

## Adherence of Coagulase-Negative Staphylococci to Plastic Tissue Culture Plates: a Quantitative Model for the Adherence of Staphylococci to Medical Devices

GORDON D. CHRISTENSEN,<sup>1,2\*</sup> W. ANDREW SIMPSON,<sup>1,3</sup> JANARA J. YOUNGER,<sup>4</sup> LARRY M. BADDOUR,<sup>2</sup>  
FRED F. BARRETT,<sup>4</sup> DENNIS M. MELTON,<sup>2</sup> AND EDWIN H. BEACHEY<sup>1,2,3</sup>

*Veterans Administration Medical Center, Memphis, Tennessee 38104,<sup>1\*</sup> and the Departments of Medicine,<sup>2</sup> Microbiology,<sup>3</sup> and Pediatrics<sup>4</sup> of the University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163*

Received 4 March 1985/Accepted 3 September 1985

The adherence of coagulase-negative staphylococci to smooth surfaces was assayed by measuring the optical densities of stained bacterial films adherent to the floors of plastic tissue culture plates. The optical densities correlated with the weight of the adherent bacterial film ( $r = 0.906$ ;  $P < 0.01$ ). The measurements also agreed with visual assessments of bacterial adherence to culture tubes, microtiter plates, and tissue culture plates. Selected clinical strains were passed through a mouse model for foreign body infections and a rat model for catheter-induced endocarditis. The adherence measurements of animal passed strains remained the same as those of the laboratory-maintained parent strain. Spectrophotometric classification of coagulase-negative staphylococci into nonadherent and adherent categories according to these measurements had a sensitivity, specificity, and accuracy of 90.6, 80.8, and 88.4%, respectively. We examined a previously described collection of 127 strains of coagulase-negative staphylococci isolated from an outbreak of intravascular catheter-associated sepsis; strains associated with sepsis were more adherent than blood culture contaminants and cutaneous strains ( $P < 0.001$ ). We also examined a collection of 84 strains isolated from pediatric patients with cerebrospinal fluid (CSF) shunts; once again, pathogenic strains were more adherent than were CSF contaminants ( $P < 0.01$ ). Finally, we measured the adherence of seven endocarditis strains. As opposed to strains associated with intravascular catheters and CSF shunts, endocarditis strains were less adherent than were saprophytic strains of coagulase-negative staphylococci. The optical densities of bacterial films adherent to plastic tissue culture plates serve as a quantitative model for the study of the adherence of coagulase-negative staphylococci to medical devices, a process which may be important in the pathogenesis of foreign body infections.

Coagulase-negative staphylococcal infections characteristically occur in the setting of an indwelling medical appliance (18). Apparently, the foreign body predisposes the patient to infection with what would otherwise be innocuous bacteria. We verified this observation in a mouse model for foreign body infection. Normal animals did not become infected when we injected coagulase-negative staphylococci under their skin. On the other hand, when the animals were prepared by implanting subcutaneous sections of intravascular catheters, one-third of the injected animals developed infections at the catheter site (8).

In 1972, Bayston and Penny first observed that many strains of coagulase-negative staphylococci form mucoid deposits on cerebrospinal fluid (CSF) shunts (3). In recent years, several groups of investigators have conducted scanning electron microscope surveys of naturally infected intravascular catheters (11, 21, 26), peritoneal dialysis catheters (23), and pacemaker leads (22, 28) and found adherent deposits of coagulase-negative staphylococci. For many pathogens, the interaction between bacterial cell and host surface determines the ability of the microorganism to colonize and infect the host (reviewed in reference 6). Considering the propensity of coagulase-negative staphylococci to produce foreign body infections, it seems likely that these microorganisms opportunistically infect medical appliances

because they preferentially adhere to the surface of the appliance.

In 1979 and 1980, we noted a large number of patients in the City of Memphis and University of Tennessee Hospitals with intravascular catheter-associated sepsis due to coagulase-negative staphylococci (4, 5). In the course of our investigations, we found that some strains coated the culture tube walls with a tenacious bacterial film or slime. Intravascular catheters immersed in the broth also became coated with slime (7). These studies suggested that staphylococci isolated from patients with intravascular catheter-associated sepsis produced more slime than did saprophytic strains (7).

We have since improved our original qualitative assessment of slime production by modifying the spectrophotometric methods used by Fletcher (9, 10) in her studies on the adherence of marine microorganisms to smooth surfaces. In this report, we quantitate the adherence of coagulase-negative staphylococci to plastic by using our original collection of coagulase-negative staphylococci from the Memphis outbreak of intravascular catheter-associated sepsis. We have also studied a collection of strains from children with CSF shunt infections and strains from adults and endocarditis.

(This paper was presented in part at the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy [G. D. Christensen, W. A. Simpson, E. H. Beachey, A. L. Bisno, and F. F. Barrett, Program Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother. abstr. no. 649, 1982].)

\* Corresponding author.

TABLE 1. Clinical collections used in this study

Collection	No. of strains (no. of isolates)	Source
Intravascular catheter-associated sepsis collection		G. D. Christensen
Pathogens	35 (49)	
Blood culture contaminants	46 (48)	
Cutaneous strains	46 (46)	
CSF shunt infection collection		F. F. Barrett
Pathogens	51 (101)	
CSF contaminants	33 (33)	
Pediatric hyperalimentation infection	1 (7)	F. F. Barrett
Adult endocarditis collection	7 (7)	A. W. Karchmer

## MATERIALS AND METHODS

**Bacterial sources.** The clinical details, microbiology, and epidemiology of our collection of coagulase-negative staphylococci from the Memphis outbreak of intravascular catheter-associated sepsis have been previously reported (4, 5). These organisms were collected between 1979 and 1980 and stored as lyophilized cultures (Table 1). For some studies, we selected four strains from this collection, RP12 (ATCC 35983), RP62A (ATCC 35984), RP14 (ATCC 35981), and SP2 (ATCC 35982), whose adherence characteristics were known from prior investigations (1, 7, 8). From 1980 to 1984 we gathered a second collection of coagulase-negative staphylococci from pediatric patients hospitalized at LeBonheur Children's Medical Center, Memphis, Tenn., with CSF shunt infections (Table 1). The clinical and microbiological features of this collection will be reported in a companion paper (J. J. Younger, G. D. Christensen, D. L. Bartley, J. C. H. Simmons, and F. F. Barrett, manuscript in preparation). In addition to these CSF shunt strains, we have also included a single strain of *Staphylococcus epidermidis* represented by seven isolates from a pediatric patient with a hyperalimentation line infection. In this manuscript we have used the term strain to refer to a distinct line of bacteria isolated from an individual patient or from experimentally infected laboratory animals. Each strain was represented by a single isolate; however, some strains were represented by multiple identical isolates. For clinical isolates, we considered a strain to be reisolated on more than one occasion if multiple isolates from a particular patient shared the same antimicrobial susceptibilities and species classification (5). For experimentally infected animals, we considered an isolate to be a daughter isolate of a laboratory parent strain if it was a coagulase-negative staphylococcus and had the same antimicrobial susceptibility pattern as the challenge organism (1, 8). The organisms from pediatric patients were maintained frozen at  $-70^{\circ}\text{C}$  in thioglycollate-glycerol broth. The final clinical collection of seven endocarditis strains was a gift from A. W. Karchmer, Harvard Medical School, Boston, Mass. (Table 1). In addition to these clinical isolates, we also measured the adherence of 126 isolates obtained from seven strains of coagulase-negative staphylococci passed through a mouse model for foreign body infection and a rat model for catheter-induced endocarditis. The type strain for *Staphylococcus epidermidis*, ATCC 14990, and strains ATCC 155 and

ATCC 31432 were obtained from the American Type Culture Collection, Rockville, Md.

