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A STUDY ON CHARACTERISTICS OF ATYPICAL MYCOBACTERIA ISOLATED FROM SPUTUM SPECIMENS IN KOREA

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INTRODUCTION

In many countries of the world, studies on atypical acid-fast bacilli and their bacteriological and clinical significance have become an important field of research since Buhler and Pollak first reported human infection with "atypical acid-fast organisms" in 1953.[1]

Interest in this field in Korea was initiated in 1960 when Lee reported isolation of 28 strains of atypical acid-fast bacilli from the sputum cultures of the patients with "active pulmonary tuberculosis".[2]

In 1970, Kim et al. reported isolation of 97 strains of atypical mycobacteria from more than 22,000 sputum samples from Seoul and its vicinity,[3], but, unfortunately, there was hardly any mention on the virulence or clinical significance of these organisms in any of these reports in Korea.

Since the pathogenicity of atypical mycobacteria isolated in Korea is in doubt, the author carried out identification porcedures and experiments on the virulence of atypical mycobacteria for the white inbred mice.

MATERIALS AND METHODS

Fifteen strains of atypical mycobacteria were obtained from the acid-fast bacilli cultures in the Central Laboratory of the Korean Tuberculosis Association from February through May, 1970. These strains were isolated on 3% Ogawa medium from sputa following digestion with 4% NaOH or on Löwenstein-Jensen medium following digestion with oxalic acid, and only those strains yielding more than 20 colonies were used in the experiment.

These strains were classified according to Runyon's classification into photochromogens (Group I), scotochromogens (Group II), nonchromogens (Group III) and rapid growers (Group IV).[4] Subtyping of the organisms were done on the bases of the growth rate, pigmentation of

the colonies, colouring response upon exposure to light [5,6], niacin test [7,8], nitrate reduction [9], catalase activity [10], Tween 80 hydrolysis [11], and arylsulfatase activity [12]. Identification of the organisms was done on the basis of several methods of reference. [13,14,15]

The reference strains utilized in the identification were human type (H37Rv TMC # 102), bovine type (TMC # 401, Revanel), avian type (TMC # 706 Mckee # 1) and other atypical mycobacteria, *M. Kanasii* (TMC # 1201, Forbes, p-1), and *M. fortuitum* (TMC # 1530, Martin, PHS # 383), obtained from the mycobacterial culture collection of the Trudeau Institute of the United States.

The experimental animals used were male mice of SM strain (body weight, 13 ± 1 g), and 8 to 10 mice were used for each strain of atypical mycobacteria tested. The inoculum was cultured in Dubos liquid medium for 7 days, and was diluted to 1 mg/ml with distilled water, and 0.2 ml of the latter (average number of viable units, 21.7×10^5) was injected into the tail vein of each mouse.

The following observations were made:

1. Death rate of the animals up to 9 weeks following inoculation.
2. Inspection (*naked eye*) of the pathological changes in the animals sacrificed two at a time from each group at three week intervals up to 9 weeks following inoculation.
3. Weights of the lung and the spleen of the animals thus sacrificed were recorded, and the relative lung weight and the relative spleen weight were calculated according to the following formulae.

$$\text{Relative lung weight} = \frac{\text{lung weight (mg)}}{\text{body weight (g)}} \times 10$$

$$\text{Relative spleen weight} = \frac{\text{spleen weight (mg)}}{\text{body weight (g)}} \times 10$$

4. Weekly colony counts; the entire lungs of the sacrificed mice were put in 2% NaOH, and liquified in universal homogenizer at 15,000 rpm, and 0.1 ml of the material thus obtained was inoculated on each of 10 Löwenstein-Jensen media and was incubated at 37°C.

RESULTS

1. Identification and Classification.

By naked-eye inspection of the isolated colonies, the growth rate and the color were determined, and according to the photochromogen test, the isolated strains were classified according to Runyon's scheme as shown in Table 1. Nine strains (60%) were identified as nonchromogens, 4 were scotochromogens (26.7%) and 2 were rapid growers (13.3%). There were no photo-

Table. 1 Atypical Strains Classified by Runyon's Scheme

	No. of Stranis Ioslated	G-I	G-II	G-III	G-IV
No. of Strains	15	0	4	9	2
%	100.0	0	26.7	60.0	13.3

chromogens.

