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Further studies on African strains of *Mycobacterium tuberculosis*. Comparison with *M. bovis* and *M. microti*

BY

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Summary — African strains of *M. tuberculosis* originating from Accra (Ghana), Dakar (Senegal), Kinshasa (Congo) and Butare (Rwanda) were examined and characters compared with *M. bovis* and *M. microti*.

The occurrence of dysgonic strains of *M. tuberculosis* was highest in Rwanda (81 percent).

Most of the West African strains are poor nitrate reducers and are inhibited by small doses of TCH (0.2-1 mcg/ml).

Nitrate reduction is more easily detected among strains from Rwanda, they are also in general less sensitive to TCH (1-5-15 mcg/ml). All kind of transitions occur between these two.

When viewed from the standpoint of numerical taxonomy, these strains are highly different from *M. microti* and *M. bovis* but closely related with *M. tuberculosis*, the differences with *M. tuberculosis* are not greater than the differences among each other so that to consider them as a different species is not justified.

Introduction

During the last ten years African strains of *Mycobacterium tuberculosis* have been studied more closely with the aid of modern *in vitro* tests. Gradually it has become clear that quite a number of strains isolated in Africa or from African patients show some differences in these tests as compared with the strains of *M. tuberculosis* we are most familiar with in Europe.

(Discussion IIe Colloque Internat. Mycob. Antwerpen, 1961, *in* Ann. Soc. belge Méd. trop. 1962, 42, p. 561; Hermans-Boveroulle M.T. *et al.*, 1965; Castets *et al.*, 1968; Meissner, Wallace, cited by Castets *et al.*, 1968.)

We have reinvestigated this problem, comparing strains isolated in Kinshasa (Congo), Dakar (Senegal), Accra (Ghana) and Butare (Rwanda).

Strains of *M. bovis* and *M. microti* were studied for comparison.

TABLE 1

Results obtained with *Mycobacterium bovis*

No.	Origin	Growth in Lebek in mm	Niacin	Nitrate reduction		Inhibition by		
				Virtanen	Tacquet	INH	TCH	Pyrazin-amid
						mcg/ml		
M 929	ATCC 19210	10	–	–	–	0.1	1	> 50
M 281	Zoo	9	–	–	–	0.1	0.75	
M 322	Zoo	8	–	–	–	0.1	0.50	
M 323	Zoo	8	–	–	–	0.1	0.75	
M 378	Zoo	10	–	–	–	0.1	0.50	
M 401	Zoo	8	–	–	–	0.1	0.20	
M 505	Zoo	7	–	–	–	0.1	0.75	
M 740	Zoo	12	–	–	–	0.1	0.75	
M 895	Zoo	7	–	–	–	0.1	1	
M 908	Zoo	8	–	–	–	0.1	1	> 50
M 927	Human	7	–	–	–	0.1	N. T.	> 50
M 1277	Human	8	–	–	–	0.1	0.5	> 50

Materials and methods

Strains examined

Two dysgonic *M. tuberculosis* strains (M 836-1344) isolated in Kinshasa, belonging to the group studied in 1965 (Boveroulle *et al.*, 1965).

Eight dysgonic *M. tuberculosis* strains recently isolated in Kinshasa by one of us (J.M.). These dysgonic strains were detected among isolates distributed as follows :

	Eugonic	Dysgonic
Pulmonary	29	6
Extrapulmonary	7	2

Eighty-four *M. tuberculosis* strains isolated in Butare — Rwanda — by one of us (L.S.).

Four dysgonic *M. tuberculosis* strains isolated in Dakar — Senegal — and provided by N. Rist, Institut Pasteur, Paris, France.

Six *M. tuberculosis* strains isolated in Accra — Ghana — and sent by Dr G. Meissner, Borstel, Germany.

Twelve *M. bovis* strains from our collection : ATCC 19210, 9 strains isolated from zoo — and slaughterhouse animals (Pattyn *et al.*, 1967) and 2 strains isolated from man.

