SLIME-PRODUCING COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM HUMAN EYE INFECTIONS

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Some strains of coagulase-negative staphylococci produce an extracellular material, slime, which mediates adherence to foreign surfaces, such as indwelling biomedical devices. It is not known if slime is involved in adherence to human tissue. Coagulase-negative staphylococci are the most common members of normal ocular flora and cause many ocular infections, although the role of slime in these infections has not been studied. Of 151 *Staphylococcus epidermis* strains isolated from ocular infection and screened for slime production, 32 % were positive. Most strains produced slime only in presense of glucose, although a few heavy slime producers did so in acetate. Antibiotic sensitivity patterns as measured by MIC_{90} did not relate to slime production. Slime composition varies among strains and does not appear to be involved in adherence to cultured human corneal epithelial cells.

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CHAPTER I

INTRODUCTION

Coagulase-negative staphylococci have arisen from relative medical obscurity to a position of importance among human pathogens. Since coagulase-negative staphylococci lack virulence factors such as hemolysins, capsules, protein A, leukocidins, hyaluronidases, and enterotoxins which are commonly associated with *Staphylococcus aureus*, they are generally regarded as nonpathogenic members of the normal skin flora. While humans usually live comfortably with these bacteria, certain circumstances allow coagulase-negative staphylococci to act as somewhat virulent opportunists.

It has been known for decades that some strains of coagulase-negative staphylococci produce an extracellular material which remains loosely associated with the cell. This material is a slime or, more specifically, glycocalyx. Glycocalyx is a general term describing the superficial polysaccharide-containing structure on the external surface of cells; for example, the cuticle of invertebrates, cell walls of plants, epithelial cell basement membranes, intercellular cement, and the carbohydrate-rich surfaces of eukaryotic and prokaryotic cells (7). Bacterial slime is loosely associated with the bacterial cell surface and has an indistinct margin. It is composed primarily of polysaccharides, but variation in slime components among strains of coagulase-negative staphylococci has been noted (47).

Slime is thought to mediate adherence to surfaces, most notably, adherence to indwelling biomedical devices (2, 7, 11, 43, 46). Slime production is considered a virulence factor (4, 6, 22, 27, 52) but other factors yet to be elucidated may be involved in the pathogenesis of coagulase-negative staphylococci (22, 45). Strains of *S. epidermidis* causing endocarditis are twice as likely to be slime producers as non-slime producers (4) and slime-producing coagulase-negative staphylococci have replaced Gram-negative bacilli as the primary cause of catheter-related sepsis (19, 30).

Coagulase-negative staphylococci can also be significant ocular pathogens. They are associated with conjuctivitis, blepharitis, and are the most common cause of endophthalmitis (13). Most infections are believed to be opportunistic as these staphylococci are the most common member of the normal aerobic ocular flora (36). Slime production has not been evaluated as a factor in the pathogenesis of ocular infection.

The scope of this research is essentially three-fold. Initially, strains of coagulase-negative staphylococci from diseased eyes and normal eyes were screened for slime production using the Christensen tube method (18). Clinical data were collected such as antimicrobial susceptibility and treatment outcome .

Strains shown to be positive for slime production were evaluated further for the ability to produce slime in the presence or absence of glucose and acetate. Several carbohydrate staining methods were used to characterize and compare the slime of selected strains. Spectrophotometric methods were used to determine total carbohydrate and presence of uronic acids in 10 selected strains.

Finally, the degree and nature of adherence to human corneal cells in vitro by some of the slime-producing staphylococci was compared to that of representative non-slime producing staphylococci.

CHAPTER II

LITERATURE REVIEW

Staphylococcus epidermidis and other coagulase-negative staphylococci, once considered harmless commensal bacterial species, have become recognized as potential human pathogens (19, 20, 22, 27, 35). This revelation became apparent with the increased usage of catheters and other prostheses, essential for the management of intensive care patients (34, 35). *S. epidermidis* is the most common cause of foreign-body associated infections (18, 19, 20, 27, 30, 42, 53, 73) and is the major human pathogen for all human coagulase-negative staphylococcal infections exclusive of the urinary tract (19, 22, 30, 52, 67). Therefore, interest in the mechanics of bacterial adherence to biomaterials has increased recently in an attempt to find ways to prevent these infections.

Bacterial Adherence Phenomena

Adherence seems to rely on hydrophobic surface moieties, but the exact mechanism is unknown (7). Hydrophobic surfaces promote prokaryotic-eukaryotic cell contact. A variety of long-range, non-specific weak interactions such as chemotaxis, London-van der Waals forces, electrostatic forces, and surface tension can attract particles to surfaces (e.g. mucosal membranes or inanimate surfaces). However, if the surface possesses the same charge as the

particle, the particle will remain just off the surface. This reversible state is known as adsorption. The energy barrier separating the particle and the surface can be overcome in several ways. In an aqueous environment, such as a mucosal surface, microbial surface hydrophobicity promotes the close association of microbes with lypophilic regions of eukaryotic cell membranes. Some staphylococci and other prokaryotic cells excrete compounds which adsorb to the surface, embed the organisms, and "glue" them onto the surface. Adherence of coagulase-negative staphylococci has been found to occur in distinct phases in a mode similar to the adherence of *Streptococcus mutans* to dental surfaces (7, 67, 74) and environmental bacteria to rocks and other surfaces (2, 14, 37). Adherent bacteria are referred to as biofilm.

Cells differ according to their location within the biofilm. Cells located near the surface of the biofilm resemble cells suspended in liquid (planktonic cells). They are larger than cells deeper within the biofilm, have better access to nutrients and oxygen and are, therefore, metabolically active. They also have few problems with the removal of toxic wastes. The cells located deeper within the biofilm are smaller, thicker walled, less metabolically active, and protected from outside influences (1, 2, 58).

Staphylococcal Adherence

In 1963 Jones et al. noted slime production by some strains of *S. epidermidis* when grown with pyruvate as the energy source (50). In 1972, Bayston and Penny studied slime production and adherence to cerebrospinal fluid (Holter) shunts (11). A decade passed before Christensen et al. published a comprehensive study on the adherent properties of coagulase-negative

staphylococci (18) which is now considered a landmark study in the field of staphylococcal slime production. Since that time, there has been extensive research on the prevalence of slime-producing coagulase-negative staphylococci including their relationship to foreign body infections (5, 6, 11, 18, 22, 30, 32, 35, 43, 45, 64, 73, 86), host response to these bacteria (35, 41, 49, 53), and the nature and composition of the staphylococcal glycocalyx itself (7, 28, 47).

This colonization is most notable in conjunction with prostheses but must occur on some living tissues as well, since infection takes place only after bacterial attachment to tissue (55, 67).

Studies have shown that adherence of staphylococci to foreign bodies takes place in several stages consisting of adherence, growth, and slime production (2, 7, 37, 39, 57, 58, 64). The infecting staphylococci eventually become embedded in a matrix of slime composed of bacteria, exopoly-saccharides, and exogenous factors (28, 57). Cell division inside the slime matrix results in the formation of microcolonies which coalesce to form biofilm (2, 20, 23, 74).

It has been shown that staphylococci and other bacteria modify their surface chemistry when a surface is encountered (58). Some proteins are expressed when staphylococci are grown on solid media that are not produced from cells grown in liquid media. The production of these proteins may represent a "surface recognition" mechanism and the proteins may be adhesins (17). Studies of the surface of coagulase-negative staphylococci have focused on the production of extracellular slime, but capsules are now being recognized

as important cell surface components in both slime-producing and slime-free bacteria (44, 82).

Slime does not appear to be essential for the early phases of adherence to inanimate surfaces (45, 51), but rather serves to establish colonization by promoting the formation of biofilm. Adherence of both slime-producing and slime-free strains of staphylococci to foreign surfaces is significantly increased by fibronectin (45, 81). A receptor for fibrinogen has been demonstrated for coagulase-positive and coagulase-negative staphylococci (45) and remarkable specificity of binding has been noted (67). Fibronectin and other macromolecules have been shown to coat intravascular devices (81). Bacterial production of slime is time dependent (18), but bacteria must bind to a surface quite instantly in order to initiate colonization. Therefore receptor mediated processes, including host-derived adhesins, are probably responsible for initial organism-substrate interactions.

A polysaccharide antigen which is rich in galactose and which probably mediates the initial attachment of *S. epidermidis* to catheter surfaces has been purified by Tojo et al. (78) This antigen (PS/A) is also present in slime-negative strains, but these strains can not accumulate on surfaces in multiple layers. However, it has also been reported (56) that many, if not most, strains of *S. epidermidis* can directly attach to plastic surfaces and that this attachment is directly mediated by PS/A. Other antigens, described below, are probably responsible for the ability of staphylococci to persist in multilayered cell clusters embedded in slime.

Christensen (23) also isolated an antigenic marker, SAA, for adherent strains of *S. epidermidis*. This antigen consists primarily of glucose (59%) and

small amounts of protein (7%) and uronic acids (10%). SAA was identified on the basis of its loss from extracts of nonslime-producing daughter strains of a slime-producing strain. The same antigen was isolated from two other plastic-adherent strains of *S. epidermidis*. As noted previously, colonization of smooth surfaces proceeds through an initial adherence phase followed by an accumulation phase. SAA was expressed only by those strains that express an accumulation phase. SAA was shown to be serologically distinct from PS/A.

A third polysaccharide antigen was identified by Mack et al. (56). This antigen was shown to be involved in the adherence of *S. epidermidis* cells to each other forming biofilm on plastic surfaces. The antigen is specific for plastic-adherent strains. Non-adherent strains were negative for the antigen which is believed to be polysaccharide in nature since its reactivity was completely abolished by periodate oxidation. Quantitative studies showed an association between a large amount of antigen on the bacterial cell surface and attachment and accumulation of cells on a solid surface.

In conclusion, these antigens provide for interactions among bacterial cells and the latter two seem to be specific for adherent strains and to allow for accumulation of cells in multiple layers. Staphylococcal antigens involved in attachment to human cells have not been fully described but probably exist as they do in other bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae*, and *Streptococcus mutans* (23, 45). It is probable that surface molecules exist on the surface of staphylococcal cells which are receptors for host cell lectins.

Adherence by Other Bacteria

Bacterial adherence is mediated by lectins. Lectin binding is described as carbohydrate-binding protein or glycoprotein. Bacterial surface molecules involved in lectin binding may be proteins or glycoproteins and host-cell receptor molecules may also be glycoproteins or glycolipids with oligo-saccharide recognition sites (67). Tissue trophism is related to the match between bacterial lectins and host-cell receptor molecules (16, 67). Many bacteria adhere to solid surfaces by means of fimbrae or pili (62, 67). Examples of bacteria that adhere to mammalian cell surfaces by means of lectin binding include *Escherichia coli* (62, 67), *Neisseria gonorrhoeae* (67), and *Staphylococcus* sp. (16, 23, 57, 67, 78).

Pseudomonads. Pseudomonads adhere by means of a surface polymer (7, 37) and fimbriae (7). The polymer was found to consist mostly of carbohydrate and protein. Log phase cultures of pseudomonads have been shown to exhibit the greatest tendancy to attach to surfaces, and there is a progressive decline in numbers of bacteria which become attached after the onset of stationary phase and during death phase. The most likely explanation for such a decline is a progressive change in the quality or quantity of surface polymer. The carbohydrate fraction contains several sugars but no uronic acids (37). The large amount of alginate exopolymer produced by strains of *Pseudomonas aeruginosa* limits diffusion and thus prevents effective control by antibiotics (58).

Streptococci. Group A streptococci form a fibrillar network which seems to mediate attachment to epithelial cells (7). In addition, lipoteichoic acid linked to M protein serves as an adhesin. *Streptococcus mutans* forms a polymeric

glucan which specifically links cells together by interaction with a glucan-binding protein on the cell surface (74) or tooth surface, leading to decay (58).

Enterobacteriaceae. Most *Enterobacteriaceae* possess fimbriae (or pili). These adhesins enable the bacterium to attach to most protozoal, fungal, and animal cells and cell products (37). Enterotoxigenic *Escherichia coli* possess a specific bacterial adhesin which recognizes and binds to eukaryotic cells by a lectin-carbohydrate mechanism (62, 67).

Marine bacteria. The ability to attach to solid surfaces has been proposed as a survival advantage for marine bacteria in that the development of a thick slime layer provides a community environment which is protected from fluctuating and often stressful conditions (37). The same could be said of any bacteria, including those which live in the relatively hostile environment of the human body.

Most aquatic bacteria appear to adhere to surfaces by means of surface polymers (37, 51). However, this may not always be the case. When aquatic bacteria were grown in a carbon-limited environment, adherence was noted although there was no extracellular polysaccharide produced (14).

Aquatic bacteria show less adherence to inert surfaces when grown under conditions of carbon excess, even though extracellular polysaccharides are produced (14, 51). These findings contradict the accepted view that extracellular polysaccharides act as mediators of attachment.

Neisseria gonorrhoeae. N. gonorrhoeae have at least two fimbrae-associated lectins which are specific for receptors on cells of the urethra and other tissues (67).

Other examples of biofilm production include water systems where potentially pathogenic bacteria (e.g. *Legionella* spp.) may be protected from chlorination and contact lenses where bacteria may induce inflammation and possible ocular infection (58).

Staphylococcal Slime Production: Prevalence and Significance

Frequency. In three studies, the occurrence of slime-producing strains of "clinically significant" coagulase-negative staphylococci was higher and more variable 44-76% (18, 53, 56) than that of environmental or cutaneous strains (35-56%). Slime production may be confined to biotype S-2. Biotype S-2 is generally equivalent to *S. epidermidis* (19). Bayston and Penny found that 70% of staphylococci isolated from CSF shunts of pediatric patients were biotype S-2 and all of these produced slime (11). Slime production has been noted in clinical isolates of *S. hominis, S. haemolyticus, S. xylosus,* and *S. saprophyticus* , but slime production among strains of *S. epidermidis* occurs with greater frequency (19, 34).

Slime production was shown to be strain stable (19, 80). The presence or absence of this characteristic was constant for strains isolated from multiple blood cultures obtained on the same day or on different days (19).

Clinical Relevance. Slime production is recognized to be a marker of coagulase-negative staphylococci capable of clinically relevant infection of blood, body fluids, and medical devices (7, 18, 20, 23, 26, 27, 48, 86), but no attention has focused on the pertinence of slime production of ocular isolates of coagulase-negative staphylococci. Slime production has been a major factor in coagulase-negative staphylococcal pathogenicity. However, significant

infections with non-adherent coagulase-negative staphylococci have also been observed (19, 57). Younger (86) found that all slime-producing strains were adherent to CSF shunts but that not all adherent strains produced slime.

It would be convenient if all clinically relevant isolates of *S. epidermidis* produced slime or possessed some other readily detectable marker such as characteristic antibiograms, phage susceptibility patterns, or biochemical reactions. However, initial attempts to find a laboratory marker that predicts pathologic potential have been largely unsuccessful (53). Recently, three distinct antigenic markers for adherent strains of coagulase-neagtive staphylococci have been described by Christensen (23), Maki (56), and Tojo (78). Two of these antigens (See references 23 and 56) were shown to be present in plastic-adherent strains of coagulase-negative staphylococci but absent in non-adherent strains.

The following is a summary of some characteristics of coagulase-negative staphylococci which have been studied in relation to clinical relevance.

Slime production seems to be unrelated to any particular biochemical reaction or to phage susceptibility. Many (up to 50%) of *S. epidermidis* strains are untypable using phage susceptibility testing (19).

Other parameters of clinical relevance include speciation (18, 19, 27) and hydrophobicity (35, 45, 59, 71). These factors can be used in combination to distinguish coagulase-negative staphylococci which are true pathogens from members of the normal flora (59). The hydrophobicity test is based on bacterial adherence to hydrocarbons (e.g., toluene or hexadecane) and measures cell surface hydrophobicity by detecting different lipophilic residues on the bacterial

surface (51, 71). Hydrophobicity correlates reasonably well with bacterial adherence in many systems (51, 67). Martin et al. (59) showed that of three markers, namely slime production, hydrophobicity, and identification of species as *S. epidermidis*, hydrophobicity was the single test with the highest positive predictive value (79%) for clinical significance. However, none of these were ocular infections. The predictive value increased to 89% when combined with identification of the species as *S. epidermidis*. Determination of species other than *S. epidermidis* showed the highest negative predictive value (78%). However, when other species of coagulase-negative staphylococci were determined to be clinically significant, they were almost always slime producers and hydrophobic.

