In vitro assays of Staphylococcus epidermidis characteristics and outcome in an endocarditis model

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OBJECTIVE: Staphylococcus epidermidis adherence to indwelling polymers is important in prosthetic valve endocarditis. Earlier studies have related streptococcal endocarditis to isolates with high levels of cell-associated hexoses. The objective of the present study was to determine if a relationship exists between an S. epidermidis isolate assay score and production/severity of experimental endocarditis. DESIGN: Groups of patient S. epidermidis isolates were screened for surface hexoses and an animal model of endocarditis with isolates testing highest and lowest on the screen was produced. Disease severity produced by 'high hexose' versus 'low hexose' organisms was evaluated. Endocarditis responding variables were bacterial vegetation weight and log10 colony forming units (cfu) and in survival tests, comparative time to death with different isolates. Bacterial characteristics were not measured. Baseline data showed a vegetation weight difference so that with a β error of 0.20 and a two-tailed α error of 0.05, a significant difference would be noted using 30 animals. A total of 64 animals was used. POPULATION STUDIED: Bacterial isolates from two patient groups (n=42 and n=68) on which in vitro assays were run. An animal model of endocarditis (n=64) was used to evaluate four selected isolates for vegetation size, log10 cfu/g, and survival time. MAIN RESULTS: In a group of S. epidermidis endocarditis animals evaluated for time of death, a significantly more rapid death time resulted in the group dosed with the high hexose-scoring organism (P<0.025). Vegetations and log10 cfu produced by test high hexose isolates averaged larger but were not significantly different. CONCLUSIONS: A significantly more rapid death rate occurs in untreated endocarditis using a high hexose isolate than with S. epidermidis with low surface hexoses. Using bacterial vegetation and cfu as endpoints, however, experimental endocarditis using patient isolates of S. epidermidis does not show the same strong correlation to bacterial surface hexoses as does streptococcal endocarditis.

Key Words: Bacteremia, Bacterial assays, Endocarditis model, Staphylococcus epidermidis

Essais in vitro sur les caractéristiques et les effets du Staphylococcus epidermidis dans un modèle

OBJECTIF: L'adhérence de Staphylococcus epidermidis aux polymères à demeure joue un rôle important dans l'endocardite au niveau de prothèses valvulaires. Des études préliminaires ont lié l'endocardite streptococcique à des isolats présentant des taux élevés d'hexoses associées aux cellules. L'objectif de la présente étude était de déterminer s'il y a un rapport entre le score obtenu avec un isolat S. epidermidis et la production/gravité de l'endocardite expérimentale. MODÈLE: Des groupes d'isolats prélevés chez des patients infectés à S. epidermidis ont été soumis à un dépistage des hexoses de surface et un modèle animal d'endocardite a été élaboré avec les isolats dont les résultats étaient plus élevés et les plus bas lors du
This study used interested in the possible relationship between viru­
venous bacteria. Negative polysaccharides have been evaluated as adhe­
by the viridans streptococci - high native valve endo­
swabs were implanted on mannitol salt agar and were 
organisms:

**Materials and Methods**

**Organisms:** *S. epidermidis* samples used for these studies included a group of 42 from adult patients at Truman Medical Center West and 68 from infants at Children’s Mercy Hospital, both in Kansas City, Missouri. Patient swabs were implanted on mannitol salt agar and were tested for catalase, hemolysis patterns, protein A, and clumping factor. Coagulase-negative staphylococcal isolates were biochemically identified using either the API Staph-Ident system (Analytab Products, Plainview, New York) or the Microscan Gram-positive combo plate (American Scientific Products, McGaw Park, Illinois). Isolates were frozen in tryptic soy broth 15% glycerol (TSB) (Remel Labs, Lenexa, Kansas) 15% glycerol and stored at -70°C. Each was streaked on blood agar and grown 24 h before being assayed, cultured in either TSB or pooled normal rabbit serum, 76 mg/dl blood glucose.

**Patient characteristics:** The adult isolates comprised one unselected box from a group of 1676 specimens collected and identified by the Truman Medical Center microbiology laboratory. A large percentage of the adult specimens were obtained from an obstetrics-gynecology clinic, and were isolated from nasopharynx, skin, blood and cervix. The adult population would be considered to be a low-infection group; few had polymeric implants or catheters (14). The pediatric bacterial samples came from: noses of healthy infants (n=21); and blood cul­
tures (n=47), 34% of which had central venous or umbilical catheters. Nine of the 47 were considered probable contaminants, 10 true sepsis, and 28 were from infants with one or more clinical signs of sepsis.