Eleven *M. microti* strains : ATCC 19422, the dassie bacillus (Wagener *et al.*, 1958) 2 strains isolated by Huitema *et al.*, (1967); 3 strains from the Institut Pasteur, Paris, France (H. Boisvert); 3 strains received from the Tuberculosis Institute, Prague (Dr Sula) (2 vaccine strains MP and M 84 and the Oxford vole strain 191); finally 1 strain isolated in our laboratory from a zoo llama (Pattyn *et al.*, 1970).

Techniques

All techniques have been published before (Pattyn *et al.*, 1964; Pattyn *et al.*, 1965; Hermans-Boveroulle *et al.*, 1965; Pattyn, 1966).

Nitrate reduction was detected by the techniques of Virtanen (1960) and Tacquet *et al.* (1966).

Some strains were also plated on Dubos oleic agar containing triton as described by Lorian (1967).

TCH sensitivity was determined by the proportion method on L-J medium either in a « long » series (0.1, 0.2, 0.5, 0.75, 1, 2.5, 5, 12.5 mcg/ml) or a « short » series (1, 5, 15, 25 mcg/ml). Some strains were also tested for sensitivity to 4-amino-diphenyl-sulfone or DDS.

TABLE 2

Characters of *M. microti*

No.	Origin	Growth on				Niacin	Nitrate reduction	Sensitivity		Amidases
		L-J	Lebek.	W. M.	Dubos O. A.			TCH	Pyraz.	
								mcg/ml		
422	Dassie	D	15	Cr	Oc	+	−	0.5		(3).5.6
935	ATCC 19422	D	7	Cr	Oc	+	−	< 0.1	< 20	3.5.6
1278	I. P. Paris	D + E	3	Cr	Oc	+	−	0.1		3.5.6
1279	Huitema (cat)	D	10	D	RR	+	−	< 0.1	< 20	3.5.6
1290	I. P. Paris	D + E	10	Cr	Oc	+	−	0.2		3.5.6
1291	Huitema (swine)	D	10	D	Oc	+	−	1	< 20	
1292	I. P. Paris	D + E	3	Cr	Oc	+	−	0.2	< 20	3.5.6
1376	Antwerp llama	D + E		Cr	Oc	+	−	0.2		
1429	Prague MP 246	D + E	10	Cr	Oc	+	−	0.2		
1430	Prague M 84	D + E		Cr	Oc	+	−	< 0.1		3.5.6
1431	Prague OV 191	D + E	7	Cr	Oc	+	−	0.1		

D = small, dome shaped colonies, dysgonic growth.
Cr = crateriform colonies.
D + E = mixed dysgonic, eugonic.

Results

1. Mycobacterium bovis

The characters of these strains were classical (table 1).

Growth is dysgonic on Loewenstein-Jensen medium.

In Lebek's agar (1968) all strains develop in a zone 7-12 mm from the surface, an occasional strain grows as deep as 15 mm.

Growth on Wagener and Mitscherlich medium (1951-52) is very dysgonic : 1 mm circular, glistening colonies develop after 1 month.

On Dubos oleic agar, colonies are of the RR type, indistinguishable from *M. tuberculosis.*

Cording appears on triton containing Dubos oleic agar.

Niacin is not produced.

Nitrate reduction is negative when tested both by the method of Virtanen and that of Tacquet.

Catalase is weakly positive (3-5 mm) or absent, thermolabile.

Of six strains tested for amidases, one strain was urease positive, the others were negative.

All strains were inhibited by 0.1-0.2 mcg INH/ml and by 0.2 to 0.75 mcg TCH/ml. However on prolonged incubation some growth occurred sometimes up to 5 mcg/ml of the latter compound.

Four strains tested were resistant to 50 mcg/ml of pyrazinamid.

Mice injected intravenously with 0.2 mg bacilli (Wet weight) develop progressive tuberculosis in spleen, liver and lungs, within 6 weeks with some animals dying during this observation period.

2. Mycobacterium microti (table 2)

On Loewenstein-Jensen medium, 2 types of growth are observed : a very dysgonic growth in 4 cases (dassie strain, ATCC 19422 and the two Huitema strains) composed of hemispheric pearly colonies; and a mixed dysgonic-eugonic growth, producing heaped up, umbilicated (« wreath shaped » Sula, 1958) crateriform colonies on a dysgonic basis in 7 instances.

