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Laboratory Review of Reference Strains of *Corynebacterium diphtheriae* Indicates Mistyped Intermedius Strains

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All biotyped strains of *Corynebacterium diphtheriae* from the American Type Culture Collection (ATCC) were compared for morphology and biochemical reactions. Biotypes of all gravis strains and most mitis strains were confirmed, but intermedius strains were found to be misclassified. New lipid-dependent intermedius strains have been deposited with the ATCC.

Anderson et al. described three types of *Corynebacterium diphtheriae* on the basis of cultural and biochemical differences and noted that the gravis strains caused the most severe cases of diphtheria (1, 2, 9). A number of characteristics, including colonial morphology on McLeod's tellurite chocolate agar that clearly distinguished the minute colonies of intermedius strains from the much larger mitis and gravis colonies, differentiated the biotypes of *C. diphtheriae*. Gravis strains were distinguished by their ability to produce acid from starch and glycogen. Other distinguishing features of the three types are summarized in Table 1. Frobisher et al. proposed a fourth type of *C. diphtheriae* named minimus (5). Ward reported the growth stimulation of intermedius and minimus strains in the presence of Tween 80 but did not realize that it was due to the available lipids (14).

While analyzing a large number of intermedius isolates from an outbreak of diphtheria in Seattle, we discovered that intermedius strain ATCC 8032 was actually a mitis strain and omitted it from the study (3). The goal of the present study was to eliminate confusion for future researchers and commercial identification systems dealing with *C. diphtheriae* biotypes.

This study included all American Type Culture Collection (ATCC) reference strains of the three biotypes of *C. diphtheriae* as well as the minimus strain, ATCC 14779 (Table 2). To ensure their authenticity, reference cultures were purchased from the ATCC in 1993. Also included were four cultures described in an earlier study in which it was shown that Seattle isolates S 394 and S 486 were indistinguishable from intermedius strains from the Centers for Disease Control; FA 386 was received from A. A. Ferris of Australia in 1949, and strain 675, originally from A. Saragea, was received from C. H. Jellard in 1980 (3).

Biotyping was done according to the methods used at the Centers for Disease Control (12). Morphologic comparisons were made from colony growth on modified McLeod's medium, a tellurite chocolate agar prepared from rabbit blood and from growth on heart infusion (HI) agar with 5% sheep blood. Beta-hemolysis was determined after 48 h on HI blood agar. Acid production from carbohydrates was tested in HI broth with bromcresol purple indicator and 1% glucose, 1% sucrose, 0.2% starch, or 0.5% glycogen. Nitrate reduction was tested in 0.2% potassium nitrate in HI broth. Strains were also tested in the 20 microcupules of the Rapid CORYNE system (bioMérieux Vitek, Inc., Hazelwood, Mo.) as described by Gavin et al., with the exception that the inocula were harvested from Trypticase soy-blood agar instead of Columbia blood agar (6). Broth growth characteristics were observed in 5-ml aliquots of HI broth. Growth stimulation by addition of 0.2% Tween 80 was determined in HI broth. Microscopic morphologies of cells grown overnight on Loeffler's slants and stained with methylene blue were compared. All cells were incubated in media at 35°C. Plates were incubated in 5% CO_2 and broth media in an aerobic atmosphere. Growth and biochemical results were recorded after 24 and 48 h.

Table 1 presents the expected morphological and biochemical reactions of the three biotypes of *C. diphtheriae* as found in descriptions by Anderson et al. (1), McLeod (1, 9), Ward (14), and Sottnek and Miller (12). Results from the 13 reference strains and 4 intermedius stock cultures are summarized in Table 2.

It is clear that the ATCC strains designated intermedius do not fulfill the criteria for this biotype. Growth on modified McLeod's medium was considered the key feature for distinguishing the morphology of the larger, coarse colonies of gravis and mitis strains from the minute, fine colonies of intermedius strains. The intermedius strains from ATCC produced the mitis-gravis colony type instead of the minute colonies that are the constant and unique feature of the intermedius type (1). The three putative intermedius strains also differed from the published descriptions of this biotype in rapid glucose fermentation, lack of lipid stimulation, good growth in HI broth, and lack of distinctly barred cells (Table 2). In addition, intermedius strain ATCC 9675 was beta-hemolytic, which is not a feature of this biotype. These three reference strains belong in the mitis group.

