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Comparison of Minitek and Conventional Methods for the Biochemical Characterization of Oral Streptococci

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Fifty-six strains of viridans streptococci were tested in the Minitek system (BBL Microbiology Systems, Cockeysville, Md.), and the results were compared with those obtained by conventional methods. An overall agreement of 98.9% was obtained when the Minitek tests were performed as follows. (i) All disks were incubated anaerobically for 48 h except for esculin and arginine, which required 5 to 7 days. (ii) The arginine disks were overlaid with 0.1 ml of sterile mineral oil even though incubated anaerobically. (iii) The Voges-Proskauer tests were performed under aerobic and anaerobic conditions. (iv) All tests for carbohydrate fermentation except for raffinose and salicin were read after the addition of 2 to 3 drops of 0.025% phenol red (pH 7.2). Of all the tests performed, only the fermentation of starch resulted in such poor agreement as to be considered unreliable. Results for inulin (unavailable in the Minitek system) were obtained by conventional methods.

Oral streptococci consist of a heterologous group of poorly defined organisms commonly referred to as the viridans streptococci, a term not totally descriptive since some oral streptococci may occasionally produce complete hemolysis or be nonhemolytic on blood agar containing sheep erythrocytes (7). Attempts to develop comprehensive schemes for serological classification of viridans streptococci have failed, and no correlation between serological and biochemical characteristics has been observed (9, 10, 13, 14, 17). Identification of viridans streptococci to species level is possible on the basis of physiological characteristics as shown by Facklam (3), who reported a 97% success rate in the identification of 1,227 clinical and 80 stock strains. The biochemical scheme proposed by Facklam is applicable after alpha- and gamma-hemolytic strains of groups B, D, N, and Q are serologically identified and excluded from the key.

Commonly used tests such as those for sugar fermentations and hydrolysis of esculin and arginine and tests for the production of indole and urease in tubed media are laborious and time consuming. To overcome these difficulties, miniaturized systems have been recently developed and shown to be accurate and dependable for characterization of *Enterobacteriaceae* (5, 8), lactobacilli (4), and anaerobes (6, 15). Because of the reported important role of biochemical characteristics in the identification of oral streptococci, we have investigated the reliability of the commercially available identification system, Minitek (BBL Microbiology Systems, [BBL], Cockeysville, Md.), by comparing it with conventional methods.

MATERIALS AND METHODS

Bacteria. Strains of microorganisms used in this study are listed in Table 1. The majority of strains were obtained from the American Type Culture Collection, Rockville, Md. Additional strains were kindly supplied by B. Gunn, Walter Reed Army Medical Center, Washington, D.C.; K. C. Gross, The New York Hospital, New York, N.Y.; L. A. Thomson, National Institute of Dental Research, Bethesda, Md.; and R. R. Facklam, Center for Disease Control (CDC), Atlanta, Ga. All cultures were maintained on 5% sheep blood agar plates at 37°C in a GasPak jar (BBL) containing disposable GasPak generators (H_2 + CO_2) (BBL) and activated catalyst replacement charges (BBL). Lyophilized cultures (American Type Culture Collection, Rockville, Md.) were reconstituted with brain heart infusion broth (Difco Laboratories, Detroit, Mich.).

Minitek procedure. The Minitek system (BBL) includes paper substrate disks (35 are available) which are placed into wells stamped in plastic plates. With this system the tests were performed as follows. Bacterial growth (24 h) from the surface of a blood agar plate was removed with a dry sterile cotton swab and suspended in 1 to 1.2 ml of Minitek enteric and nonfermenter broth. The resulting suspensions were dispensed with a 0.05-ml repeating dispenser gun into the individual wells containing the appropriate test disks,

