In this study, it is apparent that some species of opportunist mycobacteria could be further differentiated from other species of the Runyon's group by determination of differences in the pigmentation of mycobacteria grown on L-J medium containing 2.8 mg/ml gardenia powder. Among the photochromogens, *M. kansasii* was positive while *M. marinum* was negative. Group II pathogen *M. scrofulaceum* was negative while saprophyte *M. aquae* was positive. Group IV pathogen *M. fortuitum* was 3+positive at 3 dayes test while *M. smegmatis* showed partial or doubtful reaction at 5 days test. No pigmen-

tation of *M. bovis, M. tuberculosis, M. marinum, M. scrofulaceum, M. gastri* and *M. terrae* was appeaed at 7 days culture. It is of interest that johnin strain of *M. johnei*, P18, can be differentiated with 3+positive at 9 days test from strains of *M. avium* shown only 1+at 7 to 9 days test, but number of strains produced violet pigmentation (2 to 3+) was increased by the extension of cultivation time.

Generally, the production of violet pigment on L-J medium containing gardenia powder was first appeared on growing tubercle bacilli, se-con dly on medium around cultures and finally thr-oughout the medium. It suggests that the pig-mentation is possibly associated with some enzy-me or exotoxin produced by mycobadteria.

Although a result was not mentioned in the text, an interesting finding was that some strains of *M. bovis, M. tuberculosis* and *M. kansasii* grown on L-J medium containing crocin without malachite green reduced intrinsic yellow colour of crocin into egg white. This phenomenon was observed in three of 4 strains of *M. bovis*, three of 4 strains of *M. tuberculosis* and two of 4 strains of *M. kansasii* It is presumed that the crocin reduction is associated with some enzyme produced by mycobacteria.

SUMMARY

On the basis of pigment production and growth rate on L-J medium containing crocin, differen-tiation of opportunist mycobacteria belonging to photochromogens, scotochromogens, nonchrom-ogens and rapid grower has been investigated. Among photochromgens, positive pigmentation of *M. kansasii* was differentiated from negative strain of *M. marinum*. Scotochromogen *M. aquae* was positive whereas *M. scrofulaceum* was negative. Rapid grower *M. fortuitum* was positive at 3 days test whereas *M. smegmatis* was negative.

Subdivision of opportunist mycobacteria into four groups on the basis of growth rate and pigment production on L J medium containing gardenia extraction appeared to be a valuable adjunct to the Runyon's classification for the rapid presumptive identification of opportunist mycobacteria of different clinical significance.

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