Tentative Evidence of AIDS-Associated Biotype of *Mycobacterium kansasii*

ENRICO TORTOLI,[1]* M. TULLIA SIMONETTI,[1] CARLA LACCHINI,[2]
VALERIA PENATI,[2] AND PASQUALE URBANO[3]

*Microbiological and Virological Laboratory, Careggi Hospital, 50139 Florence,[1] Tuberculosis Laboratory,
Institute for Chest Disease, 20159 Milan,[2] and Institute of Microbiology,
University of Florence, 50134 Florence,[3] Italy*

Previous studies revealed heterogeneous behavior within the species *Mycobacterium kansasii* against commercially available DNA probes (Accuprobe *M. kansasii* culture identification test; Gen-Probe); several isolates, conventionally identified as *M. kansasii*, failed in fact to hybridize. Looking for a possible association with phenotypic features, we tested a fully characterized panel of 69 clinical isolates of *M. kansasii* (19 of which were Accuprobe negative) with a semiquantitative micromethod which tests for 19 enzymatic activities (Api Zym; BioMérieux). The strains were from 25 hospitals in 18 Italian towns; 20 isolates came from human immunodeficiency virus type 1-positive patients who fulfilled the Centers for Disease Control criteria for AIDS diagnosis. On the basis of the whole set of phenotypic traits, our strains clustered in two groups, allowing the differentiation of biotypes within the species. There was a perfect association between biotype 2 and hybridization failures with Accuprobe and a very significant association between this novel biotype 2 and AIDS status, which suggests that it differs in virulence.

Among mycobacteria other than tuberculosis, *Mycobacterium kansasii* is one of the species most frequently isolated from clinical specimens. Since the first description of human diseases due to *M. kansasii*, many cases have been reported, and in various areas of the United States, this species has long been the most frequently isolated mycobacterial species other than tuberculosis. The AIDS epidemic has changed the scenery, favoring the *M. avium-M. intracellulare* complex, but *M kansasii* continues to be isolated, often in association with AIDS (3, 9).

The development of commercially available DNA probes such as the Accuprobe *M kansasii* culture identification test (Gen-Probe, San Diego, Calif.) for the presumptive identification of this species from culture has dramatically simplified and shortened the procedures for its identification; the technique is now within reach of every laboratory. Recently, however, there have been reports of a number of *M kansasii* isolates which failed to hybridize with Accuprobe (2, 6, 8), thus casting doubts on the test's sensitivity.

We investigated the biochemical features of several clinical isolates of *M kansasii* from both human immunodeficiency virus (HIV)-negative and HIV-positive patients to verify whether any phenotypic character was associated with hybridization failure; it turned out that probe-negative strains belong to a previously unrecognized biotype.

MATERIALS AND METHODS

All the organisms, except two reference strains, were isolated from clinical specimens (*n* = 69) in 25 hospitals in 18 Italian towns. When more than one isolate was isolated from the same patient (six cases), all the strains were processed to verify the consistency of results, after which only one isolate per patient was included in the study.

Twenty patients were HIV type 1 positive and fulfilled the Centers for Disease Control criteria for AIDS diagnosis (1). All

strains were identified (5) as *M. kansasii* on the basis of an extended set of tests (Table 1), whose results were also assessed with a program for computer-assisted identification of mycobacteria (7). Tween 80 hydrolysis was read daily, and the day of the first observation of a positive reaction was recorded.

The hybridization test was performed on all strains according to the procedures recommended by Gen-Probe. Briefly, a 1-μl loopful of mycobacterial colonies was sonicated (Bransonic B-

TABLE l. Percentages of positive results with tests performed for standard identification of 71 (19 probe-negative and 52 probe-positive) *M. kansasii* strains

Test	% Positive	
	Probe -	Probe +
Niacin	0	2
Nitrate reduction	100	98
Heat-stable catalase	100	100
Catalase >45 mm of foam	100	100
Photochromogenicity	100	100
β-Glucosidase	0	0
Tween 80 hydrolysis (10 days)	100	100
Slow growth rate	100	100
Growth (25°C)	100	100
Growth (45°C)	0	0
MacConkey agar	0	o
Tellurite reduction	0	0
Arylsulfatase	0	0
Rough colonies	94.7	100
Urease	100	100
Resistance to:		
NaCl (5%)	0	0
p-Nitrobenzoate (500 μg/ml)	52.6	15.4
Thiophene-2-carboxylic hydrazide (5 μg/ml)	100	100
Thiacetazone (10 μg/ml)	0	0
Hydroxylamine (500 μg/ml)	15.8	2
Isoniazid (1 μg/ml)	31.6	63.5
Oleate (250 μg/ml)	0	0

