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Rapid Microbiochemical Identification of *Corynebacterium diphtheriae* and Other Medically Important Corynebacteria

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A rapid biochemical method based on the fermentation of carbohydrates, the hydrolysis of urea, and the reduction of nitrate was used to identify *Corynebacterium diphtheriae, C. ulcerans, C. pseudodiphtheriticum, C. haemolyticum, C. pseudotuberculosis, C. pyogenes, C. ovis*, the Centers for Disease Control JK group, and *Rhodococcus* (*Corynebacterium*) *equi*. With this procedure identification was confirmed for 133 stock cultures and clinical isolates of corynebacteria. Most were identified within 1 h and all were identified within 4 h after inoculation into the test substrates.

Although the incidence of diphtheria has declined steadily since the first extensive use of immunization in the 1920s, the case fatality rate of 5 to 10% has remained practically unchanged. Classical respiratory infection is still recognized occasionally, but cutaneous infections, which are becoming increasingly important in North America (1, 3, 11), represent an important reservoir of diphtheria-causing organisms (1, 11) and may be a more contagious form of disease than the respiratory form (10).

In addition to *Corynebacterium diphtheriae*, several other species of corynebacteria are recognized as causing infections in otherwise healthy persons (4, 10). *C. ulcerans* has been reported to cause classical diphtheria as well as mild respiratory disease (12), *C. haemolyticum* has been reported to cause pharyngitis as well as systemic infection (3, 9), and *C. pseudotuberculosis* has been reported to cause lymphadenitis (7, 10). *C. xerosis* (6), *C. pseudodiphtheriticum*, *Rhodococcus* (*Corynebacterium*) *equi*, and the JK group (3, 13) have been reported to cause minor infections as well as nosocomial infections in compromised patients (4, 10).

Biochemical characterization of corynebacteria generally requires 24 to 72 h, although some rapid tests which speed up carbohydrate reactions have been described (8). To reduce the sometimes prolonged reaction time of serum carbohydrate media (4), we used a rapid microbiochemical (RM) method described previously by Yong and Prytula (15) and Yong and Thompson (16) for the biochemical characterization of many of the medically important corynebacteria in 1 to 4 h.

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MATERIALS AND METHODS

Cultures. Frozen stock cultures of corynebacteria were obtained from the National Reference Centre for Diphtheria, Provincial Laboratory of Public Health, Edmonton, Alberta, Canada (61 strains), and from the Central Public Health Laboratory, Toronto, Ontario, Canada (40 strains). A further 37 clinical isolates from our own laboratories were also included after identification was confirmed by the Reference Bacteriology Laboratory of the Central Public Health Laboratory, Ontario Ministry of Health.

Isolation and identification procedures. Stock cultures were grown on Columbia agar base (Gibco Diagnostics) with 5% sheep blood. Clinical specimens were inoculated onto Columbia blood agar-Hoyle tellurite medium (Columbia agar base with 5% laked horse blood and 0.34 g of potassium tellurite per liter).

Isolates of gram-positive, catalase-positive coryneform bacilli were inoculated into Andrade carbohydrate media (5) with 10% rabbit serum and dextrose, maltose, sucrose, starch, trehalose, or glycogen. Conventional nitrate broth medium (Difco Laboratories) and Christensen urea agar slants (BBL Microbiology Systems) with 1.5% agar (agar no. 1; Oxoid Ltd.) were also inoculated.

All isolates were also inoculated into the following RM media: dextrose, maltose, sucrose, starch, trehalose, glycogen, nitrate, and urea. The RM base medium consisted of Casamino Acids (20 g/liter; certified; Difco), L-cysteine HCl (0.3 g/liter; Sigma Chemical Co.), sodium sulfate (0.3 g/liter; J. T. Baker Chemical Co.), neopeptone (25 g/liter; Difco), and phenol red (0.1 g/liter). These ingredients were added to distilled water. The complete carbohydrate media were pre-

TABLE 1. Comparison of biochemical reactions of corynebacteria tested with conventional and RM substrates

