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Comparison of Conventional and Miniaturized Biochemical Techniques for Identification of Animal Streptococcal Isolates

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Abstract. Human clinical streptococcal isolates can be identified rapidly by means of commercially available miniaturized biochemical systems, in contrast to animal and environmental isolates which may require extensive characterization using conventional methods. Streptococcal isolates (n = 548) from fresh animal feces of cattle, swine, and broiler chickens were tested by means of conventional biochemical and physiological techniques, and also with a miniaturized technique in which conventional formulations were dispensed in 0.1 ml volume into microtiter plates. Agreement of the positive feature frequencies of the two methods were compared. Results from the tolerance tests in the two methods were generally in good agreement. However, the miniaturized method tended to give false negative results in some carbohydrate fermentation tests. Agreement between the 2 methods ranged from 100% for bile esculin tests to 71% for raffinose fermentation. Cluster analysis of the conventional method data indicated that there were 11 biochemically related groups of isolates, 2 of which were identified as *Streptococcus faecalis*, and *S. morbillorum*. Half of the isolates biochemically resembled *S. faecium*. Errors of miniaturized tests occurred mainly in certain tests and in certain biochemically related clusters of isolates. The data indicate that further investigation of experimental conditions such as medium formulation and inoculum size could lead to a successful miniaturized technique for testing animal streptococcal isolates.

Introduction

Rapid, 4-hour, miniaturized, biochemical testing systems have been developed for the identification of clinical streptococcal isolates (G. S. Bosley, R. R. Facklam, and D. L. Rhoden, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C89, p. 286) [18]. In environmental and animal studies, extensive biochemical and physiological characterization of isolates is necessary for their comparison to described taxa. Large data bases of this type can be used to determine the

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incidence of established species in different environments or to describe new species.

An earlier study compared the probable identification of known strains of streptococci using a miniaturized test system [9]. Development of a reliable miniaturized system, requiring smaller quantities of media, fewer laboratory personnel, and less time, would be economically beneficial. In this report, we compare conventional biochemical feature frequencies with those obtained using a miniaturized system on isolates from animal feces.

Materials and Methods

Bacterial strains ($n = 548$) were isolated from fresh feces of healthy production cattle, swine, and broiler chickens. Fecal material (10 g) was suspended in 90 ml of a 25 mM phosphate buffer, pH 7, and appropriate dilutions were plated on KF streptococcal agar (KF) (BBL, Cockeysville, MD). Presumptive colonies were Gram stained and streaked onto heart infusion agar (HIA) (Difco Laboratories, Detroit, MI) to ensure purity. Colonies on the HIA plates were tested for catalase after incubation at 37°C for 24 hours with 3% hydrogen peroxide. Catalase-negative isolates having a streptococcal morphology were selected for further study and tested for hemolysis on 5% sheep blood agar (BBL); growth at 10°, 45°, and 50°C in Todd Hewitt broth (THB) (BBL); starch hydrolysis [3]; hippurate hydrolysis [4]; and reduction of methylene blue [5].

Twenty-one additional biochemical tests were performed on each isolate using both conventional and miniaturized techniques. The conventional tests (3–4 ml/13 × 100 mm test tube) included esculin hydrolysis in bile esculin agar (BE) (Difco) prepared according to the manufacturer's instructions but without horse serum, arginine hydrolysis (Ag) [7], growth at pH 9.6 [3], growth in 10% and 40% bile [12], growth in 6.5% NaCl [2], pyruvate utilization [7], acid production from cellobiose, glucose, glycerol, inulin, lactose, mannitol, melibiose, raffinose, sorbitol, sucrose, trehalose, salicin [3], arabinose, and xylose (1% w/v). The latter two pentoses were filter sterilized and added aseptically to sterile heart infusion broth (Difco). The media were inoculated with one drop of a 24-hour THB culture from a Pasteur pipet and read after 72 hours of incubation at 35°–37°C.