The results of various chemical tests were shown in Table 2 to 5. Among the 4 strains of scotochromogenic group, 3 were *M. scrofulaceum,* and 1 was identified as *M. flavescens* showing positive reactions to nitrate reduction test and Tween 80 hydrolysis test. Among the nine of nonchromogenic group, as shown in Table 3, 5 were Battey-avium, 3 were *M. terrae* complex showing positive reaction to nitrate reduction test and Tween 80 hydrolysis test, and 1 was *M. triviale* showing positive reaction to arylsulfatase test. The 2 rapid growers were identified as *M. fortuitum* growing on Löwenstein-Jensen medium within three days and showing positive

Table. 2 Scotochromogenic Group Identified by Various Biochemical Tests

Strain No.	Growth Within 7 days	Pigment D.	Pigment L	Niacin	Nitrate Reduct.	Catalase S.Q.	Catalase 68°C PH7	Tween 80 Hydrolysis 5dy	Tween 80 Hydrolysis 10dy	Arylsulf. 3dy	Arylsulf. 2wk	Species Identified as
443	—	Y	Y	—	—	50	+	—	—	—	—	*M. scroflaceum*
288	—	Y	Y	—	—	50	+	—	—	—	—	〃
183	—	Y	Y	—	—	50	+	—	—	—	∓	〃
535	∓	Y	Y	—	+++	50	+	±	+	—	±	*M. flavescens*

Note : — Negative
+ Trace but very weak positive
± Trace but weak positive
+ Positive
Y Yellow
D in dark
L in light
SQ Semiquantitative

Table. 3 Non-Chromogenic Group Identified by Various Biochemical Tests

Strain No.	Growth Within 7 days	Pigment D	Pigment L	Niacin	Nitrate Reduct.	Catalase S.Q.	Catalase 68°C PH7	Tween 80 Hydrolysis 5dy	Tween 80 Hydrolysis 10dy	Arylsulf. 3dy	Arylsulf. 2wk	Species Identified as
758	—	—	—	—	—	40	+	—	—	—	—	Battey-avian
447	—	—	—	—	—	40	+	—	—	—	—	〃
620	—	—	—	—	—	40	+	—	∓	—	—	〃
274	—	—	—	—	∓	40	+	—	—	—	—	〃
333	—	—	—	—	—	40	+	—	—	—	∓	〃
1278	—	—	—	—	+++	40	+	±	+	—	—	*M. terrae* Complex
906	—	—	—	—	+++	40	+	±	+	—	—	〃
61	—	—	—	—	+++	40	+	+	+	—	—	〃
130	—	—	—	—	+++	40	+	±	+	∓	+	*M. triviale*

Table. 4 Rapid Growers Group Identified by Various Biochemical Tests

Strani No.	Growth Within 7 days	Pigment D	Pigment L	Niacin	Nitrate Reduct.	Catalase S.Q.	Catalase 68°C PH7	Tween 80 Hydrolysis 5dy	Tween 80 Hydrolysis 10dy	Arylsulf. 3dy	Arylsulf. 2wk	Species Identified as
12	+	—	—	—	+++	50	+	—	∓	+	+	*M. fortuitum*
159	+	—	—	—	+++	50	+	—	—	+	+	〃