**Tryptophan (t0) assay:** This colorimetric assay (15) was used to measure alcohol-precipitated cell-adherent polysaccharide, stripped by sonication from saline-suspended coagulase-negative staphylococci which had been grown in either rabbit serum or TSB for 48 h. The precipitated carbohydrate was treated with 66% aque­
ous sulphuric acid and 1% tryptophan, heated at 100°C for 20 mins, and absorbance was read at 500 nm against a standard of 500,000 molecular weight dex­
tran. Values reported were actual peak absorbance A500. The two clinical bacterial groups (n=42 and n=68) were measured by t0 assay using four to 12 replications per isolate, and the high score isolate and low score isolate were selected from each group. These four iso­
lates, used in endocarditis studies, were all *S. epider­
midis, although many low scoring isolates were Staphylococcus hominis.

Animal model: The University Animal Care and Use Committee approved the methods (4,16) used to produce endocarditis in 280 g Sprague-Dawley rats (Sasco, Omaha, Nebraska). Thirty-two animals were used to test the adult staphylococcal isolates and 16 to test the pediatric isolates. Briefly, polyethylene sleeves (Intramedic PE-10, Clay Adams, Parsippany, New Jersey) over sterile 32 gauge hypodermic tubing were inserted into the left ventricular lumen via the right common carotid artery. The stainless steel insert was removed upon correct placement, and the polyethylene sleeve was sutured in place. About 20 h after cannula implantation, 0.5 mL containing 1 to 4x10^7 organisms (16 h growth, saline suspended) were dosed via tail vein. Animals were killed three days after bacterial challenge, and 1.5 mL blood from the dorsal aorta were drawn and cultured to confirm bacteremia. Hearts with cannulas in place were removed, placed in tissue culture cell-wells and necropsied in sequence under a 12.5x microscope. Vegetations adhering to valve leaflets and to cannulas across the valves were aseptically excised and added to pre-weighed sterile bags; wet weights were taken. Each vegetation was then macerated in the bag, serial dilutions were made and plated to blood agar. Bacterial vegetation counts were made at 24 h, and log_{10} bacteria per gram of vegetation calculated for each animal. A greater than 90% average infection rate was obtained.

In survival tests, rodents with implanted cannulas were dosed with bacteria as above. Animals were checked twice daily and the time of death recorded. Blood samples were cultured to confirm bacteremia as above.

In vitro bacterial analyses: A quantitative slime assay (17) and a bacterial hydrophobicity assay were also performed on the pediatric bacterial population (n=68). The slime assay evaluated material remaining on a glass tube after 24 h culture in TSB with 10% glucose. Decanted tubes were fixed, stained with 0.1% safranin, and heated (85°C for 1 h in 0.2 M sodium hydroxide). Colour was read at 530 nm. Hydrophobicity analysis (18) evaluated the absorbance of a 10^8/mL bacterial suspension in phosphate buffered saline before and after vortexing 2 mins with p-xylene (Merck, Darmstadt). Hydrophobicity was expressed as absorbance (600 nm) of extracted versus initial suspensions times 100.

Neutrophil phagocytosis/bactericidal assays: The adult isolates used in endocarditis survival studies were analyzed further for mechanisms of bacterial virulence. Human blood neutrophils (polymorphonuclear leukocytes [PMN]) were isolated as reported (19). Overnight cultures of the two test S epidermidis isolates were washed and suspended at 7.5x10^6 colony forming units (cfu) in Hank’s balanced salt solution (HBSS) containing 0.5% gelatin with 10% autologous serum. The organisms were cultured in duplicate tubes with and without 1.5x10^8 PMN with shaking at 37°C. Ten microlitres from each tube were sampled at 0, 30, and 60 mins, placed in 10 mL iced sterile water, and aliquots were plated on blood agar and counted after a 24 h incubation. Percentage kill was calculated as follows:

$$-\log_{10}\text{No-PMN tube cfu/mL} - \log_{10}\text{PMN tube cfu/mL} \times 100$$

An additional phagocytosis/bactericidal assay was performed on these organisms using an acridine orange crystal violet microassay as described previously (20). In this assay, S epidermidis isolates used in endocarditis survival tests were opsonized with 10% rabbit serum in HBSS, then incubated with rabbit PMN on glass slides 45 mins before staining. The living bacteria fluoresced green and the dead fluoresced red. Crystal violet quenched the fluorescence of extracellular/membrane adherent organisms so these were not visualized. Counts were made of total and nonviable S epidermidis in 100 PMN.