TABLE 1. *Strains of streptococci tested*

Identification with the Facklam key	Identification no.	Sender identification
S. salivarius	ATCC 9222	*S. salivarius*
	ATCC 9758	*S. salivarius*
	ATCC 9759	*S. salivarius*
	ATCC 13419	*S. salivarius*
	ATCC 27945	*S. salivarius*
	ATCC 25975	*S. salivarius*
	ATCC 31067	*S. salivarius*
	CDC SS-262[a]	*S. salivarius*
	206[b]	*S. salivarius*
	208[b]	*S. salivarius*
	5[b]	*S. sanguis* biotype I
	4/1212α[b]	*S. sanguis* biotype I
S. mutans	ATCC 10035	*Streptococcus* sp.
	ATCC 19641	*Streptococcus* sp.
	ATCC 19642	*Streptococcus* sp.
	ATCC 19643	*Streptococcus* sp.
	ATCC 19645	*Streptococcus* sp.
	ATCC 25175	*S. mutans*
	ATCC 27351	*S. mutans*
	ATCC 27352	*S. mutans*
	ATCC 27607	*S. mutans*
	ATCC 27947	*S. mutans*
S. sanguis biotype I	ATCC 8144	*Streptococcus* sp. Lancefield group H
	ATCC 10556	*S. sanguis*
	ATCC 10558	*S. sanguis*
	ATCC 12396	*Streptococcus* sp. Lancefield group H
	CDC SS-910[a]	*S. sanguis* biotype I
	6[b]	*S. sanguis* biotype I
	11[b]	*S. sanguis* biotype I
S. sanguis biotype II	ATCC 903	*S. mitis*
	ATCC 6249	*S. mitis*
	ATCC 10557	*S. sanguis*
	ATCC 15909	*S. mitis* type I
	ATCC 15914	*S. mitis* type VII
	CDC SS-911[a]	*S. sanguis* biotype II
	JC-74[c]	*S. sanguis* biotype II
S. mitis	ATCC 4782	*Streptococcus* sp.
	ATCC 9811	*S. mitis*
	ATCC 15911	*S. mitis* type III
S. anginosus-constellatus	ATCC 27823	*S. constellatus*
	CDC SS-1111[a]	*S. anginosus-constellatus*
	P-651[d]	*S. milleri*
	B-1823[d]	*S. milleri*
	E-5033[d]	*S. milleri*
	C-3654[d]	*S. milleri*
	P-1914[d]	*S. milleri*
	B-4534[d]	*S. milleri*
	409[b]	*S. milleri*
S. MG-*intermedius*	ATCC 27335	*S. intermedius*
	ATCC 9895	*S. mitis* type V
	ATCC 15910	*S. mitis* type II
	ATCC 15912	*S. mitis* type IV
	ATCC 15913	*S. mitis* type VI
	CDC SS-899[a]	*S.* MG-*intermedius*
	408[b]	*S. milleri*
	404[b]	*S. milleri*

[a] Obtained from CDC collection.
[b] Obtained from B. Gunn, Walter Reed Army Medical Center, Washington, D.C.
[c] Obtained from L. A. Thomson, National Institute of Dental Research, Bethesda, Md.
[d] Obtained from K. C. Gross, Laboratory of Microbiology, The New York Hospital, New York, N.Y.

overlaid with 2 drops of sterile mineral oil (API, Plainview, N.Y.) in accordance with the instructions of the manufacturer, and incubated aerobically at 37°C in a Minitek humidor for 48 h. A similar procedure was followed with the Minitek anaerobe broth, which is a supplemented Trypticase (BBL)-peptone medium based on the formulation of Lombard et al. (G. L. Lombard, A. Y. Armfield, F. S. Thompson, M. D. Stargel, and V. R. Dowell, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, C38, p. 33). Tests performed with this medium were carried out under anaerobic conditions (37°C for 48 h in a GasPak jar) without an oil overlay. All tests were performed in duplicate.

After incubation, the results were examined before and after addition of 2 to 3 drops of 0.025% aqueous phenol red. A color change to yellow or light orange was interpreted as a positive reaction for aerobically incubated streptococci. Color change to yellow with no tint of orange indicated a positive reaction for anaerobically incubated streptococci.

Conventional method. Acid production from carbohydrates was tested in tubes containing 5 ml of phenol red broth base (Difco), pH 7.2. The medium was autoclaved for 15 min at 121°C before being supplemented with filter-sterilized sugars (Difco) and soluble starch (Difco) solutions to give a 1% final concentration. Organisms were grown in tryptic soy broth (Difco) for 24 h at 37°C and incubated aerobically except for strains of *Streptococcus anginosus-Streptococcus constellatus* (*S. anginosus-constellatus*) which were incubated under 5% CO_2 (2).

