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# Identification of "*Streptococcus milleri*" Group Isolates to the Species Level with a Commercially Available Rapid Test System

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**Clinical isolates of the "*Streptococcus milleri*" species group were examined by conventional methods and a rapid, commercially available method for the identification of these strains to the species level. The levels of agreement between the identifications obtained with the commercially available system (Fluo-Card Milleri; KEY Scientific, Round Rock, Tex.) and conventional methods were 98% for 50 *Streptococcus anginosus* strains, 97% for 31 *Streptococcus constellatus* strains, and 88% for 17 isolates identified as *Streptococcus intermedius*. Patient records were also studied in order to gain information on the frequency and sites of isolation of each of the three "*S. milleri*" group species.**

The streptococci included in the "*Streptococcus milleri*" group of species are recognized as agents of serious purulent infection (9, 12, 14). While most "*S. milleri*" organisms share certain phenotypic traits, variations in their hemolytic, serological, and physiological characteristics contribute to the marked heterogeneity of strains within the group. In the four decades since Guthof (10) first applied the name "*S. milleri*" to these organisms, taxonomists have argued for and against their inclusion in a single species. Early workers (2, 4, 5) relied on phenotypic traits for classification of these streptococci, while later investigators applied molecular taxonomic techniques (3, 15). The most recent and currently accepted molecular taxonomic studies of Whiley and Beighton (15) divided the "*S. milleri*" group of streptococci into three species: *Streptococcus anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius.*

Although previous reports have examined the clinical significance of the "*S. milleri*" group as a whole or of individual species or biotypes as defined by older classification schemes, relatively few studies have examined the clinical correlations of the three species as they are currently defined. Whiley and coworkers (16) examined 153 isolates and noted that *S. intermedius* was associated with brain and liver abscesses. While *S. constellatus* and *S. anginosus* were recovered from a wider range of sources, *S. anginosus* was the predominant species isolated from gastrointestinal and genitourinary sites. Associations between *S. intermedius* and liver abscesses and *S. anginosus* and abdominal infection sites were also noted by Gómez-Garcés et al. (8) in a survey of 70 clinically significant strains. In both reports *S. anginosus* accounted for approximately 60% of the isolates studied. Isolates of this species were also the most common isolates in a study of 20 isolates that caused bacteremia (11) and 16 strains cultured from dental periapical abscesses (7).

Since most commercially available products for the identification of streptococci lack tests for the key phenotypic characteristics differentiating the "*S. milleri*" group species as they are currently defined, we evaluated a product containing rapid enzymatic tests for the differentiation of these organisms from other species. We also examined the clinical correlations of the various "*S. milleri*" group species identified in our study.

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## MATERIALS AND METHODS

**Bacteria.** A total of 106 isolates of the "*S. milleri*" group were examined. They were recovered from human clinical specimens and had previously been identified as "*S. milleri*" by using the API 20 Strep test (bioMérieux, Vitek, Hazelwood, Mo.). The three control strains used were *S. constellatus* ATCC 27823, *S. anginosus* ATCC 33397, and *S. intermedius* ATCC 27335. All strains were stored as frozen suspensions in horse blood. Thawed suspensions were maintained by routine subculturing on brucella agar plates containing 5% horse blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were incubated at 35°C in an atmosphere containing 5% $CO_2$. Growth from these plates served as inocula for all tests on the isolates.

**Biochemical tests.** Our conventional identification methods consisted of testing strains for the ability to degrade the following synthetic fluorogenic substrates: 2′-4 methylumbelliferyl (4-MU)-α-D-*N*-acetylneuraminic acid, 4-MU-*N*-acetyl-β-D-glucosaminide, 4-MU-*N*-acetyl-β-D-galactosaminide, 4-MU-β-D-fucoside, 4-MU-β-D-galactoside, 4-MU-α-D-glucoside, and 4-MU-β-D-glucoside for the detection of sialidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, β-D-fucosidase, β-galactosidase, α-glucosidase, and β-glucosidase, respectively. The substrates and reagents used in these tests were obtained from Sigma (St. Louis, Mo.). The methods of Whiley and colleagues (17) were used to perform the enzyme assays except that the strains were grown overnight at 35°C on brucella agar plates containing 5% horse blood (Becton Dickinson) in an atmosphere containing 5% $CO_2$, and the cell suspensions used in the assays were adjusted to an optical density equal to that of a no. 1 McFarland standard.