In the miniaturized procedure, the 21 biochemical media were dispensed (0.1 ml medium/well) into 96-well MIC 2000 plates (Cooke Laboratory Products, Alexandria, VA) with an Anderson Technical MIC 5000 dispenser (Anderson Technical, Inc., Denver, Co). Esculin (3.0 g/liter) broth [17] without indicator was substituted for BE in the MIC 2000 plates. Arginine hydrolysis broth was prepared similarly to the Ag medium used in the conventional tests, except that 1% D,L-arginine was used and agar was omitted. Prepared plates were sealed in plastic bags and stored at −20°C until used.

Four drops from a 24-hour THB culture were transferred to another THB tube (4 ml) and incubated at 35°–37°C for approximately 4 hours until the turbidity equaled a McFarland standard of 1 (3×10^8 colony-forming units [CFU]/ml). Two milliliters of the standardized suspension were added to 18 ml sterile distilled water in an inoculum tray (Cooke) and mixed with a sterile swab. A Dynatech MIC 2000 inoculator (Cooke) was used to inoculate each well with approximately 1.5 μl of the inoculum, giving a final inoculum concentration of 4×10^5 CFU/ml in each well. After inoculation, the wells containing the arginine medium were overlayed with sterile mineral oil and the plates were incubated at 35°–37°C for 72 hours in plastic bags.

Data from the conventional method were analyzed using the TAXON computer program [21] with simple matching coefficient and unweighted average linkage. Groups were determined by strains having at least 85% similarity. Results from the microtiter plates of these strains were then reordered to match these TAXON groups. The data from both methods were submitted to the FREAK program [19] for calculation of positive feature frequencies and hypothetical modal strains (HMS) for all strains and within each group of strains.

The conventional data from the strain most similar to the HMS were submitted to the IDDNEW program [10] matrix 7, *Streptococcus* species, for identification. IDDNEW uses positive feature

Table 1. Positive features frequencies and percent agreement between methods for all strains ($n = 548$)

Feature	Cb[a]	Mb[b]	% Agreement
Tolerance of			
40% bile	80.3	81.9	98
10% bile	83.9	82.1	97
pH 9.6	87.2	80.6	93
6.5% NaCl	85.8	81.2	95
Acid from			
Glucose	93.6	79.7	86
Pyruvate	13.0	21.0	86
Arabinose	15.7	14.1	97
Xylose	6.9	6.8	95
Salicin	80.3	79.3	98
Cellobiose	79.0	78.8	97
Lactose	79.9	79.2	98
Melibiose	58.0	50.6	91
Sucrose	83.2	71.1	86
Trehalose	80.5	78.8	97
Raffinose	42.2	17.6	71
Inulin	0.9	0.2	99
Glycerol	25.4	37.2	85
Mannitol	38.5	33.8	93
Sorbitol	25.2	26.0	97
NH_3 from arginine	76.5	77.9	95
Hydrolysis of esculin	79.4	79.4	100

[a] Cb = conventional biochemical data
[b] Mb = miniaturized biochemical data

frequencies as estimates of probabilities for given biochemical reactions by a species. For each unknown organism's biochemical pattern submitted to the program, the probability of each species within the matrix giving the same pattern is calculated.

Data from both methods were analyzed by the COMPARE program [20]. COMPARE measures the agreement between two methods of biochemical testing for binary data. A percent agreement can be calculated for each feature for the strains submitted to the program.

Results

Table 1 contains the positive feature frequencies for each test method and the agreement found between the methods for all strains. (For descriptive purposes, the conventional methods are assumed to be "more accurate.") The miniaturized method tended to give false negative results for the carbohydrate fermentation tests (especially with glucose and raffinose) and false positive results with glycerol fermentation and pyruvate utilization. Positive feature frequencies

Table 2. Positive features frequencies and percent agreement for the identified groups

