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BACTERIOLOGICAL STUDY OF *MYCOBACTERIUM HABANA*

by

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Mycobacterium habana was described by J. Valdivia Alvarez and coworkers in Bol. Higiene y Epidemiologia, *9*, 65-73 (1971), and mentioned for the first time to a larger audience in the summer of 1971 during the 21st Tuberculosis Congress in Moscow.

The original description concerns mycobacterial strains obtained in a reference laboratory in Habana, Cuba, from regional laboratories; 24 of them as pure cultures, 11 as cultures mixed with *M. tuberculosis.*

The strains were apparently not considered to be clinically significant. They were considered to belong to Runyon group III but with a very special character : production of large amounts of niacin.

We obtained 3 strains of *M. habana* from Dr. Valdivia Alvarez in March 1972, and wish to report our findings.

MATERIALS AND METHODS

These were described previously (Pattyn and Portaels, 1972).

For thin layer chromatography of mycobacterial pigments the technique described by Tarnok and Tarnok (1970) was followed, but using growth on Loewenstein Jensen medium. The malachite green pigment was revealed in each chromatogram but dit not hinder the interpretations.

The strains studied were numbered

Habana :	337-5 I	Antwerpen :	M 2902
	337 II		M 2903
	337 III		M 2904

For comparison the following strains were studied :

M. simiae strain 20 received from W. Käppler-Berlin (Weiszfeiler *et al.*, 1970);

M. simiae strain 50 received from W. Käppler-Berlin (Weiszfeiler *et al.*, 1970);

M. simiae strain 29 received from W. Käppler-Berlin (Weiszfeiler *et al.*, 1970);

M. asiaticum strain 61 receveid from W. Käppler (Weiszfeiler *et al.*, 1971);

M. asiaticum strain 64 received from W. Käppler (Weiszfeiler *et al.*, 1971).

Other strains were the subject of previous studies (Pattyn *et al.*, 1968).

RESULTS

Growth on Loewenstein Jensen medium is eugonic, smooth, readily suspendable in water.

Colony morphology on Dubos oleic agar is of the SmS type most frequently produced by *M. intracellulare.*

Pigment production on L. J. medium is of the delayed type. When the photoinduction test is performed, there is rarely some difference between the light exposed and the non light exposed cultures after 2 to 3 days. However upon further observation yellow pigment defenitely appears 1 or 2 weeks later.

This influence by light is quite different from the one we observe regularly with real photochromogenic *M. kansasii* or *M. marinum* strains.

The pigmentation is so pronounced that if the investigator dit not observe the evolution during incubation, he would definitely consider these strains as scotochromogens.

Bacterial morphology as revealed by the Ziehl-Neelsen strain is that of classical short acid fast rods.

The strains develop at room temperature, 37 °C, and 42 °C, not at 44.5 °C. There is no development on peptonagar and thus no reduction of iron ammonium citrate.

Niacin production as revealed by the Konno technique is strongly positive producing even an orange instead of the usual yellow color.

In order to make sure that this was not a false positive reaction the substance responsable was chemically identified.

The 3 strains of *M. habana* and one strain of *M. tuberculosis* were cultured in fluid TB broth (Difco). After 3 weeks the cultures were centrifuged and filtered on a Seitz filter.

Thinlayer chromatography was performed on silicagel G (0.3 mm thickness) with water eluent and detection of niacin with cyanogenbromide after treatment of the plates as described by Stahl (1967).

Bidimensional chromatography was necessary to obtain satisfactory proof of identity. The first elution being actually a clean up and the second one a comparison with the reference substance.

Thus there is no doubt that the *M. habana* strains are niacin producers.

Results of further biochemical tests are as follows :

— nitrate is not reduced
— phosphatase reaction is negative
— catalase production is high (100 mm) and not inactivated by heat (68 °C)
— the strains are inhibited by high oleate concentration
— there is no cording in albumin containing fluid medium
— the strains deaminate urea, nicotinamide, pyrazinamide; pattern 3, 5, 6 (Bönicke 1962).

The strains are fully resistant for INH (1-5 mcg/ml), streptomycin, para-amino-salicylic acid, rifampicin and 2 out of 3 for ethambutol (5 mcg/ml).

Results of animal inoculations

Mice and chickens were inoculated by the intravenous route (resp. 0.25 ml and 1 ml of a 1 mg/ml suspension) guinea pigs and 2 monkeys (*Macaca paraensis*) subcutaneously.