Strains were also tested by using the Fluo-Card Milleri test kit (KEY Scientific Products, Round Rock, Tex.). The Fluo-Card Milleri test kits were supplied by the manufacturer and consisted of filter paper strips with circles containing 4-MU-α-D-glucoside, 4-MU-β-D-glucoside, and 4-MU-β-D-fucoside. Bacterial growth from the same plates used for the conventional assays was tested with the Fluo-Card Milleri kit. The test strips were placed into incubation chambers (provided with the kit) or petri dishes, and each test circle was moistened with one small drop of distilled water. Approximately 5 to 10 colonies of bacteria were smeared in the center of each circle. The chamber was closed and incubated at 35°C for 15 min. The strips were then observed under a hand-held long-wavelength UV lamp (Wood's lamp). The appearance of an intense blue fluorescence where the organism was smeared was interpreted as a positive result. Reactions were scored from left to right in a flow chart-like arrangement of the circles on the test strip (Fig. 1). If the first circle (β-D-fucosidase) was positive, the strain was identified as *S. intermedius* and all subsequent reactions were disregarded. If the β-D-fucosidase reaction was negative but the next circle (β-D-glucosidase) was scored as positive, the isolate was identified as *S. anginosus* regardless of the reaction in the last circle. An identification of *S. constellatus* was indicated when the first two circles were negative and the third circle (α-D-glucosidase) was positive.

Isolates that demonstrated disagreement between the conventional and the Fluo-Card Milleri kit identifications were first retested by both methods, and if the identifications were again discrepant, a second test with the API 20 Strep strip was performed to confirm that the isolate was in fact "*S. milleri*."

Data on the sites of isolation of the strains tested were obtained by review of patients' microbiology records and, in some cases, their medical records.

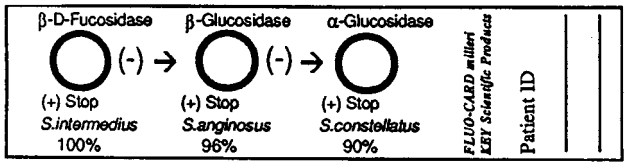


FIG. 1. The Fluo-Card Milleri test strip, composed of fluorogenic substrate-impregnated circles arranged in a flow chart-like sequence.

## RESULTS

**Comparison of conventional phenotypic test results with Fluo-Card Milleri.** By conventional methods, 50 isolates were identified as *S. anginosus* when β-glucosidase alone or β-glucosidase and α-glucosidase were the only positive tests. Thirty-one of the isolates were identified as *S. constellatus* when only α-glucosidase was demonstrated by fluorescence. Seventeen of the strains were identified as *S. intermedius* when at least three of the following were demonstrated by fluorescence: β-D-fucosidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, sialidase, or β-galactosidase. The *S. intermedius* strains also often demonstrated β-glucosidase activity or α-glucosidase activity, or both. Table 1 displays the levels of agreement between the identifications obtained by the Fluo-Card Milleri and conventional enzymatic methods. Strains displaying discrepant identifications were readily identified as one of the "*S. milleri*" group species by the Fluo-Card Milleri kit except for one strain of *S. constellatus*, which was unreactive in all three of the kit's enzyme assays.

Of the eight strains that could not be assigned to a species on the basis of their conventional method reactions, six were noted to be esculin hydrolysis negative on the API 20 Strep strip. One of these isolates was also arginine hydrolysis negative. Since the degradation of esculin and the degradation of arginine are characteristic of the majority of strains in all three "*S. milleri*" group species (15), these six isolates can be considered atypical strains. While the API 20 Strep system identified the esculin hydrolysis-negative isolates as "*S. milleri*" at the species level of identification (with no definitive biotype), the Fluo-Card Milleri kit identified them as either *S. constellatus* (four strains) or *S. anginosus* (two strains). These esculin-negative strains were isolated from infections in the abdomen (two strains), skin (two strains), blood (one strain), and neck (one strain). Their conventional reactions varied from no demonstrable activity (three strains), to β-galactosidase and α-glucosidase activities (two strains), to β-galactosidase, β-glucosidase, and β-*N*-acetylglucosaminidase activities (one strain).

The esculin hydrolysis-positive strains that were unidentified by conventional means were β-glucosidase positive. One also showed β-*N*-acetylglucosaminidase activity, and the other was additionally positive for α-glucosidase and β-galactosidase by conventional test methods. Both of these strains were identified as *S. anginosus* by the Fluo-Card Milleri kit.