Feature	*S. faecalis* 23 (126)[a]			*S. morbillorum* 8.2 (45)		
	Cb[b]	Mb[c]	% Agreement	Cb	Mb	% Agreement
Tolerance of						
40% bile	100.0	100.0	100	0.0	2.2	97
10% bile	100.0	100.0	100	6.7	2.2	95
pH 9.6	100.0	99.2	99	31.1	2.2	71
6.5% NaCl	100.0	99.2	99	15.6	2.2	82
Acid from						
Glucose	100.0	99.2	99	71.1	2.2	31
Pyruvate	56.3	77.8	54	0.0	0.0	100
Arabinose	2.4	3.2	97	0.0	0.0	100
Xylose	21.4	19.8	88	0.0	0.0	100
Salicin	100.0	98.4	98	0.0	2.2	97
Cellobiose	100.0	97.6	97	0.0	2.2	97
Lactose	100.0	98.4	98	0.0	2.2	97
Melibiose	4.0	5.6	95	2.2	2.2	95
Sucrose	99.2	97.6	98	33.3	0.0	66
Trehalose	100.0	99.2	99	0.0	0.0	100
Raffinose	1.6	4.0	97	0.0	2.2	97
Inulin	0.0	0.0	100	0.0	0.0	100
Glycerol	98.4	98.4	96	0.0	0.0	100
Mannitol	100.0	77.0	76	2.2	0.0	97
Sorbitol	100.0	96.8	96	0.0	0.0	100
NH_3 from arginine	100.0	96.0	96	0.0	0.0	100
Hydrolysis of esculin	100.0	100.0	100	0.0	0.0	100

[a] % Total (number of strains)
[b] Cb = conventional biochemical data
[c] Mb = miniaturized biochemical data

from the tolerance tests were in general agreement. The largest discrepancy (6.6%) in the 2 methods was in testing growth in pH 9.6 broth.

Agreement between the 2 methods ranged from 100% for bile esculin tests to 71% for raffinose fermentation. Sixteen of the miniaturized tests agreed with their respective conventional tests 91% of the time or better. Except for raffinose fermentation, the results of the remaining miniaturized and conventional tests were in at least 85% agreement.

To determine whether the discrepancies between methods occurred in biochemically similar groups of isolates, the TAXON program was run with data from the conventional method. The TAXON cluster analysis established 11 groups containing 98.2% (538) of the isolates tested. Identification of the isolate most similar to the HMS was attempted with the IDDNEW program.

Only 2 of the groups were identified: *Streptococcus faecalis* (126 isolates)