Microscopically, the typical hooked, curled, s and circleshaped bacilli were observed in the freshly isolated llama strain only.

However all strains showed this particular morphology in organs of experimentally inoculated mice.

In Lebek's agar, 2 strains develop in the upper 3 mm zone of the medium, the other strains in a 7-16 mm deep zone.

On Wagener and Mitscherlich medium, the 2 Huitema strains produce small, glistening, convex colonies (marked D in table 2) indistinguishable from *M. bovis*. All other strains produce heaped up, dry, rough, crateriform colonies (marked Cr in table 2). There was no colour change of the indicator.

Colony morphology on Dubos oleic agar is very characteristic for 10 strains. Young colonies are more or less round, opaque, rough, with some radially disposed bacilli at the periphery, remembering somewhat the « Xf » aspect of *M. xenopi* colonies (Pattyn, 1966). But gradually the colony takes an irregular shape while the periphery thickens considerably; by engulfing this periphery the characteristic crateriform colony, irregular in shape and with entire edges is formed. For this colony type we propose the designation « Oc » for opaque, ciliate, crateriform (figures 1, 2, 3).

With one exception, all strains examined produce typical Oc colonies.

The exception is strain 1279 that produces RR colonies similar to those of *M. tuberculosis* or *M. bovis*, although after prolonged incubation some colonies (< 10 percent of total) show a tendency to evolve into the Oc type. (That this strain is a *M. microti* is shown by its other characters).

The addition of triton to the Dubos oleic agar, does not change the aspect of the colonies, although the peripheral filaments of young Oc colonies show cording on this medium.

Niacin production was strongly positive for all strains even the dysgonic ones.

Nitrate reduction was negative both by Virtanen's and Tacquet's technique. Two strains were cultured in nitrate containing fluid medium without nitratase being produced.

Catalase is weakly positive (3-5 mm) and thermolabile.

Results for amidase reactions were obtained for 7 strains and gave a 3, 5, 6, pattern (urease, nicotinamidase, pyrazinamidase).

All strains were sensitive to 0.1 mcg/ml INH and to 0.1-0.2 mcg TCH/ml, one strain was resistant to 0.5 mcg TCH/ml but was inhibited by 1 mcg/ml.

Five strains tested were inhibited by 20 mcg/ml of pyrazinamide.

Four strains tested were resistant to 3 mcg DDS/ml.

All strains were inoculated intravenously into 3 mice each (0.2 mg bacilli).

Only one animal (inoculated with a Pasteur Institute strain) died from progressive tuberculosis.

The histological picture in the remaining mice at the end of the 6 week observation period was one of moderate to low grade involvement, particularly in the lungs, consisting of granulomas built up