Results were recorded at 48 h and at 7 and 14 days. Uninoculated media incubated for 14 days served as controls for color comparison. A color change of medium from red to yellow was recorded as a positive reaction. The medium of Niven et al. (11) was used for the determination of ammonia production from L-arginine. Development of an orange-yellow color after addition of 0.1 ml of Nessler reagent to the 48-h culture indicated ammonia production. Christensen urea broth, Simmons citrate agar, and tryptic nitrate medium (Difco), prepared according to recommendations of the manufacturer, were inoculated, and the results were examined as described by Vera and Dumoff (16) after incubation at 37°C for 48 h. Esculin broth was prepared as described previously (16). A black precipitate after 48 h of incubation indicated a positive reaction. Bacto-M.R.-V.P. broth (Difco) was inoculated for the Voges-Proskauer test, which was performed by the O'Meara modified method (12). Since inulin disks are not presently manufactured by BBL for use in the Minitek system, results from conventionally prepared inulin broth were utilized in the Facklam key for the identification of viridans streptococci.

RESULTS

Although mannitol, lactose, inulin, arginine, esculin, raffinose, sorbitol, and acetoin (Voges-Proskauer) have been described as differentiating tests for oral streptococci (3, 7), 17 additional tests (Table 2) were performed, and the results were compared with conventional methods to assess more fully the applicability of the Minitek system. All streptococcal strains tested gave a positive reaction in broth containing dextrose, maltose, mannose, and sucrose and a negative reaction in media containing arabinose, glycerol, inositol, rhamnose, xylose, and citrate, as well as negative indole and nitrate reactions. Therefore, it was obvious that these tests were of no diagnostic value for species differentiation. Also, we

TABLE 2. *Percent agreement for results obtained when 56 streptococcal strains were tested by the conventional and Minitek systems*

Differentiating test	Agreement (%)			
	Aerobic-oil overlay (except as indicated)		Anaerobic-no oil overlay (except as indicated)	
	No phenol red[a]	Phenol red	No phenol red[a]	Phenol red
Arabinose	48.2	92.8	100	100
Cellobiose	78.2	75.0	75.0	87.5
Dextrose	92.8	89.3	100	100
Glycerol	98.2	100	100	100
Inositol	98.2	100	85.7	100
Lactose	98.2	94.6	92.8	100
Maltose	91.1	91.1	100	100
Mannitol	98.2	100	96.4	100
Mannose	100	91.1	100	100
Melibiose	98.2	98.2	98.2	100
Raffinose	98.2	100	96.4	92.8
Rhamnose	100	100	98.2	100
Salicin	92.8	87.5	96.4	85.7
Sorbitol	96.4	98.2	91.1	96.4
Starch	8.9	0	16.1	8.9
Sucrose	96.4	96.4	100	100
Trehalose	91.1	91.1	100	100
Xylose	100	100	100	100
Arginine	89.3	NA[b]	100[c]	NA
Citrate	100[d]	NA	100	NA
Esculin	86.0[d]	NA	100	NA
Indole	100[d]	NA	100	NA
Nitrate	100[d]	NA	100	NA
Urea	100	NA	100[c]	NA
Voges-Proskauer	98.8[d]	NA	96.4	NA
Mean (excluding starch)	93.8	95.0[e]	96.9	98.3[f]

[a] No phenol red was added in addition to that incorporated into the disk.

[b] Not applicable.

[c] Oil overlay.

[d] No oil overlay.

[e] Mean calculated using results for noncarbohydrate substrates (arginine-Voges-Proskauer) from the aerobic-oil-no phenol red column.

[f] Mean calculated using results for noncarbohydrate substrates (arginine-Voges-Proskauer) from the anaerobic-no oil-no phenol red column. Mean value became 98.9% when tests for raffinose and salicin were read in the absence of phenol red.

did not find the four remaining sugars, cellobiose, melibiose, salicin, and trehalose, to be of diagnostic value.

