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Adherence Measured by Microtiter Assay as a Virulence Marker for *Staphylococcus epidermidis* Infections

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Staphylococcus epidermidis **strains isolated from clinical sources showed a wide range of abilities to adhere to glass and plastic materials. The degree of adherence depended on a number of factors, most notably, the composition of the growth medium. Adherence was enhanced by the addition of glucose or oleic acid to the growth medium and inhibited by serum. We have demonstrated a statistically significant association between the quantitative assessment of adherence to polystyrene tissue culture plates and clinical relevance. No such association was found when adherence was assessed by the qualitative adherence assay. Possible new approaches for assessing the clinical relevance of coagulase-negative staphylococcal isolates are discussed.**

Coagulase-negative staphylococci (CNS) are now accepted as important nosocomial pathogens in patients with indwelling medical devices. Of currently recognized CNS species, *Staphylococcus epidermidis* is most frequently associated with clinically relevant infections. It is now generally believed that the virulence of CNS is related to their ability to attach to and subsequently colonize the surfaces of implanted medical devices. While attachment is dependent on hydrophobic interactions and electrostatic attraction, colonization of the biopolymer surface is associated with an extracellular polysaccharide or slime which protects the staphylococcal cells from both host defenses (3, 17) and antimicrobial agents (12, 27).

Several studies support the view that slime production is a marker of clinically relevant infection (10–12, 15, 19, 31), and there is some evidence that infections with slime-producing strains of CNS are more difficult to eradicate than infections with slime-negative strains (12, 14, 20, 31). Information on the capacity of a clinical isolate to produce slime would therefore help clinicians evaluate its clinical relevance and devise an appropriate treatment plan for the patient.

Slime production by CNS may be determined by either qualitative or quantitative tests. The qualitative tube adherence assay described by Christensen et al. (10) depends on the visual assessment of the degree of adherence of staphylococci to the sides of glass or polyethylene test tubes. Although simple to perform and suitable for routine diagnostic use, the tube assay is subjective and not highly reproducible (11, 13). The quantitative test (11), which depends on the spectroscopic measurement of the density of stained bacterial films adherent to plastic surfaces, gives an objective measurement of the degree of adherence but is not easily adaptable for routine diagnostic use.

We have tested consecutive clinical isolates of *S. epidermidis* by both qualitative and quantitative adherence assays. In addition, we have evaluated both assays for their reproducibility. We report a statistically significant association between laboratory-assessed clinical relevance and adherence measured by the quantitative microtiter assay.

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MATERIALS AND METHODS

Bacterial strains. One hundred consecutive clinical isolates of *S. epidermidis* submitted for antibiotic susceptibility testing were used in this study. They included isolates from blood (n = 34), catheter tips (n = 39), skin (n = 12), and miscellaneous sites (n = 15). Tests for susceptibility to 11 antibiotics were performed by agar dilution and speciation by a combination of replica plating and the API Staph system (API Systems SA, La Balme les Grottes, France), as previously described (13). Resistant strains, defined as resistant to five or more antibiotics, accounted for 53 of the 100 strains. Test strains were stored on agar slopes at 4°C before assay and then transferred to skim milk at −70°C. Strains RP12 (ATCC 35983), RP62A (ATCC 35984), and SP2 (ATCC 35982), kindly donated by G. Christensen, were used as controls. Control strains were stored at −70°C or freeze dried; working cultures were maintained on nutrient agar slopes at 4°C.

Quantitative microtiter adherence assay. Qualitative and quantitative assays were set up in parallel with the same broth culture. The quantitative microtiter procedure described by Christensen et al. (11) was followed, except for the following modifications. Flat-bottomed polystyrene tissue culture plates (Linbro; Flow Laboratories) were used. Optical densities (ODs) were read on a Titertek Multiscan enzyme-linked immunosorbent assay reader at a wavelength of 600 nm. Early in the study, aspiration and washing were performed manually, but in later experiments, we used a hand-made 12-pronged aspirator connected to a low vacuum. The latter method was preferred because it was less time-consuming and resulted in more even washing with less disruption of the slime layer.