Substrate	No. tested	No. (%) of reactions in agreement
Nitrate	138	137 (99.3)
Urease	138	138 (100)
Dextrose	138	137 (99.3)
Maltose	138	138 (100)
Sucrose	138	137 (99.3)
Starch	138	135 (97.8)
Trehalose	81	81 (100)
Glycogen	81	78 (96.3)

pared with a final carbohydrate concentration of 2%. Dextrose, maltose, sucrose, and trehalose media were filter sterilized through a 0.45-μm-pore membrane filter (Millipore Corp.). Starch and glycogen media were sterilized by autoclaving at 121°C for 10 min. The nitrate broth medium was the same as that used in the conventional method (2). The urease test broth medium was prepared by the method of Stuart et al. as previously described (14).

Each medium for the RM method was prepared in a 50-ml quantity and stored in a glass dropper bottle. Microtubes (disposable borosilicate glass, 6 by 50 mm; Kimble Div., Owens-Illinois, Inc.) were sterilized by autoclaving at 121°C for 15 min. Just before use, approximately 0.025 ml (1 drop) of each sterile medium was dispersed into microtubes. The tubes were placed in microtiter V plates (Dynatech Laboratories, Inc.), and the media were allowed to warm to room temperature (22°C) before inoculation.

Inoculation method. With a 3-mm-diameter loop, one-third of a loopful of pure growth of each isolate grown for 18 to 24 h on Columbia blood agar was inoculated into each of the media. Particular attention was paid to emulsifying clumps of bacteria in the substrates by the mixing action of the loop. Thorough emulsification and mixing are necessary; otherwise, the reaction time is prolonged. After inoculation, the medium in the urea tubes was overlaid with 2 drops of sterile mineral oil. The oil overlay shortens the time required for a positive reaction.

The tests were read after the inoculated tubes had been incubated for 1 h at 36°C in a shallow water bath. A positive carbohydrate fermentation reaction was indicated by a yellow color, and a negative reaction was indicated by a red color. In the urease test, a positive reaction was indicated by a pink color, and a negative reaction was indicated by a yellow color. In the nitrate test, after the 1-h incubation period, 1 drop of sulfanilic acid reagent and 1 drop of alphanaphthylamine were added, with thorough mixing following each addition. Reading was the same as for the conventional test.

RESULTS

Table 1 shows that the percent agreement between test results obtained with conventional and RM substrates in 990 individual tests was 99.1%. Most reactions were complete within 1 h; however, some reactions were delayed and required an incubation period of up to 4 h, viz., 15 starch reactions and 1 trehalose reaction. In addition, 1 starch reaction, 5 maltose reactions, and 1 trehalose reaction were weak but were considered to be positive when compared with a negative control. A positive result for urea was considered to be a bright pink color. Traces of pink color observed with occasional strains of *C. diphtheriae* and with pigmented strains of *R. equi* were disregarded.

In comparing reactions with RM substrates (Table 2) to accepted identification tables, 938 of 946 reactions (99.2%) were as expected. Of 138 organisms examined, 133 (96.4%) were identified by the RM method. Five organisms were not identified by the RM or conventional methods or by the Reference Bacteriology Laboratory.

TABLE 2. Reactions of 133 strains of corynebacteria tested by the RM method with an incubation period of 1 to 4 h