Analysis: Differences in mean vegetation weights and mean vegetation bacterial concentrations were evaluated by Student’s t test for unpaired data. Probability of survival (untreated endocarditis) was plotted with Kaplan Meier curves and with linear regression analysis (21). Significance of in vitro tests was determined using the Asyntast statistical software package.

RESULTS

Coagulase-negative staphylococcal blood culture isolates from two groups of patients, 42 adult and 68 pediatric, were quantified by the t0 assay. The data

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**Figure 1** Cell-associated polysaccharide (tryptophan assay, t0) for one patient population, n=68. Points represent mean t0 scores (four to 12 replications of each isolate) on the ordinate, positioned on the abscissa by the same isolate score on the quantitative slime assay. No statistically significant correlation can be found between the two assays in this population, (P=0.568, slope 0.367, intercept 0.114, linear regression and correlation analysis)
Table 1: In vitro assays: Comparing isolates of Staphylococcus epidermidis

| Isolate                  | Quantitative slime* | Hydrophobicity† | Tryptophan assay (T0)† |
|--------------------------|---------------------|-----------------|------------------------|
| Group median             | 0.156               | 55.0            | 0.220                  |
| High T0 adult isolate    | 0.01                | 12.3            | 0.270                  |
| Low T0 adult isolate     | 0.114               | 54.00           | 0.117                  |
| High T0 pediatric isolate| 0.442               | 33.3            | 0.308                  |
| Low T0 pediatric isolate | 0.183               | 52.3            | 0.187                  |

*Absorbance at 530 nm; †Percentage of initial absorbance at 600 nm remaining after xylene extraction; ‡Absorbance at 500 nm; §Coagulase-negative staphylococcal patient isolates, group averages on isolates tested with 10 or more replications shown as a point of comparison.

Table 2: Endocarditis model: Comparing isolates of Staphylococcus epidermidis

| Tryptophan assay | Tests 1 to 4, adult isolates* | Vegetation weight (mg) | Log10 cfu/g | Tests 5 and 6, pediatric isolates† | Vegetation weight (mg) | Log10 cfu/g |
|------------------|-------------------------------|------------------------|-------------|---------------------------------|------------------------|-------------|
| Staphylococcus epidermidis isolate, highest T0 test | 50.13±36.0                  | 8.13±0.04              | 16.7±6.45   | 9.37±0.31                       | 13.77±5.97            | 8.86±1.87   |
| Staphylococcus epidermidis isolate, lowest T0 test | 36.23±23.2                  | 8.005±0.97             | 13.77±5.97  | 8.86±1.87                       | 13.77±5.97            | 8.86±1.87   |

*Two adult isolates were tested in four experiments, eight animals each. †Two pediatric isolates were tested in two experiments, eight animals each. All comparisons P>0.05.

The right-hand side of Table 2 details animal endocarditis models using T0 high and low isolates from the pediatric population. Again the higher T0 scoring S. epidermidis produced a somewhat larger cfu/g than the isolate scoring at the bottom of the T0 series.

Figure 2 shows mortality (time of survival) in untreated S. epidermidis endocarditis in a group of 16 animals, eight of which received the high T0 adult isolate and eight the low T0 isolate at identical infective doses. Using the S. epidermidis isolates from the adult population group, endocarditis was established using 2.2x10^7 intravenous organisms. At the end of seven days, one death had occurred in the low T0 assay bacterial group, and eight of eight had died in the high T0 assay bacteria group. This difference in mortality, even with the small populations, is significant (P<0.025 x^2 test).

Table 3 gives results of phagocytosis and neutrophil bactericidal assays performed on the two isolates showing the significant difference in endocarditis mortality. Ability to escape from phagocytosis in vivo is a classical bacterial virulence factor that has been related to surface glycocalyx. Using a bacteria to PMN ratio of 5:1, there was a 4.7% kill of the high virulence isolate and a 6% kill of the low virulence isolate. These data were not significant at 60 mins in vitro, but offered enough
difference to follow in other testing. Table 3 also shows 
the slide microassay data, determined by incubating 
rabbit PMN with opsonized high or low survival 
S epidermidis strains, then staining with acridine 
orange and crystal violet. Dead and live bacteria were 
counted in 100 PMN for each isolate, in duplicate 
slides. Comparing internalized bacteria from the 
two isolates using an ANOVA with Bonferroni post hoc analy­ 
thesis, the P values were 0.8 for dead bacteria and 0.36 for 
living.