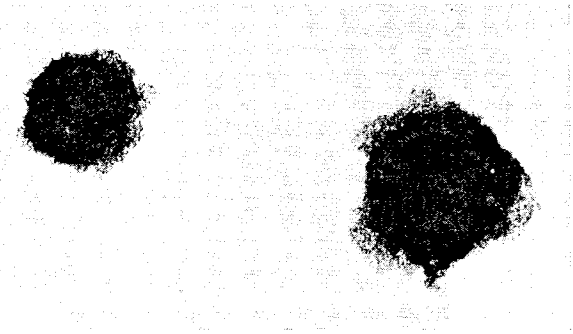

Figure 1

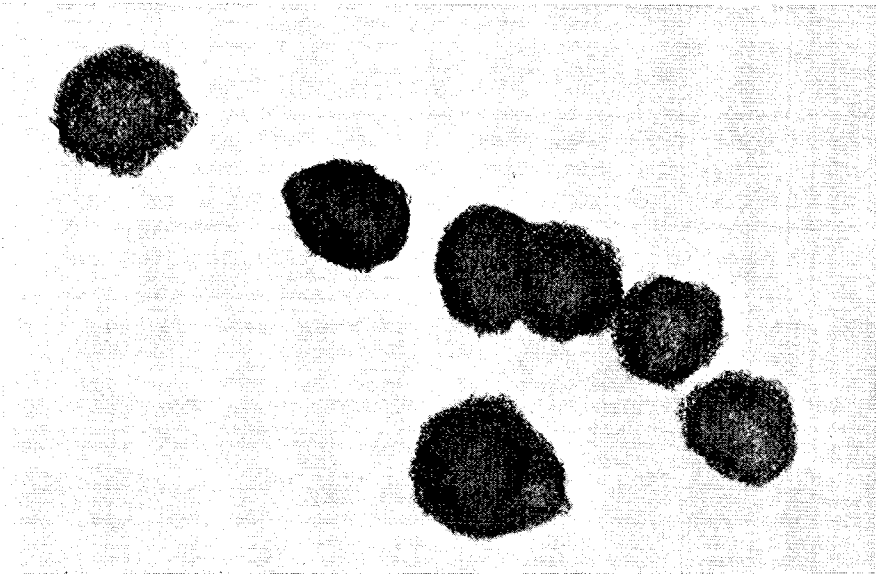

Figure 2

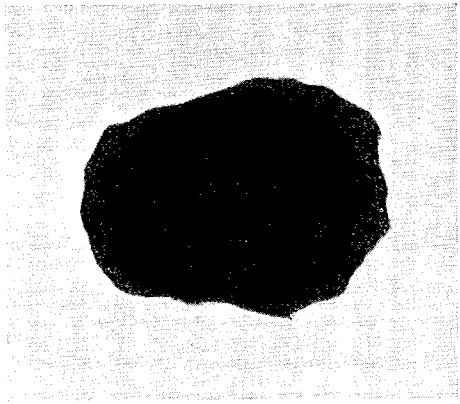

Figure 3

TABLE 3

Results on strains from Kinshasa, Dakar & Accra

No.	Origin	Growth		Niacin	Nitrate		Sensitivity		
		L. J. W. M.	Lebek		Virtanen	Tacquet	INH	TCH	Pyraz
836	Kinshasa	D	16	+	−	−	0.2	0.5	< 10
1344	Kinshasa	D	7	+	−	+	< 0.1	0.75	
1360	Kinshasa	D + E	2	+	−	+		> 2.5	
1389	Kinshasa	D		+	−	+		0.5	
1393	Kinshasa	D		+	−	+		0.2	< 10
1394	Kinshasa	D	10	+	−	+		> 5	
1395	Kinshasa	D	5	+	+	+		1	
1397	Kinshasa	D	3	+	+	+		2.5	
1546	Kinshasa	D	2	+	−	+		5-10	
1548	Kinshasa	D	8	+	−	+		0.2	
1099	Dakar	D	15	+	−	−	< 0.1	0.2	< 20
1100	Dakar	D	10	+	−	−	< 0.1	0.2	< 20
1101	Dakar	D	10	+	−	+		0 5	< 20
1102	Dakar	D		+	−		< 0.1	0.5	
1350	Accra	D	11	+	−	+	< 0.1	0.5	
1351	Accra	D	10	+	−	+	< 0.1	0.5	< 10
1352	Accra	D	10	+	−	+	< 0.1	1	< 10
1353	Accra	D	10	+	−	+	< 0.1	1	< 10
1354	Accra	D	12	+	−	+	< 0.1	0.75	< 10
1355	Accra	D	9	+	−	+	< 0.1	1	

by epitheloid cells with very few if any Langhans giant cells or lymphocytes and absence of caseation. In all cases could curled AFB be found in lungs and/or liver.

3. *Human strains from Kinshasa, Dakar, Accra* (table 3)

These strains were selected as a result of their dysgonic type of growth on Loewenstein medium, strain No. 1360 produces a mixture of eugonic and dysgonic growth; while the eugonic growth tends to predominate gradually on subculture.