By comparing two methods of performing the Minitek system with a conventional method (2) commonly used for characterizing streptococcal species (Table 2), we were able to demonstrate an overall agreement of 98.9% when the Minitek tests were performed as follows. (i) All disks were incubated anaerobically for 48 h, except for esculin and arginine, which required 5 to 7 days. (ii) The arginine disks were overlaid with 0.1 ml of sterile mineral oil even though incubated anaerobically. (iii) The Voges-Proskauer tests were performed under aerobic and anaerobic conditions. (iv) All tests for carbohydrate fermentation, except for raffinose and salicin, were read after the addition of 0.025% phenol red (pH 7.2). Results for inulin (unavailable in the Minitek system) were obtained by conventional methods. Results from this test were used in conjunction with Minitek results when applying the Facklam key.

Of all the tests performed, only the fermentation of starch resulted in such poor agreement as to be considered unreliable. Of all other tests performed under optimal conditions, the poorest agreement (87.5%) occurred with cellobiose.

After anaerobic incubation, the addition of 2 to 3 drops of 0.025% aqueous phenol red (pH 7.2) to the carbohydrate disks was found to greatly facilitate interpretation by eliminating borderline colors and thereby improving the overall agreement with conventional methods by 1.4%. Only the raffinose and salicin tests gave better agreement when no additional phenol red was added. The Minitek-anaerobic method read with phenol red exhibited an overall agreement of 98.3% with the conventionally tubed media, whereas the aerobic Minitek-oil method read with phenol red exhibited an agreement of 95.0% (Table 2). After the use of phenol red, a 5.0% decrease in overall agreement between conventional and aerobic Minitek-oil results was observed, but only a 1.7% decrease between conventional and Minitek-anaerobic methods was noted. This represents a 3.3% increase in agreement with conventional methods for the Minitek-anaerobic method.

A 100% agreement was observed for the urea test which was included in this study, since 50% of the strains of *S. salivarius* have been reported (7) to be urea positive.

We were able to correctly identify 100% of the oral streptococci tested (Table 3) by applying the Facklam key to the results obtained by using either conventional media (2) or the Minitek system. Therefore, under the conditions described, a substitution of bromocresol purple (3) by phenol red (conventional media [2] and Minitek) did not alter the reliability of the Facklam key for accurate species identification.

DISCUSSION

We have observed frequent false-negative reactions associated with certain species of oral streptococci (particularly, strains of *S. anginosus-constellatus*) in the Minitek system when performed under aerobic conditions even though the disks were overlaid with oil. Anaerobic incubation for 48 h eliminated these false-negative reactions except for the esculin and arginine tests, which required an extended incubation of 5 to 7 days. An accurate esculin test was essential in the Facklam key for the differentiation of *S. anginosus-constellatus* from *S. morbillorium*. The more luxuriant growth of most streptococci occurring under anaerobic conditions decreased the incubation period required for completion of the tests. Accurate test results were observed for *S. mutans* (ATCC 25175) within 5 h under anaerobic conditions in Lombard-Dowell medium whereas 48 h was required when inoculated Minitek enteric and nonfermenter broth was incubated aerobically. Under anaerobic conditions, the arginine disk required a 0.1-ml mineral oil overlay to prevent escape of ammonia from the medium, which resulted in false-negative or borderline reactions. Under these conditions, a 100% agreement with the conventional arginine test was obtained (after 5 to 7 days of incubation).

Since the Voges-Proskauer test depended upon the oxidation of acetylmethylcarbinol in alkaline medium to form diacetyl (1), dissolved oxygen existing in the aerobically incubated medium enhanced the pink color required for a positive reaction. Consequently, organisms capable of growing aerobically gave the strongest positive reaction under aerobic conditions. Due to poor growth aerobically, the microaerophilic oral streptococci required an anaerobic environment to facilitate the abundant growth necessary for a positive Voges-Proskauer reaction. Therefore, it is recommended that this test be performed both aerobically and anaerobically, with a color change under either condition considered a positive reaction for the Voges-Proskauer test. The difficulty encountered in reading an anaerobically incubated Voges-Proskauer test may be partially overcome through aeration by vigorous stirring after the addition of reagents.