**Microbiology.** All isolates were gram-positive, clustering cocci that produced catalase but not coagulase. Working cultures were maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood and transferred every 2 to 3 months. Species determinations were made on all clinical isolates with API Staph-Ident strips (Analytab Products, Plainview, N.Y.) and DMS StaphTrac strips (DMS Laboratories, Flemington, N.J.) as described in the instructions of the manufacturers. In the mouse foreign body infection model and the rat endocarditis model, we confirmed the identities of daughter isolates by checking their antimicrobial susceptibility patterns as previously described (1, 8).

**Slime production.** (i) **Tube method.** A qualitative assessment of slime production was determined as previously described (7). This tube method consisted of inoculating 10 ml of Trypticase soy broth (TSB; BBL Microbiology Systems) with a loopful of organisms from a blood plate culture and incubating the broth culture tube overnight (18 h) at  $37^{\circ}\text{C}$ . The culture tubes were then emptied of their contents and stained with trypan blue or safranin. Slime production was judged to have occurred if a visible film lined the walls of the tube. Ring formation at the liquid-air interface was not considered indicative of slime production. To compare observer variation with the tube method, the coded 127 strain catheter collection was examined on three separate occasions by independent observers familiar with the tube technique. Each observer estimated the amount of slime production as absent (score 0), weak (score 1), moderate (score 2), or strong (score 3). The observations were compared with each other and with spectrophotometric measurements by calculating a correlation coefficient.

(ii) **Spectrophotometric method.** Early in these studies, we noted that the adherence measurements of some of our isolates were greater without glucose than with glucose. Therefore, we measured the optical densities (ODs) of adherent bacterial films produced under both conditions and reported the data in both interval and ordinal fashions. Stationary (18-h) TSB cultures of coagulase-negative staphylococci were diluted 1:100 with fresh TSB and with TSB prepared without the usual glucose supplementation (TSB without glucose; BBL Microbiology Systems). Individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates were filled with 0.2-ml aliquots of the diluted culture. We used two brands of tissue culture plates, MicroTest III (Falcon no. 3072; Becton Dickinson, Oxnard, Calif.) and Cell Wells (no. 25860; Corning Glass Works, Corning, N.Y.); we could not detect any performance differences between the two plates. Both manufacturers sterilize the plates with gamma irradiation and electrically charge their surfaces to diminish the hydrophobicity of the polystyrene. The tissue culture plates were incubated for 18 h at  $37^{\circ}\text{C}$ . The contents of each well were gently aspirated by tipping the plate and placing the aspirator (a Pasteur pipette connected to low vacuum) tip in the lowest corner of the well. With an automatic hand pipette (100- $\mu\text{l}$  Octapette; Costar, Cambridge, Mass.), the wells were washed four times with 0.2 ml of phosphate-buffered saline (ph 7.2). Adherent organisms were fixed in place with Bouin fixative (14) and stained with Hucker crystal violet (30). Excess stain was rinsed off by placing the plate under running tap water. After drying, the ODs of stained adherent bacterial films were read with a MicroELISA Auto Reader (model MR580; Dynatech Laboratories, Inc., Alexandria, Va.). We used a

wavelength of 570 nm with the photometer switched to the single-wavelength mode (lambda test).

Adherence measurements were performed in quadruplicate and repeated at least three times; the values were then averaged. If the strain was represented by more than one isolate, the readings for the isolates were averaged together to find the reading for the strain. The MicroELISA Auto Reader has a maximum OD reading of 1.500, and many adherence determinations exceeded this value. To accommodate this limitation, we adopted the following convention: for averaging purposes, values in excess of 1.500 were considered 1.501; if three of the four determinations exceeded 1.500, the averaged value was taken as 1.501. This convention biased the data by limiting the OD of strongly adherent organisms to a specified maximum value.

For the purposes of data simplification, we used an ordinal classification for the adherence capability of individual strains of coagulase-negative staphylococci. Bacteria were divided into three categories, nonadherent, weakly adherent, and strongly adherent, based upon the ODs of bacterial films produced in TSB with and without glucose. If the ODs in both media were less than or equal to 0.120, we classified the organism as nonadherent. If the OD in either medium exceeded 0.240, then we classified the strain as strongly adherent. Strains whose maximal OD was greater than 0.120 but less than or equal to 0.240 were classified as weakly adherent. We chose the number 0.120 for a guideline because it was three standard deviations (0.023) above the mean OD (0.050) of a clean tissue culture plate stained by the previously described procedure.

The reliability of this classification was examined by using 35 clinical strains (isolated from blood and CSF) represented by 105 isolates. All but two of these strains were adherent by the preceding definition; however, 11 strains were nonadherent in TSB with glucose, and 7 strains were nonadherent with TSB without glucose. Therefore, for the purposes of the following calculations, we separately considered the adherence in TSB with glucose and that in TSB without glucose. Calculations of the sensitivity,  $a/(a + c)$ ; specificity,  $d/(b + d)$ ; positive predictive value,  $a/(a + b)$ ; negative predictive value,  $d/(c + d)$ ; and accuracy,  $(a + d)/(a + b + c + d)$  of the spectrophotometric readings were made;  $a$ ,  $b$ ,  $c$  and  $d$  refer to the number of determinations in which: the classification of the strain and the isolate agree ( $a$ ), the isolate has the classification and the strain does not ( $b$ ), the strain has the classification and the isolate does not ( $c$ ), and neither the strain nor the isolate has the classification ( $d$ ).

Interval data were used for finer comparisons between strains. For this purpose, we combined the data for adherence in TSB and adherence in TSB without glucose into a single scalar, nondimensional adherence value by using the following formula:

adherence value =

$$\sqrt{(\text{OD in TSB})^2 + (\text{OD in TSB without glucose})^2}$$

This formula simply calculated the distance between the origin and the adherence coordinates of a strain of coagulase-negative staphylococci, when the ODs of the strain in TSB and TSB without glucose were plotted as the ordinate and abscissa, respectively.

**Optical density of bacterial film versus weight of bacterial film.** A graph comparing the wet weight of the bacterial film with the OD of the bacterial film was constructed by using four strains of coagulase-negative staphylococci, RP12, RP14, RP62A, and SP2. Each strain was propagated over-

TABLE 2. Correlation between independent readers of the tube test and with the spectrophotometer classification

Comparison	Correlation coefficient <sup>a</sup>	Probability (P)
Observer 1 vs observer 2	0.5439	<0.001
Observer 1 vs observer 3	0.5962	<0.001
Observer 2 vs observer 3	0.2207	<0.01
Observer 1 vs spectrophotometer	0.6028	<0.001
Observer 2 vs spectrophotometer	0.3996	<0.001
Observer 3 vs spectrophotometer	0.6148	<0.001
Avg of observers 1 through 3 vs spectrophotometer	0.6462	<0.001

<sup>a</sup>  $n = 143$ .

night in TSB and TSB without glucose. An aliquot was centrifuged at  $5,000 \times g$  for 15 min, and the bacterial pellet was weighed. The original broth culture was then diluted to a concentration of 40 mg of bacteria (wet weight) per ml, followed by serial twofold dilutions. Eight 0.2-ml aliquots of each dilution were placed in a 96-well tissue culture plate, and the plate was spun at  $500 \times g$  for 45 min. The clarified supernatants were removed with gentle aspiration, and the remaining bacterial pellets coating the well floors were fixed, stained, and read as previously described. The DNA content of RP12, RP62A, RP14, SP2, and the three ATCC strains were determined by the method of Hanson and Phillips (12).

