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Semiautomated Technique for Identification of Subgingival Isolates

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A semiautomated approach for the characterization of subgingival bacterial isolates which economizes in media preparation, inoculation, reading, recording, and interpretation of results was tested. Test ingredients were added to a basal medium consisting of Mycoplasma broth supplemented with 5 μg of hemin, 0.5 mg of $NaHCO_3$, and 0.5 mg of L-cysteine per ml. Sterile test media were aseptically dispensed into wells of sterile microtiter plates with a MIC 2000 dispenser. Inocula were grown in broth or scraped from agar plates, dispersed, and inoculated with a MIC 2000 inoculator. After 2 to 4 days of incubation, the optical density of growth was determined with an Artek 210 vertical beam reader at 580 nm and stored on a floppy disk. Reagents were added to each well, and the changes in optical density were determined. Thresholds for positive reactions were determined after extensive preliminary studies for each test. The tests were run in duplicate on each plate and interpreted with an Artek vertical beam reader. Tests that were run in this system included: fermentation of carbohydrates, decarboxylase reactions, reduction of nitrate and nitrite, ammonia production, hydrolysis of esculin, growth in the presence of inhibitory or stimulatory substances, and indole production. Approximately 80% of all isolates from subgingival samples could be characterized by this technique. Comparisons were made between the semiautomated and conventional identification techniques. Overall reproducibility of 2,980 strains by the semiautomated and conventional techniques were 95 and 90%, respectively. There was an 86% similarity of results by the semiautomated and conventional methods. The semiautomated technique was more rapid, less expensive, and as reproducible as the conventional method of identification.

The characterization and identification of microorganisms in samples of dental plaque is an essential prerequisite for determining the etiology of various forms of dental caries, periodontal diseases, and root canal infections. Many methods have been used to identify bacterial isolates from plaque samples, including conventional biochemical characterization, serological techniques (3, 6, 7), and electrophoresis of cell constituents (1, 6, 7). Conventional characterization of bacterial isolates, including media preparation, inoculation, reading, recording, and interpretation of tests, is labor intensive and time consuming. It seems essential, therefore, that procedures be developed to accelerate the process of characterization. Efforts have been made to utilize resting cell tests and micromethods for identification. With some exceptions (4, 5, 8, 12), most methods have been directed toward the identification of the more readily cultivable clinical isolates. Members of the indigenous microbiota of the human oral cavity appear, however, to be somewhat more difficult to characterize (9, 10), and therefore, development of methods for their characterization have evolved more slowly. The purpose of this investigation was to develop and evaluate a rapid and reproducible method for the characterization and identification of bacterial isolates.

MATERIALS AND METHODS

Source of strains. In Table 1 are listed 70 ATCC reference strains characterized by both the conventional and semiautomated techniques. In Table 2 the identifications of 2,980 fresh bacterial isolates which were used to test the reproducibility of the semiautomated technique are summarized. All bacterial isolates were derived from plaque samples taken from subjects with various forms of periodontal disease. The isolates included 613 gram-positive facultative and anaerobic cocci, 1,076 gram-positive facultative and anaerobic rods, 1,213 gram-negative capnophilic to anaerobic rods, and 78 gram-negative anaerobic cocci. It should be noted that strains which do not fit existing species descriptions are grouped by cluster analysis (11), as indicated by Roman numerals (Table 2).

Media and maintenance of strains. The strains were maintained by weekly transfer on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood. Inocula were prepared by growing the organisms in Mycoplasma broth (BBL) supplemented with 5 μg of hemin, 1 mg of glucose, and 0.5 mg of filter-sterilized sodium bicarbonate per ml. Both agar plates and broth cultures were incubated at 35 to 37°C in an atmosphere of 80% N_2–10% H_2–10% CO_2. Broth inocula were incubated for 1 to 2 days. Inocula were used only if the turbidity approximated at least a no. 1 MacFarland standard.