Table 3. Positive feature frequencies for the unidentified groups

	Group 1 1.4 (8)[a]		Group 3 47.4 (20)		Group 5 0.6 (9)		Group 4 5.3 (29)		Group 6 0.9 (5)		Group 8 4.4 (24)		Group 7 3.1 (17)		Group 9 1.6 (9)		Group 11 1.1 (6)	
Feature	Cb[b]	Mb[c]	Cb	Mb	Cb	Mb	Cb	Mb	Cb	Mb	Cb	Mb	Cb	Mb	Cb	Mb	Cb	Mb
Tolerance of																		
40% bile	100.0	100.0	100.0	99.6	100.0	100.0	100.0	100.0	100.0	100.0	0.0	8.3	11.8	43.8	0.0	0.0	0.0	0.0
10% bile	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	4.2	4.2	88.2	50.0	11.1	0.0	0.0	0.0
pH 9.6	100.0	100.0	100.0	97.7	100.0	100.0	100.0	100.0	100.0	100.0	12.5	0.0	94.1	50.0	33.3	0.0	0.0	0.0
6.5% NaCl	100.0	100.0	100.0	99.2	100.0	100.0	100.0	100.0	100.0	100.0	8.3	0.0	100.0	50.0	11.1	0.0	0.0	0.0
Acid from																		
Glucose	100.0	100.0	100.0	99.2	100.0	100.0	100.0	100.0	100.0	20.0	58.3	0.0	76.5	18.8	44.4	0.0	0.0	83.3
Pyruvate	0.0	87.5	0.0	3.1	0.0	22.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arabinose	25.0	25.0	26.9	23.8	100.0	88.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Xylose	50.0	50.0	0.0	1.5	55.6	22.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.3	0.0	0.0	0.0	0.0
Salicin	100.0	100.0	100.0	99.6	100.0	100.0	100.0	100.0	100.0	20.0	0.0	0.0	5.9	6.3	0.0	0.0	0.0	0.0
Cellobiose	100.0	100.0	98.5	98.8	100.0	100.0	100.0	100.0	60.0	20.0	0.0	0.0	0.0	6.3	0.0	0.0	0.0	0.0
Lactose	100.0	100.0	100.0	99.2	100.0	100.0	100.0	100.0	20.0	20.0	4.2	0.0	5.9	6.3	0.0	0.0	0.0	0.0
Melibiose	87.5	37.5	97.3	95.0	77.8	77.8	20.7	20.7	20.0	0.0	79.2	8.3	11.8	0.0	100.0	11.1	33.3	50.0
Sucrose	100.0	100.0	95.4	93.8	55.6	55.6	0.0	0.0	100.0	20.0	83.3	0.0	82.4	31.3	44.4	0.0	16.7	83.3
Trehalose	100.0	100.0	100.0	98.1	100.0	100.0	100.0	100.0	80.0	20.0	4.2	0.0	5.9	12.5	0.0	0.0	0.0	0.0
Raffinose	100.0	12.5	68.5	32.7	22.2	11.1	0.0	0.0	20.0	0.0	100.0	4.2	0.0	0.0	88.9	0.0	16.7	33.3
Inulin	12.5	0.0	0.8	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glycerol	87.5	87.5	0.0	20.4	0.0	55.6	0.0	42.9	0.0	0.0	0.0	0.0	5.9	6.3	0.0	0.0	0.0	0.0
Mannitol	100.0	100.0	25.0	25.8	100.0	100.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0
Sorbitol	100.0	100.0	0.4	2.7	11.1	33.3	0.0	0.0	20.0	0.0	0.0	0.0	0.0	6.3	0.0	0.0	0.0	0.0
NH_3 from arginine	100.0	100.0	94.6	98.8	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0
Hydrolysis of esculin	100.0	100.0	100.0	100.0	100.0	100.0	96.6	96.6	0.0	0.0	0.0	0.0	17.6	17.6	0.0	0.0	0.0	0.0

[a] % Total (number of strains)
[b] Cb = conventional biochemical data
[c] Mb = miniaturized biochemical data

Table 4. Summary of the percent agreement between methods for each feature by group

Feature	Group											
	All	1	2[a]	3	4	5	6	7	8	9	10[b]	11
Tolerance of												
40% bile	98	100	100	99	100	100	100	68	91	100	97	100
10% bile	97	100	100	100	100	100	100	62	91	88	95	100
pH 9.6	93	100	99	97	100	100	100	43	87	66	71	100
6.5% NaCl	95	100	99	99	100	100	100	50	91	88	82	100
Acid from												
Glucose	86	100	99	99	100	100	20	25	41	55	31	16
Pyruvate	86	12	54	96	100	77	100	100	100	100	100	100
Arabinose	97	100	97	96	100	88	100	100	100	100	100	100
Xylose	95	75	88	98	100	66	100	93	100	100	100	100
Salicin	98	100	98	99	100	100	20	87	100	100	97	100
Cellobiose	97	100	97	97	100	100	60	93	100	100	97	100
Lactose	98	100	98	99	100	100	100	87	95	100	97	100
Melibiose	91	50	95	97	100	100	80	87	29	11	95	83
Sucrose	86	100	98	95	100	100	20	37	16	55	66	33
Trehalose	97	100	99	98	100	100	40	93	95	100	100	100
Raffinose	71	12	97	59	100	88	80	100	4	11	97	50
Inulin	99	87	100	99	96	100	100	100	100	100	100	100
Glycerol	85	100	96	79	57	44	100	87	100	100	100	100
Mannitol	93	100	76	98	100	100	80	87	100	100	97	100
Sorbitol	97	100	96	97	100	77	80	93	100	100	100	100
NH_3 from arginine	95	100	96	93	100	100	100	87	100	100	100	100
Hydrolysis of esculin	100	100	100	100	100	100	100	100	100	100	100	100

[a] *S. faecalis*
[b] *S. morbillorum*

and *S. morbillorum* (45 isolates). Positive feature frequencies and percent agreement between methods are shown in Table 2 for the two identified groups. In the *S. faecalis* group, the largest differences in feature frequencies, and hence the lowest level of agreement, were pyruvate utilization and mannitol fermentation. Eighteen of the feature results were in at least 95% agreement in both methods.