There is evidence that slime production is a contributing virulence factor (4, 27, 52). While slime may mediate adherence to medical devices, its main role in virulence and persistence seems to be protection against antimicrobial agents (11, 26), host defense responses (4, 41), and lysozyme (11). Slime-producing coagulase-negative staphylococci may be able to persist in the eye due to their resistance to lysozyme. *S. epidermidis* is not only the most common species of coagulase-negative staphylococci implicated in human disease (19, 21, 22, 34, 52, 67), but is the species most likely to produce slime (59), to cause endocarditis when compared to other coagulase-negative staphylococci (4), and to be adherent to both human cells (72) and to smooth surfaces (18). In any case, slime production in a clinically significant isolate should be considered in diagnosis and treatment decisions. In the future, treatment may be directed not only against the colonizing bacteria but toward the slime as well (2, 11). The concrete arguments for the clinical relevance of slime production in the

pathogenicity of staphylococci are that strains implicated in disease, especially conditions associated with colonization of a medical device, are often slime producers (11, 18, 27, 30, 53, 73).

Detection of Slime

In the Christensen test (18), slime production is detected in vitro by growing stationary staphylococcal cultures 18 to 24 h in plastic or glass tubes of tryptic soy broth (enzymatic digest of casein, soy protein, sodium and phosphate salts, and glucose [0.25% wt/vol]). The tube contents are decanted, a cationic stain such as safranin, alcian blue, or trypan blue is added and after removal of the stain, the tubes are examined for the presence and quality of stained, adherent growth (18). Stained coating on the sides of the tubes is referred to as slime, but in reality is adherent growth and presumed to be evidence of slime production. This test, while extremely valuable for the detection of slime production, is not very sensitive. The lack of sensitivity is based primarily on disagreement between individual observers in interpreting weak reactions (20, 66).

Other methods of Slime Detection

Quantitative method. Christensen (20) and Pfaller et al. (66) developed a method to quantitate slime production which measures the optical density of slime adhering to plastic tissue culture plates. After growing strains of coagulase-negative staphylococci in these plates, the contents of each well was aspirated and the wells were stained with safranin. Optical density was read with a Micro ELISA Autoreader. Strains with an optical density reading above 0.415 were considered slime-positive. This method correlates well with the

macro-tube method, is more sensitive in the detection of weakly-positive strains, and thus provides standardization when required.

Cell-associated markers. Dunne and Burd (31) developed a rapid method which does not require the production of a stainable slime layer for detection of adherent organisms. This method relies on detection of urease activity from staphylococcal cells which remain attached to a surface. The urease is detected using a commercially prepared enzyme linked immunosorbent urease reagent and measuring the intensity of a color reaction on an ELISA reader. Although this assay permits the evaluation of adherence of only urease-positive organisms, other cell-associated enzymes could serve as markers.

Slide method. Fessia (34) placed glass microscope slides in conical centrifuge tubes containing tryptic soy broth and 10^6 CFU/ml of coagulase-negative staphylococci. After 48 h incubation, the slides were removed from the broth, washed with distilled water, air-dried, and stained with safranin or acridine orange. This method allows the slides to be examined microscopically.

Rolling catheter on agar. Maki et al. (57) rolled catheters removed from patients across the surface of blood agar plates. Patient data were correlated to the number of colony forming units detected on agar and it was found that growth of ≥15 colonies was consistent with catheter-related septicemia.

Electron microscopy. Peters (64) incubated catheter sections with coagulase-negative staphylococci and then glued the sections, at timed intervals, to microscopic stubs. The stubs were coated with gold and paladium and examined with a scanning electron microscope. The staphylococci were

noted to adhere to catheters within 5-30 min after exposure and microcolonies were observed after 40-60 min. Heavy colonization occurred after 6-12 h.

There are advantages and disadvantages of every method. When grown in broth, slime-producing coagulase-negative staphylococci coagglutinate into an indispensable mass (4, 20) that cannot be centrifuged and washed without extraction. Colony forming unit determinations become less meaningful as each colony represents many organisms, but this is always a problem with staphylococci, which clump easily. More sensitive methods have been developed in order to chemically characterize staphylococcal slime, but there is yet no single generally accepted method for determination of slime production. The Christensen tube method was used in our studies because it is a rapid, simple test conducive to screening a large number of strains.

Composition of Slime

Many questions remain unanswered about the chemical make-up of the *S. epidermidis* glycocalyx. The composition varies in response to growth conditions (28, 47) and also differs from strain to strain (47).

Drewry found slime of strain RP-12 (ATCC 35983) to consist of 72% carbohydrate, 57% of which was galactose (28), but it was shown by these investigators and others (46) that agar or other high molecular weight media components accounted for the presence of galactose in the crude slime. Quie and colleagues (67) reported that staphylococcal slime is 40% carbohydrate and 27% protein. Mannose was found to be a major component of the slime produced by at least one reference strain of *S. epidermidis* (27).

Hussain has performed chemical analyses of slime produced by three strains of *S. epidermidis* isolated from catheters or blood cultures and grown on dialysis membranes to avoid contamination of galactose from the agar or on media solidified with silica gel instead of agar (47). Gel and ion-exchange chromotography were used for isolation of slime. Using various methods, slime from the three strains was shown to be composed of glycerol phosphate, glucose, glucosamine, alanine, uronate, an unidentified component, and protein which was not covalently linked to the carbohydrate polymer as summarized below:

	Strains		
Constituents[1]	RP-62A (ATCC 35984)	RP-12 (ATCC 35983)	CI543
Total carbohydrate	20	10	22
Protein	25	12	16
Glucose	13	<0.1	13
Glycerol phosphate	8	7	5
Glucosamine	10	12	15
Uronic acids	3	7	3

[1]Constituents are given as % (wt/wt) of extracted material (44).

Influence of Nutrients

Certain growth media will not support slime production. Brain heart infusion broth was unreliable and sucrose broth failed to support slime production at all in a study of 146 strains by Christensen (18).

When other carbohydrates such as fructose, lactose, maltose, sucrose, mannose, ribose, cellobiose, D-xylose, or D-galactose were substituted for glucose in TSB, variable amounts of slime production still occurred when known slime-producing strains were grown in these media. Neither mannitol,

D-arabinose, dextran, and α-methyl-mannoside supported slime production nor did slime production occur in the absence of carbohydrate (18).

Interestingly, when glucose was filter-sterilized and added to the medium instead of being autoclaved along with it, the medium would not support slime production. In addition, other broths containing glucose (Todd-Hewitt, brucella broth, and some synthetic broths) were also unable to support slime production (18). Therefore, the presence of glucose, *per se,* may not be vital for slime production, but rather, some fragment or modification of the molecule is utilized by the cell.

In the development of a complex minimal essential medium, (HHW) Hussain et al. (46) found no clear difference in the carbohydrate requirements of slime-producing strains compared to non-slime producers. Heavy slime producers gave the same results when grown in TSB as HHW, but the poor or slight slime producing strains made more slime in HHW than in TSB.

It is the general consensus among various investigators that glucose or other carbohydrates are required for slime production (18, 27, 28, 46, 57). However, Deighton and Balkau found that 17 of 100 *S. epidermidis* strains were able to produce slime when grown in TSB without glucose and tested by the Christensen method and 2 strains produced slime in TSB without glucose, but not in TSB with glucose (27). A slime-producing strain, ATCC 35984, produces slime regardless of whether glucose is present in the growth medium. However, adherence is enhanced when grown with glucose (18). Another slime-producing *S. epidermidis* strain, ATCC 35981 exhibits less adherence if cultivated in media with glucose than in glucose-deficient media (71). A polysaccharide antigen shown to be associated with adherent strains of

coagulase-negative staphylococci is present in greater amounts when the bacteria are grown in TSB with glucose compared to growth in TSB without glucose. However, the antigen was still present when the strains were grown without glucose (56). It is clear that variability in slime-production exists among strains of coagulase-negative staphylococci and that some strains differ in the ability to produce slime in various growth media.

S. aureus also produces extracellular material which is composed mainly of polysaccharides and is termed slime. The slime layer functions similiarly to a capsule in mediating attachment to surfaces (76). Carbohydrate in the growth medium does not effect slime or capsule production in *S. aureus* (61, 76).

Brown et al. (15) suggest that glucose receptors are involved in adherence to surfaces. The glucose present in the environment could bind to a surface and act as a bridge for the attachment of bacteria by the binding of the bacterial receptor sites to the glucose molecules (15).

Influence of External Elements on Slime Production

The formation of glycocalyx protects bacteria from a variety of physical, chemical, and biological stresses (2). However, some factors influence the formation and character of the glycocalyx. These are discussed below:

Atmosphere. Coagulase-negative staphylococci appear to require oxygen for the production of slime and increased CO_2 tends to reduce slime production (85). Since oxygen fluctuations occur in the human host, this could be important in patient management (9).

Lysozyme. Slime protects the bacterial cell from the action of lysozyme (11, 61).

Oleic acid. Oleic acid increases adherence, even in broths with no added carbohydrates (27).

Serum. The addition of 10% horse serum to the growth medium decreases adherence (27). Human serum and, more specifically albumin, show a striking inhibition of adherence to polymers, probably due to a decreased hydrophobicity of the interacting surfaces (45).

Host defenses and antimicrobial agents. There is evidence that the presence of slime protects coagulase-negative staphylococci from some host defenses such as chemotaxis and phagocytosis (49) and from the action of some antimicrobial agents (4, 43, 49, 58). Johnson (49) found that the capsular polysaccharides of many organisms are anti-phagocytic and staphylococcal slime may provide protection by this method. Slime isolated from *S. epidermidis* has been shown to inhibit chemotaxis by polymorphonuclear nucleophils (PMNs) when extracted slime was incubated with the PMNs and *S. epidermidis.* Additionally, phagocytosis was decreased when radiolabeled *S. epidermidis* were allowed to produce slime on a surface for 18 hours before exposure to PMNs compared to *S. epidermidis* which were immediately exposed to PMNs.

Resistance to Antimicrobial Agents

The presence of slime probably forms both a physical and ionic barrier which inhibits the access of antimicrobial agents to the bacteria (41, 43, 73). Additionally, bacteria in biofilm are frequently in a nutrient-starved state with decreased metabolism and may be rendered more resistant to antibiotics by decreased permeability of the cell membrane (1,2). Additionally, and more

likely, the antibiotic targets of slow-growing biofilm cells may become altered so that they are less accessible to antibiotics (1,2).

It has not been conclusively demonstrated that slime producing staphylococci are inately more resistant to antibiotics than nonslime-producing staphylococci when tested *in vitro* except that slime producers are more likely to be resistant to gentamicin (19, 83) and several of the slime-producing strains in the ATCC collection exhibit *in vitro* resistance to multiple antimicrobial agents (71). Bayston and Penny (11) found that 62% of slime-producing coagulase-negative staphylococci isolated from CSF shunts were multi-resistant and only 7% of nonslime-producing strains were considered to be multi-resistant. Christensen et al. also found that a higher, although not significant, proportion of "resistant strains" produce slime (19). These findings could represent a case of in vitro results which do not parallel the native in vivo state.

Coagulase-negative staphylococci and other microorganisms which display adherent properties exhibit different behavior in suspension than when grown on a solid surface. The solid surface most accurately portrays conditions which occur during infection. In fact, attachment to a solid surface is a prerequisite for infection (16, 55). Susceptibility testing is usually carried out under laboratory conditions that select against slime production (59). Therefore, in vitro susceptibility results, especially those obtained using liquid media, should be evaluated with caution (2, 55, 69).

These points were dramatically illustrated in a study by Farber et al. (33). These investigators isolated "crude slime" by removing proteins and lipids from slime-producing cultures of *S. epidermidis*. When the crude slime was added to suspensions of both slime-producing and nonslime-producing strains,

susceptibility to vancomycin (the drug of choice for treatment of indwelling catheter infection) was determined using inocula with and without the slime extract (0.5%). All isolates tested showed an increase in the MIC of vancomycin when grown in the presence of the extracted slime. In most instances, there was a four-fold increase in MIC compared to the growth without the slime. An antagonistic effect of slime was also demonstrated in time-kill studies where vancomycin activity was greatly decreased.The authors concluded that the slime probably complexes with the vancomycin, rendering it biologically inactive, or that the slime produces a physical barrier. The former seems more likely as the effect was not noted with cefazolin, clindamycin, or rifampin.

Biofilm of slime-producing coagulase-negative staphylococci is influenced by the presence of vancomycin, cephalosporins, chloramphenicol and other antimicrobial agents (30, 35, 65, 67, 71, 86). In several studies, many of the slime-positive strains tested produced a biofilm which was significantly more dense when grown in tryptic soy broth containing the drugs at sub-MIC concentrations. In contrast, other strains produced a less dense biofilm. These results are significant, not only with respect to the management of patients with biomedical devices who may be receiving antimicrobial therapy, but they also demonstrate that slime-producing strains are variable in their response to external elements.

While slime produces a barrier to most antimicrobial agents, presenting treatment difficulties in some patients, some antimicrobial agents show activity in reducing the biofilm or preventing its development. Antimicrobial agents which act on sites other than the cell wall, e.g. quinolones or fusidic acid, have been shown to reduce attachment to medical devices (32, 71).

Once a biofilm has become established on an indwelling medical device, antimicrobic therapy is usually not effective in eradicating the slime-producing coagulase-negative staphylococci from its surface even if in vitro susceptibility is demonstrated (26, 30, 86). In fact, an established biofilm is much more resistant to antimicrobial therapy than a recently developed biofilm (2). While most slime-producing coagulase-negative staphylococci are susceptible to vancomycin and rifampin, most are resistant to other antimicrobial agents such as beta-lactam antibiotics, macrolides, sulfonamides, trimethoprim, chloramphenicol, and the aminoglycosides (19, 20, 30, 86). In most cases removal of the indwelling device is indicated (26, 30, 43, 86).

The reasons for such resistance are complex and not fully understood. It is probable that reduced permeability of the bacterial cell wall plays a key role along with the simple physical barrier of the negatively-charged extracelluar slime. Additionally, cells located deep within a biofilm may be able to switch on the genetic expression of antibiotic degrading enzymes before the antimicrobial agent can penetrate and act.

Anwar et al. (1) demonstrated the resistant state of bacterial cells deep within a biofilm. Using a chemostat system to deliver regular doses of tobramycin and piperacillin to *Pseudomonas* cells, these investigators showed that planktonic cells were killed while older cells enmeshed within the biofilm could not be eradicated. The concentrations of tobramycin and piperacillin used in the study are bactericidal for *Pseudomonas* sp.; 5 µg/ml and 500 µg/ml, respectively (1).

In conclusion, MICs have little value in predicting the susceptibility of organisms which are in an established biofilm and should only be used to

identify resistant strains (1, 2).

Adherence of Staphylococci to Human Cells

Romero-Steiner et al. studied the adherence of gram-positive bacteria to human epithelial cells (68). They propose that mannose and galactose receptors on human cells are involved in bacterial adherence. Although *Staphylococcus* sp. were evaluated, specific information on staphylococcal adherence was not discussed. There is evidence that a glycoprotein component or an α–glycol containing carbohydrate on the surface of respiratory epithelial cells contributes to binding of mucin by *S. epidermidis* (70). Glycoprotein receptors have been described by Chugh (24).

The investigation of adherence of coagulase-negative staphylococci to human cells is limited pharyngeal cells, fibroblasts, and keratinocytes as summarized in Table 1. *S. epidermidis* probably has several modes of adherence. While Sanford et al (70) have described the binding of *S. epidermidis* to nasal mucosa via protein containing mucin-binding receptors, Chugh (24) has isolated a non-proteinaceous adhesin associated with adherence to pharyngeal cells. It is well known that slime produced by *S. epidermidis* and some other coagulase-negative staphylococci mediates adherence to inanimate objects (1, 7, 18), but its role in adherence to eukaryotic cells is still unclear. Slime-producing coagulase-negative staphylococci were evaluated for their ability to adhere to human fibroblast cells (8) and urinary epithelial cells (16). In these studies, slime production was not necessarily associated with adherence. It was proposed that slime production may mask the adherence of coagulase-negative staphylococci to tissue cells. Another group

of investigators found that *Staphylococcus saprophyticus* recognizes structures on tubular epithelial cells which contain N-acetyl-galactosamine. Adherence to plastic trays was reduced when the *S. saprophyticus* was grown in medium containing N-acetyl-galactosamine as compared to growth in media containing other carbohydrates. Therefore, the bacterial cells bind to N-acetyl-galactosamine rather than the plastic surface, indicating that the bacteria have specific receptors for this carbohydrate (38).

Carbonero et al. (16) found that physiochemical interactions, such as surface charge and hydrophobicity, are responsible for initial bacterial adherence to human cells while slime production may play a secondary role in the maintenence of colonization and protection from immune responses and antimicrobial agents.

Coagulase-negative staphylococci must possess the ability to adhere to ocular tissue, contact lenses, and intraocular lenses. It has been recently reported by Nissen and Speaker (63) that disposable extended wear contact lenses are associated with a shift in the predominant pathogens causing keratitis from Gram-negative to Gram-positive bacteria. The prevalence of *S. epidermidis* endophthalmitis after cataract surgery implies adherence to such lenses. Whether such adherence is receptor specific, only stereochemically specific (adsorption), or nonspecific has never been addressed. A corneal epithelial cell model developed by S.D. Dimitrijevich provides an opportunity to study the adherent properties of slime-producing staphylococci to ocular tissue.