Observation periods were : chickens and guinea pigs : 2 months, mice : 6 weeks.

None of animals died from the infection.

The guinea pigs and monkeys had no macroscopic nor microscopic lesions.

The livers of mice and chickens however revealed a considerable number of tuberculous follicles containing a moderate to small number of acid-fast bacilli.

This picture is compatible with what we regularly observe with *M. intracellulare* strains in these animals.

Results of agglutination tests

The 3 strains were all agglutinated to titer by a Darden serotype anti-serum, and not by antisera for *M. kansasii*, *M. gastri*, scotochromogens *M. avium*, and other serumtypes of *M. intracellulare*.

However there is no complete antigenic identity between *M. habana* and serotype Darden.

This is revealed first by the low titer agglutination of Darden antigen by anti-habana serum and secundly by the results of cross-absorption tests.

They reveal that *M. habana* and Darden strains possess some common but also a different antigen.

TABLE 1

RESULTS OF CROSS ABSORPTION TESTS BETWEEN *M. HABANA* AND A SEROTYPE DARDEN STRAIN

	Habana serum no absorption		Darden serum no absorption	
Antig. habana	320		160	
Darden	20		320	
absorbed by	Habana	Darden	Habana	Darden
Antig. habana	0	160	0	0
Darden	0	0	80	0

Comparison with M. simiae *and* M. asiaticum *strains*

Table 2 shows the overall cultural characters of the *M. simiae* and *M. asiaticum* strains in comparison with those observed for *M. habana*.

TABLE 2

CULTURAL CHARACTERS OF *M. HABANA*, COMPARED WITH *M. SIMIAE* AND *M. ASIATICUM* STRAINS

	M. habana	*M. simiae* 20	*M. simiae* 59	*M. simiae* 29	*M. asiaticum* 61 and 64
Pigment	slowly photo	—	light yellow	—	light yellow
Niacin	++	++	—	—	—
NO_3	—	++	—	+	—
PO_4	—	—	—	—	+
37 °C	+	+	+	+	+
42 °C	+	+	+	+	+
44.5 °C	—	—	—	—	—
INH 1 γ	R	R	R	R	R
INH 5 γ	R	R	S	S	S
TCH	R	R	R	R	R
Cat.	> 100 mm	40 mm	> 100 mm	> 100 mm	> 100 mm
Cat. 68 °C	++	30 mm	60 mm	60 mm	60-100 mm
Col.	SmS	SmS	SmS	SmS	SmS
Agglut. Darden	+	—	—	—	—
Amidases	3,5,6	3,5,6	3,5	3,5,6	5,6

Results of thin layer chromatography of pigments

Since *M. habana* strains develop a yellow pigment after a few weeks incubation, thin layer chromatography of the extracted pigment was performed on the strains *M. habana M. kansasii,* the different species composing the scotochromogenic group (Pattyn *et al.*, 1968) *M. intracellulare*, *M. simiae*, and *M. asiaticum.*

The results of this study may be summarised as follows.

The chromatographic patterns of the pigment of the 3 *M. habana* strains are very similar although there are slight reproducible differences.

The pigments of *M. habana* strains differ from those of *M. kansasii*, the scotochromogenic species (*M. gordonae*, *M. scrofulaceum*, *M. flavescens*, our « second group » scotochromogens) they are also different from those of *M. simiae* and *M. asiaticum.*

The pigments of the 3 *M. simiae* strains are not identical among each other.

DISCUSSION

Strains presently designated as *M. habana* have certainly some pecular characters. Although they are producers of huge amounts of niacin, they cannot be mistaken for *M. tuberculosis* : their aspect on LJ medium, smoothness, colony morphology, pigment production, absence of nitratase, polyresistance are sufficiently characteristic.

Unfortunately we were not in a position to apply the numerical taxonomy procedure comparing the strains with many others. The case of *M. habana* once more indicates the need for the possibility to add results of new studies to previously stored data p. ex. those of Wayne *et al.* (1971), Kubica *et al.* (1972), Meissner *et al.* (to be published).

Although *M. habana* has amidases 3, 5, 6 its pigment develops slowly and it is serologically related to *M. intracellulare*. It is difficult to relate *M. habana* to either *M. asiaticum* or *M. simiae.*

The first species as far as our results go (table 2) belongs to the species *M. intracellulare*, while *M. simiae* strains are not homogenous : they differ from each other in nitrate reduction and INH sensitivity.