**Adherence to other substrates.** In addition to the Falcon and Corning tissue culture plates, qualitative assessments of bacterial adherence were also made to Titertek 96-well microtiter plates (Linbro; Flow Laboratories, McLean, Va.), disposable polyvinyl chloride 96-well microtiter V plates (Dynatech), Lucite 96-well microtiter U plates (Dynatech), Fisherbrand snap-cap polypropylene test tubes (17 by 100 mm; Fisher Scientific Co., Pittsburgh, Pa.), Falcon snap-cap polystyrene test tubes (17 by 100 mm), and Kimax borosilicate glass culture tubes (Fisher). After overnight culture, the plates and test tubes were rinsed and stained as described above.

**Glass slides.** Glass slides with adherent bacterial films were prepared by incubating sterile glass slides in TSB and TSB without glucose seeded with RP12, RP62A, RP14, or SP2. The slides were rinsed four times, fixed with Bouin fixative, and stained with crystal violet or Alcian blue (25). Photomicrographs were taken with a Zeiss Ultraphot photomicroscope through a Planapo 100 oil immersion objective with Technical pan 2415 film (Eastman Kodak Co., Rochester, N.Y.). A no. 25 red filter was used for the Alcian blue photomicrographs.

**Animal model infections.** Infections were produced in mice and rats as previously described (1, 8).

## RESULTS

**Evaluation of the tube test.** The qualitative assessment of slime production, as originally reported by us, proved an inadequate test for bacterial adherence. In Table 2 we list the correlation coefficients obtained when the tube test readings from independent observers were compared with one another. The highly significant  $r$  values indicated that each observer witnessed the same general phenomenon. Nevertheless, the low absolute value for  $r$ , ranging from 0.2207 to 0.5936, indicated that individual observers frequently disagreed in their interpretation of the tube test. Consequently, although it is reliable for broad surveys, the tube test could not accurately gauge the ability of individual strains of

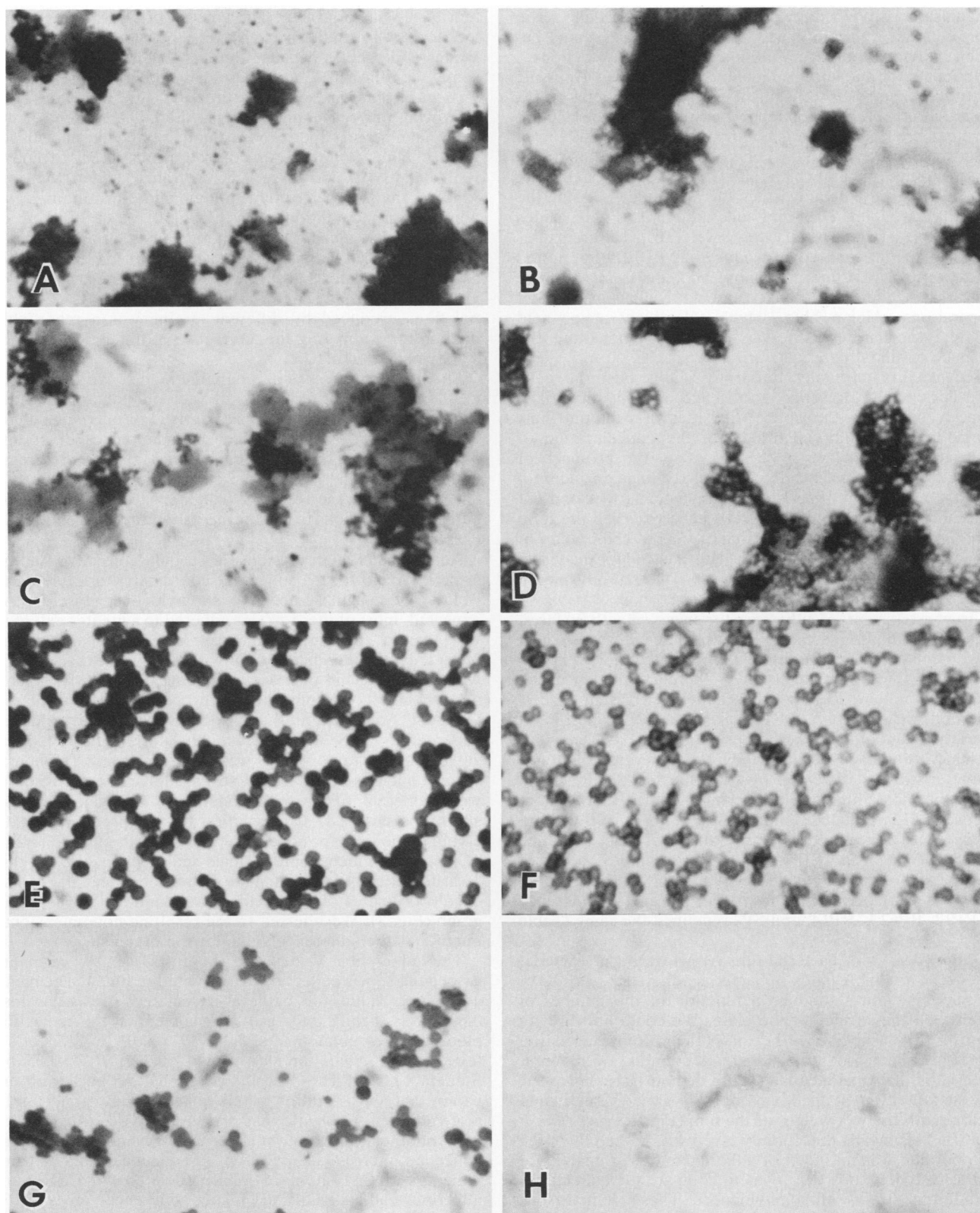


FIG. 1. Photomicrographs of glass slides with adherent bacterial films stained with crystal violet or Alcian blue. Crystal violet deeply stains the cells of adherent RP12 (A), RP62A (C), RP14 (E), and SP2 (G); crystal violet stains to a lesser extent the slimy extracellular material produced by RP12 and RP62A. Alcian blue selectively stains the extracellular mucopolysaccharides produced by RP12 (B), RP62A (D), and RP14 (F). SP2, which does not produce slime, stains poorly with Alcian Blue (H). RP12, RP62A, and SP2 were cultivated in TSB; RP14 was cultivated in TSB without glucose. Magnification,  $\times 3,150$ .

coagulase-negative staphylococci to produce slime. The source of this variability was primarily disagreements between individual observers in interpreting weak reactions.

**Rationale for the spectrophotometric technique.** By using polystyrene petri dishes stained with crystal violet and a spectrophotometer, M. Fletcher measured the adherence of marine microorganisms to smooth surfaces (9, 10). We used the same methods, but switched the substrate to plastic tissue culture plates which allowed us to automate the readings. Figure 1 demonstrates the advantage of using the stain crystal violet. Bacterial cells adherent to glass slides were deeply stained by crystal violet. To a lesser extent, extracellular slimy materials produced by RP12, RP62A, and RP14 were also stained. Because SP2 does not produce slime, extracellular materials were not visible in the SP2 preparations. Alcian blue, which selectively stains acid mucopolysaccharides (25), was used to demonstrate the presence of slimy materials.

Because crystal violet uniformly stains bacterial cells regardless of the presence or absence of slimy materials, properly speaking, the optical densities of bacterial films stained with crystal violet indicate the concentration of bacteria on the surface of the plate, not the presence of slime. We therefore considered these readings an index of the adherence of an organism to the plastic surface and not a measure of slime production. We have reserved the term slime production, on the other hand, for the elaboration of extracellular Alcian blue-staining materials and for the visual deposition of thick viscid sheets of bacteria on smooth surfaces.