Preparation of microtiter plates. Appropriate concentrations of the various substrates (Table 3) were added to tubes containing 20 ml of the basal medium, consisting of Mycoplasma broth supplemented with 5 μg of hemin per ml. The tubes were sterilized for 15 min at 121°C. Sodium bicarbonate and L-cysteine solutions were filter sterilized and aseptically added to each tube to make final concentrations of 0.5 mg/ml each. The media were aseptically dispensed in 0.1-ml portions into wells of sterile microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) with a 96-channel MIC 2000 dispenser (Dynatech). The plates were used immediately or stored frozen at −20°C for up to 3 weeks. Examples of the layout of a gram-negative and a gram-positive plate are shown in Fig. 1. Duplicate tests or multiple strains or both could be run on each microtiter plate.

Inoculation and incubation of microtiter plates. To provide sufficient volume of inocula for the desired biochemical tests, gram-positive facultative cocci, gram-negative rods,

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TABLE 1. ATCC reference strains tested by both the conventional and semiautomated methods

Gram-positive rods	ATCC no.
Rothia dentocariosa	14189, 14190 17931, 14191
Propionibacterium lymphophilum	27520
Propionibacterium acnes	06919, 06921, 11828
Propionibacterium granulosum	25564
Propionibacterium avidum	25577
Propionibacterium jensenii	04867
Eubacterium combessi	25545
Eubacterium contortum	25540
Eubacterium cylindroides	27528, 27803
Eubacterium alactolyticum	23263, 17927
Eubacterium necrogenes	25556
Eubacterium limosum	08486
Eubacterium moniliforme	25546
Eubacterium brachy	33089
Eubacterium timidum	33094, 33092
Eubacterium nodatum	33099
Eubacterium aerofaciens	25986
Bifidobacterium adolescentis	15703, 15705
Bifidobacterium dentium	27534, 27680
Bifidobacterium infantis	15697
Actinomyces viscosus	15987, 19246
Actinomyces israelii	23860, 10049
Actinomyces odontolyticus	17929, 17982
Actinomyces naeslundii	12104, 27040
Arachnia propionica	14157, 29324 29325, 29326
Bacterionema matruchotii	14265, 14266

Gram-negative rods or cocci	ATCC no.
Leptotrichia buccalis	14201, 23472, 23471
Bacteroides melaninogenicus	15930, 33184, 33185, 25845
Bacteroides melaninogenicus sp.	15032, 15033
Bacteroides intermedius	25261
Fusobacterium mortiferum	25557, 09817
Fusobacterium necrophorum	25286, 27852
Fusobacterium naviforme	25832
Fusobacterium nucleatum	25586, 10953
Eikenella corrodens	23834
Capnocytophaga ochracea	27872
Capnocytophaga sputigena	33123
Capnocytophaga gingivalis	33124
Selenomonas sputigena	33150
Veillonella alcalescens subsp. *dispar*	17748
Veillonella alcalescens subsp. *alcalescens*	14894
Veillonella parvula	10790
Viellonella parvula subsp. *atypica*	17744

and gram-positive rods and anaerobic cocci were grown in 10, 20, and 40 ml of broth, respectively. The number of biochemical tests required for characterization dictated the type of inoculum tray to be used (Fig. 1). The gram-positive inoculum tray contained one reservoir, which allowed one strain to be run in duplicate. Gram-negative inoculum trays contained two reservoirs, which allowed two organisms to be tested, each in duplicate; and the inoculum tray used for gram-positive facultative cocci had four reservoirs, which permitted four organisms to be tested in duplicate. After growth, the broth cultures were poured into the inoculum trays. A MIC 2000 inoculator (Dynatech) was used to transfer approximately 1.5 μl of inocula into each well. Gram-positive facultative cocci were incubated for 2 days, and all other strains were incubated for 5 days at 35 to 37°C in an atmosphere of 80% N_2–10% H_2–10% CO_2.

Reading and recording of results. The plates were evaluated with an Artek model 210 vertical beam reader (Artek Systems Corp., Farmington, N.Y.) at 580 nm. The Artek 210 is an enzyme-linked immunosorbent assay (ELISA) reader which scans the wells of microtiter plates and sends the optical density (OD) values of these wells to an Artek 200 computer. The system permits comparison of OD readings of the wells of inoculated plates with those of uninoculated (blank) controls. The differences in OD between the wells of inoculated and uninoculated plates were used to measure growth of the isolates in each well. Stimulation of growth by arginine or inhibition of growth by bile or antibiotics was determined by comparing the OD of the growth in the wells containing the test substances with those containing glucose. Arginine was considered to be stimulated if the ODs were greater than twice those observed in the glucose wells. Growth was considered to be inhibited by a substance if the ODs were less than half those observed in the glucose wells.