The format of the 96-well tissue culture plate was such that each clinical isolate was tested in quadruplicate in Trypticase soy broth (TSB) with the usual glucose supplementation (standard TSB; Oxoid) and also in TSB without glucose (made from Oxoid ingredients). The results given in this article are the averages of four OD readings. Each batch of assays also included one or more control strains, as well as blank wells containing uninoculated broth. Staphylococci were classified as strongly adherent (OD $>$ 0.6), weakly adherent (0.3 $\leq$ OD $\leq$ 0.6), or nonadherent (OD $<$ 0.3). The low cutoff was chosen by using the criteria described by Christensen et al. (11), i.e., it represented approximately

three standard deviations above the mean OD of uninoculated (blank) wells. We also compared OD values in medium with and without the addition of 0.13 mg of oleic acid per ml.

Qualitative tube adherence assay. The qualitative assessment of adherence to glass was performed as described by Christensen et al. (10) in standard TSB and in TSB without glucose. Because individual observers may disagree in their interpretation of the tube test (11), results were read by one observer during the entire period of the study and recorded as strong adherence (+++ or ++), weak adherence (+), or negative (equivocal or no adherence), according to the density of the adherent film. In some experiments, 10% horse serum was added to the test medium, or nutrient broth (Oxoid nutrient broth no. 2 with 3 mg of yeast extract added per ml) was substituted for TSB.

Assessment of clinical relevance. Assessment of clinical relevance was based on laboratory criteria and clinical notes and supplemented with additional clinical information if necessary. The following laboratory criteria were used. Blood culture isolates were considered clinically relevant if CNS of the same species, having the same antibiogram and capacity for slime production, were recovered from more than one blood culture set. The probability of assigning two random isolates to the same type, by using a similar typing system (antibiogram, API Staph-Ident, slime production), has been calculated to be 0.076 to 0.086 (8). Catheter tips were cultured by the semiquantitive technique of Maki et al., which distinguishes infection (15 or more colonies) from contamination (22). Conventional quantitative microscopic and cultural criteria were used to evaluate urine isolates, which were classified as clinically relevant (significant bacteriuria) or as contaminating skin flora. Isolates recovered from miscellaneous skin sites, sputum, peritoneal dialysis fluids, and other specimens were assessed on the basis of some or all of the following criteria: (i) numbers of polymorphs and staphylococci observed in smears or wet preparations, (ii) the number of staphylococcal colonies isolated on horse blood agar, (iii) whether staphylococci were recovered in pure or mixed culture, and (iv) results of other cultures from the same site.

Of the 100 strains, 35 were defined as clinically relevant, including 7, 18, 8, and 2 isolates from blood, catheter tips, urine, and peritoneal dialysis fluids, respectively.

Statistical methods. A paired Z test was used to compare the OD in TSB with and without glucose supplement, and a paired Wilcoxon test was used for the same comparison of the qualitative assessment of adherence. A χ^2 test was used for the comparison of qualitative and quantitative adherence tests. Both the association between the quantitative assessment and clinical relevance and the effect of resistance were investigated by using a logistic model and the Generalised Linear Interactive Modelling software (26).

RESULTS

Quantitative assessment of adherence of 100 *S. epidermidis* strains in TSB with and without glucose. In TSB with the usual glucose supplementation, OD readings for 100 *S. epidermidis* strains ranged from 0.041 to 2.63 (Fig. 1). By using the criteria given in Materials and Methods, 54 strains were classified as nonadherent and 23 were classified as weakly adherent. Twenty-three strong adherers were identified, including six with very high OD readings (more than twice the high cutoff value). Five of these six strains were assessed as clinically relevant. The distribution of OD readings in TSB without glucose supplementation showed a much smaller range of values. More strains were classified as nonadherent, and considerably fewer strains were classified as either strongly or weakly adherent.

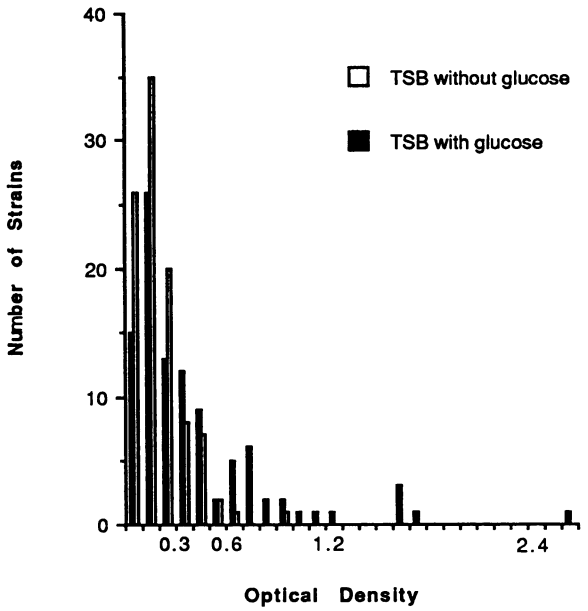

FIG. 1. ODs of 100 clinical isolates of *S. epidermidis*, tested in TSB with and without glucose supplementation. The cutoff value was 0.3 for nonadherence and 0.6 for strong adherence.

