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Carbohydrate Typing of Mycobacteria* (27030)

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Differentiation of mycobacteria species of medical interest has presented considerable difficulty due to a lack of definitive tests, especially with regard to the "atypical" or "anonymous" acid-fast organisms(1). Biochemical or metabolic testing should be preferred for precise differentiation of microorganisms, but slow-growing mycobacteria are classified at present primarily by cultural morphology and animal pathology. There are several useful biochemical tests, such as Konno's niacin test(2) and the succinamidase test(3), for distinguishing single species or strains of species. Other biochemical tests, e.g. the microcolonial neutral red and cording test(4), aid the grouping of mycobacteria. But no procedures have been reported which appear capable of aiding the typing of all kinds of mycobacteria. Organic acid utilization(5) offers some promise for general speciation, although no reactions were reported positive for the human tubercle bacillus and only one positive reaction was reported for *Mycobacterium avium*.

Carbohydrate utilization testing has not been applied generally to the mycobacteria due to technical difficulties. By adaptation of a procedure reported by Pickett and Nelson(6) for carbohydrate utilization of brucellae, differentiation of mycobacteria strains may be facilitated.

Materials and Methods. Organisms. Mycobacterial strains were inoculated from Lowenstein-Jensen egg medium (stock strains) into 50 ml amounts of TB Broth Base† with 0.5% sterile bovine albumin. No glycerol or other carbohydrate was added. Slow-growing organisms were incubated usually for 7 to 10 days, until just moderately turbid.

Preparation of Inoculum. The bacteria in 25 ml volumes of culture fluid were sedimented by centrifugation at 2,200 revolutions per minute for 3 to 5 minutes; clear supernates were poured off and discarded. Organisms sedimented from 25 ml of culture fluid were resuspended in 0.5 ml of basal medium without agar; 0.1 ml of the suspension was used as inoculum for a single carbohydrate test. The volume of packed wet cells per inoculum was 0.007-0.01 ml.

Basal Test Medium. Basal test medium for 100 tests was composed of: phenol red 4 mg, yeast extract 100 mg, K_2HPO_4 35 mg, agar 1 gm, distilled water 160 ml. The pH was adjusted to 7.0-7.2. Melted medium was dispensed in 1.6 ml quantities in screw-cap vials and autoclaved at 121°C for 15 minutes. Vials were cooled in vertical position; no slant was given the agar.

Carbohydrates. Carbohydrates were pre-

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TABLE I. Carbohydrate Reactions of Single Strains of Acid-Fast Microorganisms

Carbohydrate	Slow Growers						Rapid Growers		
	M. tuberculosis	BCG	Bovine Isolate	Swine Isolate	Scotochromogen	Photochromogen	*M. fortuitum*	*M. smegmatis*	*Nocardia asteroides*
Arabinose	−	−	−	−	−	W	−	+	W
Fructose	−	−	−	+	+	W	+	+	W
Galactose	−	−	−	−	−	−	−	+	+
Glucose	+	+	+	+	+	+	+	+	+
Mannose	−	W	+	+	+	−	+	+	−
Raffinose	−	−	−	−	−	−	−	+	−
Rhamnose	−	−	−	+	−	W	−	+	+
Sucrose	−	−	−	−	−	−	−	−	−
Trehalose	+	+	+	−	W	−	+	+	−
Xylose	−	W	−	+	W	+	−	+	−
Inositol	−	−	+	−	−	W	−	+	−

− = no acidification; + = strong acidification; W = weak acidification.

pared as 10% solutions, brought to a pH of 7.2, then sterilized by filtration. By overlayering 0.4 ml of carbohydrate solution on a 1.6 ml amount of agar base medium, final concentration of carbohydrate was 2%.

Testing Procedure. An inoculum of 0.1 ml of bacterial suspension was placed into the 0.4 ml of carbohydrate solution on top of the basal test medium. Vials were incubated at 37°C for 1 to 14 days, depending upon the strain of microorganism used and the particular carbohydrate. For prolonged incubation periods vial caps were tightened to prevent drying. Results were interpreted from change of the color of the phenol red indicator. Yellow (indicating acid production) was a positive result, red to purple was a negative. and orange was interpreted as either weakly positive or negative depending upon comparison with other carbohydrate tests with the same microorganism.

Results. Table 1 presents carbohydrate reactions by single strains of particular species or types of acid-fast microorganisms. Members of a fairly well-defined species such as *Mycobacterium tuberculosis* show similar but not always identical patterns. Strains of the same species generally resemble each other more closely in carbohydrate reactions than they resemble different species.

In the overall testing of various carbohydrates and polyhydric alcohols with many types of mycobacteria, glucose and glycerol usually gave strong positive reactions (acid production.) Weak positive reactions with other carbohydrates could, at times, be changed to stronger positive reactions by increasing the size of the inoclum, using younger organisms or modifying the medium. The acid-fast "yellow bacilli" have been reported to have generally low metabolic activities(7), and slow-growing pigmented mycobacteria often showed weak carbohydrate reactions.

Table II lists the results of testing 4 strains

TABLE II. Comparison of Carbohydrate Reactions of *M. avium* and Battey Strains

Carbohydrate	*M. avium*				Battey			
	Strain: 1	2	3	4	Strain: 1	2	3	4
Fructose	+	+	+	+	+	+	+	W
Glucose	+	+	+	+	+	+	+	+
Mannose	+	+	W	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+
Trehalose	−	−	−	−	+	+	+	+
Xylose	+	+	+	+	−	−	−	−
Adonitol	+	−	−	−	−	−	−	−
Glycerol	+	+	+	+	+	+	+	+
Inositol	−	−	−	−	−	+	−	−
Sorbitol	−	−	−	+	−	−	−	−

− = no acidification; + = acidification; W = weak acidification.

of *M. avium* and 4 strains of Battey type (non-photochromogen) organisms. These 2 types have been considered to be closely related by some workers and appear to be difficult to distinguish(8), although animal pathology clearly differentiates certain strains.