On L-J, the colonies are moist, convex, round, with a diameter of 1-2 mm, frequently bearing a central pimple. In some strains however colonies are flat, dry, more irregular, the central pimple being more evident (1360, 1393, 1394, 1348, 1351). These aspects are much more pronounced on the medium of Wagener and Mitscherlich.

For typical dysgonic strains, growth in Lebek's agar is deep.

(For comparison, five *M. tuberculosis* strains isolated in our laboratory from local patients grow only at the surface, however when glycerin was omitted from the Lebek formula, these strains also developed in a zone 8-12 mm deep.)

On Dubos oleic agar all colonies are rough, while cords develop when triton is added to this medium.

All strains were niacin positive, although some rather poorly.

Nitrate reduction with Virtanen's technique was positive for 2 strains only, however if performed on nitrate containing medium (Tacquet's technique) all but 3 strains gave positive reactions.

Some amidase patterns were determined : strains 836; 1099; 1100; deaminated ureum, isonicotinamide and pyrazinamide.

All strains tested were INH and pyrazinamid sensitive.

All Dakar and Accra strains were inhibited by 1 mcg TCH/ml or less.

This was the case for only 6 of the 10 Kinshasa strains : for 4 of them the minimal inhibitory concentration of TCH was 2-5 to 10 mcg/ml.

It may be worthwile to note that the two strains showing nitrate-reductase in the Virtanen technique were of an intermediate sensitivity for TCH (1 and 2.5 mcg/ml respectively).

There is however a tendency for the more resistant strains to develop more at the surface in Lebek's agar.

Two Dakar-, 2 Accra- and 5 Kinshasa strains were inoculated into mice (0.2 mg wet weight, intravenously). Whitin a month the animals died or were very sick, they showed extensive tuberculous lung lesions, rich in AFB.

4. Mycobacterium tuberculosis *isolated in Butare (Rwanda)*

Among 72 strains from Butare, only 13 classical eugonic *M. tuberculosis* were observed (table 4).

Fifty-nine strains or 81 percent were dysgonic on L.J.

TABLE 4

Some characters of *M. tuberculosis* from Butare (Rwanda)

Growth on L-J	Number	Nitrate reduction		TCH sensitivity		
		Virtanen	Tacquet	Concentration	Number	
Eugonic	13	13 +		1-10 (*)	4	
				> 25	6	
				N. T	3	
Dysgonic	59	46 +		1	16	78 %
				5	13	78 %
				> 25	5	
				N. T	4	
				INH-R	5	
		13 −	13 +	1	7	72 %
				5	1	72 %
				15	1	
				25	2	
				N. T	2	

(*) Numbers indicate highest concentration of TCH (in mcg/ml) at which growth occurred.

On Wagener and Mitscherlich medium, these 59 strains produced flat to slightly convex, opaque colonies, with a central pimple. In 15 cases some rough, eugonic colonies (< 1 percent) developed on a dysgonic veil.

Fourty-three strains were tested in Lebek's agar, all produced deep type growth.

On Dubos oleic agar rough colonies are produced.

Niacin was positive, although sometimes weakly.

In only 13 instances was nitrate reduction negative with the Virtanen technique, and all without exception, were strongly positive in the Tacquet technique.

More detailed observations on TCH sensitivity gave the following results :

About 3/4 of the dysgonic strains from Rwanda, be it good or bad nitrate reducers, developed in the presence of maximum 1 or 5 mcg/ml TCH, some strains develop at 15 mcg/ml, only a minority at 25 mcg/ml.

Moreover whereas the population of the dysgonic strains, originating from Kinshasa, Dakar and Lagos was rather homogenous in it's sensitivity to TCH (more than 90 percent of growth being inhibited by the low concentrations) the population of the Butare strains was much more heterogenous in this respect since frequently, 25-40 percent of the growth still developed at higher concentrations.

Discussion

Dysgonic strains of *M. tuberculosis* have now been observed in most parts of Africa (for review, see Castets *et al.*, 1968) and also in Japan (Ujiye and Nemoto, 1967). Unfortunately Japanese strains were not available for comparison.