In the *S. morbillorum* group, there were large frequency differences in the fermentation of glucose (68.9%) and sucrose (33.3%) between the conventional and miniaturized methods (Table 2). Of the tolerance tests, there were differences of 13.4% in growth in 6.5% NaCl and 28.9% in growth at pH 9.6. In all 4 tests, the errors were false negative results by the miniaturized method. Seventeen of the miniaturized features agreed with the conventional method 95% of the time or better.

The largest number of isolates (50.4%) fell into 1 of 3 groups (groups 1, 3,

and 5, Table 3) whose HMS resembled *S. faecium* according to the IDDNEW program. The conventional biochemical patterns of these groups differed from the typical *S. faecium* in at least 2 of the following tests: fermentation of arabinose, glycerol, xylose, and mannitol; and growth at 50°C. Positive feature frequency results for the 2 methods for each of the 3 pairs of groups were very close in most tests (Table 3). The greatest differences were pyruvate utilization, and the fermentation of raffinose, melibiose, glycerol, and xylose. The pyruvate (group 1) and glycerol (group 5) tests had false positive results; the errors in the raffinose, melibiose, and xylose tests were false negative reactions with the miniaturized method. Sixteen and 15 of the miniaturized tests agreed with the conventional results 100% of the time for group 1 and group 5, respectively (Table 4). Eighteen of the miniaturized tests agreed with the conventional tests 95% of the time or better for group 3 (Table 4).

According to the IDDNEW identification program, the HMS for group 4 resembled *S. durans* but differed from the typical *S. durans* in that growth did not occur at 10°C and neither glycerol nor sucrose were fermented (Table 3). Differences in positive feature frequencies for the 2 methods were limited to the fermentation of glycerol. None of the isolates in group 4 fermented glycerol with conventional methods, but almost 43% of the isolates produced acid with the miniaturized method. Of the remaining tests, only inulin agreed with the conventional results less than 100% of the time (Table 4).

The HMS for group 6 resembled *S. anginosus*. However, the test results for growth in 6.5% NaCl and at pH 9.6, lack of esculin hydrolysis, and fermentation of cellobiose were inconsistent with this identification (Table 3). Group 6 comprised the smallest of the 11 groups from TAXON analysis. The miniaturized carbohydrate fermentation tests for glucose, salicin, cellobiose, sucrose, and trehalose differed in positive frequency from the conventional method by at least 40%; all had false negative results. Twelve of the miniaturized tests were in 100% agreement with the conventional results (Table 4).

Several miniaturized fermentation test results in group 8 (Table 3) did not agree well with the conventional results because of false negative results, particularly in the glucose, melibiose, sucrose, and raffinose fermentation tests. Thirteen of the miniaturized tests agreed with the conventional method at least 95% of the time (Table 4).

The respective HMS of the remaining three groups (7, 9, and 11, Table 3) resembled *S. morbillorum* in the IDDNEW program. However, the conventional test results for each group are inconsistent with this identification for at least 3 of the following tests: growth in 10% bile and 6.5% NaCl, growth at pH 9.6, 10°, 45°, and 50°C; fermentation of melibiose and raffinose; and the inability to ferment glucose. The differences in the positive feature frequencies between the 2 methods are greatest for growth in 40% bile, 6.5% NaCl, growth at pH 9.6, and fermentation of glucose, melibiose, sucrose, and raffinose (Table 3). False positive errors occurred in the miniaturized tests with 40% bile, glucose, and sucrose. False negative results occurred with the pH 9.6, 6.5% NaCl, glucose, melibiose, sucrose, and raffinose tests. Five, 14, and 17 of the miniaturized and conventional test results agreed fully in groups 7, 9, and 11, respectively (Table 4).