Table 1. Adherence of coagulase-negative staphylococci to human cells in culture.

Type of cell	Adherence medium	Method	Incubation	Result	Ref
Fibroblasts	?	Confluent fibroblast cell sheets seeded with staph., incubated, and Gram-stained.	1 h, 37 C	Adherence of slime - strains > than one slime+.	8
Urethral epithelial cells	PBS	Confluent cells on glass slides seeded with staph., incubated, and Gram-stained	1 h 37 C	Adherence of *S. saprophyticus* > than *S. epidermidis* ; adherence not related to slime.	16
Pharyngeal epithelial cells	Minimal essential medium	Epithelial cell suspensions mixed with slime- coagulase-neg. staph., incubated, and stained with trypan blue.	30 min 37 C	All strains adhered, but strain variation was noted. Glycoprotein epithelial cells receptor and lipase-sensitive staph. adhesin described.	24
Renal tubular epithelial cells	PBS	Epithelial cells seeded with *S. saprophyticus* in microtiter plates with or without added carbohydrates, incubated with immune sera, adherence was determined using an ELISA technique.	1 h 37 C	N-acetyl-galactosamine added to the growth medium inhibited adherence, suggesting that receptors contain this carbohydrate.	38
HEp-2, keratinocytes, buccal epithelial cells	PBS	Equal amounts of bacteria and epithelial cells incubated in suspension, filtered and Gram-stained on filter.	30 min room temp.	Adherence of diphtheroids to epithelial cells > than other cutaneous bacteria. Galactose, fucose, N-acetyl-D-glucosamine, and fibronectin inhibited adherence.	68

Ferret nasal tissue	PBS	Ferrets were challenged intranasally with radiolabeled *S. epidermidis* and *S. aureus*. Tissue was recovered after 90 min.	N/A	High numbers of staph adhered. Trypsin pretreatment inhibited adherence, suggesting that mucin-binding recptors of the cocci have protein components.	70
HEp-2	Eagle basal medium with serum	Confluent monolayers inoculated with *S. epidermidis, S. aureus,* and *S. saprophyticus*. Incubated and counted adherent bacteria using phase contrast and UV microscopy.	3 h 37 C Wash, 2 h 37 C	Adherence of *S. saprophyticus* > than the other species and was the only species able to localize internally.	72

CHAPTER III

MATERIALS AND METHODS

Media, Bacteria, and Corneal Cells

Staphylococcal strains were grown on commercially prepared blood agar plates (Remel or BBL) or tryptic soy agar (Difco). The following media were used for slime detection and in nutritional studies: tryptic soy broth (Difco), nutrient agar (Difco), nutrient broth (Difco), sodium acetate agar and sodium acetate broth. All Difco media was prepared according to the manufacturer's recommendations. Sodium acetate agar and broth were prepared by the method of Trabulis, et al. (79) except 1% peptone (Difco) was added in order to support the growth of staphylococci. Media composition is described below.

Table 2. Composition of Growth Media

	TSA	TSB	NA	NB	NaAc	NaAc-B
glucose present?	no	yes (2.5%)	no	no	no	no
Carbon source/ Other nutrients	tryptone, soytone	tryptone, soytone	beef extract	beef extract	Sodium acetate	Sodium acetate
Nitrogen Source	tryptone, soytone	tryptone, soytone	peptone	peptone	monoammonium phosphate, peptone	monoammonium phosphate, peptone

Supplemented Keratinocyte Basal Medium, KBM+ (Clonetics Corp.- Modified MCDB 153 KBM™ with 0.15 mM Ca^{++}) was used for corneal epithelial cell culture. Corneal cells were initially cultivated in KBM containing 100 U/ml

All bacterial strains studied belong to an extensive collection of ocular isolates and were kindly donated by Alcon Laboratories, Inc., Fort Worth, TX. Bacterial strains were identified as *S. epidermidis* by the Vitek® System or API STAPH Trac® strips (both by bioMerieux Vitek, St. Louis, MO).

Human corneal epithelial cells were obtained from S. D. Dimitrijevich, Texas College of Osteopathic Medicine, Fort Worth, TX. Corneas were obtained from an eye bank and cells used for each adherence study were obtained from different corneas. Although all corneas were processed by Dr. Dimitrijevich, the method is briefly described here. Corneas were rinsed in calcium-free KBM and placed in dispase solution and incubated at 4 C in a six-well tissue culture plate for 24-48 h, depending on the age of the cornea. The corneas were then gently scraped with a scalpel to remove the epithelial sheet. The sheet was then incubated at 37 C for 15 min, forced through a 22 gauge needle several times, transferred to a conical tube, and centrifuged for 5 min. The supernatant was removed and 5 ml of KBM and 50µl of fibronectin was added. The mixture was resuspended and transferred to a flask coated with a 1 µg/µl solution of Collagen IV. After cells reached 90% confluency, they were harvested and dispensed into six-well tissue culture plates or Nunc brand chamberslides. Chamberslides are collagen coated glass microscope slides with plastic walls attached which form two wells. Each well can accomodate about 2 ml of medium. The plastic can be removed, leaving a slide which can be viewed microscopically.

Slime Production by Ocular Isolates

S. epidermidis strains were isolated from patients with corneal ulcers, conjunctivitis, or blepharitis and cryopreserved after determining species. One hundred fifty-one strains were screened for the ability to produce slime as described by Christensen et al. (18). Cultures were grown overnight on tryptic soy agar (TSA) at 35 C. A few colonies were transferred to 2 ml of tryptic soy broth (TSB) in polystyrene tubes and incubated for another 24 hr. The broth was decanted and 2-3 ml of safranin was added for 1 min and poured out. The tubes were inverted and examined for a stained film lining the inside of the tube. Slime varied from heavy to slight and usually extended from the tube bottom up the sides to the height of the 2 ml broth suspension (See Figures 2 and 3).

An alternative method for detecting adherence to glass was explored. Slime-producing and non-slime producing strains were grown in 2" diameter glass jars containing TSB or nutrient broth (NB). A 1" square coverslip was dropped into each jar before inoculation. Jars were incubated, without shaking, at 35 C for 18 hours. The coverslips were then removed with forceps, rinsed thoroughly with sterile phosphate buffered solution (PBS), and allowed to dry on paper towels. Coverslips were stained by dropping 2-3 drops of either safranin or trypan blue (0.1%) stain onto the slide. Excess stain was removed by rinsing with PBS and coverslips were viewed microscopically.

Thirty-two strains of coagulase-negative staphylococci were derived from normal eyes, i.e. eyes exhibiting no symptoms of blepharitis or other ocular infections, and were screened for slime production by the Christensen method.

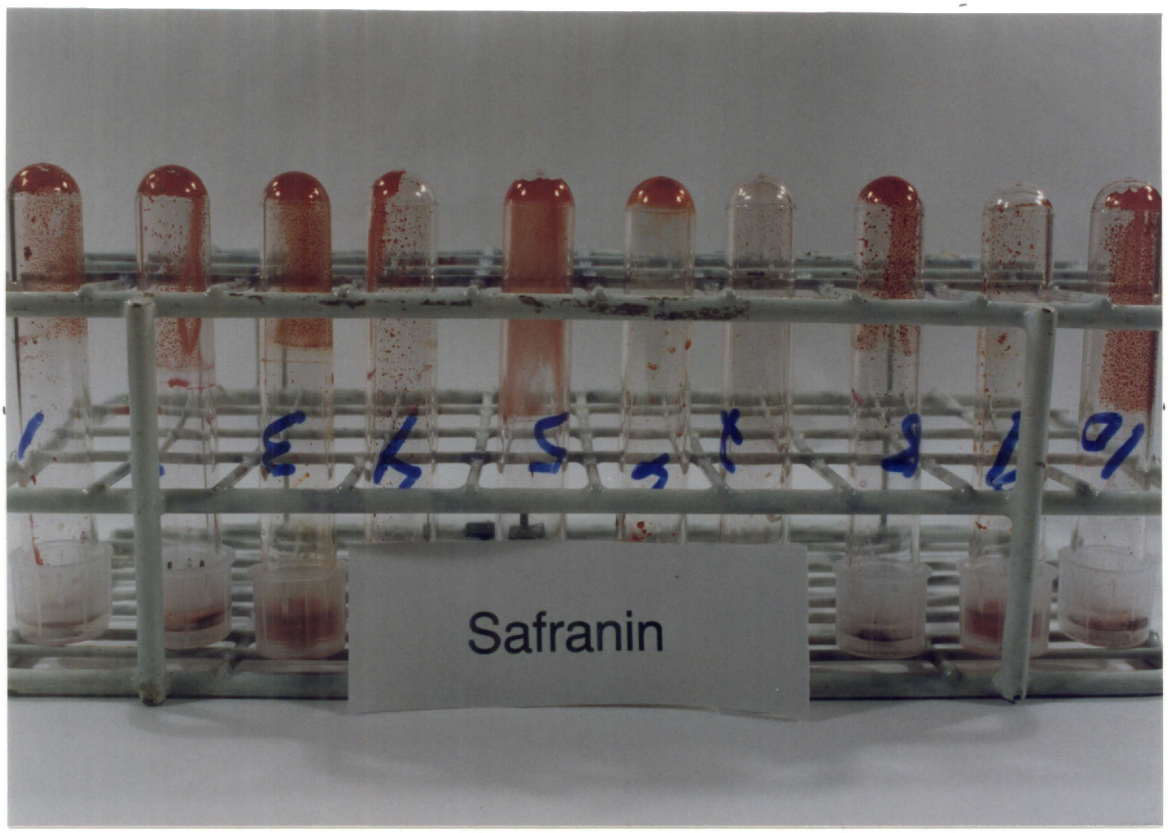


Figure 1. Tubes stained with safranin by the Christensen method.

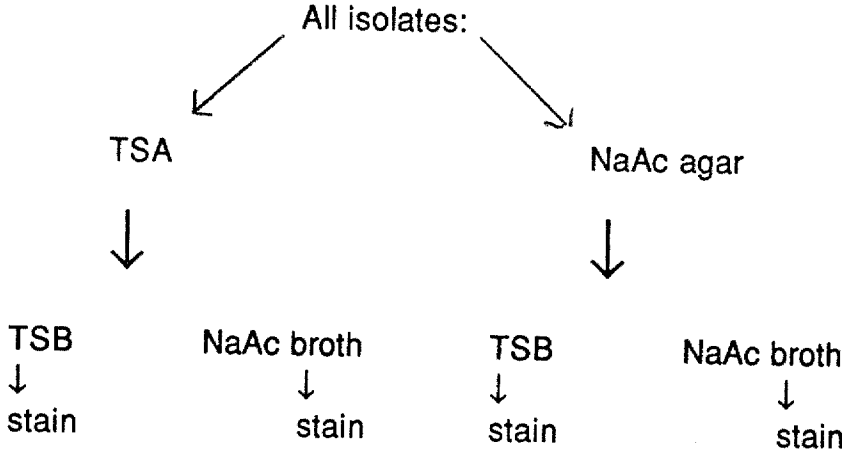
Tube 1	+++	Tube 6	-
Tube 2	+++	Tube 7	-
Tube 3	+++	Tube 8	++
Tube 4	+	Tube 9	-
Tube 5	+++	Tube 10	+++

++, +++ increasing intensity of color and thickness of stained, adherent biomass.

Effects of Glucose on Slime Production

In order to determine whether selected slime-producing staphylococci can produce slime in media without glucose, eleven slime-producing strains of *S. epidermidis* isolated from diseased eyes and one positive control organism (ATCC 35983, a blood culture isolate and one of the strains analyzed by Hussain, 47) were grown on TSA and sodium acetate agar (NaAc). NaAc agar was prepared as described by Trabulis (79) except 1% peptone was added to support the growth of staphylococci. Neither medium contains glucose.

Several colonies from each plate were transferred to TSB and to sodium acetate broth (NaAc-B). After overnight growth in either TSB or NaAc-B, the tubes were stained by the Christensen method as shown in the scheme below:

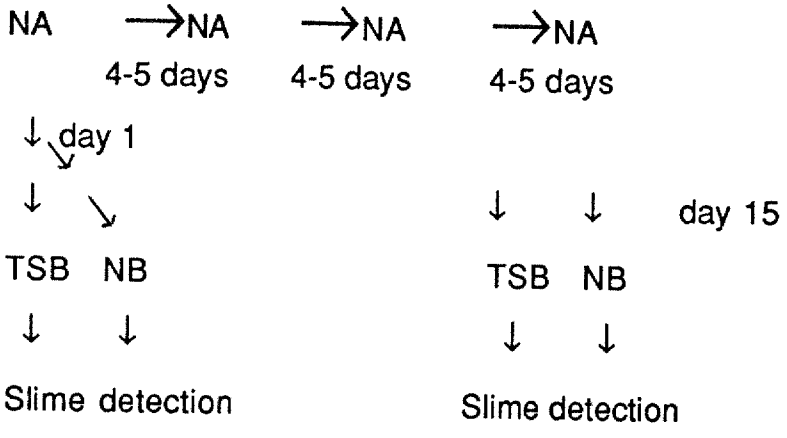


Six strains were chosen from these glucose and sodium acetate studies for further examination of nutrient effect on slime production. These strains were:

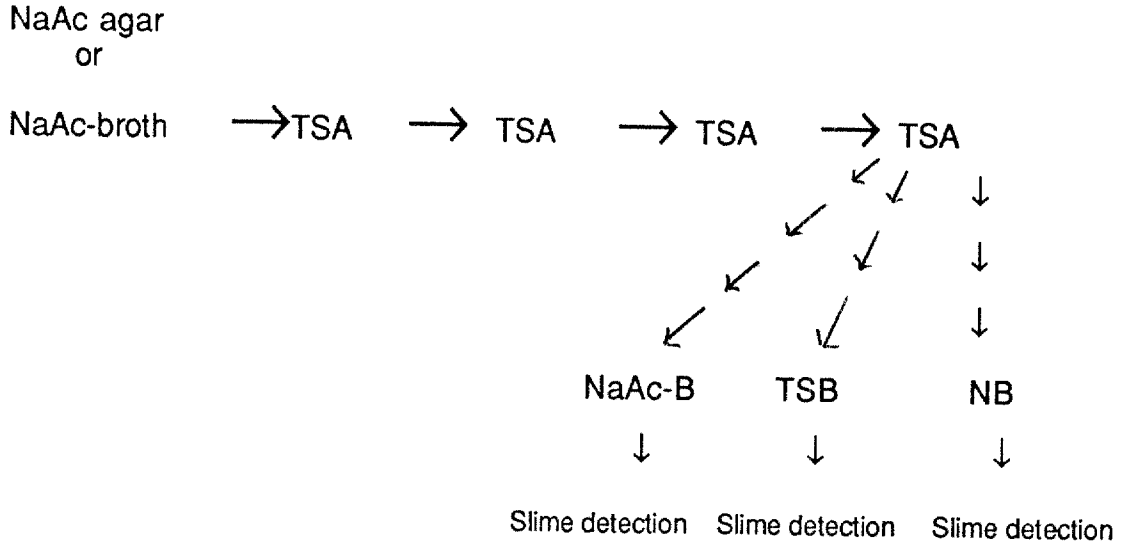
00085	heavy slime producer; continues slime production in sodium acetate broth.
00143	weak slime producer; does not produce slime in sodium acetate broth or nutrient broth.

SD22113	heavy slime producer; does not produce slime in sodium acetate broth or nutrient broth.
SD24924	moderate slime producer; produces slime in sodium acetate broth and nutrient broth.
ATCC 35983	moderate slime producer; slime contains no glucose according to Hussain (47); does not produce slime in sodium acetate broth or nutrient broth.

The effects of growth and storage on nutrient agar for long periods were studied by inoculating six slime-producers, culturing overnight at 35 C, storing at 4 C for 14 days, and subculturing to fresh nutrient agar every 4-5 days. On day 1 and day 15, each was transferred to TSB and nutrient broth (NB), incubated overnight at 35 C, and tested for slime production according to the scheme below:



The same six strains were deprived of glucose by culturing on sodium acetate agar or sodium acetate broth and then passing to tryptic soy agar at intervals of several days. Cultures were stored at 4 C during the interim. The "aged" tryptic soy cultures were then transferred to sodium acetate broth, nutrient broth, and tryptic soy broth, grown overnight, and examined for the presence of slime as shown below:



Results of the nutritional studies are summarized in Tables 7, 8, and 9.