**Visual assessments of adherence to various smooth surfaces.** Because flat-bottomed tissue culture plates were the most convenient substrate, we first established that adherence to these plates was similar to the adherence of coagulase-negative staphylococci to other materials. Visual assessments of the adherence of RP12, RP62A, RP14, and SP2 to a variety of smooth surfaces are listed in Table 3. These four strains were chosen because they had different adherence characteristics as indicated by the tube test: RP62A produced the most slime and did so in TSB and TSB without glucose; RP12 produced slime in TSB but not in TSB without glucose; RP14 produced slime in both media, but the slime was most evident in TSB without glucose; and SP2 did not produce slime in either medium. Although certain material variations were apparent, the overall pattern of adherence for RP12, RP62A, RP14, and SP2 remained the same (Table 3).

**Optical density of bacterial film versus weight of bacterial film.** To be a reliable index of bacterial adherence, the OD of the bacterial film should be a function of the number of bacteria on the surface of the plate. We could not directly confirm this association because the autoagglutination caused by slime production prevents counting of adherent bacteria by the usual means. We did demonstrate, however, that the OD of the stained bacterial film varied directly and linearly with the wet weight of the film (Fig. 2;  $r = 0.906$ ,  $P < 0.01$ ). This means that for any given strain, the OD of the bacterial film was an index of adherence. Our primary goal for these studies, however, was to compare the adherences of different strains of staphylococci. These comparisons would only be valid if the number of staphylococcal cells in a gram of bacterial film was roughly the same regardless of whether the strain produced slime and of which species of staphylococci it represented. Once again, we could not directly confirm this by counting the bacteria; however, we could count the chromosomes by measuring the DNA con-

tent of the bacterial film. The amount of DNA in a staphylococcal cell should be relatively constant for different strains and species because the DNA is primarily chromosomal, and the DNA homology for members of the *Staphylococcus epidermidis* species group is 40% or better (15). Following this reasoning, we used the DNA content of the bacterial film as an index to the number of bacterial cells per gram of film. For the four control strains (RP12, RP62A, RP14, and SP2) and three ATCC strains (14990, 155, and 31432), the DNA content under slime-producing and nonproducing conditions was  $3.7 \times 10^{-3} \pm 1.9$  mg/mg of bacteria (wet weight). This result indicated that the weight of the bacterial cell was relatively constant for different media and different strains and species of coagulase-negative staphylococci; it also indicated that measuring the ODs of bacterial films produced by different strains was a reasonable method for comparing the adherence of different strains of staphylococci to plastic.

**Stability of adherence in an animal model.** We used spectrophotometric measurements of bacterial adherence to determine the stability of this characteristic through the course of an infection. We examined the adherences of strains passed through laboratory animals in a model for foreign body infection and a model for catheter-induced endocarditis; in all cases, the resultant daughter strains retained the adherence characteristics of the laboratory parent strain (Table 4).

**Adherence of clinical strains of coagulase-negative staphylococci.** We plotted the adherence coordinates for 219 clinical isolates of coagulase-negative staphylococci (Fig. 3). As expected, most of these strains adhered to a greater extent in TSB than in TSB without glucose. A few strains, however, had a greater adherence in TSB without glucose than in TSB with glucose. This unexpected finding created a problem: under which conditions should an organism be considered adherent? Some strains, such as RP12, would be considered adherent in TSB with glucose but not in TSB without glucose, while the opposite was true for a small number of strains, such as RP14. Many strains, for example, RP62A, were adherent under both conditions; how should these strains be counted? Our solution to this problem was to combine the data for adherence in TSB with glucose and in TSB without glucose into a single scalar value. We did this by arithmetically calculating the distance between the adherence coordinates of a particular strain and the origin of the graph (Fig. 3). We referred to the resulting nondimensional number as the adherence value for the organism.

The adherence values for the intravascular catheter-associated sepsis collection are depicted in Fig. 4. The mean adherence value for catheter-associated sepsis strains, 0.602, was significantly greater than either the mean for blood culture contaminants, 0.295 ( $P < 0.01$  by Student's  $t$  test), or the mean for skin strains, 0.371 ( $P < 0.05$  by Student's  $t$  test). These results confirmed our earlier observation that sepsis strains in this collection are significantly more adherent to plastic than are saprophytic strains.

In another series of studies, we examined a clinical collection of coagulase-negative staphylococci isolated from patients with CSF shunts. Strains associated with CSF shunt infections had a significantly higher adherence value, 0.563, than the adherence value for CSF contaminants, 0.363 ( $P < 0.05$  by Student's  $t$  test) (Fig. 5).

Not all pathogenic strains of coagulase-negative staphylococci had high adherence values. A. W. Karchmer had kindly provided us with seven strains of coagulase-negative staphylococci isolated from patients with endocarditis (six

TABLE 3. Qualitative assessment of bacterial adherences to tissue culture plates, microtiter plates, and culture tubes

Strain	Adherence to <sup>a</sup> :							
	Tissue culture plates		Microtiter plates			Culture tubes		
	Falcon irradiated polystyrene	Corning irradiated polystyrene	Titertek polystyrene	Dynatech polyvinyl	Dynatech Lucite	Fisher polypropylene	Falcon polystyrene	Kimax glass
RP62A	++/++	++/++	++/++	++/++	++/++	++/++	++/++	++/++
RP12	++/-	++/-	-/-	+/-	-/-	+/-	++/-	++/-
RP14	+/++	+/++	-/-	+/+	-/-	-/+	-/+	++/++
SP2	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-

<sup>a</sup> Results are presented as adherence in TSB/adherence in TSB prepared without the usual glucose supplementation. Symbols: ++, strong adherence; +, moderate adherence; -, no adherence.

patients with prosthetic valve endocarditis and one patient with native valve endocarditis). Although a comparison group of saprophytic strains was not available, the mean adherence value of the endocarditis strains, 0.122 (range, 0.062 to 0.213), was clearly low. This finding prompted us to tally separately the adherence values of six strains isolated from our catheter-associated sepsis patients who had experienced prolonged episodes of bacteremia in the setting of an indwelling catheter. We also included in this tally one additional pediatric patient (observed in the course of the CSF shunt study) with prolonged coagulase-negative bacteremia in the setting of a hyperalimentation line. The mean adherence value for these strains, 0.305 (range, 0.111 to 0.604), was also low, roughly equal to the adherence values we obtained for saprophytic strains.

**Spectrophotometric technique and ordinal classification.** If relatively few pathogenic strains were greatly adherent, averaging their adherence values with nonadherent strains could skew the data, giving the appearance that the pathogenic strain collection as a whole was very adherent. Furthermore, it seemed likely that the distinction between

adherent and nonadherent was of far greater importance to pathogenesis than the actual degree of adherence. To avoid these potential criticisms of the preceding data, we also examined the data with an ordinal classification system. This system grouped the strains into three adherence classes based upon the ODs of their adherent bacterial films. Like the adherence value, these classifications indicated the distance between the adherence coordinates and the origin (Fig. 3). These classifications were, however, only an approximation of this distance; we obtained the approximation by dividing the graph into three portions: nonadherent (OD in both media,  $\leq 0.120$ ), weakly adherent (OD in either medium,  $> 0.120$  but  $\leq 0.240$ ) and strongly adherent (OD in either medium,  $> 0.240$ ). The reliability of this spectrophotometric classification was confirmed in two ways.