The degree of adherence of most strains of *S. epidermidis* was significantly greater in standard TSB than in TSB without glucose supplementation ($P < 0.001$, $Z = 5.9$). Only two strains had OD readings which were appreciably higher (0.3 or more) in TSB without glucose than in standard TSB.

Qualitative assessment of adherence (tube adherence assay) of 100 *S. epidermidis* strains in TSB with and without glucose. The tube adherence assay with TSB with the usual glucose supplementation classified 53 strains as strongly adherent, 19 as weakly adherent, and 28 as nonadherent (Table 1). Glucose enhanced the adherence of most strains. We identified two strains which were strongly adherent (++) in TSB without glucose but nonadherent in standard TSB. The paired Wilcoxon test on the 100 strains indicated a significant difference in adherence with the two media ($P < 0.0001$, $Z = 5.0$).

The ranges of ODs for the three groups of strains defined by the qualitative assay as strong, weak, and nonadherent are presented in Fig. 2. It is clear from the data presented that the assessments of adherence by the two types of assay were different. The quantitative assay recognized fewer strong adherers and significantly more nonadherent strains than did the tube adherence assay. Twenty strains with OD values of <0.3 in standard TSB were strongly adherent in the

TABLE 1. Qualitative assessment of the adherence of 100 strains of *S. epidermidis* to glass test tubes in TSB with and without glucose

Medium	No. of strains showing:		
	No adherence	Weak adherence	Strong adherence
TSB with glucose	28	19	53
TSB without glucose	59	24	17

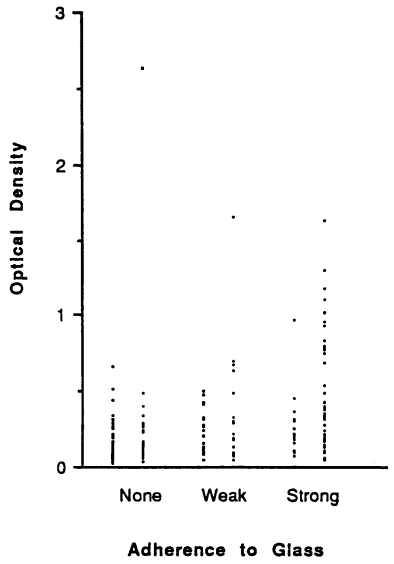

FIG. 2. ODs for *S. epidermidis* strains classified according to their adherence to glass. The columns on the left of each pair represent tests performed in TSB without glucose; those on the right were performed in standard TSB. The cutoff value for strong adherence was 0.6 and for nonadherence was 0.3.

corresponding tube adherence assay. It is interesting to note that only 3 of these 20 strains were assessed as clinically relevant by our laboratory-based criteria. Conversely, we identified one strain with an OD value of 2.63, which on initial testing by the qualitative assay produced a heavy ring of stained material at the air-liquid interface but no slimy film. According to the usual criteria for adherence, this strain was classified as nonadherent. In two subsequent tube assays, however, a densely adherent film was noted. We have also occasionally observed ring formation without the production of a slimy film by the positive control strain, RP62A. These observations have led us to believe that heavy slime deposits may sometimes become detached from glass, leading to a false-negative reading.

Association between quantitative assessment of adherence and clinical relevance. A logistic model was used to assess the association between the clinical relevance of a strain and the quantitative assessment of adherence, taking into account the site and whether or not the strain was resistant to five or more antibiotics. Clinical relevance was associated with an increase in adherence, as measured by quantitative methods ($P < 0.005$, $\chi^2 = 12.6$), but was not associated with antibiotic resistance. In contrast, clinical relevance was not associated with adherence as measured by the qualitative criteria.