Other work (37) has shown marine bacterial slime production is inhibited during growth in increasing concentrations of glucose. The same six slime-producing strains of *S. epidermidis* were grown on TSA containing no glucose, TSA with 7.0 g/L glucose, and TSA with 25 g/L glucose. Colonies from each of these media were transferred to NB, TSB with 2.5 g/L glucose, TSB with 7.0 g/L glucose, and TSB with 25 g/L glucose which were incubated as previously described and stained by the Christensen tube method (Table 10).

Our studies have demonstrated variability in the slime producing ability among strains of coagulase-negative staphylococci. For example, a few strains can produce slime in sodium acetate broth. It remained to be determined whether slime production is constitutive or strictly related to growth media. Therefore, two strains which possess the ability to produce slime in sodium acetate broth, 00085 and SD12783, were passed daily to glucose-free media, alternating between tryptic soy agar and sodium acetate agar. Slime production was tested daily in both tryptic soy broth and nutrient broth (See Table 9).

Comparative Staining of Slime

Several staining methods were used to qualitatively determine and compare composition of the slime layers of selected strains of coagulase-negative staphylococci. Strains which showed a higher degree of adherence when grown in media without glucose were tested, as well as strains which produced slime only in media containing glucose. The stains in addition to safranin, the basic stain used for initial detection of slime, were periodic acid Schiff (PAS) stain, calcofluor white, ruthenium red, and the semi-quantitative method of Blumenkrantz (12), used to detect the presence of uronic acid moieties.

Periodic acid Schiff (PAS) stain. The PAS stain is a qualitative technique for the demonstration of carbohydrate moieties in biological specimens. Periodic acid oxidizes carbon-carbon bonds where the adjacent carbons are one of the four following groups: 1,2-dihydroxy, 1-hydroxy-2 keto, 1-hydroxy-2-primary, amine, 1-hydroxy-2-secondary amines. The resultant aldehydes are demonstrated by condensation with the Schiff reagent. The condensation product between Schiff's reagent and aldehydes is initially colorless. However, when the aldehyde-reagent complex is neutralized with sodium carbonate, a deep pink color is observed.

Calcofluor white stain. Calcofluor white stain was used to detect ß-1,4 linkages. When used as fluorochromes, such brighteners produce green-yellow fluorescence in the presence of cellulose, ß-1,4 glucans, pectin, and other carboxylated polysaccharides. The brightener fluorochromes almost all types of cell walls, but usually not cell contents.

Ruthenium red stain. Ruthenium red is a polycationic stain which is specific for acid polysaccharides (16, 25, 37) and also phospholipids and soluble fatty acids (37).

Blumenkrantz method. This staining method detects hexosamine and uronic acid, components of all acid mucopolysaccharides. The method becomes quantitative by the use of spectrophotometric comparison of solubilized mucopolysaccharides normalized to total carbohydrate (29).

Periodic acid Schiff (PAS) stain

Reagents:

1. Schiff reagent:
   4.55g/L basic fuchsin
   86.5 ml/L N HCl
   4.55 g/L potassium meta-bisulfite.
2. Periodic acid solution:
   5 g/L periodic acid
3. Light green SF yellowish solution:
   0.3 g/L light green SF yellowish
   0.3 ml/L acetic acid
4. Sodium carbonate solution:
   9.5 g/L sodium carbonate

PAS procedure. Test strains were grown 24 h at 35 C in polystyrene tubes. The broth was decanted and 2 ml periodic acid was added and allowed to stand for 10 min. The stain was discarded and the tubes were rinsed with distilled water. Schiff reagent was then added to each tube and allowed to stand for 15 min. After rinsing tubes again, they were filled with sodium carbonate working solution. After 5 min, the tubes were rinsed and light green SF yellowish was added for 10-30 sec. The stain was decanted and distilled

water was used to rinse tubes. Finally, the tubes were filled with light green SF yellowish and allowed to stand for 10-30 seconds. After a final rinse, the tubes were observed for a deep pink color.

Calcofluor white (Fluorescent brightener 28) . Strains were grown on glass slides immersed in tryptic soy broth by the method of Fessia (34). A 0.01% solution of calcofluor white in distilled water was prepared and used to flood slides for 1 min (or longer to obtain more fluorescence). Slides were rinsed and air-dried in a dark chamber before viewing with a fluorescent microscope.

Ruthenium Red. Test strains were grown 18 h in TSB at 35 C in polystyrene tubes. The broth was decanted and adherent growth on the sides of the tubes were stained with a 1% ruthenium red solution. Christensen safranin staining was performed concurrently. Each tube was examined for the presence of stained material.

Blumenkrantz method.

1. *m*-hydroxydiphenyl (resorcinol) solution: 0.15% *m*-hydroxydiphenyl solution in 0.5% NaOH. The solution is stable at 4°C, but should not be exposed to light.
2. sulfuric acid/tetraborate solution: 0.0125M solution of tetraborate in concentrated sulfuric acid.
3. cetyltrimethylammonium bromide (CTAB) solution: 5% solution of cetyltrimethylammonium bromide in distilled water.

Detection of uronic acids and total carbohydrate in slime: Uronic acids in slime were detected by the method of Teller, et al. (77) as modified by Blumenkrantz, et al. (12). Total carbohydrates were detected by phenol-sulfuric acid as described by DuBois (29). Test strains were grown 24 h at 35 C in 30 ml

TSB in plastic, conical cetrifuge tubes. Slime was scraped from the tube sides with a sterile policeman, vortexed, and the centrifuged. The supernatant was decanted to another centrifuge tube. The volume and consistency of the pellet was noted. The supernatant was brought to pH 5 by the addition of 1 N HCl.

The tubes were placed in an ice water bath and 1 ml of 5% cetyltrimethylammonium bromide (CTAB) was added to each and mixed well. The tubes were allowed to remain in the water for 30 min so that precipitation of the uronic acids could occur. The tubes were then centrifuged for 15 min at 2500 rpm.

The supernatant was discarded and the pellet was resuspended in 30 ml of 95% ethanol saturated with sodium chloride in order to dissociate the CTAB from the uronic acids and to remove other organic contaminants. Each tube was then centrifuged for 15 min. This was repeated 2 more times. The supernatant was discarded after the final wash. The particulate matter was dissolved in 2.4 ml distilled water. All of the precipitate was went into solution after vortexing, but 0.5 N NaOH could have been added to aid in dissolution.

Two ml of the extracted slime solution was transferred to 20 mm glass cuvettes for total carbohydrate determination. The method is described below. The remaining 0.4 ml was transferred to 10 mm cuvettes for the detection of uronic acids.

Glucuronic acid standards were prepared in distilled water at the following concentrations (in µg/ml): 0.05, 0.10, 0.20, 0.40, 0.80, 1.60, 3.20. Each standard (0.4 ml) was transferred to cuvettes and treated concurrently with the extracted slime samples.

In the uronic acid procedure, 2.4 ml of the sulfuric acid/tetraborate

solution was added to each tube and kept cold in an ice bath. The tubes were then shaken in a 100 C water bath for 5 min and cooled in ice water. Forty µl of *m*-hydroxydiphenyl solution was added to each tube. The tubes were shaken and observed for color change. A blank sample was prepared using 2.4 ml of the tetraborate solution and 40 µl of the m-hydroxydiphenyl solution. The absorbance of the blank was subtracted from the total absorbance of the samples. Absorbance was measured at 520 nm on a Spectrophotometer 20.

Total carbohydrate determination: Glucose standards were prepared in the concentrations (in µg/ml): 10, 20, 30, 40, 50,60, and 70. Two ml of each standard was transferred to 20 mm glass cuvettes and 0.05 ml phenol was carefully added to each tube. In a safety cabinet, 5 ml of concentrated sulfuric acid was added to each tube. A bulb was used to ensure fast delivery into the center of each tube. The tubes were allowed to stand for 10 min and then were shaken and placed in a 25-30 C water bath until cool. Absorbance readings were taken at 490 nm. A blank sample was prepared using distilled water instead of a glucose solution. The absorbance of the blank was subtracted from the total absorbance of the samples.

Staphylococcal Adherence to Corneal Epithelial Cells

In all adherence experiments, strains of coagulase-negative staphylococci isolated from human eyes and a slime-positive control strain (ATCC 35983) were used. Although all research on staphylococcal slime has been conducted using strains isolated from biomedical devices or blood cultures, ocular isolates are significant since they can colonize intraocular lenses and cause endophthalmitis (42). *S. epidermidis* is the most common

cause of endophthalmitis (13) and is implicated in about 16% of bacterial keratitis cases (10). Coagulase-negative staphylococci may also be important in the colonization of contact lenses, and they are significant isolates in conjunctivitis and blepharitis. These experiments are the first using ocular disease strains and ocular cells in culture.

Procedure I. Adherence in 24-well plates: This procedure was done to determine the suitability of the usual growth plates for adherence studies, the optimal incubation time and survival of the corneal cells. Human corneal epithelial cells were grown in a plastic 24-well tissue culture plate. Upon receipt, the cells were about 7 days old and had begun to differentiate. Cells were incubated under 5% CO_2 until used in adherence assays. Antibiotic free KBM (KBM^-) was substituted for (KBM^+) and in some experiments, cells were fed with (KBM^+) until sufficient growth for bacterial adherence was obtained. The corneal cells were not confluent in all areas of the slide, but some cell-cell contact was observed.

The following strains of *S. epidermidis* were used initially for adherence to corneal epithelial cells in Procedures I and II:

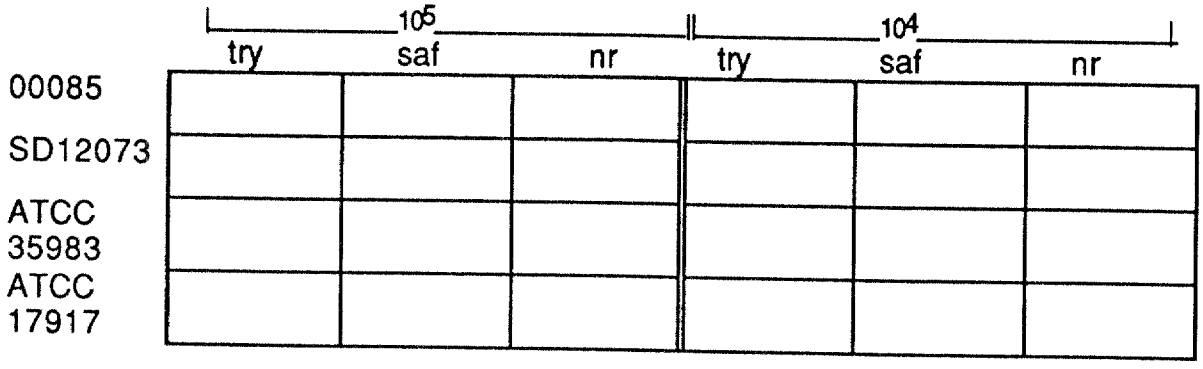
00085	slime produced in $glucose^+$ and $glucose^-$ media
SD12073	slime produced $glucose^+$ only
ATCC 35983	slime produced in $glucose^+$ only, but slime does not contain gucose (47)
ATCC 17917	no slime produced

Each staphylococcal strain was recovered from liquid nitrogen and grown 24 h on tryptic soy agar. Bacterial suspensions in KBM^- of $1x10^8$ CFU/ml were diluted to $1x10^7$ CFU/ml and $1x10^6$ CFU/ml. Ten µl (10^4 and 10^3 CFU) of each diluted suspension was added to each well (See Figure 2). The plate was gently rotated and then incubated for 1 hour at 35 C with 5% CO_2. After incubation, the contents of each well were aspirated and gently rinsed three times with KBM^- to remove nonadherent bacteria. Trypan blue and safranin were added to appropriate wells as indicated in Figure 2 and then rinsed with sterile PBS after 2-3 minutes. Each well was observed for the exclusion of trypan blue since this stain is absorbed by dying or dead cells. The wells were also observed for the presence of safranin to detect the presence of living or dead cells.

The neutral red (NR) assay was performed according to the method of Babich et al. (3) except that NR incorporation into the lysosomes of viable cells was determined microscopically rather than spectrophotometrically. The method consists of removing growth media from the wells of the tissue culture plate and adding diluted (1:100) neutral red. The tissue culture plates were then incubated at 37 C for 3 hours to allow uptake of the dye. The cells were examined under a light microscope to determine the number of cells which incorporated the dye.

Procedure II. Adherence to corneal cells on glass slides. In order to better visualize the bacteria under a light microscope, corneal epithelial cells were grown on collagen coated glass microscope slides with attached plastic wells (Nunc brand chamber slides). These slides not only allow the epithelial cells to grow on glass, a condition which provides much better growth, but also

Figure 2. Cell culture plate and staining scheme for corneal epithelial cells in six-well tissue culture plates.



try-trypan blue
saf-safranin
nr-neutral red

provides a convenient method of obtaining the cells on a flat surface suitable for viewing microscopically.

It was hoped that the cells would form a confluent monolayer on the slides, but this was not acheived after 7 days. In fact, many of the cells were no longer attached to the slides at that time. By day 10, only 2-6 cells/LPF remained. Nevertheless, the remaining cells were infected by adding 10 µl of either a $1x10^7$ or $1x10^6$ bacterial suspension to the wells. Each suspension was prepared in KBM^- and TSB. The incubation was lengthened to 4 h.

The chamber slides were incubated at 35 C in a non-CO_2 incubator for 4 hours. After incubation, growth media (KBM^- or TSB) were decanted, plastic wells were removed, and the slides were rinsed 3 times with sterile PBS. After air-drying, the slides were Gram stained by the usual method. The same strains were tested as in Procedure I.

Procedure III. Adherence for 24 h. Coagulase-negative staphylococci do not produce slime in vitro until they have been incubated for about 6 h (49), so

five strains, all ocular isolates, including two, 00085 and SD12073, which were used for Procedures I and II, were evaluated for adherence to corneal epithelial cells after 24 h incubation in KBM$^-$.

The bacteria were grown for 24 h in TSB, diluted in saline, and $1x10^4$ CFU/ml were added to wells of chamberslides (10 µl/well). One uninoculated well was used as a control. Each bacterial strain was also inoculated in 2 ml of KBM$^-$ to determine growth and slime production in the corneal cell medium. One tube of uninoculated growth medium was included to serve as a sterilty control. Tubes and chamber slides were incubated 24 h at 35 C with 5% CO_2. After incubation, samples from each well were cultured in TSB to determine the presence of viable bacteria. Each well was then rinsed three times with PBS to remove non-adherent bacteria. The slides were then Gram stained and examined for the number of bacterial cells adhering to the corneal epithelial cells. The KBM$^-$ tubes were observed for bacterial growth and stained with safranin in the same manner as previously described.

Procedure IV. Effect of lysozyme on adherence. Corneal cells were inoculated with the same five strains used in procedure III and the same procedure was used with the following exceptions:

- Each strain was incubated at 35 C in KBM$^-$ containing 1 mg/ml sterile lysozyme and in KBM$^-$ without lysozyme for 2 hours. Bacteria not treated with lysozyme were used as controls.
- After incubation, each strain was washed with PBS and centrifuged, whether or not it had been treated with lysozyme. Each was washed and centrifuged 3 times and then resuspended in KBM$^-$.
- Bacteria (10^6 CFU/ml) were inoculated into chamberslides.

CHAPTER IV

RESULTS

Screening of Ocular Isolates

One-hundred and fifty-one ocular isolates of coagulase-negative staphylococci, believed to be *S. epidermidis,* were confirmed using either the Vitek® System or API® Staph TRAC (both by bioMerieux Vitek, Hazlewood, MO). All strains were identified as *Staphylococcus epidermidis* except a weak slime producer, 00143, which was used in nutritional and adherence studies (Tables 7, 8, 10, and 13), was later determined to be *S. simulans.* Antibiotic susceptibility testing had previously been performed by this investigator.

Of these isolates, 25 (17%) were strongly adherent to the sides of polystyrene tubes when stained and thus considered heavy slime producers (Tables 3 and 4). Eighteen of 151 strains (12%) were moderately adherent, while 4 (3%) were weakly adherent. Figure 3 shows examples of heavy, moderate, and weakly stained biomass representative of slime production by the Christensen method (18). A total of 47 strains (32%) of these ocular isolates produced some level of slime. Other investigators have determined the frequency of slime production of coagulase-negative staphylococci isolated from a variety of sources to be from 29-53% (18, 27, 86) so the frequency of slime production among ocular isolates may be slightly lower than other clinical isolates, especially foreign body related infections.

The coverslip method of slime detection did not produce expected results. Staphylococcal cells adhered to all coverslips regardless of the ability of the strain to produce slime. It is possible that electrostatic forces between the glass coverslips and the bacterial cells were not overcome with the PBS rinse. In addition, the coverslips were placed on the bottom of the jars where most of the bacteria could have accumulated. When the Christensen tube method is employed, the tubes containing strains of non-slime producers settle at the bottom of the tube and are stained while the sides of the tube show no stained film. Fessia (34), using a similiar method, placed microscope slides into jars of broth in a standing position and was able to distinguish slime-producing staphylococcal strains. Christensen (18) reported that slime-producing strains of *S. epidermidis* adhere equally well to glass and plastic.