Table. 5 Result of Observations on Mice Infected with Atypical Mycobacteria

Species	Strain No.	No. of Mice Observed	Viable Unit Infected ($\times 10^5$)	Death Rate	Macuroscopic Findings	L/B Weight Ratio	S/B Weight Ratio
M. scrofla.	443	10	48	0	0	122	135
//	288	10	38	0	0	110	89
//	183	10	19	0	0	129	118
M. flaves.	545	9	24	0	0	120	103
Batt-avium	759	10	28	0	0	139	65
//	447	10	21	0	0	140	83
//	620	10	27	0	0	104	112
//	274	8	18	0	0	134	87
//	333	10	33	0	0	108	89
M. terrae	1278	10	18	0	0	98	65
//	906	10	19	0	0	118	66
//	61	10	11	0	0	130	59
M. triviale	130	10	6	0	0	127	123
M. fortuit	12	10	14	0	0	112	112
//	159	10	22	0	0	88	70
*M. bovis**		10	13	100.	+++	718	162
*M. avium**		10	18	0	0	86	not tested
*M. kansasii**		10	10	0	0	103	//
*M. fortuit.**		10	36	0	0	112	54
M. TB(H37Rv)*		10	18	90	+++	620	141
Control		8	0	0	0	116	44

Note : *Reference Starin L/B Lung and Body
Control not infected S/B Spleen and Body

Table. 6 Viable Units of Atypical Mycobacteria in Experimental Mice Lungs at Three-Week Intervals

Species	Strain No.	After 24 hr	3rd wk	6th wk	9th wk
M. scrofla.	443	56, 200	0	0	0
//	288	34, 000	0	0	0
//	183	14, 200	0	0	0
M. flaves.	545	8, 600	0	0	0
Batt-avium	759	50	0	0	0
//	447	34, 000	1, 500	0	0
//	620	35, 500	160	0	0
//	274	68, 500	1, 100	220	86
//	333	42, 500	1, 000	21	0
M. terrae	1, 278	78, 500	12, 500	3	0
//	906	12, 700	6, 300	11	1
//	61	92, 700	230	0	0
M. triviale	130	97, 500	1, 500	0	0
M. fortuit.	12	32, 500	8	0	0
//	159	600	0	0	0

reaction to arylsulfatase test.

2. Pathogenicity for mice.

Table 5 shows the results of 9 weeks observation of the mice inoculated with the atypical acid-fast bacteria and the reference strains. None of the experimental animals died within the 9 weeks of observation except those inoculated with the reference strains of H37Rv and *M. bovis*. Also the organs of the sacrificed mice did not show any pathological changes except those from the animals infected with the reference strains of H37Rv and *M. bovis*. showing severe granulomatous changes in the lungs. There was no appreciable difference in the relative lung weights between the study and the control groups, but only the spleen weight of the study group increased as compared with the uninfected control group.

In order to observe the persistence of the bacteria in the lungs, the viable units were counted at 24 hours, 3 weeks, 6 weeks, and 9 weeks as shown in Table 6, and there was a rapid decrease in the numbers of bacteria with the passage of time. *M. scrofulaceum* and *M. flavus* completely disappeared from the lungs within 3 weeks, but other groups of organisms showed persistence at 3rd week in considerable numbers. At 6th week, only a few colonies of Battey-avium 274, 333, and *M. terrae* 1,278 and 906 were obtained, but other strains completely disappeared, and at 9th week only Battey-avium 274, and *M. terrae* 906 survived in small numbers whereas all other strains disappeared from the lung tissue.

DISCUSSION

Since the first report of isolation of atypical mycobacteria from the sputum cultures of suspected tuberculosis patients in Korea in 1960,[2] epidemiological and bacteriological works have been published in this country. They uniformly reported recovery of many strains of atypical acid-fast bacilli belonging to Groups II, III, and IV according to Runyon's classification[2,3]

However, little information is available as to the clinical significance and virulence of the isolated atypical mycobacteria in Korea. The author's work, up to the time of this writing, represents the only animal experiment to evaluate the virulence of the atypical mycobacteria isolated from human sputa in Korea.

The white mice were used in this study for the possible superiority of the mice to the guinea pigs as experimental animals for the virulence study of atypical mycobacteria.[4]

The isolated strains of atypical mycobacteria in this study belonged to Group II, (26.7%), Group III (60%) and Group IV (13.3%) according to Runyon's classification. This is generally similar to the findings of other workers in Korea, especially the notable absence of the strains belonging to Group I, which are considered to be more virulent than others,[2,3] and the relative preponderance of strains belonging to Group III.[3,16]

As shown in Table 5, none of the strains of atypical mycobacteria tested caused death of the infected mice within the nine week period of observation, and only the relative spleen weight somewhat increased in the study group. The number of viable units cultured from the lung tissue of the infected mice rapidly decreased with the passage of time, and almost completely disappeared by the 9th week of observation.