_____
Corresponding author. Phone: 39-55-4277343. Fax: 39-55-4223895.

TABLE 2. Day of Tween 80 hydrolysis positivization of
Accuprobe-positive and -negative *M. kansasii* strains

Probe result	No. of strains Tween 80 positive on day:							
	1	2	3	4	5	6	7	8
Positive	34	11	4	2	1			
Negative		3	2	4	2	1	2	4

1200E4) in tubes containing glass beads and lysing reagent and heated for 10 min at 95°C. Hybridization with a lyophilized DNA probe and the selection step (after the addition of the provided specific reagent) were both performed at 60°C for 15 and 8 min, respectively. Hybridization results were read with a PAL/AccuLDR luminometer (Gen-Probe) after 5 min at room temperature and were expressed as PAL light units (PLU). According to the manufacturer's cutoff, samples producing signals greater than 899 PLU were considered positive. The hybridization test was repeated on eight of the strains that gave a negative reaction; they all scored negative again.

A further biochemical characterization was performed with a semiquantitative micromethod (Api Zym; BioMérieux, Marcy l'Etoile, France) which tests for 19 enzymatic activities. A heavy mycobacterial suspension, with turbidity between McFarland standards 5 and 6, was prepared from each isolate that had been subcultured on slants of Middlebrook 7H11 for no more than 4 weeks at 37°C in a CO_2-enriched atmosphere. Each cupole of the strip, containing an enzymatic substrate, was inoculated with 65

μl of the mycobacterial suspension carefully dispersed by vortexing with several glass beads. The strips were incubated overnight at 37°C, and the results were read after the addition to each microtube of reagents Zym A and Zym B (fast blue BB reaction). The intensity of the colored reaction was scored, per the color chart enclosed in the kit, on a scale from 0 (negative) to 5: 1 corresponds to roughly 5 nmol, 2 corresponds to 10 nmol, 3 corresponds to 20 nmol, 4 corresponds to 30 nmol, and 5 corresponds to 40 nmol and more.

RESULTS

The hybridization test gave positive results, with PLU values far above the cutoff, with only 52 of the 71 strains (73.2%), including the two reference ones. The mean of these strains was 8,070.25 ± 1,939.82 PLU, with a range of 3,466 to 10,432 PLU. On the contrary, the PLU values of the remaining 19 isolates were extremely low; the mean of these strains was 59.68 ± 26.47, with a range of 31 to 148. Both groups were clearly outside the suggested repeat range. Results were reproducible among multiple isolates from the same patient and with duplicate testing.

The variability of phenotypic characters conventionally used for the identification of *M. kansasii* was very limited among both the probe-positive and probe-negative strains (Table l); only the presence of one nitrate-negative strain and an unquestionably niacin-positive one merits any comment. Niacin accumulation by *M. kansasii* is unusual, but it has been reported in several instances (3).

TABLE 3. Distribution of binary responses to the tests which were not 100 or 0% positive[a]