Staphylococcal isolates from normal eyes (Table 6) were shown to have a very low frequency of slime production. Only 3 of 32 (9%) strains produced slime and the slime production was low to moderate.

The staphylococcal strains studied for slime production were isolated from patients enrolled in clinical studies. Therefore, clinical data such as antimicrobial susceptibility and efficacy of antimicrobial treatment were available. Susceptibility data was compiled and MIC_{90}s of various antimicrobial agents were determined (See Tables 3 and 4). An MIC_{90} is the concentration of an antimicrobial agent that inhibits growth of 90% of bacterial strains when tested using conventional in vitro susceptibility test methods. Treatment outcome was based on individual physician judgements.

Susceptibility to most antimicrobial agents did not differ significantly among slime-positive and slime-negative strains. However, a higher proportion

of slime-positive strains were resistant to oxacillin. The MIC_{90} of oxacillin against slime-positive strains was 3.12 µg/ml while it was only 0.39 µg/ml against slime-negative strains. Gentamicin resistance has been reported to be associated with slime-positive strains (19) but no correlation was shown in this investigation.

Table 3. *Staphylococcus epidermidis* isolated from blepharitis patients

Specimen number	Slime	Treatment[a]	Microbiological Outcome[b]	Op[c]	Gent[d]	Ery[e]	Tob[c]
SD0487	-	cip	erad	1.0	0.13	0.5	0.13
SD0497	-	cip	erad	0.5	32.0	128	32.0
SD10798B	-	pla	prolif	0.25	0.13	0.5	0.13
SD15805B	-	cip	erad	0.13	0.13	128.0	0.13
SD17560	-	pla	erad	0.25	0.13	0.25	0.13
SD13825	-	cip	reduc	0.13	0.13	0.13	0.13
SD14573B	-	tob	persis	0.13	0.13	0.25	4.0
SD14573C	-	tob	persis	0.13	8.0	>128.0	8.0
SD13860	++	cip	persis	nd	nd	nd	nd
SD16250	+++	pla	prolif	nd	nd	nd	nd
SD10586	-	tob	erad	1.0	0.13	0.13	0.25
SD11389A	-	cip	reduct	0.13	0.13	0.25	0.13
SD11394	+	pla	persis	0.13	0.13	0.5	0.13
SD11401B	-	cip	erad	0.13	64.0	>128.0	32.0
SD11428	-	cip	erad	0.25	0.25	0.25	0.5
SD11958A	+++	cip	no data	0.13	1.0	64.0	2.0
SD12069	-	cip	erad	0.25	0.13	0.13	0.13
SD12073B	+++	pla	reduc	0.13	0.13	>128.0	0.13
SD12143A	-	cip	erad	0.25	0.13	2.0	0.13
SD12155A	-	pla	no data	2.0	0.13	0.13	0.25
SD12162A	-	pla	erad	0.13	0.13	0.13	1.0
SD14243	-	cip	erad	0.13	0.13	>128.0	0.13
SD14257	+++	cip	erad	2.0	0.13	128.0	0.5

Specimen number	Slime	Treatment[a]	Microbiological Outcome[b]	Cip[c]	Gent[d]	Ery[e]	Tob[c]
SD15072	+++	pla	reduc	0.13	0.13	0.25	0.13
SD16261	+	cip	erad	nd	nd	nd	nd
SD17671	+++	cip	erad	0.25	0.13	0.25	0.5
SD2937A	++	cip	reduc	0.13	0.13	0.25	0.13
SD11930	+++	cip	reduc	nd	nd	nd	nd
SD11258	+++	tob	persis	nd	nd	nd	nd

Table 4. *Staphylococcus epidermidis* isolated from corneal ulcers

Strain	Slime	Outcome[a]	Cip[b]	Tob[c]	Cep[d]	Ox[e]
00045	-	Improved	0.10	3.12	0.20	0.20
00047	-	Cured	0.20	≤0.05	0.10	0.20
00049	-	Cured	0.10	nd	0.20	0.20
00055	-	Cured	0.10	≤0.05	0.20	0.20
00085	+++	Cured	≤0.05	≤0.05	≤0.05	0.20
00089A	-	Cured	≤0.05	≤0.05	≤0.05	≤0.05
00089B	-	Cured	≤0.05	≤0.05	≤0.05	≤0.05
00129	-	Cured	0.10	0.39	0.10	0.10
00143A	+	Cured	0.10	≤0.05	0.20	0.20
00163A	-	Cured	0.20	≤0.05	0.10	0.20
00163B	-	Cured	0.20	≤0.05	0.10	0.20
00228	-	ND	0.05	≤0.05	≤0.05	≤0.05
00301A	-	Cured	0.20	≤0.05	0.10	0.20
00303	-	Cured	0.10	≤0.05	≤0.05	0.10
00307	+++	Cured	0.10	≤0.05	0.10	0.10
00551	+++	Cured	0.10	≤0.05	0.10	0.20
00555	++	Cured	0.20	3.12	0.10	0.10
00557B	-	Cured	0.20	≤0.05	0.10	0.10
00559A	-	Cured	0.20	0.10	0.20	0.10
00559B	-	Cured	0.10	0.10	0.20	0.20
00560	-	Cured	nd	nd	nd	nd

Strain	Slime	Outcome[a]	Cip[b]	Tob[c]	Cep[d]	Ox[e]
00561A	-	Cured	0.20	0.10	0.10	0.20
00561B	-	Cured	0.20	0.10	0.20	0.10
00561C	-	Cured	0.10	0.10	0.20	0.10
00693	+++	Cured	0.10	25.0	0.78	1.56
00811	+	Cured	0.20	≤0.05	0.10	0.20
01107A	-	ND	0.39	0.10	0.10	0.20
SD25381D	++	Cured	nd	nd	nd	nd
D307	-	ND	0.13	32.0	nd	nd
D399	+++	ND	0.13	16.0	nd	nd
D454	+++	ND	0.13	129.0	nd	nd
D497	+++	ND	0.13	129.0	nd	nd
SD22063	+++	ND	0.20	3.12	nd	1.56
SD22066	-	Cured	0.20	0.39	nd	0.20
SD22069	-	Cured	0.20	0.39	nd	0.20
SD22074	-	Cured	0.10	0.10	nd	0.20
SD22103	-	Cured	0.10	>3.12	nd	3.12
SD22105A	++	Cured	0.20	>3.12	nd	3.12
SD22107	-	Cured	0.20	0.20	nd	0.20
SD22112	-	ND	nd	nd	nd	resistant
SD22113	+++	Cured	0.20	>3.12	nd	>3.12
SD22132B	-	ND	0.50	nd	nd	nd
SD22211	-	Cured	0.20	0.10	nd	0.20
SD22222	-	Cured	0.20	0.39	nd	0.10
SD22223B	-	Cured	0.20	0.39	nd	0.20
SD22225	-	Cured	0.20	0.39	nd	0.20
SD22238	-	Cured	0.20	0.39	nd	0.20
SD23021	-	Improved	0.20	>3.12	nd	0.20
SD23022	-	Unchanged	0.20	0.39	nd	0.20
SD23063	-	Cured	0.10	0.39	nd	0.39
SD23086	-	Cured	0.20	0.39	nd	0.20
SD23087B	-	Improved	0.20	3.12	nd	0.20
SD23090B	-	Cured	0.20	6.25	nd	0.78

Strain	Slime	Outcome[a]	Cip[b]	Tob[c]	Cep[d]	Ox[e]
SD24051	-	Cured	0.20	0.39	nd	0.10
SD24055	-	Cured	0.20	0.10	nd	0.20
SD24056	-	Cured	0.20	0.20	nd	0.20
SD24073	-	Cured	0.20	3.12	nd	0.20
SD24180	-	Cured	0.20	0.20	nd	0.20
SD24185	+	Cured	0.20	0.10	nd	0.20
SD24188	-	Improved	3.12	25.0	nd	25.0
SD24193A	-	Cured	0.10	0.39	nd	0.20
SD24946A	+++	ND	0.20	>3.12	nd	3.12
SD24947	+++	ND	nd	nd	nd	nd
SD24951B	-	Improved	0.20	≤0.05	nd	0.39
SD25383	+++	Cured	0.20	0.39	nd	0.20
SD25384A	++	Cured	0.20	0.39	nd	0.20
SD25386B	+++	Cured	0.20	0.10	nd	0.20
SD25387B	+++	Cured	0.20	0.39	nd	0.20
SD27234A	+++	Improved	0.39	0.10	nd	0.20
SD27247A	++	Cured	0.20	0.20	nd	0.20
SD27247B	++	Cured	0.20	0.20	nd	0.20
SD28951	-	Improved	0.20	3.12	nd	0.20
SD28955	-	Improved	0.20	0.10	nd	0.20
SD28958	+++	Cured	0.78	0.20	nd	0.10
SD28959A	++	Improved	0.10	0.39	nd	0.20

[a]Outcome is based on the physicians' judgement after antimicrobial therapy.

[b]Cip=ciprofloxacin, [c]Tob=tobramycin, [d]Cep=cephalothin, [e]Ox=oxacillin

nd=not done ND=no data

\+ light slime production

++ moderate slime production

+++ heavy slime production

Table 5. Comparison of MIC_{90}s of various antimicrobial agents against coagulase-negative staphylococci.

Ciprofloxacin			
MIC90		% Susceptible	
S+	S-	S+	S-
0.25	0.39	100	100
Tobramycin			
MIC90		% Susceptible	
S+	S-	S+	S-
25.0	8.0	84	92
Gentamicin			
MIC90		% Susceptible	
S+	S-	S+	S-
*	8.0	100	82
Erythromycin			
MIC90		% Susceptible	
S+	S-	S+	S-
*	>128	57	65
Oxacillin			
MIC90		% Susceptible	
S+	S-	S+	S-
3.12	0.39	86	93
Cephalothin			
MIC90		% Susceptible	
S+	S-	S+	S-
*	0.2	100	100

S+ Slime-positive

S- Slime-negative

%susceptible is based on the breakpoint which is the concentration of an antimicrobial agent required to kill bacteria or inhibit their growth. Bacteria able to grow at concentrations above the breakpoint are considered to be resistant to a particular antimicrobial agent.

Table 6. Evaluation of slime production of coagulase-negative staphylococci from normal eyes

Strain	Slime production	Strain	Slime production
1	–	308A	–
2	–	308B	–
4	–	312A	++[1]
5	–	312B	–
6	–	320	–
7	–	331	–
9	–	331-2	–
10	–	336A	+[1]
11	–	336B	–
12	–	336C	–
13	–	342	+[1]
14	–	348A	–
16	–	348B	–
223	–	352	–
301A	–	TG-OS	–
301B	–	TG-OD	–

– negative; +weakly positive-very low slime production; ++weakly positive-low to moderate slime production

[1]Upon evaluation of the three subjects with slime-producing coagulase-negative staphylococci, the following was noted:
Subject 312 also carried *Staphylococcus aureus*, albeit in low numbers.
Subject 336 was colonized with other coagulase-negative staphylococci which did not produce slime.
Subject 342 also carried an enormous (TNTC) number of *Achromobacter xylosoxidans*.
A. xylosoxidans is occasionally implicated in ocular infection.

Nutritional studies

In general, staphylococci grown in TSB produce slime and those grown in NaAc-B do not (Table 7). However, 4 of the 12 strains were unable to produce slime in TSB after having grown on the nutrient poor NaAc. Two strains (SD12783 and 00085) were able to produce slime in NaAc-B after having grown on TSA and one of these (SD12783) was able to produce slime growing solely on NaAc media. Both of these strains can also produce slime in Mueller Hinton broth (a nutritionally poor medium) which has been prepared at half strength.

Table 7. Effect of glucose on slime production.

Isolate	TS agar → TS broth	TS agar→ NaAc broth	NaAc agar→ TS broth	NaAc agar→ NaAc broth
D497	++	++	-	-
D399	+++	+++	-	-
SD15072	+++	+++	-	-
SD12783	+++	+++	++	++
SD14257	+++	-	-	-
SD12073	+++	-	-	-
SD11958	+++	++	-	-
00085	+++	+++	++	-
00693	+++	+++	-	-
SD25381	+++	-	-	-
00143	++	-	-	-
ATCC35983*	++	+++	-	-

+++heavy slime layer produced, ++light slime layer produced, -no slime layer
*Known slime producer used as positive control.

After four passes to nutrient agar over a period of 14 days, all six slime-producing strains tested could produce slime when transferred to tryptic soy broth. However, when transferred to nutrient broth, only one strain (00085) retained slime-producing ability. This strain consistently produces heavy slime in tryptic soy broth and produces a moderate amount of slime in sodium acetate broth. The other strains tested were unable to continue slime production after growth and storage at 4 C on tryptic soy agar. Christensen (18) has reported similiar loss of slime production in the absence of glucose.

NaAc media and TSA (both media contain no added sugar) produced similiar results in a second study. Strains which were grown on NaAc media were passed four times to TSA and tested for slime production. All six strains could produce slime upon transfer to TSB but only two (00085 and SD24924) could produce slime in both NaAc-B and NB (Table 8).

When two strains having the ability to produce slime in sodium acetate broth (00085 and SD12783) were deprived of glucose by daily passage on tryptic soy agar or sodium acetate agar, the ability to produce slime was maintained over six passages when tested in tryptic soy broth. However, when tested in nutrient broth (Table 9), SD12783 immediately lost slime producing ability and strain 00085 was capable of weak slime producing ability during the first 2 days of passage to tryptic soy agar and sodium acetate agar, but then lost the ability.

Increasing glucose concentrations

The results of slime production by staphylococci in the presence of high concentrations of glucose are shown in Table 10. Although the results are not striking, a trend toward inhibition of slime production is noted. When strains are

cultivated for 1 day in the presence of 25 g/L glucose and then transferred to TSB containing 7 or 25 g/L glucose, slime production, while not absent, was shown to decrease significantly in all six strains tested. Strain SD24924, which retains the ability to produce slime in the absence of glucose, appears more sensitive than the others to inhibition of slime production by high glucose concentration. ATCC 35983 also has an individualistic response to prior growth in higher glucose concentrations. The inhibitory effect occurs at a lower concentration than SD24924 (i.e., 7.0 g/L glucose in TSB) but is alleviated in an even higher concentration (25 g/L glucose in TSB).

Table 8. Slime-producing strains of *S. epidermidis* grown without glucose for 14 days

	NA→TSB[a]	NA→NB[b]	NaAc→TSA→ TSB[c]	NaAc→TSA→ TSB[d]	NaAc→TSA→ NaAc-B[e]
00085	+	+	+	+	+
00143	+	-	+	-	-
SD22113	+	-	+	-	-
SD24924	+	-	+	+	+
SD22063	+	-	+	-	-
ATCC 35983	+	-	+	-	-

TSB contains 2.5 g/L glucose.
Media containing no glucose:
NA- nutrient agar NaAc- sodium acetate agar
NB- nutrient broth NaAc-B- sodium acetate broth
[a] 4 passes to NA over 14 days; overnight growth in TSB.
[b] 4 passes to NA over 14 days; overnight growth in NB.
[c] Strains grown on NaAc for 5 days then passed 4 times to TSA over 12 days; overnight growth in TSB.
[d] Strains grown on NaAc for 5 days then passed 0 times (00085) or 4 times (SD24924) to TSA over 7 days (00085) or 12 days (SD24924); overnight growth in NB.
[e] Strains grown on NaAc for 5 days then passed 4 times to TSA over 7 days (00085) or 12 days (SD24924); overnight growth in NaAc-B.

Table 9. Loss of slime production.

		00085		SD12783	
Day	Medium	TSB	NB	TSB	NB
1	NaAc ↓	+	+/-	+	-
2	TSA ↓	+	+/-	+	-
3	NaAc ↓	+	-	+	-
4	TSA ↓	+	-	+	-
5	NaAc ↓	+	-	+	-
6	TSA	+	-	+	-

TSA- tryptic soy agar TSB- tryptic soy broth
NaAc- sodium acetate agar NB- nutrient broth
+/- weakly positive

Comparative staining

The results of comparative staining are presented in Table 11. Only 7 of the strains produced detectable slime using the Christensen method. All strains were shown to contain carbohydrates when the adherent slime layer was stained using the periodic Schiff stain. Calcoflour white failed to detect any carboxylated polysaccharides (e.g., cellulose) in any of the strains. All but 2 strains (D497, ATCC 35983) were positive for ruthenium red staining, indicating the presence of acidic polysaccharides in these strains, but the differences among strains between the two cationic stains, safranin and ruthenium red, implies strain differences in the acidic polysaccharides (Figure3).