(i) After classifying the catheter-associated sepsis collection by this method, we compared the spectrophotometric classification with the readings of independent observers of the tube test (Table 2). The *r* values for observers were highest when their observations were compared with the spectrophotometric classification. Furthermore, the highest

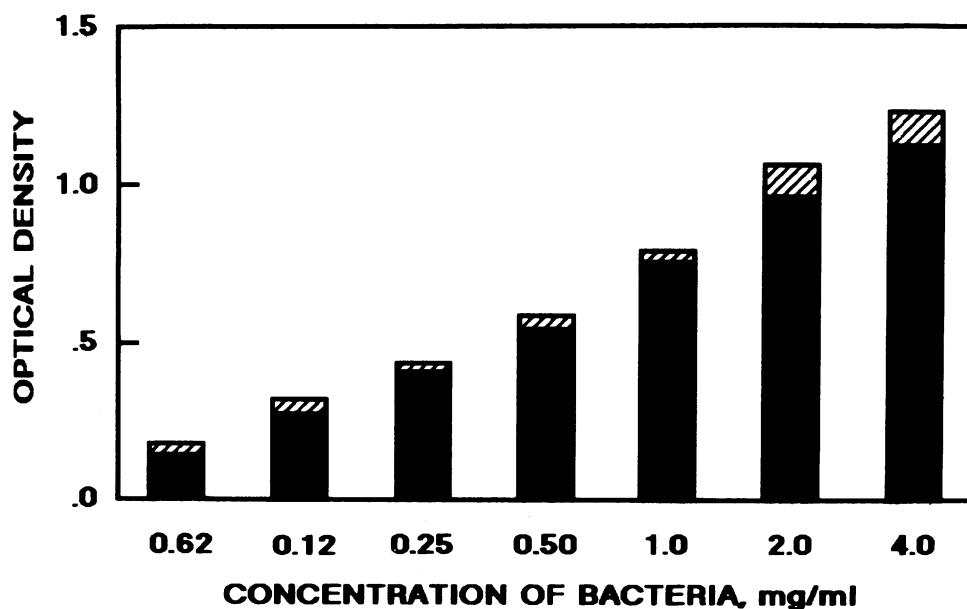


FIG. 2. Bar graph of optical density versus bacterial concentration on the surface of the tissue culture plate. Symbols: ■, mean OD; ▨, standard error of the mean.

TABLE 4. Adherence values of strains passed through animals<sup>a</sup>

Strain (no. of isolates)	OD in:		Adherence value
	TSB	TSB without glucose	
Mouse foreign body infection model			
SP2			
Daughter animal isolates (8)	0.066 ± 0.033	0.071 ± 0.029	0.099 ± 0.040
Parent laboratory strain	0.046	0.045	0.083
RP12			
Daughter animal isolates (29)	0.230 ± 0.080	0.096 ± 0.036	0.253 ± 0.083
Parent laboratory strain	0.322	0.055	0.327
RP62A			
Daughter animal isolates (44)	1.501	0.475 ± 0.076	1.576 ± 0.023
Parent laboratory strain	1.501	0.541	1.596
Rat catheter-induced endocarditis model			
RE19			
Daughter animal isolates (8)	1.477 ± 0.067	0.502 ± 0.168	1.566 ± 0.104
Parent laboratory strain	1.501	0.437	1.563
RP23			
Daughter animal isolates (30)	1.501	0.329 ± 0.068	1.538 ± 0.015
Parent laboratory strain	1.501	0.334	1.538
SP18			
Daughter animal isolates (3)	0.305 ± 0.093	0.265 ± 0.228	0.420 ± 0.202
Parent laboratory strain	0.526	0.222	0.571
PC			
Daughter animal isolates (4)	0.139 ± 0.016	0.080 ± 0.060	0.164 ± 0.045
Parent laboratory strain	0.128	0.061	0.142

<sup>a</sup> Each value for daughter animal isolates is expressed as the mean ± standard deviation.

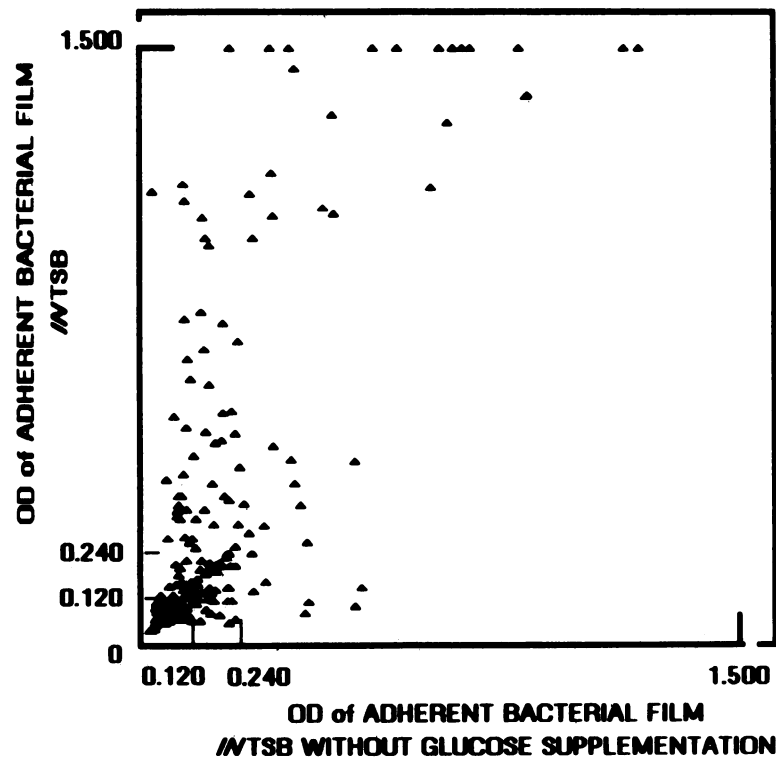


FIG. 3. The adherence coordinates (see the text) for 219 clinical strains of coagulase-negative staphylococci are displayed here. For most, but not all strains, adherence was greater in glucose-rich medium than in glucose-poor medium.