The frequency of dysgonic strains of *M. tuberculosis* from Africa was again high in the present series : 18 percent among the Kinshasa strains (as compared with 10 percent in the 1965 study, difference which is not statistically significant). However the higher frequency of dysgonic strains in extra pulmonary tuberculosis, that was significant in the 1965 study, was not found in the group of strains discussed in this paper. (And when the two groups are added, the significance also disappears).

The frequency of dysgonic strains among isolates from Rwanda is still much higher : 81 percent, but there is also a greater diversity among the strains.

TABLE 5

Comparison of characters between strains studied

	M. tuberc.	*M. bovis*	*M. microti*	African strains				
				a	b	c	d	e
Growth on L. J	E	D	D + E	D	D	D	E	D
Colonies on OAA	R	R	Oc	R	R	R	R	R
Niacin	+	−	+	+	+	+	+	+
Nitrate (Virtanen)	+	−	−	−	−	−	−	−
Nitrate (Tacquet)		−	−	−	+	+	+	−
Lebek	s	d	d	d	d	d	d	d
Amidases	3.5.6	3	3.5.6	3.5.6	3.5.6	3.5.6	3.5.6	3.5.6
TCH S	> 25	< 1	< 1	< 1	< 1	> 1	< 1	> 1
Pyrazinamide	< 20	> 50	< 20	< 20	< 20	< 20	< 20	< 20
Microscopy	r	r	c	r	r	r	r	r
Mouse virulence	+	+	−	+	+	+	+	+
Natural host	H	C	M	H	H	H	H	H

E = eugonic; D = dysgonic; R = rough; Oc = opaque, crateriform; r = rods; c = curved; s = surface; d = deep; H = human; C = cattle; M = mice.

The most common characteristic of the African strains is their dysgonic type of growth which is more evident on Wagener and Mitscherlich medium as was pointed out earlier (Hermans-Boveroulle *et al.*, 1965).

Other characters are variable : nitratase, as shown by the 59 strains from Rwanda of which 46 were positive in Virtanen's test, and all 13 others in Tacquets, whereas among the strains from West Africa only a minority were positive in Virtanen's technique and 3 remained negative even when grown in the presence of nitrate.

Sensitivity for TCH is mostly very high, growth being inhibited by concentrations as low as 0.1 or 0.2 mcg/ml.

However all transitions exist between this high sensitivity to TCH and resistance to 25 mcg/ml (which is a common character of *M. tuberculosis* isolated in our laboratory from European patients) some strains even are built up by a heterogenous population in this respect.

There is no correlation between the dysgonic type of growth, complete absence of or low nitratase activity and degree of TCH sensitivity.

Some of the eugonic strains from Rwanda were also inhibited by lower concentrations of TCH.

Our results on nitrate reduction obtained with the Tacquet method are at variance with those of Castets *et al.* (1968) who mentioned that of 21 strains they studied only 3 gave a faint positive reaction, whereas we found a positive reaction in 2 strains out of 3.

This discrepancy may be due either to the incubation time (Tacquet *et al.*, 1966, indicate that : « *the Griess reactive should be added after abundant growth is obtained* ») and/or to aeration of the cultures; it is a well know fact that this influences most biochemical reactions of mycobacteria.

The differences observed between the two techniques revealing nitrate reduction may be the result of nitratase being an inducible enzyme in *M. tuberculosis*. This is not the case for *M. bovis* nor *M. microti* for after prolonged cultivation of some *M. bovis* and *M. microti* strains in liquid medium containing nitrate, nitrite was produced in very low quantity by the *M. bovis* and not at all by the *M. microti* strains.

Table 5 shows a comparison of the differential characters of the strains studied, with the African strains put into 5 groups.

In an attempt to apply numerical taxonomy on these data, *the number of identical characters* was entered in a similarity table (table 6) (instead of calculating the percentage similarity values on a total of 10 characters.

TABLE 6

Similarity table for the strains studied

	M. tbc	b	c	d	a	e	mic	bov
b	7							
c	8	9						
d	8	9	9					
a	7	9	8	8				
e	8	8	9	8	9			
mic	3	4	3	5	6	4		
bov	2	5	5	4	7	6	3	

Number of identical characters between pairs of strains. Total number of characters taken into consideration = 10.