Test reagents and their uses are as follows: bromthymol blue (0.5 mg/ml), acid from carbohydrates, urease, decarboxylases; Nessler's reagent, ammonia production; Kovac's reagent, indole production; ferric ammonium citrate, esculin hydrolysis. The test reagents were added to the appropriate wells of inoculated and uninoculated (control) plates, and the OD of the plate, media, bacterial growth, and reagents were determined in a second scan. Subtraction of the OD of the plate, media, and growth (initial scan) provided a measure of the OD of the test reagent in each well. This value was compared with the reading of the ODs of the reagents in the uninoculated plate. An increase or decrease of the OD above or below certain predetermined thresholds was interpreted as a positive result. For example, the threshold for hexose fermentation was determined by measuring the OD in bromthymol blue-containing wells in which the pH dropped one unit as measured by a glass electrode. This value was shown to be 0.49 times the OD of the bromthymol blue in the uninoculated plate. Thus, if the OD in the bromthymol blue-containing uninoculated well was 1.0, then the threshold values assigned would be 0.49. ODs of the bromthymol blue in the inoculated wells below this value would be considered positive; above this value they would be considered negative. The thresholds were optimized for each substrate and recorded in the computer to permit well-by-well evaluations. The OD threshold values for positive reactions for various

TABLE 2. Summary of identifications of fresh isolates used to test reproducibility of semiautomated methods

Facultative isolates	No. of strains	Anaerobic isolates	No. of strains
Cocci (691 isolates)		Cocci (691 isolates)	
Streptococcus intermedius	210	*Peptostreptococcus micros*	35
Streptococcus morbillorum	39	Unidentified	42
Streptococcus sanguis II	79	*Veillonella paravula*	78
Streptococcus constellatus	6		
Streptococcus acidominimus	2		
Streptococcus uberis	26		
Streptococcus sanguis I	28		
Streptococcus mitis	44		
Streptococcus mutans	7		
Streptococcus salivarius	4		
Staphylococcus aureus	6		
Staphylococcus epidermidis	32		
Unidentified	85		
Gram-negative rods (1,213 isolates)		Gram-negative rods (1,213 isolates)	
Actinobacillus actinomycetemcomitans	50	*Bacteroides gingivalis*	45
Haemophilus aphrophilus	21	*Bacteroides intermedius*	196
Capnocytophaga ochracea	153	*Bacteroides melaninogenicus*	9
Capnocytophaga gingivalis	40	Other black-pigmented *Bacteroides* spp.	56
Capnocytophaga sputigena	18	*Fusobacterium nucleatum*	276
Unidentified *Capnocytophaga* spp.	17	*Fusobacterium gonidiaformans*	80
		Selenomonas sputigena	17
		Anaerovibrio lipolytica	5
		Bacteroides buccae-oris	3
		Bacteroides oralis	17
		Unidentified	211
Gram-positive rods (1,076 isolates)		Gram-positive rods (1,076 isolates)	
Actinomyces spp.		*Propionibacterium* spp.	
I	122	XV	39
II	40	XXVII	12
III	25	XXVIII	18
IV	21	*Eubacterium* spp.	
V	39	VI	7
VII	36	XVI	11
VIII	75	XXIV	59
IX	70	XXV	43
X	110	XL	4
XI	9	*Lactobacillus* spp.	
XII	10	XVII	8
XIII	25	XIX	54
XIV	9	XXIX	13
XVIII	15	XXX	7
XX	23	XXXII	6
XXI	17	XXXIII	4
XXII	10	XXXIV	3
XXIII	16		
XXXV	3		
XXXVI	5		
XXXVII	4		
XXXVIII	4		
XXXIX	4		

tests are as follows: acid from carbohydrates (except pentoses), <0.49; acid from pentoses, <0.45; indole and nitrate reduction, >6.0; esculin hydrolysis, >20.0; ammonia production, >10.0; nitrite reduction, >2.0.