Reliability of microtiter adherence assay. Repeated quantitative assays on control strains SP2 (nonadherent) and RP62A (strongly adherent) invariably resulted in OD readings within the expected ranges (Table 2). Strain RP12 (moderately adherent in standard TSB, nonadherent in TSB without glucose) behaved as expected in unsupplemented TSB. Glucose enhanced the adherence of strain RP12, but not sufficiently to push OD readings into the positive category. The most consistent and reliable results were obtained for strain RP62A.

Assays were performed on 76 clinical *S. epidermidis* isolates in standard TSB and in TSB without glucose (152 assays) and then repeated after storage at −70°C for 1 year. A total of 96 (63%) paired results were in complete agreement, and 10 (7%) were in strong disagreement (strong positive versus negative). The remaining 46 (30%) strains were classified as weakly adherent on one occasion and negative on the other. Thus, the quantitative assay usually discriminated clearly between strongly adherent strains and nonadherent strains but was less reliable in the weakly adherent range.

Reliability of the tube adherence assay. On the basis of a total of 70 tube tests performed on the three control strains in supplemented TSB and on 74 tests in TSB without glucose, the tube adherence assay had a sensitivity of 78% and a specificity of 72%. The reliabilities of the test for strains RP62A, RP12, and SP2 were 85, 72, and 74%, respectively.

The interassay variability of 74 clinical *S. epidermidis* isolates was assessed simultaneously and under the same conditions described in the previous section for the microtiter assay, i.e., storage at −70°C between assays. Complete agreement (positive and positive or negative and negative) between pairs of assays were observed in 66% of the assays. Major discrepancies (negative versus strongly positive) and minor discrepancies (negative versus weakly positive) were noted in 22 and 12% of paired results, respectively. In another study, duplicate tube tests on 40 freshly isolated CNS strains, stored on slopes at 4°C and minimally subcultured, showed 90% agreement.

Effect of medium and other variables on adherence. Strongly adherent strains were nonadherent when the tube assay was performed with nutrient broth or in the presence of 10% horse serum. Oleic acid significantly enhanced adhesion to polystyrene tissue culture plates in standard TSB ($P < 0.01$, $Z = 2.8$) (data not shown). In TSB without glucose, the addition of oleic acid enhanced adherence but the

TABLE 2. Range and variation of OD readings obtained by repeated testing of control strains SP2, RP12, and RP62A[a]

Strain[b]	Medium	No. of tests	OD				
			Range	Lower quartile	Median	Upper quartile	Coefficient of variation
SP2	TSB	18	0.05–0.17	0.08	0.11	0.16	0.37
	TSB without glucose	15	0.04–0.24	0.06	0.10	0.20	0.61
RP12	TSB	21	0.05–0.46	0.12	0.19	0.28	0.52
	TSB without glucose	21	0.03–0.30	0.08	0.13	0.18	0.53
RP62A	TSB	21	1.60–2.81	2.32	2.62	2.74	0.18
	TSB without glucose	21	0.87–1.60	0.95	1.07	1.26	0.21

[a] The interpretive breakpoints for strong and weak adherence were 0.6 and 0.3, respectively.
[b] Strain SP2 was nonadherent, RP12 was adherent in TSB only, and RP62A was adherent in both media.

difference was not statistically significant ($0.05 < P < 0.1$; $Z = 1.9$) (data not shown). Adherence also appeared to be influenced by small variations in the composition of the growth medium and, in the case of the tube assay, by different types of glass used in the manufacture of test tubes.

DISCUSSION

The emergence of CNS as significant nosocomial pathogens is a relatively recent event which reflects the increasing use of indwelling catheters and prosthetic devices in modern medical and surgical practice.

Recently, much attention has been given to the mechanisms whereby CNS attach to, colonize, and persist on biomaterials. Initial reversible attachment is mediated by hydrophobic interactions between protein groups on the bacterial cell surface and the biopolymer surface, which is also hydrophobic in nature. These forces overcome the relatively weak repulsive forces which operate between two negatively charged surfaces. Exopolysaccharide production by the attached cells contributes significantly to the second irreversible attachment phase, which culminates in the formation of microcolonies that are firmly adherent to the polymer surface (18). Staphylococci encased in a matrix of slime are resistant to several host defense mechanisms (3, 17) and to the action of antimicrobial agents (12, 27). Metabolically inactive forms are probably responsible for the well-known ability of CNS to persist on catheter materials and for the difficulty of eradicating these infections (2, 5). Recent in vitro and in vivo demonstrations that the expression of both exopolysaccharide (7) and small-colony, metabolically slow forms (1, 2, 4, 5) is dependent on environmental factors have led to the hypothesis that multiple CNS phenotypes may function synergistically, at least in endocardial infections (4, 5).