So far as this study goes, *M. tuberculosis* and *M. microti* differ in 6 to 8 characters from each other, whereas the african strains differ from *M. tuberculosis* and among each other in 1 or 3 characters only. Varieties b and a differ from *M. tuberculosis* in 3 characters Group a is the dysgonic, niacin positive, nitratase negative, TCH sensitive one, described in the first paper from this laboratory on the subject (Hermans-Boveroulle *et al.*, 1965) and called provisially « *intermediate* » (between *M. tuberculosis* and *M. bovis*).

Table 6 shows indeed that this group has also 7 characters in common with *M. bovis*.

Since the differences with *M. tuberculosis* are not greater than among themselves we should consider the African strains as belonging to the species *M. tuberculosis* therefore we cannot agree with Castets *et al.*, (1969) who described the African strains as *M. africanum*.

Is it not normal that some differences between bacterial strains are encountered when more of these from different geographical origin are studied for a greater number of characters ?

It may be worthwile to recall that, decades ago, differences among *M. tuberculosis* strains from England were also described by Griffith (1924).

Mitchinson *et al.* (1960) and Subbaiah *et al.* (1960) showed the Asian strains of tuberculosis to differ from European strains with respect to guinea pig virulence and hydrogen peroxyde sensitivity.

One may even wonder if a detailed study of *M. tuberculosis* strains from other parts of the world would not reveal other differences.

In the meantime, it is necessary for the bacteriologist to be familiar with these variations occurring among *M. tuberculosis* strains originating from different continents, as was stressed by Castets *et al.* (1968).

Résumé — **Nouvelle étude de souches africaines de** *M. tuberculosis*. **Comparaison avec** *M. bovis* **et** *M. microti*.

Des souches africaines de *M. tuberculosis* isolées à Accra (Ghana), Dakar (Sénégal), Kinshasa (Congo), et Butare (Rwanda) furent examinées et comparées avec des souches de *M. bovis* et *M. microti*.

La prévalence des souches dysgoniques était la plus élevée au Rwanda (81 p. cent).

La plupart des souches d'Afrique Occidentale sont de faibles producteurs de nitratase et sont inhibées par de faibles concentrations de TCH (0,2-1 mcg/ml).

La réduction du nitrate est la plus aisément détectée parmi les souches rwandaises, qui sont en général aussi moins sensibles au TCH (1-5-15 mcg/ml). Des souches, à caractères intermédiaires, entre ces deux variétés, existent.

Au point de vue de la taxonomie numérique ces souches sont significativement différentes de *M. bovis* et *M. microti*; par contre elles sont fort semblables à *M. tuberculosis*, dont elles ne diffèrent pas plus qu'elles ne diffèrent entre elles, ce qui ne permet pas de les considérer comme une espèce propre.

Samenvatting — **Nieuwere studie van Afrikaanse stammen van** *M. tuberculosis*. **Vergelijking met** *M. bovis* **en** *M. microti*.

Afrikaanse stammen van *M. tuberculosis* afkomstig van Accra (Ghana), Dakar (Senegal), Kinshasa (Congo) en Butare (Rwanda) werden vergeleken met *M. bovis* en *M. microti*).

De frekwentie van dysgone *M. tuberculosis* stammen was het hoogst in Rwanda (81 ten honderd).

De meeste Afrikaanse stammen produceren zeer weinig nitratase en worden geremd door kleine concentraties TCH (0,2-1 mcg/ml). Nitraatreductie is gemakkelijker aan te tonen bij de Rwandese stammen die tevens over 't algemeen ook minder gevoelig zijn voor TCH. Allerlei overgangsvormen komen voor.

De Afrikaanse stammen van *M. tuberculosis* verschillen voldoende van *M. bovis* en *M. microti*; ze zijn sterk verwant met *M. tuberculosis* waarvan ze niet méér verschillen dan onder mekaar, wat dus niet toelaat ze als een afzonderlijke species te beschouwen.

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