TABLE 1. Comparison of "*S. milleri*" species group isolate identifications by conventional methods and with the Fluo-Card Milleri kit

| Species (conventional identification) | No. tested | No. (%) agreement | Fluo-Card Milleri identifications of discrepant strains (no. of strains) |
|---|---|---|---|
| *S. anginosus* | 50 | 49 (98) | *S. intermedius* |
| *S. constellatus* | 31 | 30 (97) | No identification |
| *S. intermedius* | 17 | 15 (88) | *S. anginosus*, *S. constellatus* |
| No conventional identification | 8 | | *S. anginosus* (4), *S. constellatus* (4) |

TABLE 2. Sites of isolation of species of the "*S. milleri*" group

| Clinical source(s) | No. of isolates identified as: | | | |
|---|---|---|---|---|
| | *S. anginosus* | *S. constellatus* | *S. intermedius* | Total |
| Head and neck | 3 | 3 | 3 | 9 |
| CNS[a] | 0 | 0 | 1 | 1 |
| Respiratory tract | 5 | 5 | 2 | 12 |
| Urogenital | 4 | 1 | 0 | 5 |
| Gastrointestinal tract | 4 | 2 | 2[b] | 8 |
| Abdominal or pelvic sites | 6 | 8 | 2 | 16 |
| Perirectal abscess | 5 | 1 | 0 | 6 |
| Skin and soft tissues | 9 | 6 | 3 | 18 |
| Blood | 12 | 3 | 3 | 18 |
| Site unknown | 2 | 2 | 1 | 5 |
| Total | 50 | 31 | 17 | 98 |

[a] CNS, central nervous system.
[b] Includes one liver abscess.

**Distribution of strains.** Of the 98 strains identified by the conventional fluorogenic method, 5 were from unknown sites. The sites of infection from which the remaining 93 strains were originally isolated are given in Table 2. Overall, *S. anginosus* was the most frequently isolated species, and it was particularly prominent among the blood, urogenital, and perirectal abscess isolates. *S. anginosus* and *S. constellatus* were isolated at approximately equal frequencies from abdominal and pelvic and from gastrointestinal sites. One strain each isolated from infections of the central nervous system or liver was identified as *S. intermedius.*

## DISCUSSION

The identification of "*S. milleri*" group isolates to the species level is a time- and labor-intensive process involving the performance of multiple enzyme reactions. Most convenient-to-use commercial systems either identify "*S. milleri*" group strains as biotypes within a single species ("*S. milleri*") or use species designations (*S. anginosus*, *S. constellatus*, *S. intermedius*) as defined by older taxonomy schemes. One recent study (8) used a newer commercially available system that is able to identify "*S. milleri*" isolates to the species level by using the currently accepted taxonomy. That study used the Rapid ID-32 Strep system (bioMérieux, La Balme les Grottes, France), which incorporates assays for β-glucosidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase. The authors of that report examined sites of isolation and reported on the antibiotic susceptibilities of their "*S. milleri*" group strains, but they did not compare their rapid identification method with the conventional methods of Whiley and colleagues (17).

The product that we evaluated, the Fluo-Card Milleri kit, uses tests for α-D-glucosidase, β-D-glucosidase, and β-D-fucosidase to identify "*S. milleri*" group species. In our study of 106 clinical isolates we noted acceptable levels of agreement between the Fluo-Card Milleri kit identifications and those yielded by conventional methods. During the course of the study the Fluo-Card Milleri test was repeatedly performed on the three American Type Culture Collection control strains on 7 different days and demonstrated 100% reproducibility in tests with those strains. We performed the Fluo-Card Milleri test according to the package insert, but found that increasing

the inoculum size that was smeared onto the test circles from the 1 colony suggested by the manufacturer to 5 to 10 colonies increased the level of visible fluorescence significantly and made the test much easier to read. Of the eight strains not identified by the conventional enzymatic method that we used, six displayed atypical phenotypic reactions (15). Genetic studies would be necessary to accurately identify these strains with aberrant results, which were readily identified as either *S. anginosus* or *S. constellatus* by the Fluo-Card Milleri kit.

Overall, our data suggest that the Fluo-Card Milleri kit can be used successfully to accurately identify most clinical isolates of *S. anginosus*, *S. constellatus*, and *S. intermedius*. Identification of "*S. Milleri*" strains with this product is rapid, and the product is simple to use. It can provide species differentiation of isolates that have already been identified as members of the "*S. milleri*" group by presumptive tests (1, 6, 13).