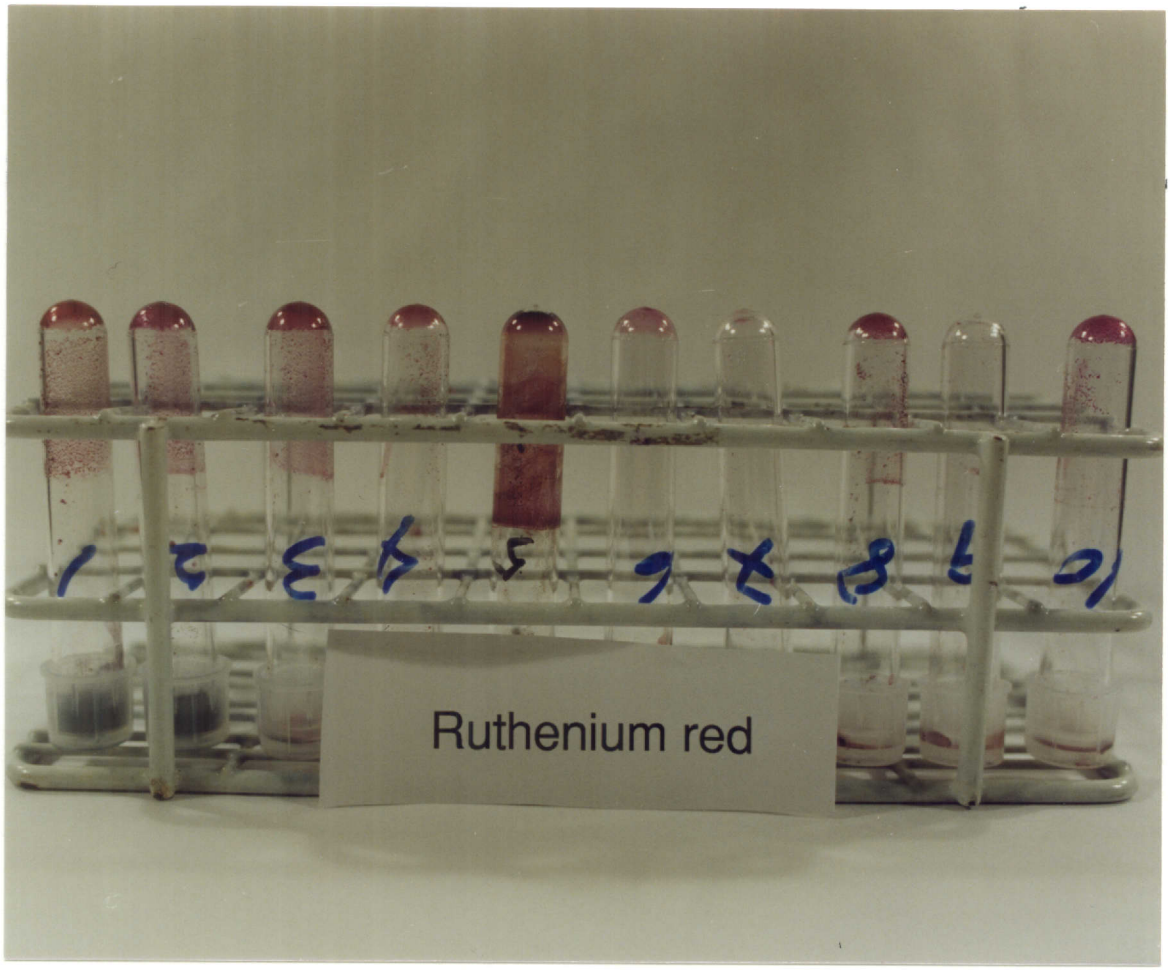


Figure 3. Tubes stained with ruthenium red.

Table 10. Effect of increased glucose concentration on slime production as determined by Christensen method.

	Strain	NB[1] (no glucose)	TSB [2] (2.5 g/L glucose)	TSB (7.0 g/L glucose)	TSB (25 g/L glucose)
From TSA[3] (no glucose)	00085	+	++++	++++	+++
	00143	-	++++	++++	++++
	22113	-	++++	++++	++++
	24924	+	++++	++++	+++
	22063	-	++++	++++	++++
	ATCC 35983	-	++	+	+
From TSA (7.0 g/L glucose)	00085	++	++++	+++	+++
	00143	++	++++	++++	+++
	22113	+	-	++++	++++
	24924	+	+++	++	++
	22063	-	+++	+++	+++
	ATCC 35983	++	+	+	+
From TSA (25 g/L glucose)	00085	+	++	++	++
	00143	+	+++	+++	+
	22113	+	+++	++	+
	24924	+	++	+	-
	22063	-	++++	++	+
	ATCC 35983	+	+	-	+

[1]NB-Nutrient Broth (Difco)

[2]TSB-Tryptic Soy Broth (BBL)

[3]TSA-Tryptic Soy Agar (Difco)

+	light slime production
++, +++	moderate slime production
++++	heavy slime production

Table 11. Comparative staining of selected slime-producing strains.

Strain	Stain			
	Safranin (Christensen method)	Ruthenium red	PAS	Calcofluor white
D399*	+++	+++	+	-
SD15072*	+++	+	+	-
SD12783*	+++	+	+	-
SD11958*	+	+	+	-
00085*	+++	+	+	-
D497*	-	-	+	-
ATCC 35983*	-	-	+	-
SD14257†	++	+++	+	-
SD12073†	-	+	+	-
SD25381†	+++	+++	+	-

* Strains able to produce slime in broth containing sugar as well as broth containing no sugar

†Strains able to produce slime in broth containing sugar but not in broth without sugar. (see Table 3)

+ light stained material

++ moderate stained material

+++ heavy stained material

Uronic acid and carbohydrate present in staphylococcal slime was quantitated relative to standards which were tested concomitantly. The absorbance of the processed standards were plotted (Figs. 4 and 5) and absorbances of of the extracted slime samples were determined (Table 12). The amount of uronic acid present in the slime of the 10 strains tested was calculated relative to the total amount of carbohydrate present. The slime from strains SD14257 and D497 contain more carbohydrate than the other strains. The slime of other strains contain about equal amounts of carbohydrate. The amount of uronic acid present was calculated relative to total carbohydrate present in the samples. The two strains (SD14257 and D399) shown to produce

slime with higher levels of uronic acid based on standards were also shown to contain higher levels of uronic acid relative to total carbohydrate present. The other strains produce slime containing 0.2-1% uronic acid/total carbohydrate.

Table 12. Quanitation of carbohydrate and uronic acid in staphylococcal slime.

Strain	Absorbance (uronic acid)	µg uronic acid (see fig 4)	Absorbance (carbohydrate)	µg carbo-hydrate (see fig 5)	uronic acid/total carbohydrate
D399*	0.091	1.4	1.700	60	2.3%
SD15072*	0.058	0.5	1.700	60	0.8%
SD12783*	0.038	0.3	1.600	55	0.5%
SD11958*	0.046	0.4	1.700	60	0.6%
00085*	0.040	0.3	1.700	60	0.5%
D497*	0.040	0.3	1.820	>70	>0.4%
ATCC 35983*	0.038	0.3	1.700	60	0.5%
SD14257†	0.097	1.6	1.890	>70	>2.3%
SD12073†	0.017	0.1	1.700	60	0.2%
SD25381†	0.070	0.6	1.700	60	1.0%

* Strains able to produce slime in broth containing sugar as well as broth containing no sugar

†Strains able to produce slime in broth containing sugar but not in broth without sugar (see Table 3).

Adherence to corneal epithelial cells

All wells of the tissue culture plate, used in Procedure I, had trypan blue and safranin present after rinsing. Due to the condition of the cells, the neutral red staining technique gave limited results. Most corneal epithelial cells retained some neutral red, but it was difficult to determine whether the dye had been taken up by the lysosomes. The nuclei of the cells were stained red and

Figure 4. D-Glucuronic Acid Standard Curve

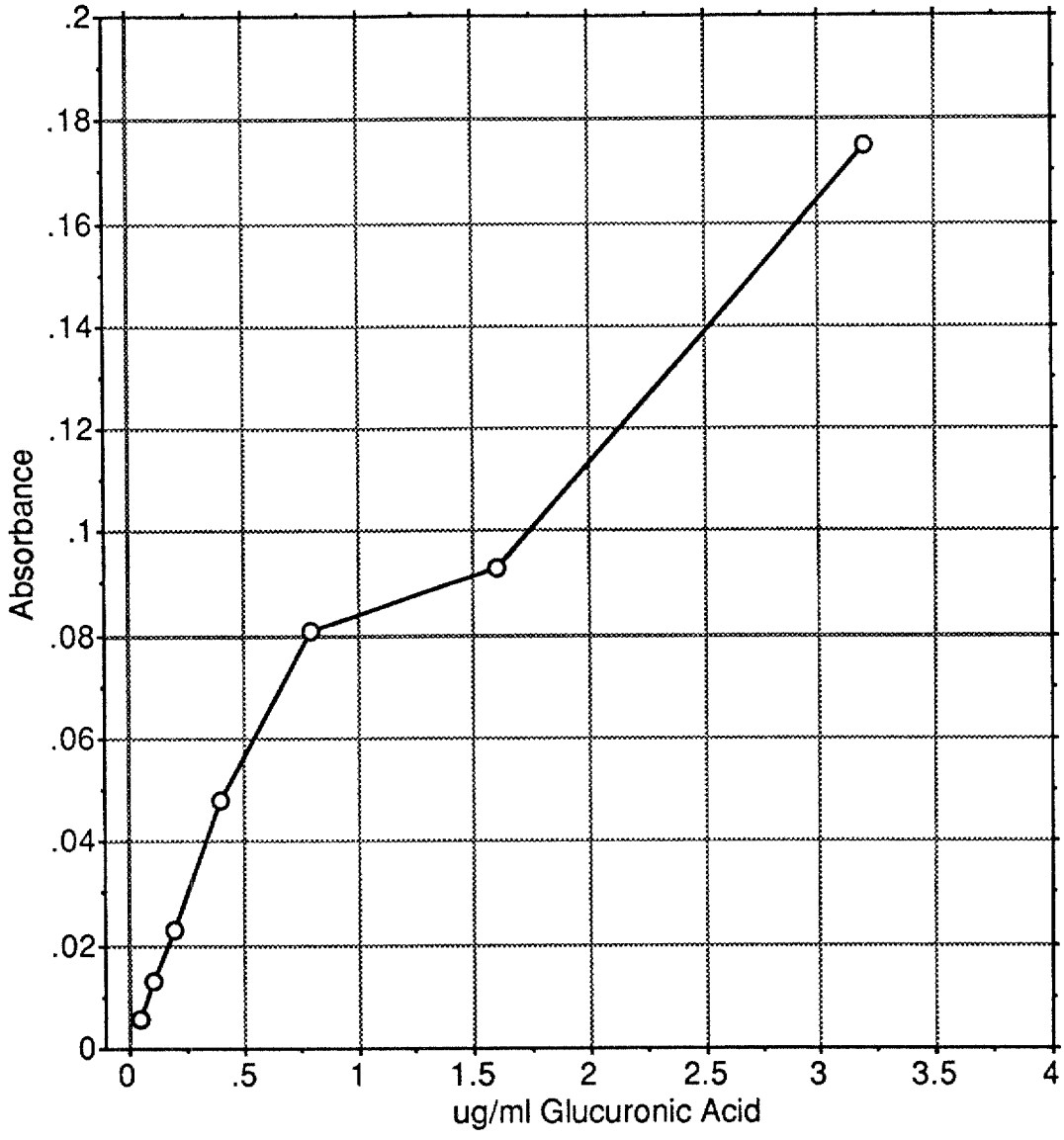
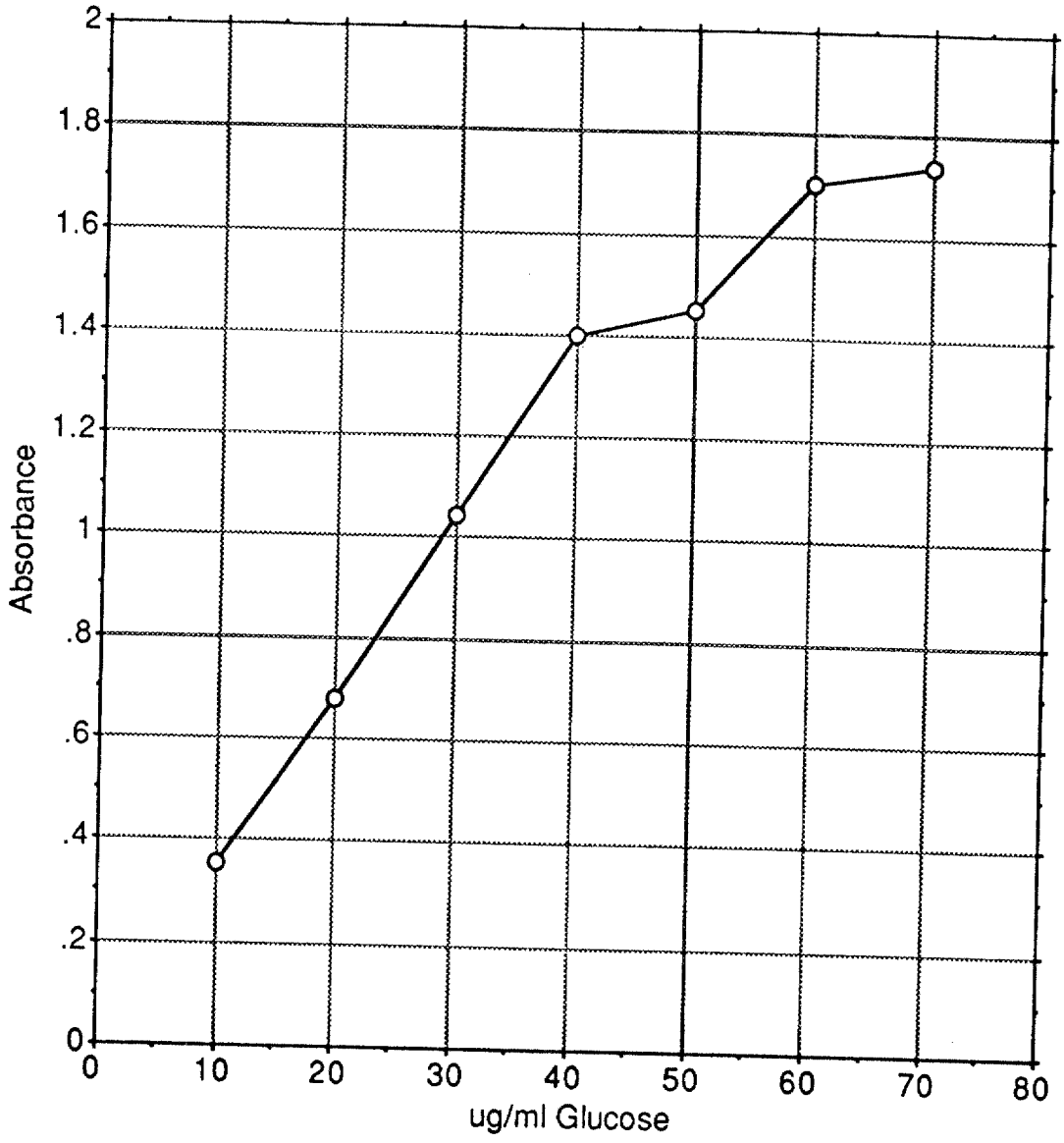


Figure 5. Carbohydrate Standard Curve



the cytoplasm was stained very light red. However, since the trypan blue was taken up by the cells, they were only weakly viable.

Adherence of staphylococci to corneal epithelial cells (Table 13)

Two days after receiving corneal cells in Nunc chamberslides, visible bacterial contamination was noted in one of the six wells. Two days later, all wells showed heavy bacterial contamination. Upon culture of the growth medium in the wells, a pure culture of diphtheroids was isolated. Since *Corynebacterium* sp. have been shown to be adherent to buccal epithelial cells, HEp-2 cells, and human epidermal keratinocytes (68) and so the contaminated slides could be put to some use, the slides were Gram stained by the usual method. Before staining the slides were rinsed three times with sterile phosphate buffered saline to remove nonadherent bacteria.

The diphtheroids did indeed adhere to the surfaces of the corneal epithelial cells in large numbers (>100 bacterial cells per epithelial cell) resembling clue cells. Since the slides had been washed, no bacterial cells were detected that were not attached to the epithelial cells.

When the experiment was repeated with fresh cells, adherence to the epithelial cells by the staphylococci was poor. Although some adherence to the corneal cells was evident, staphylococcal cells were also noted attached to the glass slide even though the washing steps were identical.

Better results were obtained when the cells were incubated with bacteria overnight. Growth occurred in broth inoculated at the same time, so it was assumed that the staphylococci had grown in the chamber slides. Johnson found that slime accumulation takes from 6-12 hours when grown in broth (49)

so slime and growth were tested by subculturing chambers before the slides were rinsed and stained.