Production of large amounts of exopolysaccharide by CNS isolated from catheter-related sepsis is therefore one factor which should predict clinical relevance. Slime production is a relatively stable characteristic of many CNS strains (10). Although selection of slime-negative variants from slime-positive cultures has been achieved in vitro, it requires multiple passages under conditions which are unfavorable for slime production (7).

The 100 clinical *S. epidermidis* isolates described in the present study displayed a range of adherence capabilities in standard TSB medium. Some strains failed to adhere to either glass test tubes or polystyrene trays, while other strains were strongly adherent to both solid supports. We have confirmed the results of other studies (9, 10, 28) which indicate that adhesion is medium dependent, being enhanced by the addition of glucose or oleic acid to the growth medium but inhibited in nutrient broth or in the presence of 10% horse serum.

Although several studies indicate a role for the slime test as a marker of clinically relevant infection with CNS, others have not agreed. Bayston and Penny (6) first reported that strains of CNS causing shunt infections frequently grew as an adherent film on the sides of test tubes. Subsequently, Christensen et al. (10) reported a significant association between slime production and catheter-related sepsis. Several more recent studies (11, 12, 15, 19, 31) support the view that extracellular slime production is an important marker for clinically significant infections associated with a prosthetic device. In other studies, slime-positive CNS have been no more prevalent among disease isolates than slime-negative strains (13, 14, 20, 24, 30). There is recent evidence, however, that infections with slime-producing strains are more difficult to treat, often requiring catheter removal, than infections with strains that do not produce slime (12, 14, 20, 31). While most of the studies cited above based their assessment of slime production on the qualitative tube assay, two of them (11, 31) evaluated the microtiter assay as a marker of clinically relevant infection. In one study (11), the mean adherence value for strains causing catheter-related sepsis was significantly greater than either the mean for blood culture contaminants or the mean for skin strains. In another study of central nervous system shunt infections (31), pathogens were significantly more adherent than cerebrospinal fluid contaminants. In the present study, we have demonstrated a significant association between clinical relevance and adherence assessed by the microtiter assay.

Reports on the reproducibility of the tube adherence assay have varied. Christensen et al. (11), using strains from a lyophilized collection, noted that observers differed in their interpretation of the tube test, with correlation coefficients between pairs of observers ranging from 0.2 to 0.6. Davenport et al. (12) performed repeated tube tests over a 4-week period on seven randomly selected CNS strains. Intraassay, interobserver, and intraobserver reproducibilities were excellent for six of the seven strains, but one strain appeared to change from a slime-negative reaction to a weakly slime-positive reaction. Diaz-Mitoma et al. (14) also reported excellent reproducibility (96%) when they evaluated 60 fresh blood culture isolates of CNS. After storing these strains for periods of up to 14 weeks at −70°C, reproducibility decreased only slightly (90%). In our hands, the tube assay was less reliable than suggested by the studies cited above. Reproducibilities of assays performed on control strains RP62A, RP12, and SP2 were 85, 72, and 74%, respectively. Moreover, major discrepancies occurred in 22% of duplicate tests on clinical isolates. It is possible that storage of these strains at −70°C between tests may have altered their adherence capabilities, because duplicate tube tests on fresh strains showed 90% agreement.

In an evaluation of their microtiter assay, Christensen et al. (11) reported sensitivity and specificity between 76 and 91% and 81 and 96%, respectively. Pfaller et al. (29) also found a modified microtiter assay to be highly reproducible. Within-day and between-day coefficients of variation ranged from 0.06 to 0.26. Slime-negative strains showed more variation in their OD values than the slime-positive strains examined. The results of the present study confirm that the microtiter assay for determining adherence is highly reliable in detecting strongly adherent strains of *S. epidermidis*. Strain RP62A, a strongly adherent control strain, consistently gave high OD readings, and there was an interassay coefficient of variation of around 0.20. The test was also highly reproducible for strongly adherent clinical isolates. Weak or moderate adherence, which is characteristic of strain RP12 and is also observed with 20 to 25% of clinical isolates of *S. epidermidis*, was more difficult to reproduce. This degree of adherence may not be as important clinically as strong adherence.