The four intermedius strains from our stock culture collection produced the minute (<0.5-mm-diameter) colonies that are characteristic of the intermedius type on McLeod's medium and performed exactly as described in Table 1. However, the fermentation of glucose which occurred after overnight incubation of the Rapid CORYNE strips was not evident in the conventional media after incubation for a week. Without a lipid supplement such as Tween 80 or serum, the glucose broths of intermedius strains only

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TABLE 1. Expected morphology and differential biochemical reactions[a] of *C. diphtheriae* biotypes

Biotype	Colony diam (mm)[b]	β-Hemolysis[c]	Fermentation		Lipid stimulation[d]	Broth growth[e]	Microscopic morphology[f]
			Glucose	Starch and glycogen			
Intermedius	0.5	−	Slow[g]	−	+	Light, finely granular growth settles to leave clear supernatant	Barred, often long and clubbed
Gravis	2–4	Var	Rapid	+	−	Marked pellicle over clear broth is typical, but all variations occur	Short forms that tend to stain uniformly
Mitis	2–4	+	Rapid	−	−	Uniform heavy turbidity; later, a soft pellicle forms	Long forms with metachromatic granules

[a] The positive (+) reactions indicate approximately 99% reactivity. −, no reaction; Var, variations.
[b] After 48 h on modified McLeod's medium at 37°C (12).
[c] Beta-hemolysis of colonies after 48 h on sheep blood agar (9).
[d] HI broth supplemented with 0.2% Tween 80 (14).
[e] Description from McLeod, who used nutrient broth (9).
[f] Methylene blue stain of overnight growth on Loeffler's slants (9).
[g] Slow fermentation, weak acid production, or no change within 24 h (12). For the present study, the weak pH change after 7 days resulted in a slight change to grey-purple instead of yellow and was considered negative.

changed to a grey-purple color instead of the bright yellow color produced by the other biotypes.

With some minor exceptions, the five mitis and four gravis strains from ATCC conformed to the expected criteria. At variance with some of the criteria was mitis strain ATCC 9061, which fermented starch and glycogen, a gravis trait, though it did not have the microscopic morphology of that biotype. Strains that fermented starch and glycogen were

TABLE 2. Characteristics of reference strains of *C. diphtheriae* biotypes[a]

Biotype and strain[b]	Results at 48 h			Lipid stimulation	Biochemical type[c]	Broth growth type	Cell morphology[d]	Rapid CORYNE profile no.[e]	Revised biotype[f]
	Colony diam (mm)		β-Hemolysis						
	McLeod[g] >0.5	Blood agar[h] >1.0							
Intermedius									
ATCC 8032	+	+	−	−	M	M	M?	1010324	M
ATCC 9675	+	+	+	−	M	M	M	1010324	M
ATCC 11050	+	+	−	−	M	G	M?	1010324	M
S 394	−	−	−	+	I	I	I	1010324[i]	I
S 486	−	−	−	+	I	I	I	1010324	I
FA 386	−	−	−	+	I	I	I	1010324	I
675	−	−	−	+	I	I	I	1010324	I
Minimus, ATCC 14779	+	−	−	+	I	I	I?	1010324	I or M
Gravis									
ATCC 8028	+	+	−	−	G	G	G	1010326	G
ATCC 9059	+	+	+	−	G	G	G	1010326	G
ATCC 9060	+	+	−	−	G	M	G	1010326	G
ATCC 11049	+	+	−	−	G	M	G	1010326	G
Mitis									
ATCC 8024	+	+	+	−	M	M	M?	1010324	M
ATCC 8026	+[j]	+	+	−	M	M	M?	1010124	M
ATCC 9061	+	+	−	−	G	M	M?	1010326	G
ATCC 9673	+	+	+	−	M	M	M?	1010324	M
ATCC 11051	+	+	+	−	M	M	M?	1010324	M

