DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM INTRACELLULARE* BY RING DIFFUSION METHOD

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(Received December 8, 1979)

The susceptibility testing of 86 strains of *Mycobacterium intracellulare,* comprising 49 strains of human isolate, 21 of swine isolate and 16 of environmental isolate, to 10 kinds of anti-tuberculous drugs was performed, using ring diffusion method (HIRAMINE, S.: J. Med. Technol., 15 : 271~273, 1971). *M. tuberculosis* H 37 Rv strain served as the control.

Susceptibility to the first choice anti-tuberculous drugs : The minimal inhibitory concentration (MIC) of SM, PAS and INH against 86 strains of *M. intracellulare* were : SM, 20 μg/ml to 45 strains, 200 μg/ml to 38 strains, namely 200 μg/ml or less to 83 strains (97%) in total ; PAS, 10 μg/ml or less to 2 strains (2%) ; INH, 5 μg/ml or less to 19 strains (22%). The SM, PAS and INH completely inhibited the growth of H 37 Rv strain of *M. tuberculosis* at MIC of 20, 1 and 0.1 μg/ml, respectively.

Susceptibility to the second choice anti-tuberculous drugs : All the drugs tested completely inhibited the growth of H 37 Rv strain of *M. tuberculosis* at MIC : KM, 25 μg/ml ; CS, 20 μg/ml ; TH, 25 μg/ml ; EB, 2.5 μg/ml ; VM, 25 μg/ml ; CPM, 25 μg/ml ; and RFP, 10 μg/ml. The *M. intracellulare* strains inhibited to grow completely at the concentration noted above were few as follows, with the exception of 66 strains (77%) to RFP : KM, 24 strains ; CS, 3 strains ; TH, 2 strains ; EB, 3 strains ; VM, 5 strains ; and CPM, 1 strain. A consideradle number of strains were barely inhibited to grow at the high concentration : KM, 100 μg/ml to 53 strains (62%), 1,000 μg/ml to 8 strains (9%), namely 61 strains (71%) in total ; CS, 40 μg/ml to 59 strains (69%) ; VM, 100 μg/ml to 51 strains (59%) ; CPM, 100 μg/ml to 61 strains (71%). In the case of TH and EB, however, susceptible strains were limited as follows : TH, 50 μg/ml to 17 strains (20%) ; EB, 5 μg/ml to 13 strains (15%). The cumulative numbers of susceptible strains at the maximal inhibitory concentrations tested were as follows : KM, 1,000 μg/ml or less, 85 strains (99%) ; CS, 40 μg/ml or less, 62 strains (72%) ; TH, 50 μg/ml or less, 19 strains (22%) ; EB, 5 μg/ml or less, 16 strains (19%) ; VM, 100 μg/ml or less, 56 strains (65%) ; CPM, 100 μg/ml or less, 62 strains (72%) ; and RFP, 50 μg/ml or less, 83 strains (97%).

It is concluded that many strains of *M. intracelluare* were sensitive to RFP, whereas few strains were susceptible to SM, KM, CS, VM, and CPM. However, the susceptibility of this bacterium to anti-tuberculous drugs was generally lower than that of *M. tuberculosis.* These findings nearly coincided with those of previous investigators who used the routine dilution method.

Mycobacterium intracellulare is, in Japan, an important causative agent of the pulmonary infections with mycobacteria other than tubercle bacilli. It seems rather difficult to expect a therapeutic effect on the infection with this mycobacterium, because its susceptibility to various anti-tuberculous drugs is generally low. For the treatment of this infection, therefore, the following regimen is usually advocated at present : first, a combined administration of two or more drugs which have proved more or less effective by preliminary susceptibility testing to various anti-microbial agents especially to anti-tuberculous drugs, second, surgical treatment, when it is indicative.

As the anti-tuberculous drug increases in number, susceptibility testing of tubercle bacilli and nontuberculous mycobacteria to these drugs has become laborious and time-consuming task, when the routine dilution method is used. Then many attempts have been made to find a simpler technique for this purpose[1]. The ring diffusion method devised recently by HIRAMINE[2,4] is simple and economical, since susceptibility testing of tubercle bacilli to two or three kinds of antituberculous drugs can be made on one plate culture medium. The results obtained by this method are reported to be consistent with those by the dilution method in more than 90%. Previously, we tested the susceptibility of tubercle bacilli to streptomycin, para-aminosalicylic acid and isoniazid by HIRAMINE's method and confirmed these points[5].

The present study was designed to investigate susceptibility testing of *M. intracellulare* to various anti-tuberculous drugs by the use of this new method.

