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Development of the Quantitative Micro-Test for Slime Production by Coagulase-Negative Staphylococci

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The macro-test for slime production by coagulase-negative staphylococci was adapted to a spectrophotometric micro-test assay to obtain more objective and quantitative information on slime production. A total of 135 isolates of coagulase-negative staphylococci (70 macro-test-positive and 65 macro-test-negative) were tested by both methods. The quantitative micro-test optical density readings were (mean ± SD) 1.176 ± 0.294 and 0.130 ± 0.095 for the macro-test-positive and -negative groups, respectively. The micro-test was reproducible and demonstrated quantitative differences in slime production among the different species of coagulase-negative staphylococci. Aside from *Staphylococcus epidermidis*, the majority of the coagulase-negative staphylococci had very low optical density readings, indicating little or no slime production under the conditions employed in this assay. This test allows one to study the relative production of slime by different strains and species of coagulase-negative staphylococci, and may be useful in studying the effects of different conditions, such as antibiotic exposure, on slime production.

The pathogenic role of coagulase-negative staphylococci in a variety of clinical situations has been well established (1–4). Of particular concern is the rising incidence of coagulase-negative staphylococcal bacteremias in association with long-term indwelling central venous catheters (4). Both in vitro and in vivo studies of these catheter-associated strains of coagulase-negative staphylococci have demonstrated the production of a viscous extracellular material, or slime, which appears to mediate the adherence of these isolates to catheters. The studies suggest that these staphylococci may be uniquely adapted to adhere to smooth surfaces (5–8).

Although we and others have found the macro-tube test for slime production to be simple and reliable in classifying coagulase-negative staphylococci as slime-positive or slime-negative, the test is limited in application due to its subjective and qualitative nature (5, 6, 9, 10). Christensen et al. (11) noted variability between observers in interpreting tests of weakly positive strains of coagulase-negative staphylococci. This suggests that a more objective, quantitative assay may be desirable for use in future studies of coagulase-negative staphylococci.

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Christensen et al. (11) reported their experience with a quantitative method for measuring adherence of coagulase-negative staphylococci to plastic tissue culture plates. We have modified this assay to reflect both adherence and subsequent slime production by coagulase-negative staphylococci.

Materials and Methods

Microbiological Investigations. A total of 135 isolates of coagulase-negative staphylococci were used in this study. These organisms consisted of isolates from blood and normally sterile body fluids obtained from patients hospitalized at the University of Iowa Hospitals from March 1983 to August 1984. All isolates were characterized as gram-positive clustering cocci that were catalase-positive or coagulase-negative. Speciation was accomplished using the Staph Ident System (Analytab Products, USA) according to the manufacturer's instructions. Stock cultures were stored in skim milk (Difco Laboratories, USA) at −20 °C. Working cultures were maintained on blood agar plates (Gibco Laboratories, USA).

Qualitative Macro-Method. Isolates were examined for slime production using a qualitative technique, as previously described (5, 9). Briefly, glass tubes containing 5 ml of Trypticase Soy Broth (TSB; *BBL Microbiology Systems*, USA) were inoculated to a density of 1×10^5 CFU/ml using organisms harvested from a 24 h growth on blood agar plates. The inoculum size was confirmed by a quantitative plating method. The tubes were incubated at 35 °C for 48 h, after which the contents were aspirated and the tubes stained with safranin. Slime production was judged to be present if a visible safranin-stained film lined the walls of the tube.

Formation of a ring at the liquid-air interface was not considered indicative of slime production. In general, results were recorded as positive (+) or negative (−) for slime production; however, 52 isolates of *Staphylococcus epidermidis* were also classified in a semi-quantitative manner using the following graded estimates of slime production: strong (3+), moderate (2+), weak (1+), or absent (0). In all cases the macro-method was performed in parallel with the quantitative micro-method described below. All readings of the macro-tube tests were performed by an individual who was blinded to the results of the companion quantitative micro-method test.

