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 $\log_{10}$ 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, $\log_{10}$ 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 $\log_{10}$ 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 associés 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

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dépistage. La gravité de la maladie produite par les organismes à hexoses élevés versus hexoses bas a été évaluée. Les variables relatives à l'endocardite ont été: le poids de la végétation bactérienne, les unités formant colonie à $\log_{10}$, des épreuves de survie et le délai comparatif avant la survenue du décès avec différents isolats. Les caractéristiques bactériennes n'ont pas été mesurées. Les données de départ ont montré une différence telle au plan du poids de la végétation qu'avec une erreur ß de 0,20 et une erreur α bilatérale de 0,05, une différence significative aurait été notée avec l'emploi de 30 animaux. En tout, 64 animaux ont été utilisés. **POPULATION ÉTUDIÉE:** Isolats bactériens de deux groupes de patients (n=42 et n=68) chez qui on a procédé à des essais *in vitro*. Un modèle animal d'endocardite (n=64) a été utilisé pour évaluer quatre isolats sélectionnés à l'égard de la taille de la végétation, du nombre d'unités formant colonies/gram et du temps de survie. **RÉSULTATS PRINCIPAUX:** Dans un groupe d'animaux atteints d'endocardite à *S. epidermidis*, évalués à l'égard du temps de décès, celui-ci est survenu nettement plus rapidement dans le groupe ayant reçu un organisme à hexoses élevés ($p<0,025$). Les végétations et les unités formant colonies produites par les isolats à hexoses élevés ont été en moyenne plus considérables, mais n'ont pas été nettement différentes. **CONCLUSIONS:** Le taux de décès survient nettement plus rapidement dans l'endocardite non traitée lors de l'emploi d'isolats à hexoses élevés que lors de l'emploi de *S. epidermidis* avec hexoses de surface faibles. À l'aide de paramètres relatifs à la végétation bactérienne et aux unités formant colonies cependant, l'endocardite expérimentale à partir d'isolats de *S. epidermidis* ne démontre pas la même corrélation forte par rapport aux hexoses de surface bactériens comparativement à l'endocardite streptococcique.

STAPHYLOCOCCUS EPIDERMIDIS, BECAUSE OF ITS HIGH tendency to colonize polymeric implants, is a major factor in human prosthetic valve endocarditis (25 to 30% of cases). Occurrence of native valve endocarditis caused by these organisms in humans is infrequent; it is reported in 1 to 3% of cases (1). *S epidermidis* and other coagulase-negative staphylococci have been used by us (2) and by others (3,4) in the animal model of endocarditis. *S epidermidis*, in our tests, virtually always produced cardiac vegetations in the presence of an indwelling polymeric arterial catheter and 10^7 intravenous bacteria. Our earlier work has shown that the propensity towards endocarditis production in humans by the viridans streptococci – high native valve endocarditis producers – was related to high scores by isolates on a colorimetric test for cell-associated hexoses (5-7). It was of interest to determine if this assay for cell-adherent bacterial polysaccharides would also reflect behaviour of different isolates of *S epidermidis* in the animal endocarditis model. Coagulase-negative polysaccharides have been evaluated as adhesion or virulence-promoting substances both in vivo and in vitro (8-13). Since the tryptophan assay for cell-adherent hexoses (tØ assay) is rapid and can be used for screening large numbers of isolates, we were interested in the possible relationship between virulence in the animal endocarditis model and quantity of bacterial surface hexose as determined by this assay. This study used *S epidermidis* isolates selected from two clinical populations because of their extreme values in the tØ assay.