Discussion

The majority of the results for all isolates from the miniaturized and conventional biochemical methods were in agreement (Table 4). However, there are some unique experimental conditions associated with miniaturized methods that are of no concern in conventional "macro" methods. In miniaturized systems, small volumes (0.1 ml) of test media are inoculated with small volumes (1.5 μl) of culture to prevent dilution of the media and the number of cells within the inoculum can be varied by the investigator. In contrast, conventional liquid media (in quantities of 3–4 ml or more) are generally inoculated with 1 or more drops of an overnight culture; the "size" of the inoculum, or number of cells, is not as critical.

Another problem with miniaturized methods is the difficulty of discerning color changes of pH indicators in a small volume and depth of medium. This difficulty is increased when the pH is lowered close to the transition range of the indicator. The general tendency of the miniaturized carbohydrate fermentation tests to yield false negative results may be due to the lack of color or contrast in color in the wells. In addition, some of the false positive pyruvate utilization tests may be due to the difficulty of discerning "yellow" from "yellow-green" in the small volumes used.

Facklam pointed out that glycerol fermentation was designed for anaerobic testing and may have little value when tested aerobically [3]. In this investigation, acid production from glycerol was variable in groups related to *S. faecium* or *S. durans* (Table 3), but not with *S. faecalis* (Table 2). Aerobic production of acid from glycerol has been variable for *S. faecium* in other investigations [8, 11]; only anaerobic production of acid from glycerol is valuable for separating *S. faecalis* and *S. faecium*. *S. faecium* will lower the pH of glycerol broth to approximately 5.4 under aerobic conditions [8]. This is very close to the value (pH 5.2) at which the indicator used here, bromcresol purple, is yellow. Apparently, glycerol fermentation, as described in this report, is not adaptable to a miniaturized system. A different pH indicator for the miniaturized carbohydrate fermentation tests (e.g., chlorphenol red) may alleviate the problem of discerning a positive reaction.

The good general agreement between the 2 test methods indicates that these potential problems may affect only certain biochemical tests or certain groups of biochemically related streptococci. For example, miniaturized raffinose fermentation results had the lowest overall percent agreement with the conventional results (71%, Table 4). However, in 4 of the groups defined by the TAXON program, the agreement of the raffinose fermentation results for both methods was at least 97% and, in 2 additional groups, the agreement was at least 80% (Table 4). The overall agreement between the 2 methods for glycerol fermentation results was only 85% with essentially all of the errors in groups 3, 4, and 5 (Table 4). Growth in 40% bile salts showed excellent overall agreement (98%) between methods; most of the errors are found in group 7 (Table 4). Examination of the other biochemical test results shows a similar localization of the errors to related groups of isolates.

Nine of the 11 groups of isolates determined by cluster analysis could not be identified by the IDDNEW program. The positive feature frequencies in

the matrix *Streptococcus* species represent a composite of American Type Culture Collection strains for 27 species of streptococci using 35 features [10]. Because the feature frequencies are derived from strains of diverse ecological sources (e.g., human clinical strains and dairy products), the probability of identifying these isolates from healthy animals is reduced. These groups biochemically resemble groups of previously described isolates [1, 6, 9, 13, 16], but differences in methods make comparison difficult. Earlier workers also found groups of isolates that did not fit the established patterns of recognized species and designated these groups as "*S. faecium*-like," "atypical," or simply "unidentified" [1, 6, 14, 15].

The present results indicate that the miniaturized technique can accurately reproduce the majority of the conventional reactions of the animal streptococci. With further research on media formulation, inoculum size, and interpretation of results, we conclude that a miniaturized system is feasible. Development of such a system would be cost effective and thus useful in characterizing streptococci for a variety of epidemiological and ecological studies.

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