Discussion. Carbohydrate utilization has had extended use in general and medical microbiology for differentiating species or strains. Investigations of carbohydrate utilization by slow-growing mycobacteria have seldom been reported, and such investigations often produced uncertain results. The massive inoculum non-growth testing method employed in the present study provides the following advantages over earlier methods: 1) the test is sensitive to slight acid production because of the use of phenol red which changes color significantly around the neutral pH of 7, 2) nitrogenous constituents in the test medium are largely eliminated, thus preventing the masking of weak acidification by basic byproducts of nitrogen metabolism, 3) results can be obtained more rapidly than with growth tests, and 4) the test is read simply by noting the color of the indicator.

In the non-growth carbohydrate testing, large inocula are used. The bacilli are grown in a rich medium and are harvested in their logarithmic phase of growth which provides a high rate of metabolic activity. The cells in a dispersed condition present a large surface area capable of magnifying weak acidification to a point of simple visualization by means of a pH indicator. Earlier techniques depended upon the growth of microorganisms to show utilization of carbohydrate. Growth methods have the disadvantage that lack of growth might be due to several factors other than lack of carbohydrate utilization. A comparison of results from single strains of slow-growing and rapid-growing mycobacteria (Table I) indicates the potential usefulness of carbohydrate typing for separating species and types.

M. avium, Battey type non-photochromogens (isolated from human patients), and certain acid-fast bacilli isolated from swine resemble one another in colonial morphology and sometimes in animal pathogenicity. Phage typing distinguishes some of these strains(9), although the majority of such microorganisms are not susceptible to the phages available at present. Bojalil and Cerbon(10) studied carbohydrate utilization of *M. avium* and non-photochromogens (e.g., Battey types) using growth and the change of bromcresol purple indicator to represent positive reactions; only glucose and mannose of 18 carbohydrates tested gave positive reactions with some strains of each type of organism. Results reported here (Table II) suggest that *M. avium* and Battey strains may be differentiated by the carbohydrates trehalose and xylose in non-growth tests. Kubica and Beam(11) report that the arylsulfatase test readily distinguishes avian tubercle bacilli from Battey strains. Carbohydrate typing by the massive inoclum technique usually offers the advantage of yielding at least some positive test results, so that an organism is not differentiated by a negative test alone, as with the arylsulfatase test in which avian bacilli are negative. Although only a few strains of avian and Battey types have thus far been investigated with the non-growth carbohydrate procedure, all strains have given differentiable patterns of results.

Table III suggests that the trehalose and xylose reactions may be of further interest with regard to possible correlation to pathogenicity. Practically all proven and suspect human pathogens gave trehalose positive, xylose negative reactions.

The validity of the non-growth carbohydrate testing is indicated by the similarity of results

TABLE III. Potential Correlation of Carbohydrate Reactions and Pathogenicity.

Carbohydrate Reactions and Types of Mycobacteria	Probable Pathogenicity for Humans	Pathogenicity for Chickens
Trehalose +, Xylose − Human tubercle bacilli, some bovine isolates, Battey strains, *M. fortuitum* strains.	Pathogenic	Non-pathogenic
Trehalose −, Xylose + *M. avium*, some swine isolates.	Non-pathogenic	Pathogenic

obtained from both non-growth and growth techniques with rapid-growing mycobacteria species such as *M. smegmatis* and *M. fortuitum* which have had extensive previous carbohydrate investigation by growth techniques(12).

Some mycobacteria will surely be found to yield reactions intermediate to those of well-defined species. Also, some strains definitely belonging to a particular species may show individual reactions. Such "aberrant" or "peculiar" reactions should not occasion confusion; these reactions should lead to further study towards distinguishing organisms and towards correlating metabolism with various characteristics. Thus, special carbohydrate reaction differences between strains of the same species may correlate to differences in morphology, pathogenicity, and serology.

Summary. 1. A massive inoculum non-growth carbohydrate testing procedure provides results not obtained previously with slow-growing mycobacteria. 2. The results obtained indicate usefulness of carbohydrate typing for aiding differentiation of species and strains of acid-fast organisms. 3. The trehalose and xylose reactions may correlate with pathogenicity for certain types of mycobacteria.

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Modification of Solubility of Cholesterol During Incubation with Growing Cells of *Aerobacter aerogenes.** (27031)

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During the course of experiments involving the catabolism of C^{14}—labeled cholesterol by growing cells of *Aerobacter aerogenes*, a significant transfer of label into the acid fraction was noted. Subsequent studies indicated that this phenomenon was due, in large part, to production during cell growth of some factor which modified the solubility of cholesterol, rather than to a conversion of the sterol itself to an acidic product.

Methods. A strain of *A. aerogenes* described earlier(1) was grown on a sterile medium containing 1% agar, 1.5% Difco Nutrient Agar, 0.5% yeast extract, and 0.01% colloidal cholesterol(1) labelled with C^{14}, at 35°C for 2 to 4 weeks, either in screw cap bottles containing vials of strong alkali as CO_2 traps, or in cotton plugged flasks. Both randomly labelled cholesterol prepared by incubating

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