Organism	No. positive (no. tested) for:							
	Nitrate reduction	Urease	Carbohydrate fermentation					
			Dextrose	Maltose	Sucrose	Starch	Trehalose	Glycogen
C. diphtheriae biotype *gravis*	28 (28)	0 (28)	28 (28)	28 (28)	0 (28)	28 (28)	0 (22)	22 (22)
C. diphtheriae biotype *intermedius*	10 (11)	0 (11)	11 (11)	11 (11)	0 (11)	0 (11)	0 (3)	0 (3)
C. diphtheriae biotype *mitis*	30 (30)	0 (30)	30 (30)	30 (30)	0 (30)	0 (30)	0 (15)	0 (15)
C. diphtheriae biotype *mitis*, belfanti strain	0 (17)	0 (17)	17 (17)	17 (17)	0 (17)	0 (17)	0 (2)	0 (2)
C. ulcerans	0 (7)	7 (7)	7 (7)	7 (7)	0 (7)	6 (7)	7 (7)	1 (7)
C. pseudodiphtheriticum	29 (29)	29 (29)	0 (29)	0 (29)	0 (29)	0 (29)	0 (16)	0 (16)
R. equi	2 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (1)	0 (1)
C. haemolyticum	0 (2)	0 (2)	2 (2)	2 (2)	1 (2)	0 (2)	1 (1)	0 (1)
C. pseudotuberculosis	2 (4)	2 (4)	4 (4)	4 (4)	0 (4)	0 (4)	0 (4)	0 (4)
C. pyogenes	0 (1)	0 (1)	1 (1)	1 (1)	0 (1)	0 (1)	1 (1)	1 (1)
Centers for Disease Control group JK	0 (2)	0 (2)	1 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)

DISCUSSION

Hollis et al. (8) have proposed the use of a rapid fermentation test in a 4-h method for the identification of fastidious gram-positive rods, but they tested only some of the corynebacteria.

We chose to use conventionally accepted substrates (4) to demonstrate and confirm the reliability and reproducibility of the RM method, even though constitutive enzymes are relied upon to carry the reactions to completion. For 98.4% of the reactions the expected result was available within 1 h of incubation. In only 1.6% of the reactions was a prolonged incubation period of 4 h required, during which the nutritionally rich medium supported the necessary additional growth of the organisms. Reactions requiring 4 h of incubation were generally not critical to the identification of the corynebacteria. The starch reactions were important in identifying the *gravis* biotype of *C. diphtheriae*; however, when they were delayed, the glycogen reactions provided sufficient information in 1 h to complete the identification. The identifications were confirmed after 4 h when the starch reactions were completed.

Good correlation (99.1%) was found between the RM and conventional biochemical tests, but conventional tests required at least 24 h and often 48 h of incubation. In nine individual tests there was disagreement between results of the RM and conventional tests. In the nitrate test, there was one discrepancy in which the RM test was positive but the conventional test was negative. The organism was not reactive in any of the other substrates and was not identified by the Reference Bacteriology Laboratory. In the dextrose test, one group JK isolate produced acid in the conventional test but not in the RM test. In the sucrose test, one *C. pyogenes* isolate produced acid in the conventional test but not in the RM test. Starch testing produced three discrepancies in which the conventional test was positive but the RM test was negative. The isolates were identified as one strain of *C. pyogenes*, for which starch is not important for identification, and two strains of *C. diphtheriae* biotype *mitis*, which is not usually considered to be starch positive. The last two reactions were not reproducible and were considered to be false-positive. Glycogen testing showed discrepancies in three tests, all conventional test positive and RM test negative. Two of the three isolates were *C. pyogenes* and *C. haemolyticum*, for which glycogen testing is not usually performed, and the other isolate was *C. diphtheriae* biotype *mitis*, otherwise typical and not usually considered to be glycogen positive.

Included in the series of stock cultures supplied to us by the Central Public Health Laboratory was a toxigenic strain of *C. diphtheriae* biotype *intermedius* that was nitrate negative; however, all other characteristics were typical. This is an unusual finding, and the organism could be confused with the belfanti strain of *C. diphtheriae* biotype *mitis*.

We advocate RM identification of coryneform organisms on the basis of hemolysis and catalase testing and the reactions presented in Table 2. We consider the key reactions to be nitrate, urea, dextrose, maltose, and starch with sucrose, trehalose and glycogen being optional but useful in confirming identity. In our laboratories, we also use growth characteristics on Hoyle tellurite medium, H_2S production on modified Tinsdale medium when isolates are stabbed into the surface, and toxigenicity testing as adjuncts in confirming identity.

In summary, we believe that RM tests provide a practical and economical diagnostic tool for the rapid differentiation of corynebacteria.

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