The data could be stored on floppy disc, output as positive or negative results to a printer, or interpreted as features in probabilistic identification or cluster analysis programs.

RESULTS

An example of a microtiter plate inoculated with a gram-negative isolate is shown in Fig. 2. The sharp test results permitted well-by-well evaluation with the ELISA reader.

Comparisons of the reproducibility of each test were made between the semiautomated and conventional techniques with 70 ATCC reference cultures (Table 4). Each test was run in duplicate on separate occasions by both techniques. The overall reproducibility when conventional techniques were utilized was 90%, and with the semiautomated system it was 95%. Overall similarity between the two methods was found to be 86%. In the conventional system, only three substrates (adonitol, rhamnose, sucrose) demonstrated <80% reproducibility. However, only arabinose and galactose demonstrated an overall similarity of <80% between the two systems tested. Overall, the semiautomated technique appeared to be more reproducible than the conventional system of characterization.

The reproducibility of each test with 2,980 fresh subgingival plaque isolates in the semiautomated system is summa-

TABLE 3. Tests performed in the semiautomated system

Test	Gram-positive rods and anaerobic cocci	Gram-negative rods	Gram-positive facultative cocci
Acid from			
Adonitol	+		
Amygdalin	+	+	
Arabinose	+	+	
Cellobiose	+	+	
Dextran	+		
Dulcitol	+		
Esculin	+	+	
Fructose	+	+	
Galactose	+	+	
Glucose	+	+	+
Glycerol	+		
Glycogen	+	+	
Inositol	+		
Inulin	+		+
Lactose	+	+	+
Maltose	+	+	
Mannitol	+	+	+
Mannose	+	+	
Melizitose	+		
Melibiose	+	+	+
Raffinose	+	+	+
Rhamnose	+		
Ribose	+		
Salicin	+	+	+
Sorbitol	+		
Starch	+	+	
Sucrose	+	+	
Trehalose	+	+	+
Xylose	+	+	
Ammonia production	+		
Indole	+	+	
Nitrate reduction	+	+	+
Nitrite reduction	+		
Arginine decarboxylase	+		
Ornithine decarboxylase	+		
Lysine decarboxylase	+		
Urea	+		
Arginine stimulation	+		
Ammonia from arginine	+		+
Voges-Prauskauer	+		
Growth in 10% bile	+	+	+
Esculin hydrolysis	+	+	+

rized in Table 5. Half of the tests showed >95% reproducibility, and all but three tests showed >90% reproducibility. The tests which were more variable were esculin hydrolysis, ammonia production from arginine, and ammonia produced from the basal medium. The reproducibility of these tests in the microtiter system was slightly lower than in conventional tests in our laboratory (about 90%). However, there was an overall agreement of 95% between all tests run in the semiautomated system. There appeared to be no major differences in the reproducibility of tests for different categories of organisms.

DISCUSSION

The present investigation indicates that biochemical characterization of human subgingival plaque isolates by a semiautomated technique was at least as reproducible as conventional biochemical characterization. Tests performed in microtiter plates have been utilized by others (1, 2, 12, 13). For example, Allen et al. (1) used the MIC 2000 system for the characterization of anaerobic isolates from clinical infections. They found >90% agreement of microdilution tests with tests performed with a conventional system. In addition, reproducibility of duplicate reactions in their microtest system was 96%. Their findings were virtually identical to those of this study.

The major advantages of the semiautomated procedure were savings in media and time. The time requirements for the biochemical characterization of isolates may be divided into three phases: (i) media preparation and storage, (ii) inoculation of tests, and (iii) reading, recording, and interpretation of results (Table 6).

Media preparation and dispensing were facilitated by performing tests in microtiter plates. The use of microtiter plates required smaller amounts of media (0.1 ml), which could be dispensed rapidly and aseptically. A single plate of 96 wells can be filled with the test media within 1 s, and up to 150 plates could be dispensed from a single set of dispensing tubes within 1 h.