Two studies have directly compared the qualitative and quantitative adherence assays. Christensen et al. (11) reported a correlation coefficient of 0.65 between averaged tube test results and the spectroscopic classification of strains. In their evaluation of a modified microtiter assay, Pfaller et al. (29) reported a sensitivity and specificity of 100 and 98%, respectively, compared with the tube assay. The 135 isolates used in their study included a large number of

strongly slime-positive and clearly negative strains, but few strains in the weakly adherent category.

In the present study, which included clinical isolates with a wide range of adherence capabilities, agreement between the qualitative and quantitative assays was poor, with significantly more strains being classified as nonadherent by the quantitative assay. Indeed, 20 strains which were deemed strongly adherent by the quantitative assay were nonadherent according to our quantitative criteria. The discrepancies between the results obtained by the two types of assay could not be resolved by altering the cutoff points used in the quantitative test (Fig. 2). Possible explanations for these divergent results are that adherence mechanisms for glass and plastic surfaces may be different or that scratches and imperfections of glass test tubes, traces of grease, or minor media variations may influence the ability of staphylococci to attach to and colonize surfaces. Alternatively, the recent demonstration of phenotypic variation by CNS (7) opens the possibility that surface characteristics responsible for adherence may be switched on and off, depending on test conditions. Finally, the subjectivity associated with the visual assessment of adherence may affect the reliability of the tube assay.

Whatever the explanation, we have demonstrated a statistically significant association between clinical relevance and the degree of adherence to polystyrene plates, as determined by the quantitative assay, but were unable to demonstrate such an association for the tube adherence assay. We therefore believe that the microtiter assay predicts clinical relevance more reliably than the tube adherence assay.

This study highlights our present inability to make a confident prediction regarding the virulence of any clinical CNS isolate on the basis of methods which are appropriate for a clinical laboratory.

The tube adherence assay is simple to perform and inexpensive, but our study confirms its subjectivity, poor reproducibility, and inability to reliably predict clinical relevance. Quantitation of adherence by the method described by Christensen et al. (11) or one of its variations (29) or extensions (16) does eliminate subjectivity. In our hands, however, the microtiter assay is less reproducible than desired for a routine diagnostic test. Moreover, the requirement for gentle washing makes it difficult to automate, and the assay does not lend itself to the testing of single isolates.

Given that slime is an important virulence factor which mediates irreversible attachment of staphylococci to biomaterials, an alternative approach to assessing adhesion may be to chemically determine the amount of staphylococcal exopolysaccharide produced by clinical isolates. While the precise chemical nature of *S. epidermidis* slime has yet to be determined, mannose is a major component of the slime produced by at least one reference strain. Reactivity with mannose-specific lectins in a gel diffusion or slide agglutination assay has therefore been proposed as a marker for slime (21). Preliminary work in our laboratory indicates that agglutination of staphylococcal cells with the mannose-specific lectin concanavalin A failed to differentiate adequately between slime-positive and slime-negative strains (S. R. Pearson, unpublished observations). Further investigation of lectin agglutination, as well as of other chemical methods of quantifying slime, may lead to the development of a simple yet specific test for CNS virulence.

Among other cell surface components associated with CNS virulence are the proteins that mediate early hydrophobic bonding to biopolymers. A direct relationship exists between cell surface hydrophobicity and the attachment of CNS to Teflon catheters in vitro (25), and one recent study has reported that cell surface hydrophobicity, determined by adherence to xylose, is predictive of clinical relevance (23). Development of more specific and simpler chemical methods awaits elucidation of the chemical nature of the proteins which mediate hydrophobic bonding.

The metabolically dormant small-colony phenotype is clearly relevant to persistence of endocardial infection, both in humans and in experimental animals (1, 4, 5). It is possible that estimation of the proportion of small-colony morphotypes in primary cultures from other types of infection could provide useful information regarding persistence and clinical relevance.

Finally, it is important to note that the adherent mode of growth differs markedly from growth in liquid media in the laboratory (27). The in vivo expression of virulence factors, including exopolysaccharide (11), the small-colony phenotype (2, 4, 5), and probably surface hydrophobic proteins as well, is dependent on environmental conditions. Future tests for virulence markers should be performed directly on biofilm material or under laboratory conditions which mimic conditions encountered by CNS in vivo.

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