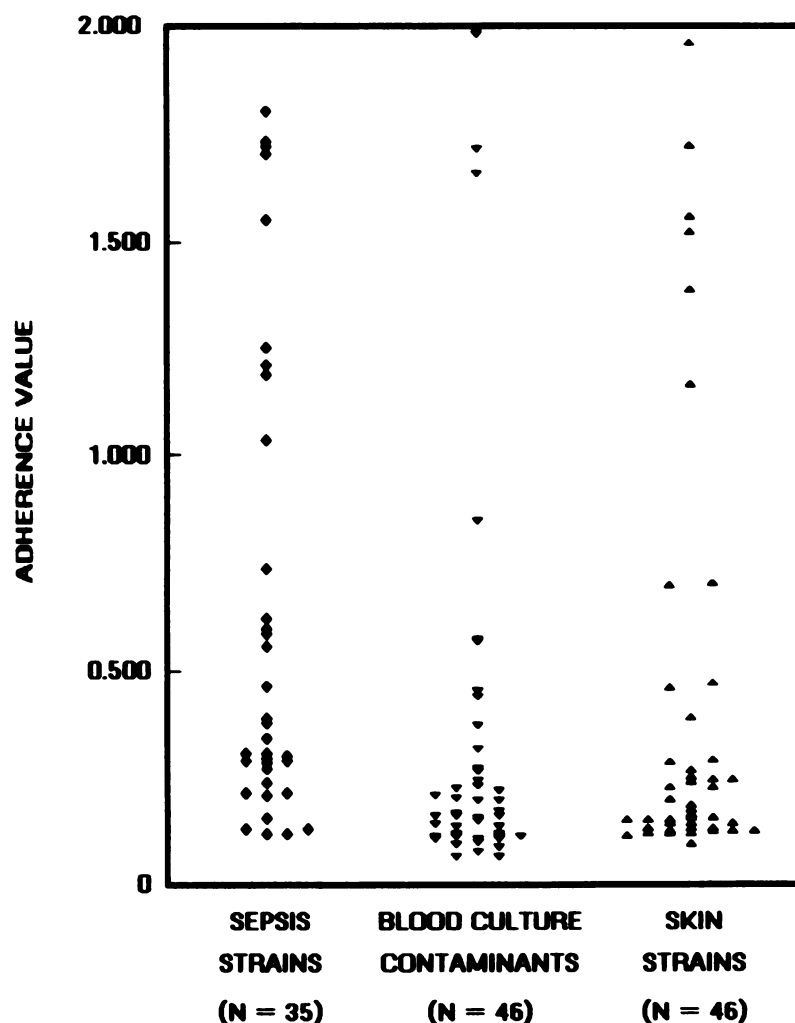


FIG. 4. Scattergrams of adherence values (see the text) for strains from the intravascular catheter-associated sepsis collection. The mean value for sepsis strains, 0.602, was significantly greater than the mean value for blood culture contaminants, 0.295 ( $P < 0.01$  by Student's  $t$  test), or the mean for skin strains, 0.371 ( $P < 0.05$  by Student's  $t$  test).

$r$  value, 0.6462, was obtained by averaging the readings of all three observers and comparing the result to the spectrophotometric classification. Thus, the tube test and the spectrophotometric classification were examining the same phenomenon, with the spectrophotometer providing the more reliable classification.

(ii) Included in the collection of strains reported here were 35 strains represented by 105 isolates. We compared the classification of each isolate (with the ODs in TSB and TSB without glucose considered separately) to the strain classification (average value for all isolates of the strain) (Table 5). With this analysis, we found that the accuracy of the spectrophotometric classification ranged between 83 and 91% with a sensitivity between 76 and 91% and a specificity between 81 and 96%. These relatively high performance values further confirmed the stability of the adherence capability of an organism through the course of an infection.

By using the spectrophotometric classification, we grouped isolates from the catheter-associated sepsis collection into the three adherence categories, once again finding significantly more sepsis strains than blood culture contaminants and skin strains to be strongly adherent ( $P < 0.001$ ;  $\chi^2_{(2df)} = 17.31$ ) (Fig. 6). Applying these same techniques to

the CSF shunt infection collection yielded similar results ( $P < 0.01$ ;  $\chi^2_{(2df)} = 10.26$ ) (Fig. 7).

## DISCUSSION

Investigations into the pathogenesis of coagulase-negative staphylococcal foreign body infections have focused upon the process by which bacteria colonize the surface of the appliance. Investigators have used a variety of experimental techniques for counting the number of staphylococci adhering to a surface. For example, Barrett radiolabeled 14 strains of *Staphylococcus epidermidis* in a study of adherence to the silicone rubber from which CSF shunts are constructed (2). Ludwicka et al. measured the adherence of five strains of *Staphylococcus epidermidis* by monitoring the release of ATP by bacterial cells (19). Sheth et al. modified the clinical methods of Maki et al. (20) to study the adherences of eight strains of coagulase-negative staphylococci to intravascular catheters. Their method consisted of immersing sections of intravascular catheters in bacterial suspensions, rinsing the catheters, and then rolling them over blood agar plates. Detached bacteria were counted as CFU per square centimeter of the catheter surface (29). Hogt et al. simply counted attached bacteria under a light microscope (13). Pulverer and



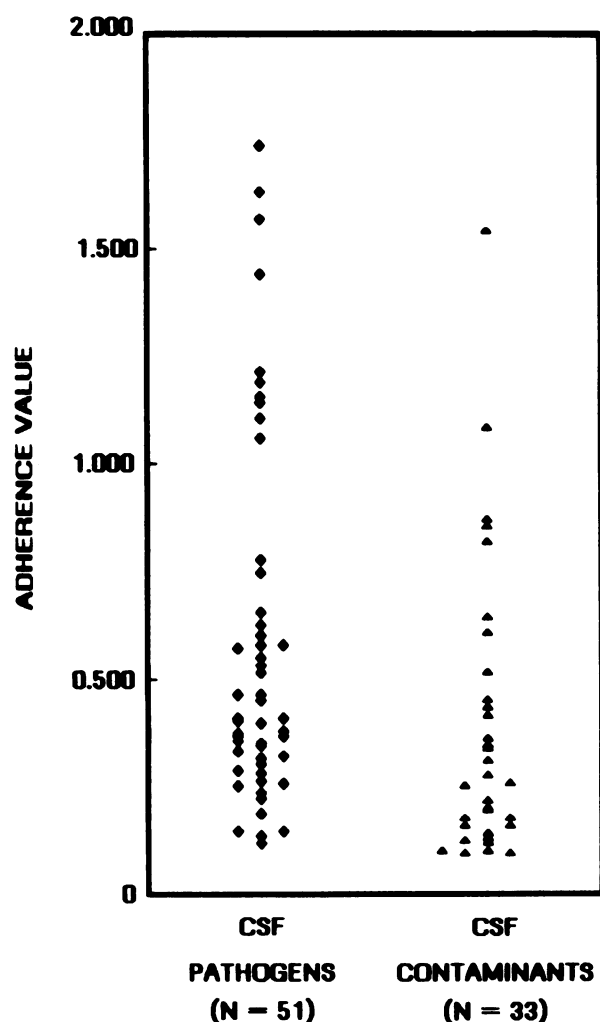


FIG. 5. Scattergrams for adherence values for strains from the CSF shunt infection collection. The mean for CSF pathogens, 0.563, was significantly higher than the mean for CSF contaminants, 0.363 ( $P < 0.05$  by Student's  $t$  test).

co-workers have not quantified their data, but they have made extensive scanning electron microscope surveys of the in vitro colonization of intravascular catheters by coagulase-negative staphylococci (16, 17, 27).

Although there are advantages and disadvantages to every method, slime-producing coagulase-negative staphylococci raise special problems regarding enumeration. When grown in broth to encourage slime production, these microorganisms coagglutinate into an indispersible mass of bacteria. Consequently, slime-producing strains cannot be centrifuged or washed, as was required in the methods of Barrett (2), Sheth et al. (29), Ludwicka et al. (19), and Hogt et al. (13). Slime production also interferes with counting procedures that rely upon CFU such as used by Sheth et al. (29), since a single CFU may represent a single organism or a cluster of many organisms. Hogt avoided these problems by selecting for study three strains that were not slime producers. The remaining investigators either raised their organisms under conditions that did not encourage overt slime production or did not address this particular issue.

Perhaps the most important consideration is the time scale under which slime production takes place. Since virtually nothing has been published regarding the mechanisms by which coagulase-negative staphylococci adhere to surfaces, we must draw inferences from other systems. We believe that the adherence of *Streptococcus mutans* to dental surfaces bears many resemblances to the adherence of coagulase-negative staphylococci to smooth surfaces. Staat et al. (31; also reviewed in reference 6) have convincingly argued that *Streptococcus mutans* adheres in two phases by different mechanisms. First there is rapid attachment followed later by slow cellular accumulation and microcolony formation mediated by cell surface carbohydrates. Our unpublished observations indicated that many strains of coagulase-negative staphylococci also adhered to smooth surfaces in two distinct phases: an early adherence phase that reached a maximum at 2 h and a late accumulation phase that reached a maximum of 6 h (G. D. Christensen, W. A. Simpson, E. H. Beachey, A. L. Bisno, and F. F. Barrett, Program Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 649, 1982). The advantage of examining bacterial films after 18 h as opposed to 2 h (or less) (2, 13, 19, 29) was that our observations included the contributions of both early adherence and late accumulation to the colonization of the plastic surface. In these observations, however, a distinction was not made between adherence and accumulation.