At this moment *M. habana* strains can be considered as a bona fide new mycobacterial species whose minidefinition (Cowan and Steel, 1965) could be : slowly mesophilic photochromogenic mycobacterium producing considerable amount of niacin; nitrate reduction and phosphatase are negative; they are highly resistant for INH, and possess a high thermostable catalase. On Dubos oleic agar SmS colonies are produced, amidase pattern is 3.5.6. (Bönicke, 1962).

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DISCUSSIE — DISCUSSION

H. Boisvert : Mon attention avait été attirée par une étude collective, publiée à propos de *M. simiae* (Karasseva et Weiszfeiler) et j'avais demandé la souche au Dr Schröder. Assez rapidement j'ai identifié une souche qui provenait de Dakar au Sénégal. Il s'agissait d'une ménagère, mais il n'y avait qu'un seul isolement. Dans mes fiches j'ai ensuite retrouvé des études qui avaient été faites sur des malades provenant pour la plupart également de Dakar, qui avaient pour particularité d'avoir une uréase et de ce fait, nous les avons recherchées chaque fois, des amidases en 3, 5, 6. Un de ces cas est particulièrement intéressant, puisqu'il venait d'un sanatorium de la région parisienne; il s'agissait alors d'une mycobactériose pulmonaire et j'avais identifié à cette époque la souche comme *M. intracellulare.* Ce malade avait vécu quatre ans en Afrique. J'ai isolé d'autre part parmi les souches isolées du singe par le Dr Coletsos, une souche correspondant à *M. simiae.* J'avais parlé de ces différentes souches au Dr Runyon, qui m'a dit qu'il s'agissait de *M. habana* et m'a envoyé la souche 4.238. J'ai fait une étude comparative entre *M. simiae* D29 et deux ou trois autres souches que j'avais identifiées et je suis convaincu qu'il s'agit de la même espèce. A première vue cette espèce ressemble à *M. intracellulare* pigmenté; elle présente deux particularités : les amidases en 3, 5, 6 avec l'uréase positive par conséquent. J'ai pu voir que l'hydrolyse du Tween 80 était négative, de même que l'arylsulphatase et la β-galactosidase; le niacine-test par contre était fortement positif. Les souches étaient fortement résistantes à tous les antibiotiques, sauf la Cycloserine.

H. Huitema : If I did not misunderstand, comparative PPD tests have so far not been carried out.

I suggest that in order to clarify the allergenic relationship between *M. habana* and other mycobacteria, this method should be used in the first instance.

A further step might be the preparation of a specific sensitin.

J. Weiszfeiler : Je suis très intéressé par la communication sur *M. habana* et ce que nous avons entendu du Dr Boisvert. Les souches de *M. simiae* paraissent par leur réaction enzymatique et par leur caractère très semblable à *M. habana* et je pense qu'on devrait procéder à une comparaison de ces souches. Je parlerai dans ma communication encore plus de cette question, mais je proposerais aussi de faire une comparaison de façon tout à fait objective de ces deux espèces.

G. Meissner : Dr Pattyn has said that these strains have amidase-spectrum 3, 5, 6. I cannot imagine that these strains should be *M. intracellulare* strains. I think that it must be something else.

W. Käppler : In our laboratory we have also studied strains of *M. habana* and according to the results I think *M. habana* is related to the monkey strains of Weiszfeiler, perhaps to *M. simiae*, but we have no results with serotyping.

G. Meissner : I have another question to Dr Valdivia. He said that he had met in patients a tuberculin reaction of 15 mm etc., but was it a human or bovine tuberculin or a tuberculin of *M. habana* ?

J. A. Valdivia : It was prepared from PPD RT23 from Copenhagen.

G. Meissner : A very high reaction for a conreaction in the patients. The difficulty with even these patients is that they may have had in former times a real tuberculosis and perhaps this tuberculin reaction also may be an explanation for the high number of mixtures in the sputum with *M. tuberculosis* and *M. habana.* It may be that after treatment the *M. habana* has remained as the only habitat.

S. R. Pattyn : We have only tested the classical anti-tuberculous agents : INH, PAS streptomycin, ethambutol and rifampicin. The strains were found to be resistent, except one of the strains that was inhibited by 5 microgram of ethambutol which is a relatively high concentration. I could say that with the exception of the niacin test, these strains differ in all other characters so much from *M. tuberculosis* that they cannot be mistaken.