					Response to:							No. of strains			Phenotype
Ini	αfu[b]	pNB	Try[b]	Chy[b]	Hyd	Var[b]	Rou	Nit	Nia	Alp[b]		Total	Probe +	AIDS[c]	
+	+	+	+	+	-	+	+	+	+	+		1	1	1	Aa
+	+	+	+	+	-	+	+	+	-	+		3	3	0	Bb
+	+	+	+	-	-	+	+	+	-	+		3	3	1	Cc
+	+	-	+	+	+	+	+	+	-	+		1	1	0	Dd
+	+	-	+	+	-	+	+	+	-	+		1	13	2	Ee
+	+	-	+	-	-	+	+	+	-	+		8	8	0	Ff
+	+	-	-	-	-	+	+	+	-	+		2	2	0	Gg
+	+	-	-	-	-	+	+	+	-	-		1	1	0	Hh
+	+	-	-	-	-	-	+	+	-	+		1	1	0	Ii
+	-	+	+	+	+	+	+	+	-	+		1	0	0	Jj
+	-	+	+	+	-	+	+	+	-	+		1	0	1	Kk
+	-	+	-	-	-	+	+	+	-	+		1	0	0	Ll
+	-	-	+	+	-	+	+	+	-	+		1	0	0	Mm
+	-	-	+	-	+	+	+	+	-	+		1	0	0	Nn
+	-	-	+	-	-	+	+	+	-	+		1	0	0	Oo
-	+	+	-	-	-	+	+	+	-	+		1	1	0	Pp
-	+	-	+	+	-	+	+	+	-	+		9	9	3	Qq
-	+	-	+	+	-	+	+	-	-	+		1	1	0	Rr
-	+	-	+	-	-	+	+	+	-	+		6	6	1	Ss
-	+	-	-	-	-	+	+	+	-	+		2	2	0	Tt
-	-	+	+	+	-	+	+	+	-	+		2	0	1	Uu
-	-	+	+	-	-	-	+	+	-	+		1	0	1	Vv
-	-	+	-	-	+	+	+	+	-	+		1	0	1	Ww
-	-	+	-	-	-	+	+	+	-	+		1	0	1	Xx
-	-	+	-	-	-	-	+	+	-	+		2	0	2	Yy
-	-	-	+	+	-	+	+	+	-	+		4	0	4	Zz
-	-	-	-	-	-	+	+	+	-	+		1	0	0	Ab
-	-	-	-	-	-	+	-	+	-	+		1	0	1	Ac

[a] Tests: Ini, isoniazid tolerance; αfu, α-fucosidase; pNB, *p*-nitrobenzoate tolerance; Try, trypsin; Chy, chymotrypsin; Hyd, hydroxylamine tolerance; Var, valine arylamidase; Rou, rough colonies; Nit, nitrate reduction; Nia, niacin; Alp, alkaline phosphatase.
[b] Semiquantitative test results were coded + or - for clarity only; all information was used in the cluster analysis.
[c] AIDS, isolated from AIDS patients.