MATERIALS AND METHODS

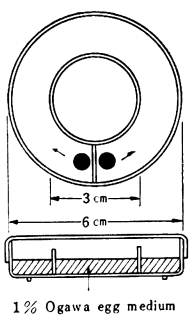
Organisms

Eighty-six strains of *M. intracellulare* stocked in our laboratory were used : they comprised 49 strains of human isolate, 21 of swine isolate and 16 of environmental isolate. *Mycobacterium tuberculosis* H 37 Rv strain served as the control.

Plate culture medium for ring diffusion method

Plate culture media were prepared in the following manner. Three ml of 1% OGAWA egg medium were poured into the central disk of 3 cm in diameter which was made in the plastic Petri dish of 6 cm in diameter and 1.5 cm in height ; 9 ml of the same medium were poured into the outer ring of 1.5 cm in width. The plates were then sterilized at 90°C for 60 minutes (Fig. 1). All the plates were prepared by Kobayashi Pharmaceutical Co., Osaka and stored at 4°C.

Fig. 1 Ring diffusion medium



The bacterial suspension was spread on the medium in the outer and inner rings of the plastic Petri dish. (●) indicates the disks containing drugs.

Drug susceptibility testing

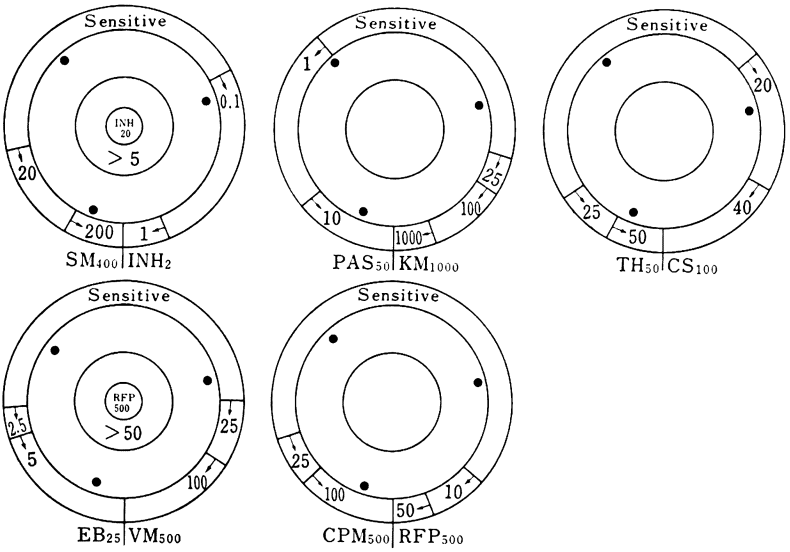
One loopful of 2-week old culture grown on 1% OGAWA egg slant was scraped off in a sterile bottom flask of 100 ml containing 40 glass beads and dispersed in 1 to 2 drops of saline by hand shaking and suspended in 4 ml of saline. It was then transferred to a test tube of 15×170 mm and kept at room temperature for 5 minutes to precipitate bacterial clumps. The bacillary suspension was adjusted to 0.15 of optical density at 540 nm of Spectronic 20 (Shimazu Manufacturing Co., Tokyo). A sponge with a stick pre-sterilized in gas (Kobayashi Pharmaceutical Co., Osaka) was absorbed fully the bacterial suspension, and it was pressed gently against the internal wall of a test tube so that a little amount of fluid escaped from the sponge. The bacterial suspension was then spread on the medium in such a way as passing the sponge over the surface of the plate. After the surface of the plate was dried enough, with the lid of the Petri dish partially opened, disks containing the following drugs were placed and pressd gently onto the plate with a sterile forceps. All the procedures were done in a safty hood. After 3 weeks incubution at 37°C, the susceptibility to the drugs was recorded by the evaluation table of Kobayashi Pharmaceutical Co. which was made according to HIRAMINE's paper (Fig. 2). The names and the concentration of the drugs contained in the disks (Eiken Kagaku, Tokyo) were as follows : streptomycin (SM), 400 μg ; isoniazid (INH), 2 μg and 20 μg ; para-aminosalicylic acid (PAS), 50 μg ; kanamycin (KM), 1,000 μg ; ethionamide (TH), 50 μg ; cycloserine (CS), 100 μg ; ethambutol (EB), 25 μg ; viomycin (VM), 500 μg ; capreomycin (CPM), 500 μg ; and rifampicin (RFP), 500 μg.