A simple capsule evaluation (carbon plus safranin 
staining of a thin smear) was performed on several 
saline-washed isolates from the pediatric bacterial 
group, including the two t0 assay 'extreme value' 
strains. There was a rather high agreement between 
this test and the t0 assay (80%), with the high t0 
isolates receiving a two or greater capsule score, while 
the lowest t0 isolates were all negative.

The four endocarditis isolates showed hydrophobic 
differences. The high t0 assay isolates were strongly 
hydrophobic, whereas the low t0 isolates scored 
around the median of the large test series.

**DISCUSSION**

These results demonstrate that the cell-adherent 
heoxse sugars of coagulase-negative staphylococci 
can be quantified by the assay which described and 
correlated with virulence (endocarditis production) in 
streptococci. The use of this t0 assay to select staphy­ 
lococcal isolates that produce a more virulent endo­ 
carditis, however, is not so clear cut. The isolates 
selected represented well-tested group extremes, all 
typed as S epidermidis. In no series was the difference 
in vegetable weight or log10/g bacterial growth statisti­ 
cally different between groups, although bacterial 
growth and vegetable weight were consistently greater 
in the high t0 S epidermidis-dosed groups. It was of 
interest in necropsy that the quantity of actual valve 
vegetation (as opposed to the catheter-associated vege­ 
tation with valve contact) was strikingly less than the 
valve-adherent growth observed in streptococcal endo­ 
carditis rats which were being necropsied at similar 
times for unrelated studies. Local effect of the catheter 
in vivo experiments has been reported (11,15) but the 
definite bacterial differences in valve versus catheter 
vegetation noted with our necropsy procedure again 
suggests how very important the catheter is to vegeta­ 
tion size.

In a test of endocarditis time of survival (Figure 2), a 
significantly more rapid death rate occurred (eight of 
eight versus one of eight at one week) when the adult 
group t0 test high isolate was compared with the t0 low 
test isolate. Although in vitro growth curves with quan­ 
titative plating were not performed for all isolates, early 
tests showed that bacterial growth in vitro was faster in 
some high t0 scoring coagulase-negative staphylococci. 
It is possible that, given the same intravenous challenge 
dose of bacteria, more rapid growth also occurred in 
vivo with the high t0 isolate, producing more deaths. 
Additional experiments were performed to evaluate 
phagocytosis resistance of those isolates, using two 
types of assay. In neither in vitro test was PMN inter­ 
nalization and killing significantly different between 
strains, but PMN killing rates of 4.7% versus 6% in 1 h 
may indicate a difference that could, in vivo, show up 
as a host survival difference. Phenotypic variants of 
bacteria producing endocardial infections have also 
been noted (3) and the resultant bacteria exhibit differ­ 
ences in virulence.

To investigate further the differences in these endo­ 
carditis strains of S epidermidis, hydrophobicity and 
slime measurements were made. Three of the four 
S epidermidis test isolates were average or below aver­ 
age slime producers. The tests on washed cells of a few 
isolates, staining for capsule with carbon and safranin, 
showed that capsule score was higher more often than 
was slime score in the high t0 assay isolates (Figure 1).

The time-of-survival endocarditis tests, using the 
two adult population S epidermidis isolates, show sig­ 
nificant differences in the death rates of the groups. The 
isolates (Table 2) differ widely in both hydrophobicity 
and in the t0 assay for cell-associated polysaccharides. 
Wadström (22) has shown that staphylococci with high 
cell surface hydrophobicity bind to plastic polymers in 
higher numbers than do cells with low hydrophobicity. 
Since the animal endocarditis model uses a polyethyl-
ene catheter across the aortic valves, a higher bacterial adherence to the catheter in vivo could contribute to the higher death rate with the hydrophobic bacterial isolate. Similarly, Hazen (23) in ex vivo experiments showed that hydrophobic cells were more likely than hydrophilic cells to seed and colonize various organs of an infected host.

Bacterial polysaccharides themselves have occasionally been considered to be more or less hydrophobic than proteins (10). Methyl-sugars and acetyl groups bound to the polysaccharide polymer are responsible for changing surface tension; such groups on a bacterial surface may elicit host in vivo response to hydrophobicity. Whether or not polysaccharide-related hydrophobicity occurs in these isolates and if such characteristics are responsible for in vivo virulence is to be clarified with further evaluation of these diverse coagulase-negative staphylococci.

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