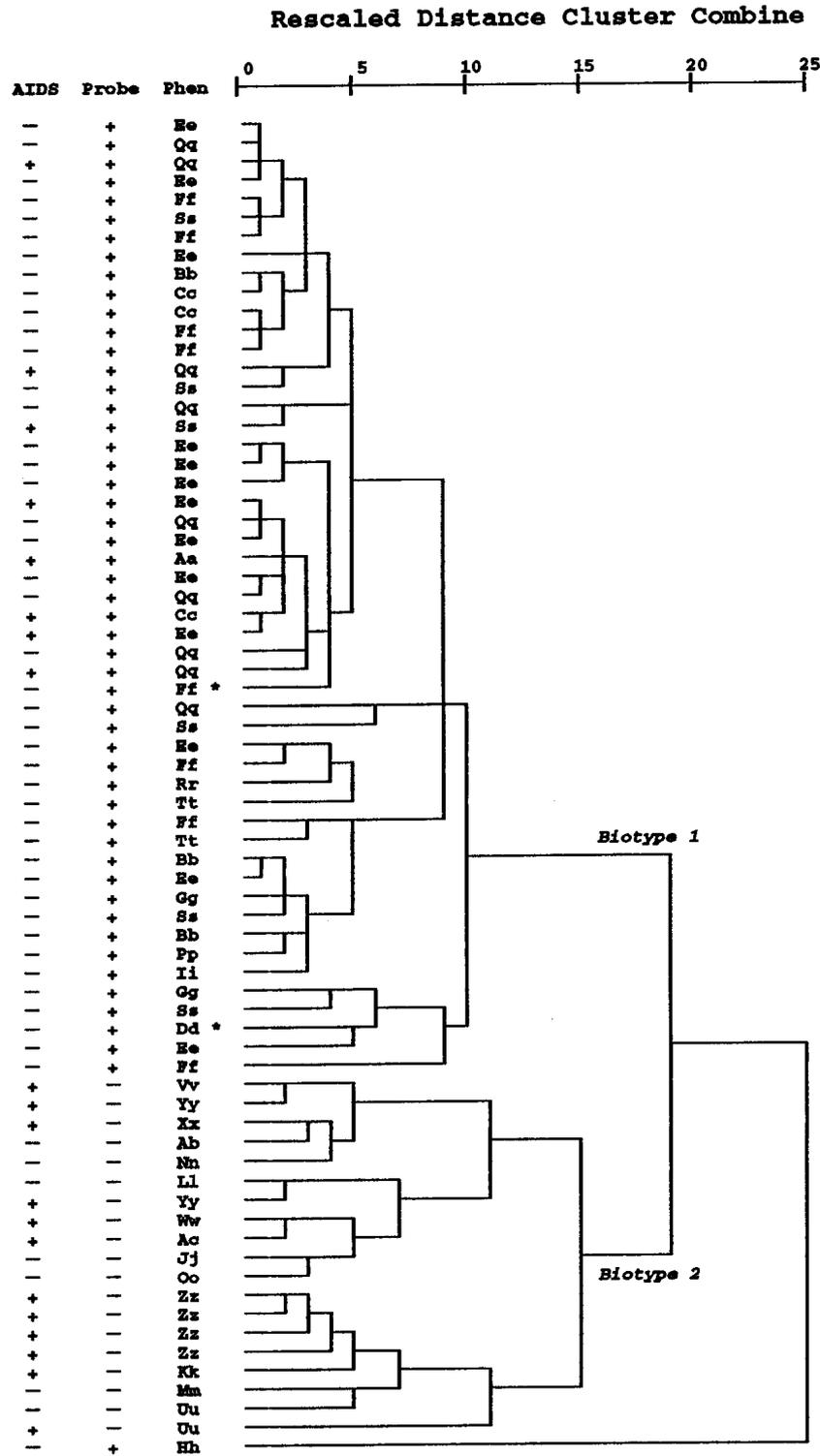## Rescaled Distance Cluster Combine

FIG. 1. Cluster analysis of the whole set of phenotypic traits for our collection of *M. kansasii* strains. Clustering was performed by the Baverage method with squared Euclidean distances. AIDS, AIDS status; Phen, phenotype label; *, reference strain.

Tween 80 hydrolysis, one of the most relevant features for *M. kansasii* identification to the species level, was positive with all isolates, but to different degrees: the hydrolysis was clearly more rapid with probe-positive strains (Table 2).

Of the enzymatic activities investigated with Api Zym, five (α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, and α-mannosidase) were absent with all the strains, whereas nine (C4 esterase, C8 esterase lipase, C14 lipase, leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase were constantly found. The remaining five activities; were, on the contrary, variable: alkaline phosphatase was positive

TABLE 4. Association between *M. kansasii* biotypes and AIDS status of patients ($\chi^2 = 15.69586$; $P = 0.00007$)

Biotype(s)	AIDS (no. of isolates)		Total
	-	+	
1 and 3	44	8	52
2	7	12	19
Total	51	20	71

for 70 strains, valine arylamidase for 67, trypsin for 57, α-fucosidase for 52, and chymotrypsin for 37 (Table 3). Except for α-fucosidase, these variabilities are difficult to evaluate, as the quantitative extent of the activities was very heterogeneous and some positive reactions were very scanty.

The α-fucosidase reaction was either strongly positive (approximately 20 nmol or more) or negative (5 nmol or less). Moreover, there was a strict association between this enzyme activity and Accuprobe hybridization: all 52 probe-positive strains scored high while probe-negative strains scored 0 (in 17 cases) or 1 (in 2 cases).

Cluster analysis of the whole series of phenotypic traits (4) for our collection of *M. kansasii* gave the dendrogram in Fig. 1, which shows how the strains cluster in two major groups, thus suggesting that there are at least two biotypes. One clinical isolate was clearly distinct from the two groups; it was probe positive and lacked alkaline phosphatase and cystine arylamidase. On account of its uniqueness, it is not considered further here.

The largest group embraced 51 strains, including the two reference ones, all of which regularly hybridized with Accuprobe, and may well represent the typical *M. kansasii* strain. The second cluster, which we call biotype 2 or variant *M. kansasii*, included 19 strains, none of which hybridized with Accuprobe. In a search for the traits which most contributed to the biotype, we found that α-fucosidase activity was high for biotype 1 but low or absent for biotype 2; in addition, Tween 80 hydrolysis was rapid for biotype 1 but slow for biotype 2. It must be stressed that the hybridization results did not contribute to clustering.