RESULTS

Susceptibility to the first choice anti-tuberculous drugs

The minimal inhibitoy concentrations (MIC) of SM, PAS and IMH against 86 strains of *M. intra-*

Fig. 2. Judgement table for susceptibility testing by ring diffusion method



1. Petri dish is placed on the judgement table and the zero points (●) are set. Judgement for sensitivity and resistance (complete and incomplete) are done by examining the extension of bacillary growth to certain concentration area and by the difference of extension of bacillus as compared to control at the time when the bacillus is beyond the bordering line.
2. The presence or absence of resistance to 5 μg of INH (or 50 μg of RFP) is determined only by either the presence or absence of growth of bacillus. The concentration of each agent is shown as μg/ml.

Table 1 Susceptibility of 86 strains of *M. intracellulare* to the first choice antituberculous drugs

Drugs	MIC (μg/ml)	Number of strains	Total
SM	20	45(52%)	83(97%)
	200	38(44%)	
PAS	1	1(1%)	2(2%)
	10	1(1%)	
INH	0.1	0	19(22%)
	1.0	1(1%)	
	5.0	18(21%)	

Table 2 Susceptibility of 86 strains of *M. intracellulare* to the second choice antituberculous drugs

Drugs	MIC (μg/ml)	Number of strains	Total
KM	25	24(28%)	85(99%)
	100	53(62%)	
	1,000	8(9%)	
CS	20	3(3%)	62(72%)
	40	59(69%)	
TH	25	2(2%)	19(22%)
	50	17(20%)	
EB	2.5	3(3%)	16(19%)
	5.0	13(15%)	
VM	25	5(6%)	56(65%)
	100	51(59%)	
CPM	25	1(1%)	62(72%)
	100	61(71%)	
RFP	10	66(77%)	83(97%)
	50	17(20%)	

cellulare were summarized in Table 1 : SM, 20 μg/ml to 45 strains, 200 μg/ml to 38 strains, namely 200 μg/ml or less to 83 strains (97%) in total ; PAS, 10 μg/ml or less to 2 strains (2%) ; INH, 5 μg/ml or less to 19 strains (22%). The SM, PAS and INH completely inhibited the growth of H37Rv strain of *M. tuberculosis* at MIC of 20, 1, and 0.1 μg/ml, respectively (data not shown).

Susceptibility to the second choice anti-tuberculous drugs

All the drugs tested completely inhibited the growth of H37Rv strain of *M. tuberculosis* at MIC : KM, 25 μg/ml ; CS, 20 μg/ml ; TH, 25 μg/ml ; EB,

2.5 μg/ml ; VM, 25 μg/ml ; CPM, 25 μg/ml ; and RFP, 10 μg/ml. The MIC of these drugs against 86 strains of *M. intracellulare* were summarized in Table 2. The strains inhibited to grow completely at the concentration noted above were few as follows, with the exception of 66 strains (77%) to RFP : KM, 24 strains ; CS, 3 strains ; TH, 2 strains; EB, 3 strains ; VM, 5 strains ; and CPM, 1 strain. A considerable number of straine were barely inhibited to grow at the high concentration : KM, 100 μg/ml to 53 strains(62%), 1,000 μg/ml to 8 strains (9%), namely 61 strains(71%) in total ; CS, 40 μg/ml to 59 strains (69%) ; VM, 100 μg/ml to 51 strains (59%) ; CPM, 100 μg/ml to 61 strains (71%). In the case of TH and EB, and EB, however, susceptible strains were limited as follows : TH, 50 μg/ml to 17 strains (20%) : EB, 5 μg/ml to 13 strains (15%). The cumulative numbers of susceptible strains at the maximal inhibitory concentrations tested were as follows : KM, 1,000 μg/ml or less, 85 strains (99%) ; CS, 40 μg/ml or less, 62 strains (72%) ; TH, 50 μg/ml or less, 19 strains (22%) ; EB, 5 μg/ml or less, 16 strains (19%) ; VM, 100 μg/ml or less, 56 strains (65%) ; CPM, 100 μg/ml or less, 62 strains (72%) ; and RFP, 50 μg/ml or less, 83 strains (97%).

DISCUSSION

The *in vitro* susceptibility of *M. intracellulare* to various anti-tuberculous drugs is lower than that of *M. tuberculosis*. On the application of chemotherapy for this infection, therefore, it is necessary to choose a combination therapy with drugs which have proved a little or more effective by susceptibility testing to various anti-tuberculous drugs. It is the reason why any simpler method for susceptibility testing to various anti-tuberculous drugs is required.