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 cultures (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 (tØ) 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% aqueous 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 dextran. Values reported were actual peak absorbance A_{500}. The two clinical bacterial groups (n=42 and n=68) were measured by tØ assay using four to 12 replications per isolate, and the high score isolate and low score isolate were selected from each group. These four isolates, 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 microsurgical scope. 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

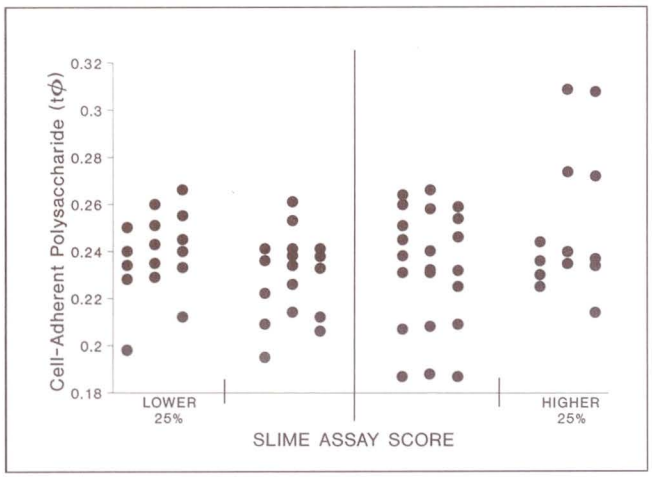

Figure 1) *Cell-associated polysaccharide (tryptophan assay, tØ) for one patient population, n=68. Points represent mean tØ scores (four to 12 replications of each isolate) on the ordinate, positioned on the abscissa by the same isolate score on the quantitative slime assay. No statistically significant 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)*

0.5% gelatin with 10% autologous serum. The organisms were cultured in duplicate tubes with and without $1.5x10^6$ 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:

$$\frac{\text{(No-PMN tube cfu/mL – PMN tube cfu/mL)}}{\text{zero time No-PMN tube cfu/mL}} \times 100$$

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 Asystant statistical software package.

RESULTS

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

TABLE 1
In vitro assays: Comparing isolates of *Staphylococcus epidermidis*

Isolate	Quantitative slime*	Hydrophobicity†	Tryptophan assay (tØ)‡
Group median§	0.156	55.0	0.220
High tØ adult isolate	0.01	12.3	0.270
Low tØ adult isolate	0.114	54.00	0.117
High tØ pediatric isolate	0.442	33.3	0.308
Low tØ 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*

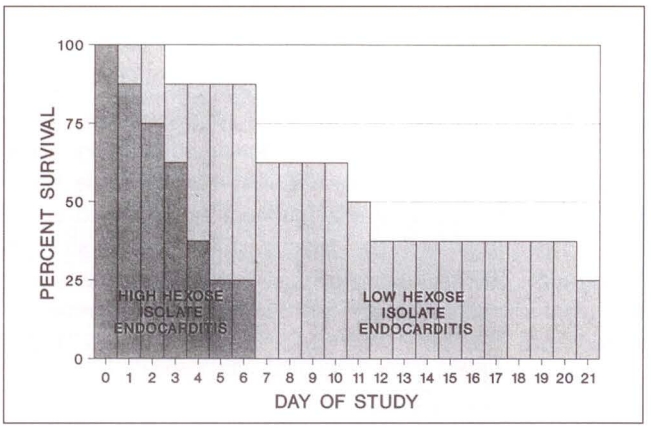

Figure 2) *Numbers of deaths in untreated* Staphylococcus epidermidis *endocarditis in the rodent animal model. Line of best fit to animal death time generated by the Asystant statistical package. The difference between groups was significant (P<0.025)*

TABLE 2
Endocarditis model: Comparing isolates of *Staphylococcus epidermidis*

	Tests 1 to 4, adult isolates*		Tests 5 and 6, pediatric isolates†	
Tryptophan assay	Vegetation weight (mg)	Log_{10} cfu/g	Vegetation weight (mg)	Log_{10} cfu/g
Staphylococcus epidermidis isolate, highest tØ test	50.13±36.0	8.13±1.04	16.73±6.45	9.37±0.31
Staphylococcus epidermidis isolate, lowest tØ test	36.23±23.2	8.005±0.97	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*

spread was wider using serum-grown isolates compared with TSB culture, and the endocarditis test strains were selected from serum-cultured assay runs. Figure 1 illustrates the tØ assay data compared with slime production by the same isolate from pediatric bacterial samples. Using averaged data, isolates from each population with the highest and lowest tØ assay values were selected for further testing.