The microtiter plate system also has the advantage of requiring less storage space than conventional systems. Prepared plates may be stored at −20°C for 3 weeks with no

TABLE 4. Percent reproducibility of conventional and semiautomated techniques[a]

Substrate	% Reproducibility by the following techniques:	
	Conventional	Semiautomated
Adonitol	79	100
Amygdalin	96	100
Arabinose	96	67
Cellobiose	88	96
Dextran	96	96
Dulcitol	100	100
Esculin	100	96
Fructose	83	96
Galactose	88	83
Glucose	92	100
Glycerol	88	96
Inositol	92	100
Inulin	92	100
Lactose	88	96
Maltose	83	100
Mannitol	92	92
Mannose	83	100
Melezitose	92	100
Melibiose	88	96
Raffinose	83	96
Rhamnose	96	100
Ribose	75	92
Salicin	88	92
Sorbitol	88	100
Starch	83	96
Sucrose	75	100
Trehalose	92	100
Xylose	96	100
NO_3 reduction	92	100
NO_2 reduction	88	88
Lysine decarboxylase	96	100
Ornithine decarboxylase	100	100
Arginine decarboxylase	92	92
Urease	100	96
Indole	92	83

[a] A total of 70 ATCC reference strains were used, with an overall reproducibility of 90% for the conventional technique and 95% for the semiautomated technique.