If an investigation into the pathogenesis of coagulase-negative staphylococcal infections considers slime production as a potential virulence factor, the method used by the investigator for counting bacteria should avoid the problems of washing and CFUs. Ideally, the method should also

TABLE 5. Reliability of spectrophotometric classifications<sup>a</sup>

Spectrophotometric classification	Test characteristic <sup>b</sup>				Accuracy <sup>c</sup>
	Sensitivity	Specificity	Positive predictive value	Negative predictive value	
Strongly adherent	85.2 (75/88)	95.8 (114/119)	93.8 (75/80)	89.7 (114/127)	91.3 (189/207)
Weakly adherent	76.4 (55/72)	85.9 (116/135)	74.3 (55/74)	87.2 (116/133)	82.6 (171/207)
Nonadherent	80.8 (38/47)	90.6 (145/160)	71.7 (38/53)	94.2 (145/153)	88.4 (183/207)
Strongly or weakly adherent	90.6 (145/160)	80.8 (38/47)	94.2 (145/153)	71.7 (38/53)	88.4 (183/207)

<sup>a</sup> Calculated with the data from 35 strains from blood and CSF, represented by 105 isolates. Adherence classifications in TSB and in TSB without glucose were considered separately; the adherence classification for each isolate was compared with the adherence classification for the strain. See the Materials and Methods section for details of calculations.

<sup>b</sup> Values are expressed as percentages. Numbers in parentheses represent data from which the percentages were calculated.

<sup>c</sup> The denominator for this calculation is 207 rather than 210 because the OD data for bacterial films produced in TSB without glucose were unavailable for one strain, which was represented by three isolates.

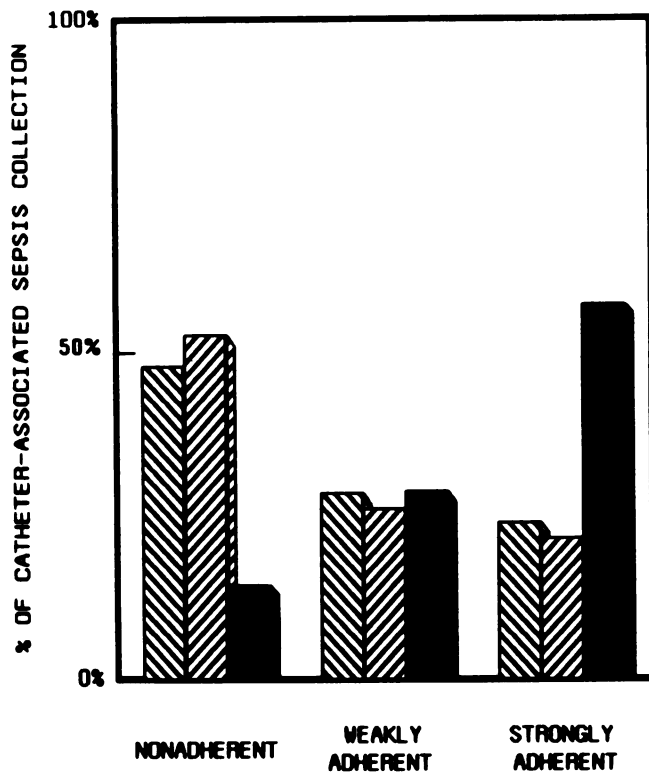


FIG. 6. Bar graph of the spectrophotometric classification of adherence when applied to the intravascular catheter-associated sepsis collection. A greater proportion of the strains associated with sepsis are strongly adherent than are skin strains and blood culture contaminants ( $P < 0.001$ ;  $\chi^2_{(2df)} = 17.31$ ). Symbols: skin strains; blood culture contaminants; sepsis strains.

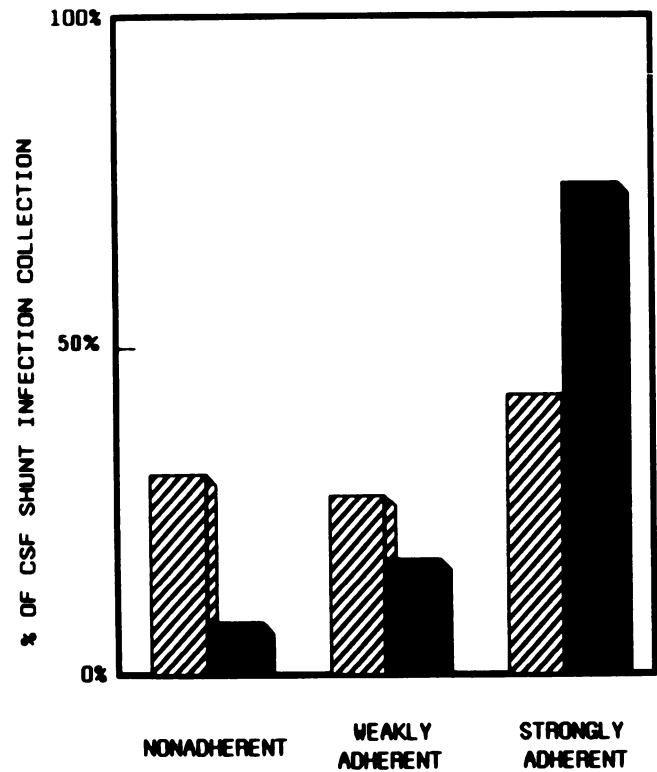


FIG. 7. Bar graph of the spectrophotometric classification of adherence when applied to the CSF shunt infection collection. A greater proportion of the CSF pathogens were strongly adherent than the CSF contaminants ( $P < 0.01$ ;  $\chi^2_{(2df)} = 10.26$ ). Symbols: CSF contaminants; CSF pathogens.

include a time scale that measures accumulation as well as attachment. It appears that these goals can be accomplished by the method we report here.

Our data also indicate that this technique can serve as a reliable quantitative tool for comparing the adherences of different strains of coagulase-negative staphylococci. Such studies are needed to confirm or refute the pathogenic significance of adherence. To facilitate such investigations, we have placed our four control strains, RP14, SP2, RP12, and RP62A, into the American Type Culture Collection (ATCC numbers 35981, 35982, 35983, and 35984, respectively).

A recent report by Needham and Stempsey (24) failed to find any association between slime production and pathogenic strains. In this study, bacteria were isolated from a variety of specimens obtained from a variety of patients. Our study included only blood culture isolates and was limited to patients with intravascular catheter-associated sepsis. Since the distinction between pathogenic strains and nonpathogenic strains for both studies rests entirely upon clinical criteria, the differences in methodology between the two studies may explain the different results. We tested our previous observation by applying the spectrophotometric measurements to a second, similar, but not identical, clinical collection of coagulase-negative staphylococci. Our finding that in pediatric CSF shunt patients, as in adult intravascular catheter-associated sepsis patients, pathogenic strains tend to be slime producing further buttresses our conclusions.