Table 1 gives data on in vitro assays performed on the four *S epidermidis* isolates. Tested by the qualitative slime screen, three of the four *S epidermidis* strains used to produce endocarditis were slime-negative, although the groups tested 60% slime-positive. By the quantitative slime assay, there was no pattern; one high tØ isolate was much below the median, whereas others were average.

Table 2, showing data separated by the source of the infective bacterial strains, presents the mean vegetation weights and bacterial counts of cfu/g of vegetation, taken from valve leaflets and from valve-contacting catheters in the rodent model of endocarditis. There was a marginal difference in vegetation weight and *S epidermidis* cfu/g between the two isolates from the adult patient sources (P<0.1), with the higher tØ assay *S epidermidis* isolate producing a larger vegetation and greater growth.

The right-hand side of Table 2 details animal endocarditis models using tØ high and low isolates from the pediatric population. Again the higher tØ scoring *S epidermidis* produced a somewhat larger cfu/g than the isolate scoring at the bottom of the tØ 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 tØ adult isolate and eight the low tØ isolate at identical infective doses. Using the *S epidermidis* isolates from the adult population group, endocarditis was established using 2.2×10^7 intravenous organisms. At the end of seven days, one death had occurred in the low tØ assay bacterial group, and eight of eight had died in the high tØ assay bacteria group. This difference in mortality, even with the small populations, is significant ([P<0.025] χ^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

TABLE 3
Neutrophil phagocytosis/killing of isolates used in endocarditis survival

Organism		Human PMN phagocytosis Log_{10} cfu/mL 0 min	30 mins	60 mins	Intracellular killing Total staphylococcus dead per 100 PMN†
High mortality	+PMN	6.04	5.89	5.81*	3688/825
	0 PMN	6.01	6.07	6.09	
Low mortality	+PMN	6.21	6.12	5.98*	4866/973
	0 PMN	6.11	6.15	6.18	

*PMN Polymorphonuclear leukocytes. *Percentage kill by human neutrophils (PMN) of the high mortality* Staphylococcus epidermidis *was 4.7% and of the low mortality* S epidermidis *6% at 60 mins incubation using the formula outlined in methods. †A larger number of low mortality organisms were internalized by PMN compared with the high mortality organisms, but the difference in kill was not significant, P>0.05*

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 analysis, 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 tØ assay 'extreme value' strains. There was a rather high agreement between this test and the tØ assay (80%), with the high tØ isolates receiving a two or greater capsule score, while the lowest tØ isolates were all negative.

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

DISCUSSION

These results demonstrate that the cell-adherent hexose 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 tØ assay to select staphylococcal isolates that produce a more virulent endocarditis, 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 vegetation weight or log_{10}/g bacterial growth statistically different between groups, although bacterial growth and vegetation weight were consistently greater in the high tØ *S epidermidis*-dosed groups. It was of interest in necropsy that the quantity of actual valve vegetation (as opposed to the catheter-associated vegetation with valve contact) was strikingly less than the valve-adherent growth observed in streptococcal endocarditis rats which were being necropsied at similar times for unrelated studies. Local effect of the catheter in 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 vegetation 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 tØ test high isolate was compared with the tØ low test isolate. Although in vitro growth curves with quantitative plating were not performed for all isolates, early tests showed that bacterial growth in vitro was faster in some high tØ 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 tØ 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 internalization 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 differences in virulence.

To investigate further the differences in these endocarditis strains of *S epidermidis*, hydrophobicity and slime measurements were made. Three of the four *S epidermidis* test isolates were average or below average 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 tØ assay isolates (Figure 1).

The time-of-survival endocarditis tests, using the two adult population *S epidermidis* isolates, show significant differences in the death rates of the groups. The isolates (Table 2) differ widely in both hydrophobicity and in the tØ 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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