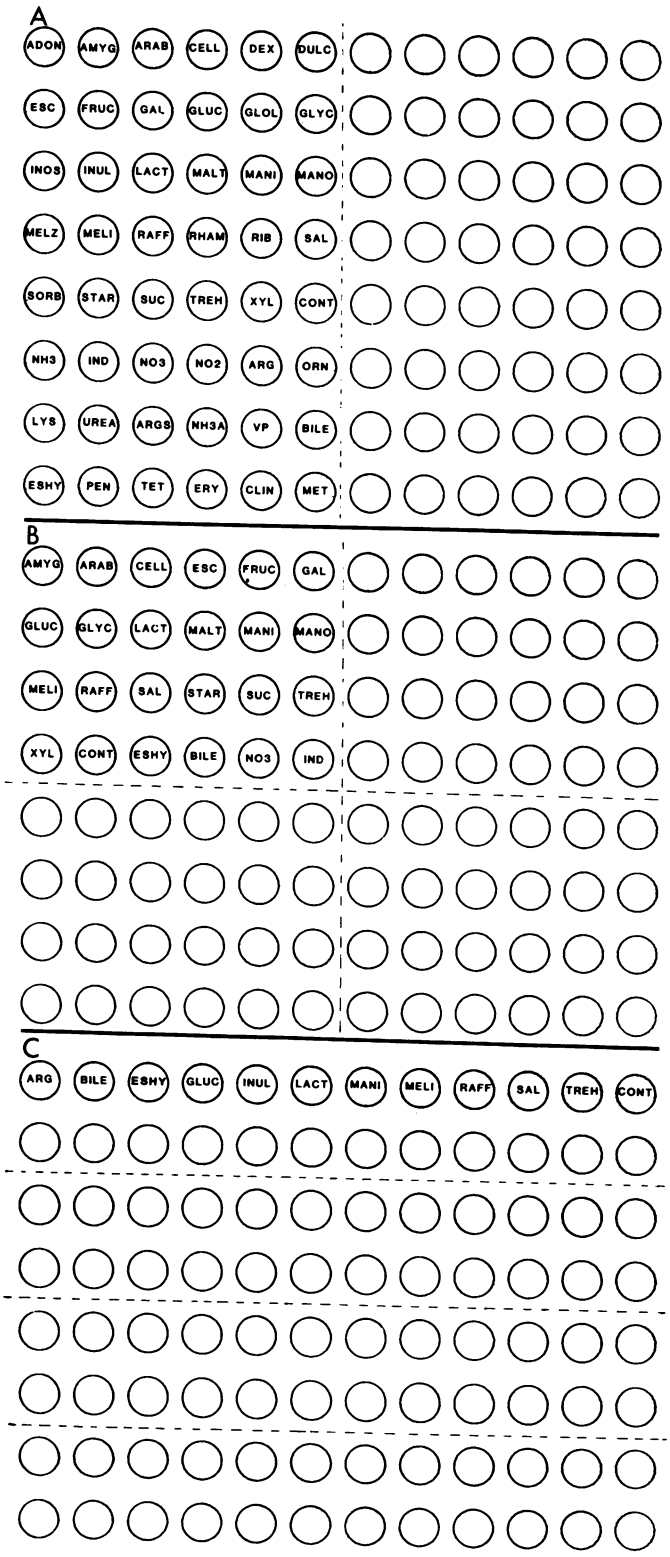

FIG. 1. (A) Format of a microtiter plate showing the biochemical tests used for the characterization of gram-positive rods. The duplicate tests for the strain are run on the right side of the plate. (B) Format of a microtiter plate showing the biochemical tests used for the characterization of gram-negative rods. Two strains can be run in duplicate on each plate, with one organism on the left side and one on the right side. (C) Format of a microtiter plate showing the biochemical tests used for the characterization of streptococci. Four strains can be run in duplicate on each plate. Abbreviations: ADON, adonitol; AMYG, amygdalin; ARAB, arabinose; CELL, cellobiose; DEX, dextran; DULC, dulcitol; ESC, esculin; FRUC, fructose; GAL, galactose; GLUC, glucose; GLOL, glycerol; GLYC, glycogen; INOS, inositol; INUL, inulin; LACT, lactose; MALT, maltose; MANI, mannitol; MANO, mannose; MELZ, melizitose; MELI, melibiose; RAFF, raffinose; RHAM, rhamnose; RIB, ribose; SAL, salicin; SORB, sorbitol; STAR, starch; SUC, sucrose; TREH, trehalose; XYL, xylose; CONT, control; NH3, ammonium production; IND, indole production; NO3, nitrate reduction; NO2, nitrite reduction; ARG, arginine decarboxylase; ORN, ornithine decarboxylase; LYS, lysine decarboxylase; UREA, urease; ARGS, arginine stimulation; NH3A, ammonium from arginine; VP, Voges-Proskauer; BILE, growth in the presence of bile; ESHY, esculin hydrolysis; PEN, growth in the presence of penicillin; TET, growth in the presence of tetracycline; ERY, growth in the presence of erythromycin; CLIN, growth in the presence of clindamicin; MET, growth in the presence of metronidazole.

TABLE 5. Percent reproducibility of tests in the semiautomated system[a]

Test	% Reproducibility
Glucose	99
Sucrose	99
NO_2 reduction	99
Lysine decarboxylase	99
Voges-Prauskauer	99
Fructose	98
Lactose	98
Maltose	98
NO_3 reduction	98
Ornithine decarboxylase	98
Indole	98
Mannose	97
Arginine decarboxylase	97
Urease	97
H_2O_2 decomposition	96
Galactose	96
Ribose	96
Trehalose	96
Inositol	95
Inulin	95
Sorbitol	95
Amygdalin	94
Cellobiose	94
Glycogen	94
Melibiose	94
Raffinose	94
Xylose	94
Adonitol	93
Melizitose	93
Rhamnose	93
Starch	93
Arabinose	92
Glycerol	92
Salicin	92
Esculin	91
Dextran	91
Dulcitol	90
Mannitol	90
Esculin hydrolysis	88
NH_3 from arginine	83
NH_3 produced	82

[a] A total of 2,980 isolates were tested. The overall agreement of the tests was 95%.