Kiehn et al. (8) reported a 97.2% agreement for the Minitek urea test when compared with

TABLE 3. *Comparison of results obtained for key differentiating tests in the identification of viridans streptococci obtained by conventional and Minitek methods*

Differentiating test	Method	% Identified as positive (+) or negative (−)						
		S. mutans	*S. sanguis* I	*S. salivarius*	*S.* MG-*intermedius*	*S. sanguis* II	*S. mitis*	*S. anginosus-constellatus*
Mannitol	Facklam[a]	100 (+)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
	Carlsson[b]	100 (+)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
	Minitek-LD[c]	100 (+)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
	Minitek-EN[d]	100 (+)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Lactose	Facklam	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (−)
	Carlsson	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (−)
	Minitek-LD	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (−)
	Minitek-EN	100 (+)	71.0 (+)	100 (+)	100 (+)	83.3 (+)	100 (+)	100 (−)
Inulin	Facklam	NA[e]	100 (+)	100 (+)	100 (−)	100 (−)	100 (−)	NA
	Carlsson	NA	100 (+)	100 (+)	100 (−)	100 (−)	100 (−)	NA
	Minitek-LD	ND[f]	ND	ND	ND	ND	ND	ND
	Minitek-EN	ND	ND	ND	ND	ND	ND	ND
Arginine	Facklam	NA	100 (+)	100 (−)	NA	NA	NA	NA
	Carlsson	NA	100 (+)	100 (−)	NA	NA	NA	NA
	Minitek-LD	NA	100 (+)	100 (−)	NA	NA	NA	NA
	Minitek-EN	NA	67 (+)	100 (−)	NA	NA	NA	NA
Esculin	Facklam	NA	NA	NA	100 (+)	100 (−)	100 (−)	100 (+)
	Carlsson	NA	NA	NA	100 (+)	100 (−)	100 (−)	100 (+)
	Minitek-LD	NA	NA	NA	100 (+)	100 (−)	100 (−)	100 (+)
	Minitek-EN	NA	NA	NA	80 (+)	100 (−)	100 (−)	89 (+)
Raffinose	Facklam	NA	NA	NA	NA	100 (+)	100 (−)	NA
	Carlsson	NA	NA	NA	NA	100 (+)	100 (−)	NA
	Minitek-LD	NA	NA	NA	NA	100 (+)	100 (−)	NA
	Minitek-EN	NA	NA	NA	NA	100 (+)	100 (−)	NA

[a] Results obtained by Facklam (3), using heart infusion broth (Difco) and a bromocresol purple indicator.
[b] Results obtained with Carlsson method (2), using phenol red broth base (Difco).
[c] Results obtained with the Minitek system under anaerobic conditions, using Lombard-Dowell (LD) medium (anaerobic broth) and phenol red indicator when appropriate (BBL product information sheet).
[d] Results obtained with the Minitek system, using enteric-nonfermenter broth (EN), phenol red indicator, and oil overlay when appropriate (BBL product information sheet).
[e] Not applicable (nondifferentiating test).
[f] Not done.

the conventional Christensen urea medium for urease produced by the *Enterobacteriaceae*. They observed false-negative urea reactions with *Serratia, Enterobacter,* and *Citrobacter* species. In a similar study, Hansen et al. (5) reported a 98.9% agreement. Of the oral streptococci, only certain strains of *S. salivarius* were urease positive (7). We found 42% of the *S. salivarius* strains tested to be urease positive and observed a 100% agreement in results obtained by comparing the two methods.

Of all the tests performed, only the fermentation of starch resulted in such poor agreement as to be considered unreliable.

Although 100% agreement for all substrate disks and all organisms may not be attainable, a 98.3% agreement is sufficiently high as to offer a reasonable substitute for the more expensive conventional methods, the utilization of which is more cumbersome and time consuming. As is reasonably expected, proper application and familiarity with the system are required. It has been suggested (3) that past failures to successfully use biochemical tests to differentiate oral streptococci occurred because sufficiently large groups of tests were not used. The Minitek system allows the small laboratory easy access to many tests since the manufacturer claims a 2-year shelf life for the disks, which conveniently require very small amounts of refrigerator and

incubator space. The miniaturized Minitek micromethod provides a fast method for the identification of oral streptococci that is easily performed by the clinical microbiology laboratory.

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