Quantitative Micro-Method. The quantitative assay was performed using sterile, polystyrene, 96-well flat-bottomed tissue culture plates (Corning Glassware, USA). Each well was filled with a 0.2 ml aliquot of TSB containing 1×10^5 CFU/ml of the test organism. The inoculum suspension was prepared as described for the macro-tube test, and the inoculum size was verified by quantitative plate counts. The tissue culture plates were sealed to retard evaporation, and incubated at 35 °C in air for 48 h. The contents of each well were gently aspirated, and the wells were washed two times with 0.2 ml of phosphate-buffered saline (pH 7.2). The wells were stained with safranin for 30 s, after which the stain was aspirated, and the optical densities of the individual wells were read with a Micro ELISA Autoreader (Dynatech, USA) at a wavelength of 490 nm. A blank reading (stained well containing sterile TSB) was obtained for each plate and subtracted from the experimental readings. The optical density determinations for each isolate were performed in triplicate and repeated between one and eight times. The values were then averaged. The Micro ELISA reader used in these studies has a maximum optical density reading of 1.500. Several isolates had optical density readings exceeding this value. For averaging purposes optical density values of ≥ 1.500 were regarded as 1.500 although this assumption biased the data by limiting the optical density of strong slime-producing strains to a specified upper limit. The within-day and between-day variability of the quantitative micro-test for slime production was evaluated with four strains (two macro-tube positive and two macro-tube negative) of coagulase-negative staphylococci. Within-day variability was evaluated by testing each strain a total of three to five times on the same day. The between-day variability was determined by testing these same strains for slime production on three to five separate days over a four week period.

Statistical Analysis. Statistical analysis was performed using Student's t test. Values of $p < 0.05$ were considered significant. Sensitivity, specificity, and predictive values were calculated as described by Galen and Gambino (12).

Results

Of the 135 isolates tested, 70 were positive by the macro-method and 65 were negative (Figure 1). The quantitative micro-test optical density readings for these isolates (mean ± SD) were 1.176 ± 0.294 and 0.130 ± 0.095 ($p < 0.05$) for the macro-test positive and negative groups, respectively. Based on these data, a slime-negative isolate was defined as having a micro-test optical density reading of 0.415 or less, and a slime-positive isolate as having an optical density reading of greater than 0.415. The number 0.415 was chosen because it was three standard deviations (one SD = 0.095) above the mean (0.130) for the macro-test negative isolates (Figure 1). Application of this breakpoint reveals a sensitivity and specificity of 100% and 98%, respectively, by the micro-test in the classification of isolates as slime-positive versus slime-negative.

A breakdown of the 135 isolates of coagulase-negative staphylococci by species is shown in Table 1. Overall, 96 (71.1%) of the isolates were *Staphylococcus epidermidis.* The range of optical density readings was quite broad for *Staphylococcus epidermidis, Staphylococcus warneri, Staphylococcus hominis,* and *Staphylococcus sciuri.* This is in contrast with the extremely narrow range observed with *Staphylococcus haemolyticus,* and to some degree with *Staphylococcus simulans* and *Staphylococcus capitis.* With only one exception (one strain of *Staphylococcus capitis*), all of the strains in the latter group had optical density readings well below the slime-positive cutoff value of 0.415.

Table 2 provides a comparison of the results of the macro-tube test, read in a semi-quantitative manner, with those obtained with the quantitative micro-test on 52 different isolates of *Staphylococcus epidermidis.* It is apparent that, although the macro-test can be read in a graded fashion, there is considerable overlap between groups and no significant quantitative difference between the weakly positive group (macro 1+; optical density = 0.185) and the negative group (macro 0, optical density = 0.113, $p > 0.05$).

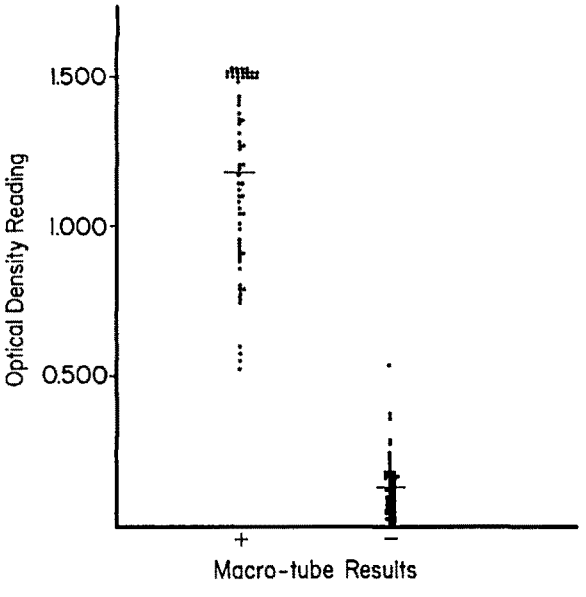

Figure 1: Scattergram of the optical density readings obtained for 135 isolates (70 macro-test positive and 65 macro-test negative) of coagulase-negative staphylococci.