In short, the fifteen strains of atypical mycobacteria belonging to Groups II, III and IV recovered from the sputum cultures of the radiologically diagnosed cases of "pulmonary tuberculosis" failed to cause demonstrable disease in mice when inoculated intravenously. The results of this study could at least partly be explained by the fact that there was no strain ascribable to Group I (photochromogen) which is generally considered to be the most virulent of all the atypical mycobacteria.[4] It is interesting to note, in this connection, that there is some similarity in the group distri- bution between the strains of atypical mycobacteria isolated from the soil samples[16] and those obtained from the human sputum cultures in Korea,[3] which show relative preponderance of Group III strains and absence of Group I strains. It is also noted, in this context, that atypical mycobacteria were cultivated from sputum samples of 7.5% of 290 apparently healthy men in tropical Australia, and most of the strains belonged to Groups II and III.[17] However, the possibility remains that these apparently innocuous organisms may cause opportune infection depending upon the condition of the host.

Another point to consider is that the author's experimental model including the route of inoculation may be unsatisfactory. While the author's experimental animals did not show any sign of disease, Blitek-Golc reported 50% mortality in the mice infected with Group III strains, but the route of inoculation was not mentioned in the abstract.[18] Japanese workers reported successful production of tuberculous lesions in the testicles of rabbits inoculated with atypical mycobacteria,[19] and even in guinea pigs inoculated by intraepididymal route.[20] Therefore, it is possible that pathological lesions could have been produced in the author's mice if various other routes of inoculation had been utilized.

Still another point to consider is finding the most suitable experimental animal for viulence tests of atypical mycobacteria. The success of any experimental animal inoculation of mycobacteria depends to a considerable extent upon the choice of an appropriate test animal. Recently, Choi et al. showed that Korean chipmunk proved to be a reliable experimental animal for *M. tuberculosis* even with the lower infectious dose (less than 5.8×10^3), the establishment of infection becoming manifest in the second week of inoculation.[21] In the author's opinion, further studies with atypical mycobacteria should be tried not only by various routes of inoculation in mice but also on some other experimental animals such as Korean chipmunk.

SUMMARY AND CONCLUSION

Fifteen strains of atypical mycobacteria obtained from sputum cultures of clinical cases of "tuberculosis" were classified into Groups II, III and IV by Runyon's classification. There was notable absence of Group I strains.

None of the test strains inoculated into mice by intravenous route for virulence test produced pathological lesions in mice, and the atypical mycobacteria rapidly disappeared from the lung tissue of the animals.

It is concluded that these strains of atypical mycobacteria from the sputum samples in Korea were of no significant pathogenicity in mice when inoculated by the tail vein, and that future trials should utilize other routes of inoculation or other experimental animals such as Korean chipmunk.

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韓国において喀痰から分離された非定型抗酸性菌の特長について

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韓国で1970年２月より５月迄に喀痰から分離された抗酸性菌の内，15株の非定型抗酸性菌の性状を検討した。その結果，Runyon 分類で第１群に属する菌株はなく，第２群４株３株（*M. scrofulaceum* ３株，*M. flavescens* １株），第３群９株（Battey-avium 型５株，*M. terrae* 群３株，*M. triviale* １株），及び第４群２株（*M. fortuitum*）が認められた。第１群がなく，第３群が最も多い点は従来の韓国での報告と一致する。

更に上記15株につき，その 10^5〜10^6 をマウス静脈に接種したが，９週間の間に死亡マウスはなく，肺内の生菌数も速かに減少した。非定型抗酸菌の毒力検定には上記の如き慣用法以外の方法，例えば動物を変える（Korean chipmunk を使用する）等をとるべきではないかと考える。