All of the slides, after rinsing and staining, still had 2-3 corneal epithelial cells/high power field (hpf). The quantity and presentation of the staphylococcal cells was quite variable (See Table 13).

Surprisingly, only one of the five strains (SD22222) was recoverable on blood agar after 24 h incubation with the corneal cells. The staphylococci probably do not thrive particularly well in the keratinocyte basal medium although some effect of the corneal cells on the bacteria cannot be ruled out. In fact, when the strains were grown overnight in 2 ml KBM, all five were recovered on blood agar upon subculture from KBM.

To test the effect of an inhibitory corneal cell product, the strains were grown overnight in KBM which had been in contact with corneal epithelial cells for several days. Only 2 of the 6 strains grew in this medium (see below). However, because the cell culture medium contained 50 μg/ml gentamicin these 2 strains may be gentamicin resistant while the other 4 strains are susceptible. Approximately 72% of *S. epidermidis* strains are susceptible to gentamicin (54).

Strains used:

00085	heavy slime producer
00143	weak slime producer (This strain was later noted to be *S. simulans)*
SD12073	produces slime in media without glucose
ATCC 17917	nonslime producer

ATCC 35983 slime producer

SD22222 nonslime producer

The ability to produce slime in Keratinocyte Basal Medium (KBM) was also determined. Slime production was verified in TSB at the time of testing, as noted above. None of the strains could produce slime in this tissue culture medium.

One strain, SD12073, exhibited a different adherence pattern after 2 h exposure to lysozyme. Approximately 100 bacterial cells/hpf adhered to corneal epithelial cells without lysozyme treatment while only 50 bacterial cells/hpf adhered after lysozyme treatment so apparenly lysozyme treatment exposes a specific adhesin.

Table 13. Staphylococcal adherence to corneal epithelial cells in chamber slides

Strain	Procedure II[a] 4 h incubation	Procedure III 24 h incubation	Procedure IV No lysozyme	Procedure IV Lysozyme
00085 (S)[c]	<10 bacteria/cell[b]	No bacteria detected	>200 bact./epithelial cell. Most associated with epi. cells.	>200 bact./epithelial cell. Most associated with epi. cells.
SD12783 (S)	n.t.	10 bact./epithelial cell. All associated with epi. cells.	n.t.[d]	n.t.

Strain	Procedure II 4 h incubation	Procedure III 24 h incubation		Procedure IV Lysozyme
SD23063 (S)	n.t	1 bact./epithelial cell. All associated with epi. cells.	n.t	n.t
SD12073 (S)	<10 bacteria/ epithelial cell. Most associated with epi. cells	n.t.	100 bact./epithelial cell. All associated with epi. cells	50 bact./epithelial cell. All associated with epi. cells
ATCC 35983 (S)	<10 bacteria/ epithelial cell. Most associated with epi. cells	n.t.	>200 bact./epithelial cell. Associated with cells and slide.	>200 bact./epithelial cell. Associated with cells and slide.
00143 (WS)[d]		>100 bact./epithelial cell. Associated with epi. cells and slide.	>500 bact./epithelial cell. Associated with epi. cells and slide.	>500 bact./epithelial cell. Associated with epi. cells and slide.
SD22222(NS)[e]		>100 bact./epithelial cell. Associated with cells and slide.		

ATCC 17917 (NS)	<10 bacteria/ epithelial cell. Most associated with epi. cells		<10 bact./epithelial cell. All associated with epi. cells	<10 bact./epithelial cell. All associated with epi. cells

[a] cells were heavily contaminated (>100 bacteria/cell) with diphtheroids.

[b] bacterial amounts represent average counts of at least 20 epithelial cells.

[c](S)- slime producerws

[d](WS)- weak slime producerns

[e]- non-slime producer

Photographs of corneal epithelial cells are shown in Figure 7. Staphylococci adhering to corneal epithelial cells are shown in Figures 8-12. These photographs illustrate the variability among strains of coagulase-negative staphylococci in adherence to different lines of corneal epithelial cells. (i.e. corneal cells from different individuals.) Some of the variability in adherence may relate to receptors on the corneal cells, rather than the adhesins of the bacteria, which could, in fact, be identical to one of the previously identified adhesins (23, 56, 78).

A nonslime-producing strain SD22222 (Figure 8) adhered to both the corneal epithelial cells and the glass slide (nonspecific adherence). A weak slime-producing strain, 00143 (Figure 10), adhered in high numbers and in clusters to 3 different lines of corneal epithelial cells and also to the glass slide. However, nonspecific adherence was not limited to nonslime-producing or weak slime-producing strains. ATCC 35983, a slime-producing strain isolated from a catheter, is shown in Figure 11 also adhering in high numbers to corneal

epithelial cells and to the glass slide. A slime-producing strain, 00085, shown in Figure 9 did not adhere to corneal epithelial cells in two studies, but was shown to be adherent to a third line of corneal epithelial cells (See Table 13). In the third study, adherence was specific, i.e., all staphylococcal cells were associated with corneal epithelial cells and few or none adhered to the glass slide. Another nonslime-producing strain, ATCC 17917 displayed specific adherence (See Figure 12).

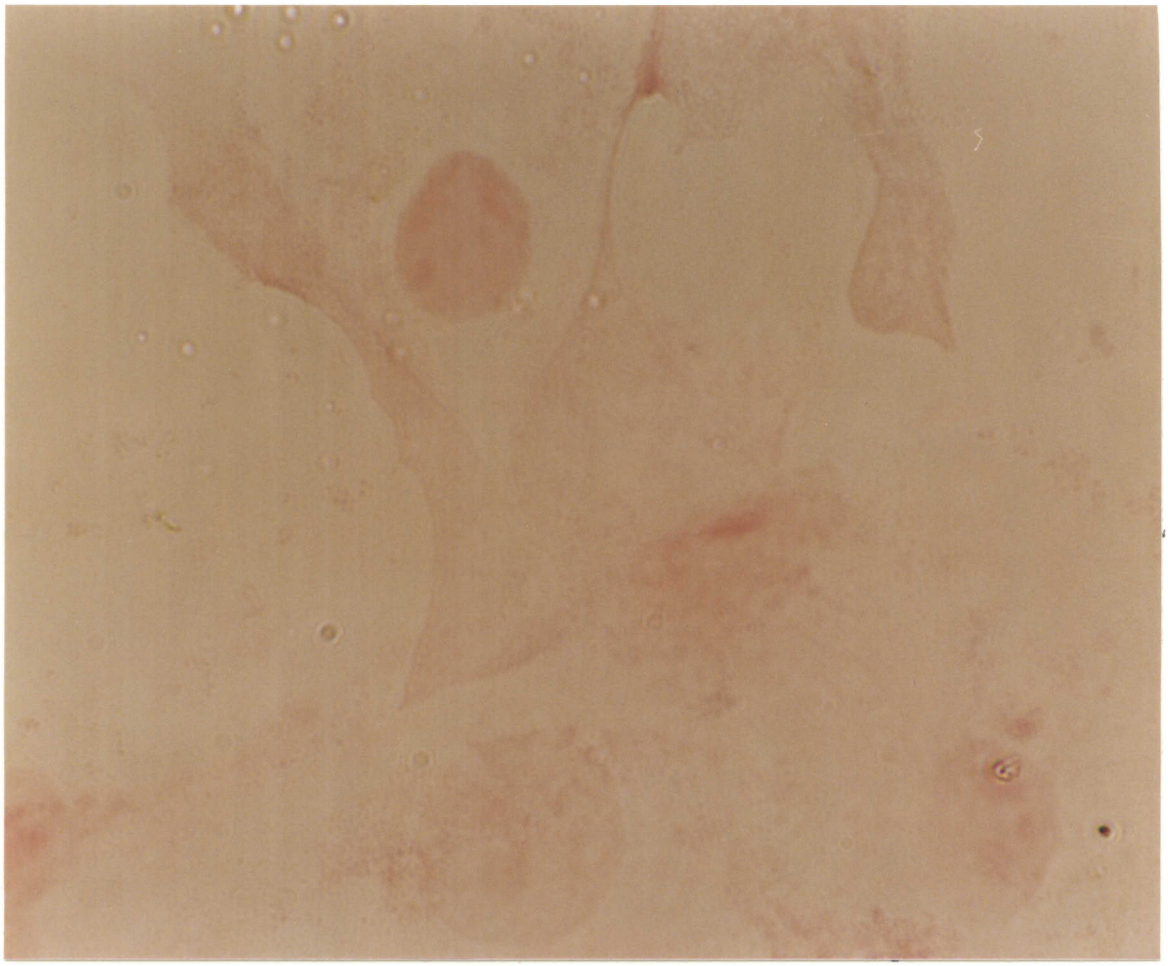


Figure 6. Corneal Epithelial Cells

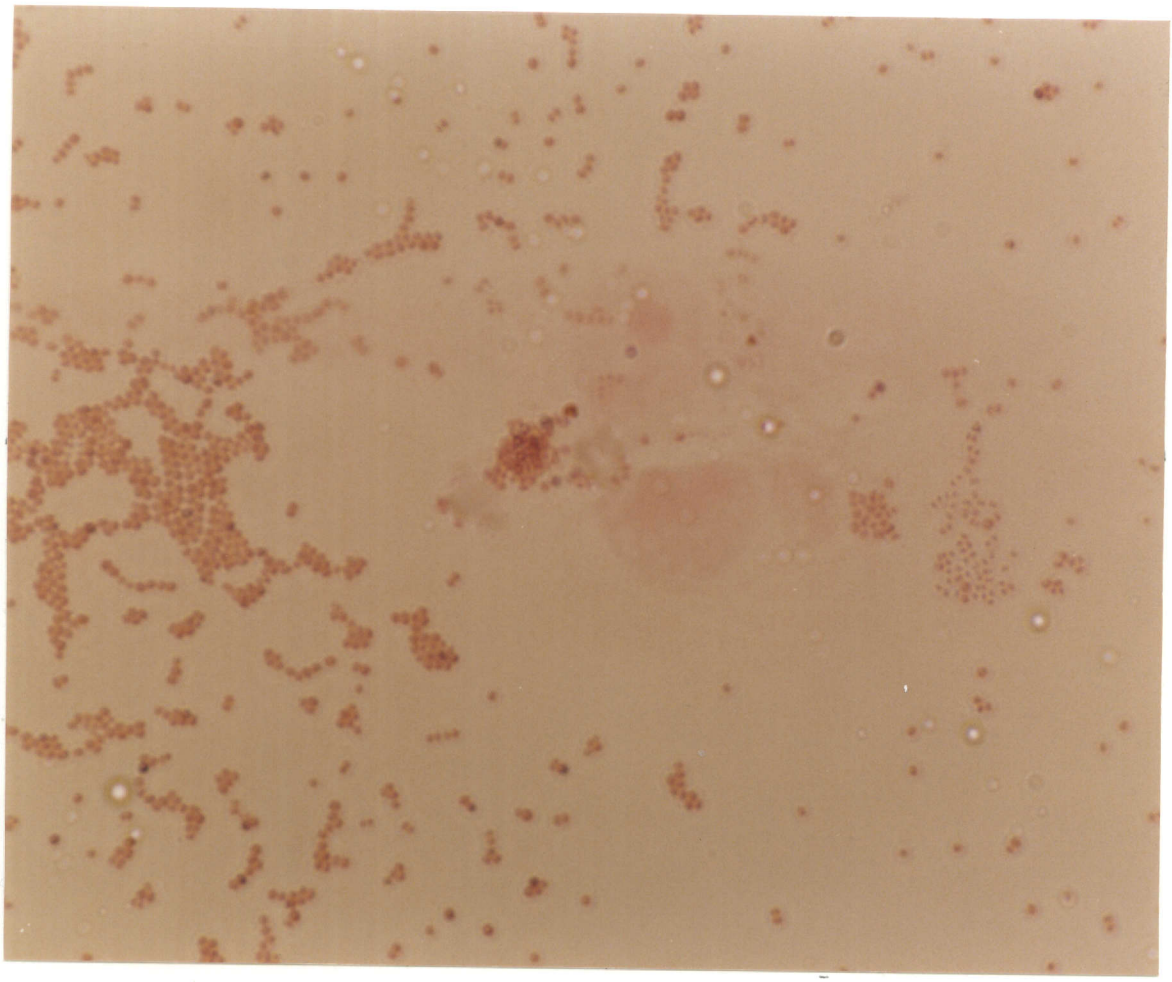


Figure 7. Slime-producing *S. epidermidis* adhered to corneal epithelial cells (nonspecific adherence)

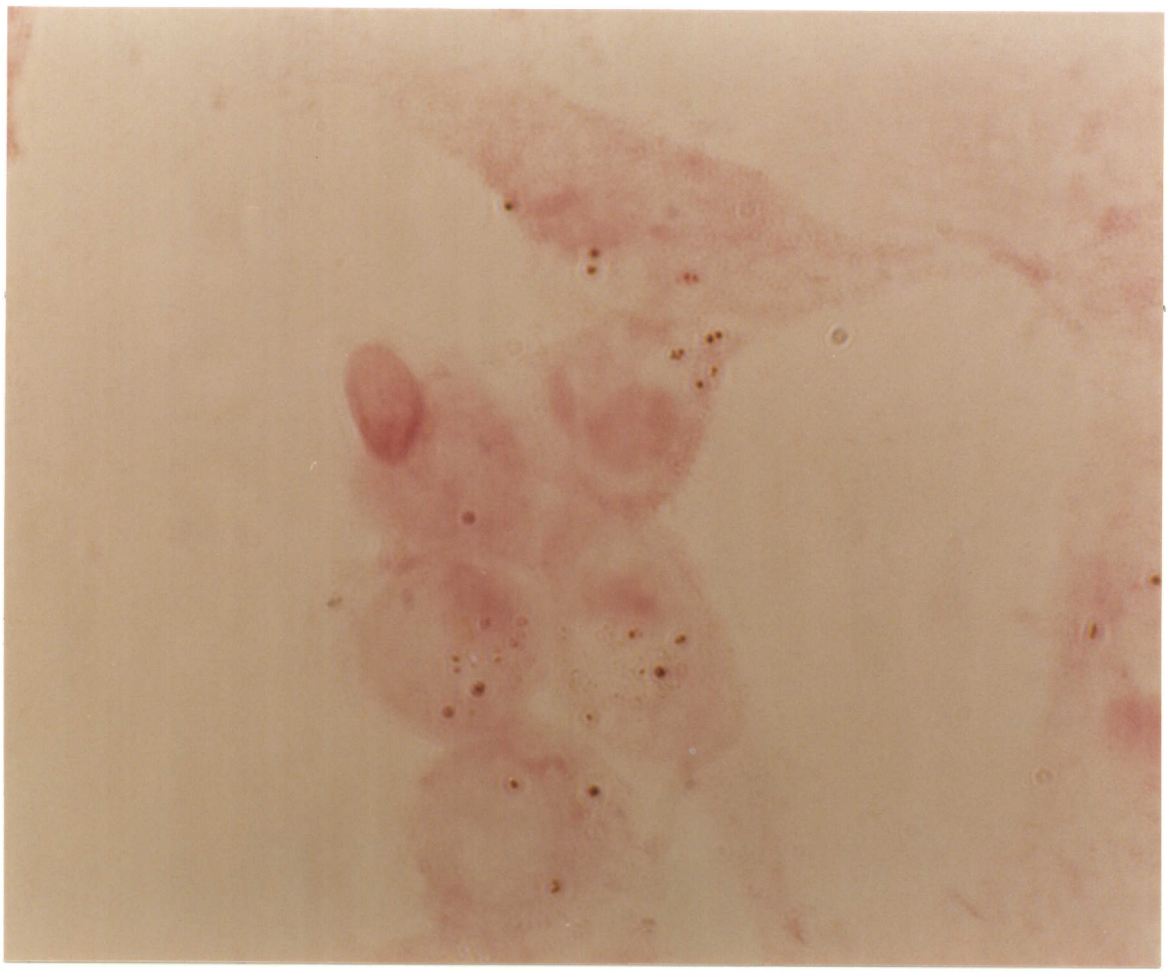


Figure 8. Slime-producing *S. epidermidis* adhered to corneal epithelial cells (specific adherence)