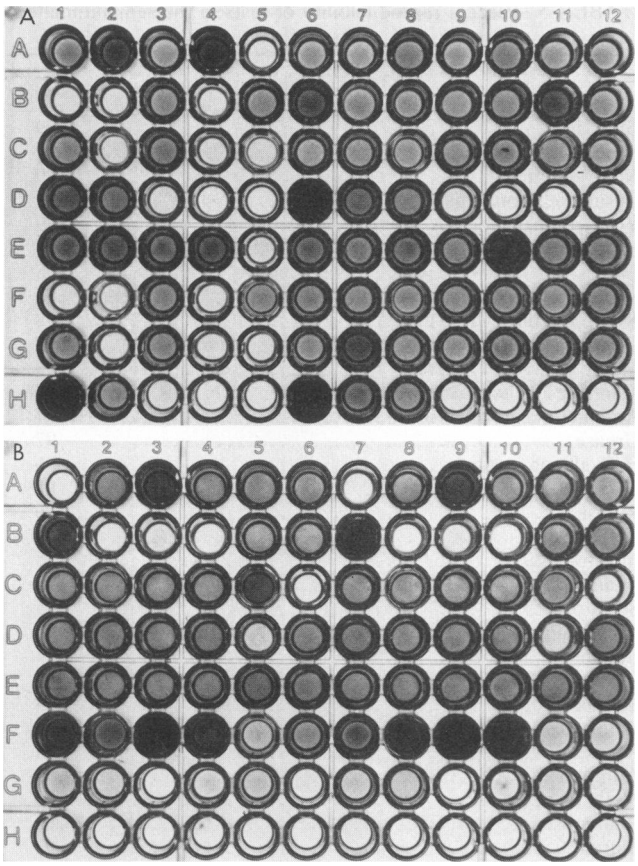

FIG. 2. (A) Example of a gram-negative rod test plate (for layout of wells, refer to Fig. 1B). The right half of the plate is an uninoculated control. The grey wells indicate the presence of bromthymol blue indicator. Other indicators (see text) were added to wells D9, D11, D12 and H9, H11, H12. The left half was inoculated with a strain of *Bacteroides intermedius*. Rows A through D and E through H are duplicates. Positive reactions include: acid production from fructose (A5, E5), glucose (B1, F1), glycogen (B2, F2), maltose (B4, F4), raffinose (C2, G2), starch (C4, G4), and sucrose (C5, G5); and indole production (D6, H6). The intensity of the indole reaction in the photograph was enhanced by an optical filter. (B) Example of a gram-positive rod test plate inoculated with a strain of *Propionibacterium acnes* (for layout of wells, refer to Fig. 1A). Columns 1 through 6 and 7 through 12 are duplicates. Positive reactions include: acid production from adonitol (A1, A7), fructose (B2, B8), galactose (B3, B9), glucose (B4, B10), mannose (C6, C12), ribose (D5, D11); indole production (F2, F8); and nitrate reduction (F3, F9). The intensities of the indole and nitrate reactions were enhanced in the photograph by an optical filter. Growth in the presence of bile and the antibiotics cannot be observed in the photograph.

evidence of diminished performance. Although not tested, it seems likely that plates could be stored without deterioration for longer periods at a lower temperature.

Inoculation time was considerably decreased. The pins of the inoculator could simultaneously inoculate 96 wells, be resterilized, and the cycle repeated in 1 min. Thus, the inoculation of large numbers of tests and bacterial strains could be accomplished more rapidly than by most conventional methods.

Perhaps the greatest savings in time was gained by the reading, recording, and interpretation of results. The time required to manually read and record 96 tests far exceeds that needed by the computer-linked reader. This is especially

TABLE 6. Comparison of conventional and semiautomated techniques

Comparison factor	Conventional	Semiautomated
Vol per test	2 ml	0.1 ml
Reproducibility	90%	95%
Time to inoculate (per 100 tests)	15 min	1 min
Time to dispense (per 100 tests)	20 min	1 min
Time to read and record (per 100 tests)	40 min	4 min
Initial investment for specialized equipment (1984)	0	$42,000[a]

[a] For the MIC 2000, approximately $25,000; for the Artek vertical beam reader, $17,000.

significant if pH electrodes are used to measure acid from carbohydrates. In addition, the factors of fatigue and human error increase with the performance of large numbers of tests by conventional methods.

If computer-facilitated identification programs are used without the ELISA reader, then manual data entry into a computer requires considerable time. If such identification programs are not used, time is also required to match test characteristics to identification profiles in books or charts. This becomes quite significant if large numbers of tests are performed for each strain. Data read by an ELISA reader and interpreted and stored in a computer have the advantage of being readily available to programs for report generation, cluster analysis, or computer-assisted identification.

The disadvantages of the system appear to include the failure of certain fastidious anaerobes to grow in the test medium as presently formulated. However, the same strains are also difficult to grow in conventional systems (9). The initial cost of equipment in quite high (Table 6); however, this should eventually be defrayed by savings in media and labor.

Greater than 80% of the isolates from subgingival plaques could be characterized with this system. The semiautomated system is more rapid, is as reproducible as conventional identification methods, and has replaced conventional techniques in our laboratory.

ACKNOWLEDGMENTS

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