The within-day and between-day variability of the quantitative micro-test for slime production with four strains of coagulase-negative staphylococci is presented in Table 3. Both within-day and between-day coefficients of variation were $< 20\%$ for the two slime-positive strains tested. The within-day coefficients of variation observed with the two slime-negative strains were comparable to those seen with the slime-positive strains. The between-day coefficients of variation were slightly greater with the slime-negative strains. The observed variability was not sufficient to cause a change in designation from slime-negative (optical density $\leqslant 0.415$) to slime-positive (optical density > 0.415).

Table 1: Comparison of slime production by different species of coagulase-negative staphylococci.

Species	n	Optical density range
Staphylococcus epidermidis	96	0.017–1.5
Staphylococcus haemolyticus	16	0.019–0.186
Staphylococcus warneri	7	0.036–1.5
Staphylococcus hominis	5	0.060–1.261
Staphylococcus simulans	5	0.205–0.372
Staphylococcus capitis	3	0.125–0.576
Staphylococcus sciuri	2	0.032–1.341
Staphylococcus saprophyticus	1	0.040

Table 2: Comparison of semi-quantitative macro-tube readings versus optical density readings with *Staphylococcus epidermidis*.

Macro-tube reading	n	Optical density		
		Range	Mean ± SD	P
3+	21	0.463–1.5	1.254 ± 0.325	–
2+	10	0.351–1.5	0.813 ± 0.311	< 0.05[a]
1+	6	0.031–0.495	0.185 ± 0.179	< 0.05[b]
0	15	0.031–0.194	0.113 ± 0.057	> 0.05[c]

[a]For the comparison with 3+. SD = standard deviation.
[b]For the comparison with 2+ or 3+.
[c]For the comparison with 1+.

Discussion

Although slime production is clearly related to the ability of coagulase-negative staphylococci to adhere to smooth surfaces, the assay described in the present study is designed to measure the production of a slimy surface film formed subsequent to adherence to the plastic surface. It is not designed to measure specific adherence properties as described by Christensen et al. (11). Our data indicate that the micro-test can serve as a reliable, quantitative technique for classifying coagulase-negative staphylococci as slime-positive or slime-negative. Furthermore, the comparison with the semi-quantitative macro-test results (Table 2) demonstrates the usefulness of a quantitative assay in classifying isolates which appear weakly positive by the macro-test. We found that the majority of the weakly positive isolates had very low optical density readings, and could easily be distinguished from moderate or strongly positive isolates with the quantitative micro-test (Table 2).

The application of the micro-test revealed quantitative differences in slime production by the different species of coagulase-negative staphylococci (Table 1). Aside from *Staphylococcus epidermidis*, the majority of the coagulase-negative staphylococci had low optical density readings (< 0.415), indicating little or no slime production under the conditions employed in this assay. This was particularly striking for *Staphylococcus haemolyticus*, and may partially explain why this species of coagulase-negative staphylococci is rarely involved in opportunistic infectious processes despite its frequent isolation as a human commensal (13, 14).

Although arbitrarily defined, coefficients of variation of 10% or less are usually considered excellent, and values of less than 20% are considered acceptable for many microbiological assays (15, 16). As presented in Table 3, with one exception, the within-day and between-day coefficients of variation for the quantitative micro-test were well within the arbitrary limit of 20%, indicating that this assay was reproducible for both slime-positive and slime-negative strains. It is important to note that, regardless of the variability of the optical density values for each organism, each of

Table 3: Within-day and between-day variability of the quantitative micro-test.

Strain	Macro-test result	Within-day		Between-day	
		Mean ± SD	Coefficient of variation (%)	Mean ± SD	Coefficient of variation (%)
148–8	3+	1.174 ± 0.220	18.7	1.257 ± 0.177	14.1
149–7	3+	1.355 ± 0.134	9.9	1.422 ± 0.087	6.1
180–14	–	0.157 ± 0.019	12.1	0.137 ± 0.024	17.5
168–4	–	0.109 ± 0.009	8.3	0.127 ± 0.033	26.0

the slime-positive and slime-negative isolates tested in Table 3 were reliably classified by the quantitative micro-test as positive or negative based on the break-point value of 0.415. Thus, the test provides an objective and reproducible means of categorizing isolates as slime-positive or slime-negative.