In our studies, strains that caused endocarditis proved to

be less adherent than strains from any other group. Furthermore, when we examined the strains in our collection from patients with prolonged episodes of bacteremia, these strains also were not particularly adherent. These findings suggest that the pathogenesis of coagulase-negative staphylococcal infections is a complex process, of which slime is only one component. For example, it may be that adherence is primarily important in colonization, while the continuation of a deep infection relies upon other mechanisms that discourage adherence. Alternatively, infections with organisms that produce slime may be more difficult to diagnose with blood cultures than are infections from organisms that do not produce slime because the daughter organisms are less likely to break away from the parent colony and seed the blood stream. By using an experimental animal model for catheter-induced endocarditis, Baddour et al. have found that, in comparison to the species designation, slime production was not a major virulence factor (1). Nevertheless, in continuing studies, by using a phenotypically manipulated organism and both the rat model for catheter-induced endocarditis and the mouse model for foreign body infection, we have found that the virulence of the organism does indeed vary with its ability to adhere to plastic tissue culture plates (L. M. Baddour, G. D. Christensen, A. L. Bisno, W. A. Simpson, M. G. Hester, and E. H. Beachey, 24th ICAAC, abstr. no. 328, 1984).

#### ACKNOWLEDGMENTS

This work was supported by research funds from the U.S. Veterans Administration and Public Health Service research grants AI-10085

and AI-13550 from the National Institute of Allergy and Infectious Diseases. G.D.C. is the recipient of a Research Associate Award from the U.S. Veterans Administration. J.J.Y. is the recipient of a postdoctoral traineeship award (AI-07238) from the National Institute of Allergy and Infectious Diseases. D.M.M. was the recipient of a Medical Student Research Fellowship grant from the U.S. Public Health Service (AM-07405).

We thank Charlean Luellen for excellent technical assistance.

#### LITERATURE CITED

1. Baddour, L. M., G. D. Christensen, M. G. Hester, and A. L. Bisno. 1984. Production of experimental endocarditis by coagulase-negative staphylococci: variability in species virulence. *J. Infect. Dis.* **150**:721-727.
2. Barrett, S. 1983. Staphylococcal infection of plastic inserts; a method to measure staphylococcal adhesion. *Br. J. Clin. Prac.* **37**(Suppl. 25):81-85.
3. Bayston, R. and S. R. Penny. 1972. Excessive production of mucoid substance in staphylococcus SHIA: a possible factor in colonisation of holter shunts. *Dev. Med. Child Neurol.* **14**(Suppl. 27):25-28.
4. Christensen, G. D., A. L. Bisno, J. T. Parisi, B. McLaughlin, M. G. Hester, and R. W. Luther. 1982. Nosocomial septicemia due to multiply antibiotic resistant *Staphylococcus epidermidis*. *Ann. Intern. Med.* **96**:1-10.
5. Christensen, G. D., J. T. Parisi, A. L. Bisno, W. A. Simpson, and E. H. Beachey. 1983. Characterization of clinically significant strains of coagulase-negative staphylococci. *J. Clin. Microbiol.* **18**:258-269.
6. Christensen, G. D., W. A. Simpson, and E. H. Beachey. 1985. Microbial adherence in infection, p. 6-23. In G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), *Principles and practice of infectious diseases*. John Wiley & Sons, Inc., New York.
7. Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachey. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.* **37**:318-326.
8. Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachey. 1983. Experimental foreign body infections in mice challenged with slime-producing *Staphylococcus epidermidis*. *Infect. Immun.* **40**:407-410.
9. Fletcher, M. 1976. The effects of proteins on bacterial attachment to polystyrene. *J. Gen. Microbiol.* **94**:400-404.
10. Fletcher, M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. *Can. J. Microbiol.* **23**:1-6.
11. Franson, T. R., N. K. Sheth, H. D. Rose, and P. G. Sohnle. 1984. Scanning electron microscopy of bacteria adherent to intravascular catheters. *J. Clin. Microbiol.* **20**:500-505.
12. Hanson, R. S., and J. A. Phillips. 1981. Chemical composition, p. 348. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*, American Society for Microbiology, Washington, D.C.
13. Hogt, A. H., J. Dankert, J. A. deVries, and J. Feijen. 1983. Adhesion of coagulase-negative staphylococci to biomaterials. *J. Gen. Microbiol.* **129**:2959-2968.
14. Ivey, M. H. 1980. Laboratory procedures in parasitology, p. 2194. In A. C. Sonnenwirth and L. Jarett, (ed.), *Gradwohl's clinical laboratory methods and diagnosis*, 8th ed. The C.V. Mosby Co., St. Louis.
15. Kloos, W. E. 1980. Natural populations of the genus *Staphylococcus*. *Annu. Rev. Microbiol.* **34**:559-592.
16. Locci, R., G. Peters, and G. Pulverer. 1981. Microbial colonization of prosthetic devices. I. Microtopographical characteristics of intravenous catheters as detected by scanning electron microscopy. *Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B* **173**:285-292.
17. Locci, R., G. Peters, and G. Pulverer. 1981. Microbial colonization of prosthetic devices. III. Adhesion of staphylococci to lumina of intravenous catheters perfused with bacterial suspensions. *Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B* **173**:300-307.
18. Lowy, F. D., and S. M. Hammer. 1983. *Staphylococcus epidermidis* infections. *Ann. Intern. Med.* **99**:834-839.
19. Ludwicka, A., B. Jansen, T. Wadstrom, and G. Pulverer. 1984. Attachment of staphylococci to various synthetic polymers. *Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* **256**:479-489.
20. Maki, D. G., C. E. Weise, and H. S. Sarafin. 1977. A semiquantitative culture method for identifying intravenous-catheter-related infection. *New Engl. J. Med.* **296**:1305-1310.
21. Marrie, T. J., and J. W. Costerton. 1984. Scanning and transmission electron microscopy of in situ bacterial colonization of intravenous and intraarterial catheters. *J. Clin. Microbiol.* **19**:687-693.
22. Marrie, T. J., and J. W. Costerton. 1984. Morphology of bacterial attachment to cardiac pacemaker leads and power packs. *J. Clin. Microbiol.* **19**:911-914.
23. Marrie, T. J., M. A. Noble, and J. W. Costerton. 1983. Examination of the morphology of bacteria adhering to peritoneal dialysis catheters by scanning and transmission electron microscopy. *J. Clin. Microbiol.* **18**:1388-1398.
24. Needham, C. A., and W. Stempsey. 1984. Incidence, adherence, and antibiotic resistance of coagulase-negative *Staphylococcus* species causing human disease. *Diagn. Microbiol. Infect. Dis.* **2**:293-299.
25. Pearse, A. G. E. 1968. *Histochemistry, theoretical and applied*, 3rd ed., vol. 1, p. 344-349 and 672. Little, Brown & Co., Boston.
26. Peters, G., R. Locci, and G. Pulverer. 1981. Microbial colonization of prosthetic devices. II. Scanning electron microscopy of naturally infected intravenous catheters. *Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B* **173**:293-299.
27. Peters, G., R. Locci, and G. Pulverer. 1982. Adherence and growth of coagulase-negative staphylococci on surfaces of intravenous catheters. *J. Infect. Dis.* **146**:479-482.
28. Peters, G., F. Saborowski, R. Locci, and G. Pulverer. 1984. Investigations on staphylococcal infection of transvenous endocardial pacemaker electrodes. *Am. Heart J.* **108**:359-365.
29. Sheth, N. K., H. D. Rose, T. R. Franson, F. L. A. Buckmire, and P. G. Sohnle. 1983. *In vitro* quantitative adherence of bacteria to intravascular catheters. *J. Surg. Res.* **34**:213-218.
30. Sonnenwirth, A. C. 1980. Stains and staining procedures, p. 1380. In A. C. Sonnenwirth and L. Jarett (ed.), *Gradwohl's clinical laboratory methods and diagnosis*, 8th ed. The C.V. Mosby Co., St. Louis.
31. Staat, R. H., S. D. Langley, and R. J. Doyle. 1980. *Streptococcus mutans* adherence: presumptive evidence for protein-mediated attachment followed by glucan-dependent cellular accumulation. *Infect. Immun.* **27**:675-681.