[a] See Table 1 for descriptions of the expected characteristics. +, present; −, absent.
[b] The non-ATCC cultures were originally described in reference 3.
[c] Based on the conventional fermentation reactions shown in Table 1. M, mitis; I, intermedius; G, gravis.
[d] Cells that did not resemble the gravis or intermedius descriptions in Table 1 were considered mitis. M?, metachromatic granules were rare or absent. I?, cells were barred but did not have long or clubbed forms.
[e] Described by Gavin et al. (6).
[f] Conclusions regarding biotypes are based on all characteristics shown in this table.
[g] After 48 h on modified McLeod's medium, colony diameters of all gravis and mitis strains ranged from 1.0 to 3.0 mm, the minimus colony diameter was 1.0 mm, and the colony diameters of strains confirmed as intermedius were <0.5 mm.
[h] After 48 h on blood agar, colony diameters of all gravis and mitis strains ranged from 1.2 to 2.5 mm; the minimus and confirmed intermedius colony diameters were 1.0 mm.
[i] If the very weak maltose reactions of the minimus and intermedius strains were read as negative, the profile number would have been 1010304.
[j] Based on the few colonies that this inhibited strain produced on modified McLeod's medium.

typed as gravis. Gravis strains ATCC 9060 and 11049 did not produce pellicles in broth. The minimus strain behaved largely like an intermedius type, as previously suggested by Johnstone and McLeod (8), although it grew better than intermedius strains on McLeod's medium. With the exception of the glucose reaction of the intermedius strains already mentioned, the Rapid CORYNE reactions agreed with all conventional tests and in addition showed that all strains had *N*-acetyl-β-glucosaminidase. The maltose reactions of the true intermedius strains were very weak. All profile numbers were listed in the profile index as *C. diphtheriae*. However, the Rapid CORYNE system does not distinguish the intermedius biotype from the mitis type.

The cellular fatty acids of all strains in this study were analyzed by gas-liquid chromatography and the software of the Microbial Identification System (Newark, Del.) (data not shown). The system correctly biotyped 10 of the 12 strains identified as mitis or gravis in Table 2. The Microbial Identification System was unable to identify as *C. diphtheriae* our four intermedius strains or strain ATCC 8026. Our data from many biotyped *C. diphtheriae* strains have been given to the Microbial Identification System for updating their data base.

Because we found that the ATCC contains no representative of the *C. diphtheriae* intermedius biotype, strains S 394 and 675 have been deposited with the ATCC and assigned catalog numbers 51279 and 51280, respectively. Our findings for the four mistyped strains have been submitted to the ATCC with the confirmed intermedius biotype of NCTC strains 3987 and 5011 (data not shown).

The results of this study raise questions regarding the stability of the biotypes of *C. diphtheriae*. Frobisher et al. noted that the variability of *C. diphtheriae* biotypes, including gravis and mitis, is well known (5), but McLeod and others concluded that the biotypes of *C. diphtheriae* are extremely stable over long periods (8–10). In our experience, intermedius strains have been very stable (3).

Because modified McLeod's medium is quite different from the original recipe, the detailed colonial differences that McLeod noted between gravis and mitis strains were not included in this study. Instead, we relied on the descriptions by Hermann (7) and the monograph by Sottnek and Miller, who used modified McLeod's medium (12).

The tiny, clear colonies on routine blood agar that are characteristic of lipid-dependent corynebacteria represent the most convenient characteristic for distinguishing the intermedius variety from the eugonic gravis and mitis types. This enables clinical laboratories to differentiate the intermedius strains from the other types without the inconvenience of preparing a special medium.

Although the mitis biotype usually prevails during nonepidemic periods (3, 11, 13), the ability to recognize the three biotypes of *C. diphtheriae* can provide useful epidemiologic data about diphtheria outbreaks (3, 4).

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