Table 4 shows a very significant association between biotype 2 and AIDS status. Among the 51 isolates from non-AIDS patients, only 7 (13.7%) belonged to biotype 2 (and scored negative with Accuprobe), while among the 20 isolates from AIDS patients, the majority (12 isolates or 60%) belonged to biotype 2 and scored negative with Accuprobe ($\chi2 = 15.69586$; $P = 0.00007$).

DISCUSSION

In short, we have found that *M. kansasii* strains may be grouped in at least two biotypes, mainly on the basis of α-fucosidase and Tween 80 hydrolysis; most importantly, one of the biotypes does not hybridize with Accuprobe, a fact which jeopardizes the accurate assessment of its prevalence.

The presence of *M. kansasii* strains that fail to hybridize with commercially available genetic probes has already been reported (2, 6), and we have noticed that the frequency of Accuprobe-negative *M. kansasii* strains is high in Italy (8).

Two recent papers analyzed the genetic heterogeneity within the presently standing species *M. kansasii* and suggested that it should be split into two subspecies on the basis of restriction fragment length polymorphism and the variability of their 16S rRNA sequences (6), as well as the presence in one of them of insertion sequence-like elements (11).

It remains to be established whether our new biotype or variant corresponds to one of the subspecies proposed on the basis of the genomic analyses discussed above. At any rate, it is of interest that it seems particularly prevalent among isolates from AIDS patients, which forecasts an increase in its frequency. The association with AIDS status also suggests that the two biotypes might differ in virulence, with biotype 2 less able to overcome natural resistance mechanisms and behaving in a more opportunistic manner. That two varieties of *M kansasii* that differ in clinical significance might exist has already been suggested on entirely different grounds (10).

Further studies are needed to solve the taxonomic puzzle of *M. kansasii*, and some effort should be spent in the search for more conserved sequences to be used as targets for diagnostic probes. In the meantime, it seems wise not to rely solely on Accuprobe for species identification of photochromogenic mycobacteria and to submit all probe-negative isolates to the traditional identification routine in specialized laboratories.

REFERENCES

1 **Centers for Disease Control.** 1987. Revision and case definition of AIDS for surveillance purposes. Morbid. Mortal. Weekly Rep. 36(Suppl.):1S-15S.

2. **Lebrun, L., F. Espinasse, J. D. Poveda, and V. Vincent-Levy-Frebault**. 1992. Evaluation of nonradioactive DNA probes for identification of mycobacteria. J. Clin. Microbiol. 30:2476-2478.

3. **Nachamkin, I., R. R. MacGregor, J. L. Staneck, A. Y. Tsang, J. C. Denner, M. Wiliner, and S. Barbagallo.** 1992. Niacin-positive *Mycobacterium kansasii* isolated from immunocompromised patients. J. Clin. Microbiol. 30:1344-1346.

4. **Norusis, N. J.** 1986. SPSS/PC+ base manual. SPSS Inc., Chicago.

5. **Roberts, G. D., E. W. Koneman, and Y. K Kim.** 1991. *Mycobacterium, p.* 304-339. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.

6. **Ross, B. C., K. Jackson, M. Yang, A. Sievers, and B. Dwyer.** 1992. Identification of a genetically distinct subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. 30:2930-2933.

7. **Tortoli, E., V. Boddi, and V. Penati.** 1992. Development and evaluation of a program and probability matrix for the computer-aided identification of non-tuberculous mycobacteria. Binary Comput. Microbiol. 4:200-203.

8. **Tortoli, E., M. T. Simonetti, C. Lacchini, V. Penati, C. Piersimoni, and V. Morbiducci.** 1994. Evaluation of a commercial DNA probe assay for the identification of *Mycobacterium kansasii*. Eur. J. Clin. Microbiol. Infect. Dis. 13:264-267.

9. **Valainis, G. T., L. M. Cardona, and D. L. Greer.** 1991. The spectrum of *Mycobacterium kansasii* disease associated with HIV-1 infected patients. J. Acquired Immune Defic. Syndr. 4:516-520.

10. **Wayne, L. G.** 1962. Two varieties of *Mycobacterium kansasii* with different clinical significance. Am. Rev. Respir, Dis. 86:651-656.

11. **Yang, M., B. C. Ross, and B. Dwyer.** 1993. Identification of an insertion sequence-like element in a subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. 31:2074-2079.