In summary, we have developed a quantitative micro-test for slime production by coagulase-negative staphylococci. The test agrees quite well with the qualitative macro-test, and is useful in providing an objective, quantitative assessment of slime production by isolates which appear weakly positive by the macro-test. The test will facilitate comparison of slime production by different species and strains of staphylococci, and may be useful in studies comparing the effects of different antibiotics and culture conditions on slime production by coagulase-negative staphylococci. Finally, this test for slime production may be a useful tool in future clinical studies of the pathogenesis of coagulase-negative staphylococcal infections.

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References

1. **Christensen, G.D., Bisno, A.L., Parisi, J.T., McLaughlin, B., Hester, M.G., Luther, R.W.:** Nosocomial septicemia due to multiple antibiotic-resistent *Staphylococcus epidermidis*. Annals of Internal Medicine 1982, 96: 1–10.
2. **Wade, J.C., Schimpff, S.C., Newman, K.A., Wiernik, P.H.:** *Staphylococcus epidermidis*: An increasing cause of infection in patients with granulocytopenia. Annals of Internal Medicine 1982, 97: 503–508.
3. **Winston, D.J., Dudnick, D.V., Chapin, M., Ho, W.G., Gale, R.P., Martin, W.J.:** Coagulase-negative staphylococcal bacteremia in patients receiving immunosuppressive therapy. Archives of Internal Medicine 1982, 143: 32–36.
4. **Sherertz, R.J., Falk, R.J., Huffman, K.A., Thomann, C. A., Mattern, W.D.:** Infections associated with subclavian Uldall catheters. Archives of Internal Medicine 1983, 143: 52–56.
5. **Christensen, G.D., Simpson, W.A., Bisno, A.L., Beachy, E.H.:** Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infection and Immunity 1982, 37: 318–326.
6. **Christensen, G.D., Parisi, J.T., Bisno, A.L., Simpson, W. A., Beachy, E.H.:** Characterization of clinically significant strains of coagulase-negative staphylococci. Journal of Clinical Microbiology 1983, 18: 258–269.
7. **Franson, T.R., Sheth, N.K., Rose, H.D., Sohnle, P.G.:** Scanning electron microscopy of bacteria adherent to intravascular catheters. Journal of Clinical Microbiology 1984, 20: 500–505.
8. **Peters, G., Locci, R., Pulverer, G.:** Adherence and growth of coagulase-negative staphylococci on surfaces of intravenous catheters. Journal of Infectious Diseases 1982, 14: 479–482.
9. **Davenport, D.S., Massanari, R.M., Pfaller, M.A., Bale, M.J., Streed, S.A., Hierholzer, W.J.:** Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. Journal of Infectious Diseases 1986, 153: 332–339.
10. **Ishak, M.A., Groschel, D.H.M., Mandell, G.L., Wenzel, R.P.:** Association of slime with pathogenicity of coagulase-negative staphylococci causing nosocomial septicemia. Journal of Clinical Microbiology 1985, 22: 1025–1029.
11. **Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M., Beachey, E.H.:** Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology 1985, 22: 996–1006.
12. **Galen, R.S., Gambino, S.R.:** Beyond normality: the predictive value and efficiency of medical diagnosis. John Wiley & Sons, New York, 1975.
13. **Kloos, W.E.:** Coagulase-negative staphylococci. Clinical Microbiology Newsletter 1982, 4: 75–79.
14. **Parisi, J.T.:** Coagulase-negative staphylococci and the epidemiological typing of *Staphylococcus epidermidis*. Microbiology Reviews 1985, 49: 126–139.
15. **Pfaller, M.A.:** Immunoassays for measurement of antimicrobial agents in body fluids. In: Jorgensen, J.H. (ed.): Automation in clinical microbiology. CRC Press, Boca Raton, Florida, 1987, p. 121–137.
16. **Stobberingh, E.E., Houben, A.W., Van Boven, C.O.A.:** Comparison of different tobramycin assays. Journal of Clinical Microbiology 1982, 15: 795.