Previously, we[5] performed comparative studies on dilution method and ring diffusion method in drug susceptibility testing of *M. tuberculosis* (33 strains), and reported that the either result was nearly consistent with each other, rate of correspondence being 88% for SM, 20 μg/ml ; 97% for SM, 200 μg/ml ; 91% for PAS, 1 μg/ml ; 94% for PAS, 10 μg/ml ; 91% for INH, 0.1 μg/ml, 97% for INH, 1 μg/ml ; 94% for INH, 5 μg/ml. Our data were quite similar to those of HIRAMINE[2,4].

In the present study, we have adopted solely ring diffusion method for susceptibility testing of *M. intracellulare* to various anti-tuberculous drugs and compared the results with those of previous investigators.

The H 37 Rv strain of *M. tuberculosis* was completely inhibited to grow with the first (SM, PAS, and INH) and the second (KM, CS, TH, EB, VM, CPM, and RFP) choice anti-tuberculous drugs at the minimal concentrations tested as indicated in the Guide for Examination of Tubercle Bacillus[1]. However, there were no strains of *M. intracellulare* showing such high suceptibility to these drugs except for RFP which showed complete inhibition against 66 of 86 strains (77%) at the minimal concentration tested, 10 μg/ml. According to several authors[5-7], *M. intracellulare* was resistent to most anti-tuberculous drugs, although few strains were sensitive slightly to SM, VM, CS, KM and EB.

Except for EB, a similar tendency has been observed in our present study using ring diffusion method. As for RFP, RYNEARSON *et al.*[9] described that it had less effect against this bacterium grown on 7 H 10 agar. On the other hand, KUZE *et al.*[8] demonstrated that RFP was generally the most active against this bacterium cultured in DUBOS liquid medium, although the effect was variable depending upon strains. We have observed a similar tendency in the present study where ring diffusion method was used.

In conclusion, ring diffusion method is of use for susceptibility testing to anti-tuberculous drugs not only of *M. tuberculosis* but also of *M. intracelluare*, although further studies are necessary as for EB.

SUMMARY

The susceptibility testing of 86 strains of *M. intracellulare* to 10 kinds of anti-tuberculous drugs was performed, using ring diffusion method. It was shown that many strains of this bacterium were sensitive to rifampicin, whereas few strains were susceptible to streptomycin, kanamycin, cycloserine, viomycin, and capreomycin. However, the susceptibility of *M. intracellulare* to anti-tuberculous drugs was generally lower than that of *M. tuberculosis*. These findings nearly coincided with those of previous investigators who used the routine dilution method.

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リング拡散法による *Mycobacterium intracellulare* の薬剤感受性試験

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Mycobacterium tuberculosis H 37 Rv 株を対照とし，*M. intracellulare* 計 86 株（ヒト由来 49 株，ブタ由来 21 株，自然界由来 16 株）の 10 種の抗結核剤，streptomycin (SM), isoniazid (INH), para-aminosalicylic acid (PAS), kanamycin (KM), ethionamide (TH), ethambutol (EB), cycloserine (CS), viomycin (VM), capreomycin (CPM) および rifampicin (RFP) に対する感受性試験をリング拡散法（平峰　繁：臨床検査，15 : 271, 1971）で検討した結果，概略以下述べるような知見を得た。

1) *M. tuberculosis* はリング拡散法によっても結核菌検査指針（室橋豊穂ら：64～70 頁，日本公衆衛生協会，東京，1972）に記載された諸種薬剤の最低濃度によって完全な発育阻止がみられた。

2) *M. intracellulare* では供試薬剤の最高稀釈濃度で完全発育阻止のみられた菌株は SM (≦200 μg/ml), KM (≦1,000 μg/ml) および RFP (≦50 μg/ml) ではそれぞれ 83 株 (97%), 85 株 (99%) および 83 株 (97%) と極めて高率に，また CS (≦40 μg/ml) および CPM (≦100 μg/ml) では各 62 株 (72%), VM (≦100 μg/ml) では 56 株 (65%) と比較的高率にみられたが，菌株の由来の別なく，*M. tuberculosis* よりも一般に低い感受性を示したことは汎く慣用されている稀釈法による成績と何ら選ぶところはなかった。なお，他の薬剤 (PAS, INH, TH および EB) に対しては感受性菌株の頻度は極めて低率 (2～22%) であった。

3) 上記のリング拡散法による *M. intracellulare* の諸種抗結核剤に対する感受性試験成績はこれら薬剤による本菌感染症の臨床効果（山本正彦：薬物療法，9, 19, 1976）とよく一致するもののようであった。