When we examined the species distribution and sites of isolation of 93 of our strains, we found, as have other investigators (8, 11, 16), that *S. anginosus* (51% of our strains) was more frequently isolated than the other two species combined. *S. anginosus* was more commonly isolated from the urogenital system as noted previously (16). We found *S. anginosus* more commonly (five of six isolates) in perirectal locations (a site not specified in previous publications). *S. anginosus* was also the most frequent isolate from blood cultures in our study (12 of 18 isolates); a similar finding was noted by Jacobs and colleagues (11) in a study of 20 blood culture isolates (16 strains were *S. anginosus*). Although prior studies have noted an increased frequency of *S. anginosus* among abdominal "*S. milleri*" group isolates, we saw approximately equal numbers of *S. constellatus* and *S. anginosus* when isolates from abdominal and gastrointestinal sites were considered together. The prevalence of *S. anginosus* among clinical "*S. milleri*" group isolates may reflect the more widespread colonization by this species compared with that by *S. constellatus* or *S. intermedius*, or it may indicate enhanced pathogenic mechanisms among *S. anginosus* strains.

*S. intermedius* was isolated from the single cultures of central nervous system and liver abscess specimens in our study; this species has been previously associated with infections at these sites (8, 16). No significant difference was noted between the frequency of species isolated from head and neck, respiratory, or skin and soft tissue sites in our study. The accumulation of data on the associations between the currently described "*S. milleri*" group species and the sites and types of infections from which they are isolated will add to our understanding of the role of these organisms in disease.

## REFERENCES


1. **Bucher, C., and A. von Graevenitz.** 1984. Differentiation in throat cultures of group C and G streptococci with identical antigens. Eur. J. Clin. Microbiol. **3:**44–45.
2. **Colman, G., and R. E. O. Williams.** 1972. Taxonomy of some human viridans streptococci, p. 281–299. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases: recognition, understanding, and management. Academic Press, Inc., New York.
3. **Coykendall, A. L., P. M. Wesbecher, and K. B. Gustafson.** 1987. "*Streptococcus milleri*," *Streptococcus constellatus*, and *Streptococcus intermedius* are later synonyms of *Streptococcus anginosus*. Int. J. Syst. Bacteriol. **37:**222–228.
4. **Facklam, R. R.** 1977. Physiological differentiation of viridans streptococci. J. Clin. Microbiol. **5:**184–201.
5. **Facklam, R. R.** 1984. The major differences in the American and British *Streptococcus* taxonomy schemes with special reference to *Streptococcus milleri*. Eur. J. Clin. Microbiol. **3:**91–93.
6. **Facklam, R. R., and J. A. Washington II.** 1991. *Streptococcus* and related catalase-negative gram-positive cocci, p. 238–257. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
7. **Fisher, L. E., and R. R. B. Russell.** 1993. The isolation and characterization of milleri group streptococci from dental periapical abscesses. J. Dent. Res. **72:**1191–1193.
8. **Gómez-Garcés, J.-L., J.-I. Alos, and R. Cogollos.** 1994. Bacteriologic characteristics and antimicrobial susceptibility of 70 clinically significant isolates of *Streptococcus milleri* group. Diagn. Microbiol. Infect. Dis. **19:**69–73.
9. **Gossling, J.** 1988. Occurrence and pathogenicity of the *Streptococcus milleri* group. Rev. Infect. Dis. **10:**257–285.
10. **Guthof, O.** 1956. Ueber pathogene "vergrunende Streptokokken." Streptokokken-Befunde bei dentgenen Abszessen und Infiltraten im Bereich der Mundhole. Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. 1 Orig. 1 **166:**553–564.
11. **Jacobs, J. A., H. G. Pietersen, E. E. Stobberingh, and P. B. Soeters.** 1994. Bacteremia involving the "*Streptococcus milleri*" group: analysis of 19 cases. Clin. Infect. Dis. **19:**704–713.
12. **Piscitelli, S. C., J. Shwed, P. Schreckenberger, and L. H. Danziger.** 1992. *Streptococcus milleri* group: renewed interest in an elusive pathogen. Eur. J. Clin. Microbiol. Infect. Dis. **11:**491–498.
13. **Ruoff, K. L., and M. J. Ferraro.** 1986. Presumptive identification of "*Streptococcus milleri*" in 5 h. J. Clin. Microbiol. **24:**495–497.
14. **Van der Auwera, P.** 1985. Clinical significance of *Streptococcus milleri*. Eur. J. Clin. Microbiol. **4:**386–390.
15. **Whiley, R. A., and D. Beighton.** 1991. Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus* as distinct species. Int. J. Syst. Bacteriol. **41:**1–5.
16. **Whiley, R. A., D. Beighton, T. G. Winstanley, H. Y. Fraser, and J. M. Hardie.** 1992. *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* (the *Streptococcus milleri* group): association with different body sites and clinical infections. J. Clin. Microbiol. **30:**243–244.
17. **Whiley, R. A., H. Fraser, J. M. Hardie, and D. Beighton.** 1990. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "*Streptococcus milleri* group." J. Clin. Microbiol. **28:**1497–1501.