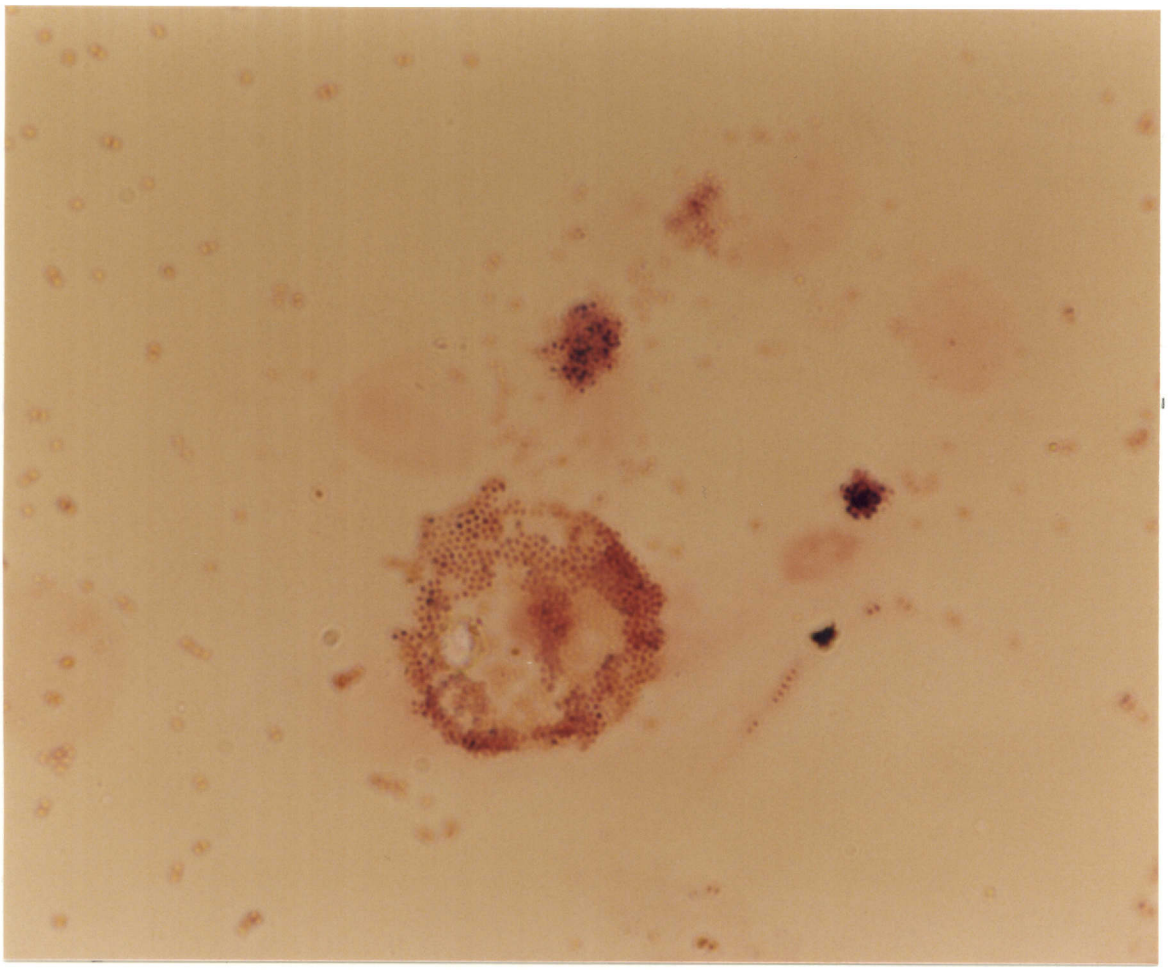


Figure 9. Weak slime-producing *S. epidermidis* adhered to corneal epithelial cells (nonspecific adherence)

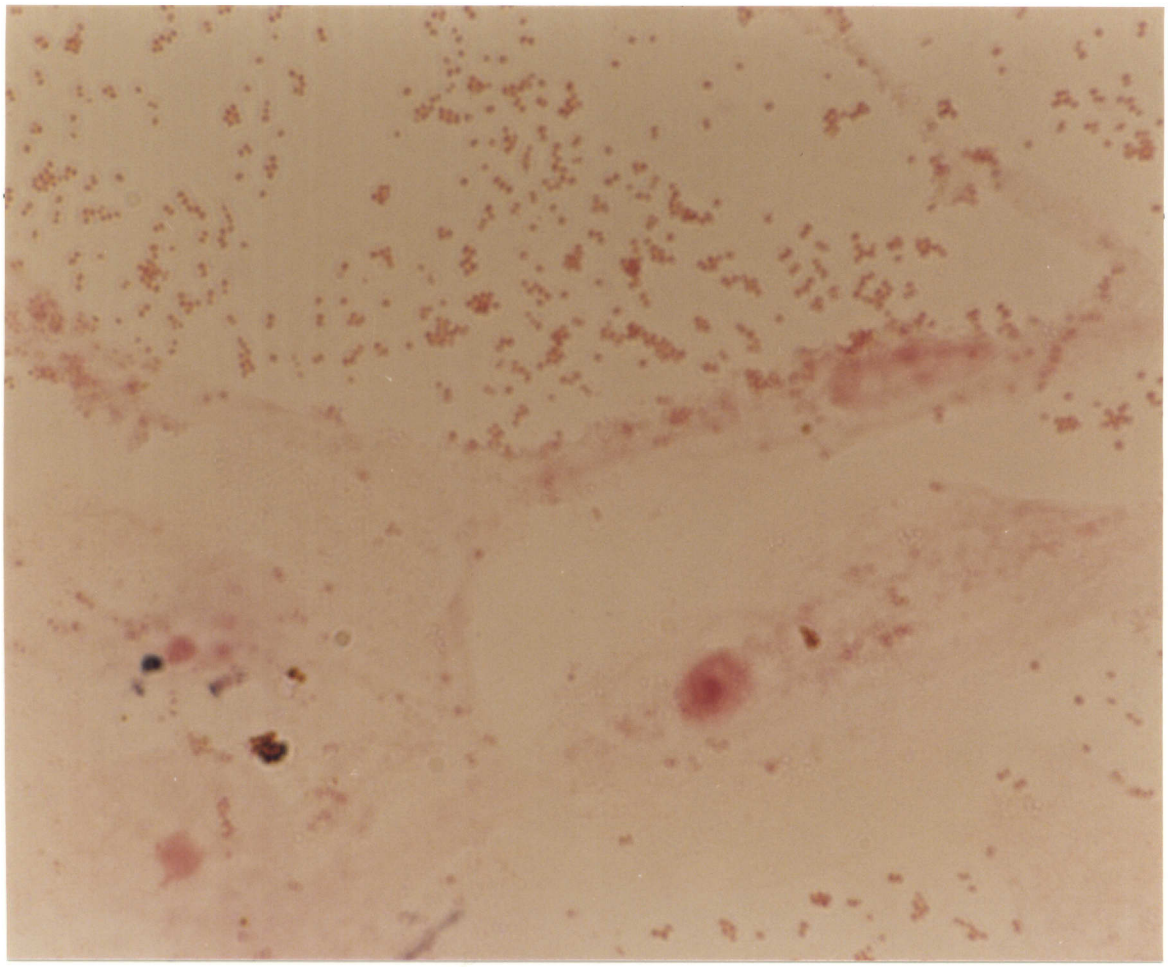


Figure 10. Slime-producing *S. epidermidis* adhered to corneal epithelial cells (nonspecific adherence)

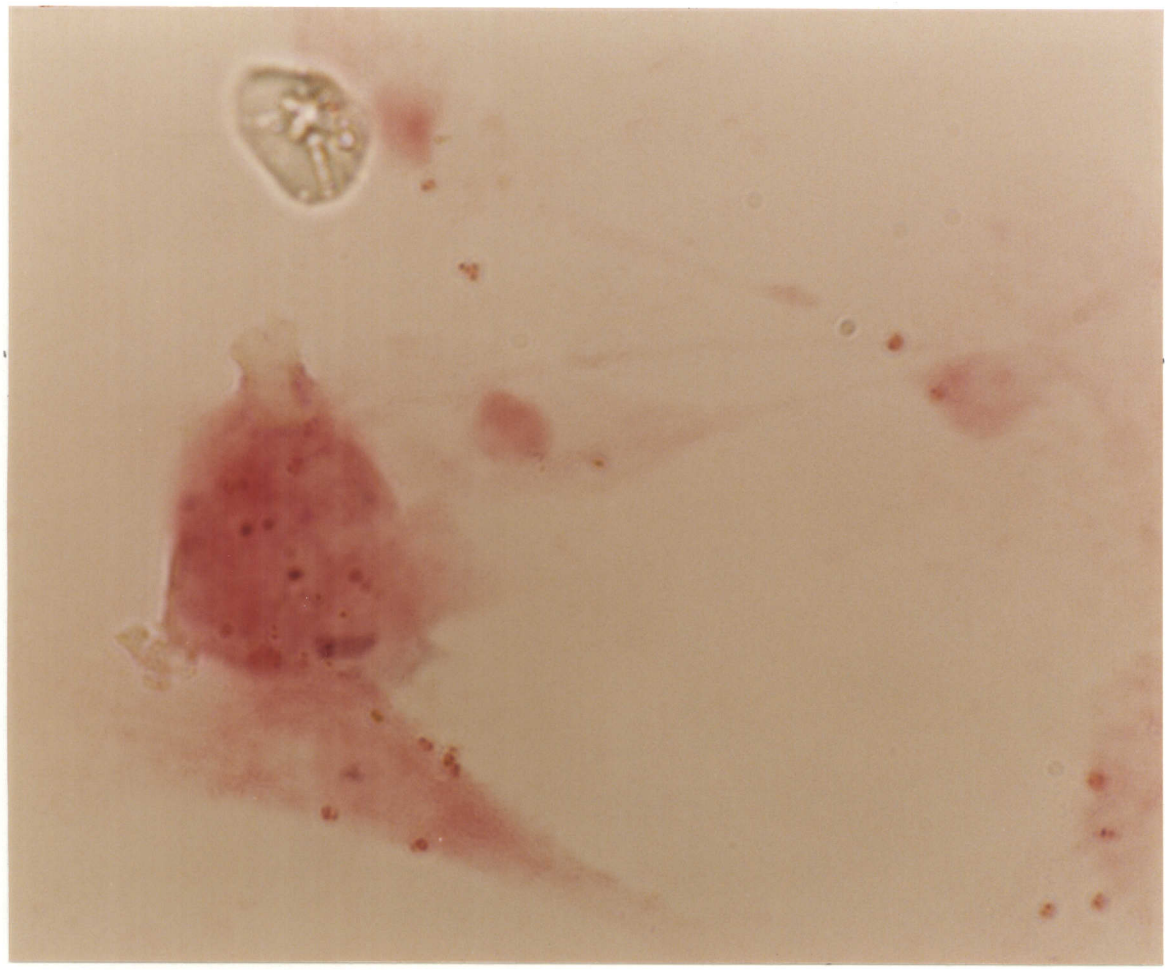


Figure 11. Nonslime-producing *S. epidermidis* adhered to corneal epithelial cells (specific adherence)

CHAPTER V

Discussion

This is the first time a large collection of ocular isolates of coagulase-negative staphylococci has been examined for the ability to produce slime. The incidence of slime production among 151 ocular isolates of coagulase-negative staphylococci from diseased eyes was 47 (31%), which is lower than the incidence found by other investigators (18, 27, 86) for cutaneous strains and much lower than isolates of "clinically significant" systemic infections. Only 3 of 32 strains (3%) from normal eyes produced low levels of slime and, interestingly, in these cases the slime-producing *S. epidermidis* strains were associated with other bacterial species in the eye (Table 6). Perhaps the presence of other persistent organisms in the eye triggers synthesis of slime by *S. epidermidis*. This possibility should be investigated. Interactions between normal cutaneous flora and staphylococci such as bacteriocins and lysozyme have been documented.

The Christensen tube method employed in our studies is not as sensitive as newer methods for slime detection, so the actual number of slime producers in our collection may be slightly higher. Christensen (20) reported that coagulase-negative staphylococci grown in TSB in polystyrene tissue culture plates, stained with crystal violet, and read on an ELISA reader provided higher

sensitivity and reliability than the tube method. The tube method was chosen for practicality in screening large numbers of isolates.

Under normal circumstances, the corneal epithelium acts as a natural barrier which must be breached by bacteria. However, an overwhelming inoculum or an immunosuppressed cornea can lead to corneal infection by many bacteria including *S. epidermidis*. The presence of a foreign body is important to the pathogenic capability of *S. epidermidis*. The combination of a foreign body such as a contact lens and a cornea which has been scraped or otherwise compromised can increase the virulence. Contact lenses and surgical instruments such as sutures or intraocular lenses also promote the ability of coagulase-negative staphylococci to cause infection (75).

Apparently the ability of microorganisms to infect the eye of a contact lens wearer depends upon lens composition and the length of time the lens is worn. Nissen and Speaker (63) noted that patients wearing disposable extended wear contact lenses were more likely to have corneal ulcers caused by Gram-positive organisms than other types of lenses. It has also been reported that lens hygiene is not as important in the prevention of ocular infection as was once thought (75). Contact lens data on the patients from which these strains were isolated were not available. Information should be obtained on the presence and types of contact lenses worn by the patients and related to our data on slime production

There were no outstanding differences in antimicrobial susceptibility patterns among slime-producing and nonslime-producing strains of ocular coagulase-negative staphylococci except for a slight trend toward higher MICs of oxacillin against slime-positive strains. Effective microbiological treatment,

i.e., the eradication or reduction of coagulase-negative staphylococci after antimicrobial treatment, were virtually equal, 89% and 91% of slime-positive and slime-negative strains, respectively.

The composition of staphylococcal slime has been satisfactorily analyzed in only a few strains. It is clear from the work of Hussain (47), Drewry (28), and Quie (67) that there is some variability among strains and that slime contains mostly glucose and glucosamine and 25% or less protein. Drewry (28) showed that the galactose, previously reported to be a major constituent of slime, was actually contamination from the agar which contains large amounts of galactose.

Quantitative and semi-quantitative staining methods used in this study have confirmed the presence of carbohydrates in 10 strains of slime-producing staphylococci. Ruthenium red staining demonstrated the presence of acidic mucopolysaccharides in most strains. Some variability among strains tested was noted between the safranin and ruthenium red staining. However, the slime from all strains tested demonstrated consistent periodic Schiff staining. We have also shown that uronic acid, a component of all acid muco-polysaccharides, exists in slime-producing coagulase-negative staphylococci in low and variable amounts compared to the amount of total carbohydrate present in slime (Table 12).

It is well documented that TSB enhances slime production in some strains of coagulase negative staphylococci. We have shown that some strains can produce slime in other media, even after having been glucose starved by passing to glucose-free media for relatively long periods of time. One strain (00085), isolated from a human corneal ulcer, and one strain (SD24924) from a

conjuctivitis patient who maintained *S. epidermidis* in the eye even after several days of treatment with topical tobramycin produce slime after growing in a medium containing no sugar. However, when stringently passed on solid media with no glucose, such as NaAc, they both lose the ability to form stainable biomass on the sides of plastic tubes.

Since some strains retained slime producing ability in the absence of glucose, it was hypothesized that some strains use a *de novo* pathway to produce slime, perhaps via a cycle involving formation of glycocalyx material from acetate or other precursors, deriving materials from dead cells during and beyond stationary phase, or by producing precursors of gluconeogenesis via the glyoxylate cycle. *E. coli* has been shown to employ this mode of gluconeogenesis when acetate is the sole carbon source (40, 60). We tested these theories by passing two strains (00085 and SD12783) which were shown to produce slime in glucose-free media to NaAc and TSA on alternating days for 6 days and testing slime production in TSB and NB daily. Heavy slime production was maintained in both strains when tested in TSB but was lost immediately by SD12783 and by 00085 on the second day of testing in NB. It has been shown that NB does not enhance slime production but that some strains of coagulase-negative staphylococci can produce slime in TSB without glucose (18) so either of these strains may be able to maintain slime producing ability in this medium.

These results provide evidence for short term storage of glucose or other precursors of slime formation among some strains of coagulase-negative staphylococci but discounts gluconeogenesis from acetate.

Slime has been widely reported to mediate attachment to surfaces and the multitude of reports demonstrating the significance of slime-producing staphylococci in clinical cases cannot be ignored (11, 19, 20, 22, 48, 52, 59, 64, 86) despite some conflicting reports. For example, Kotilainen (52) found that half of the coagulase-negative staphylococci isolated from septic patients at one hospital were slime-negative while Younger (79) reported 88% of *S. epidermidis* strains from cerebral spinal fluid shunts produced slime.

Slime was initially thought to mediate adherence to inanimate surfaces, and it does indeed have some role in such attachment. However, other factors such as bacterial cell surface hydrophobicity are also important. Staphylococcal cells can grow within the matrix of the slime and produce an adherent layer on a smooth surface. If the surface is a medical device in a human, the slime also plays a protective role against host immune responses and antimicrobial agents. Slime production is probably a stationary phase stress response.

The adherence of slime-producing coagulase-negative staphylococci to human tissue appears to be distinct from that of adherence to inanimate objects and is not well understood. Investigations of adherence of staphylococci is summarized in Table 1. Carbonero (16) found that slime production by *S. epidermidis* and *S. saprophyticus* is not a significant factor in adherence to human epithelial cells. Chugh (24) reported that 8 nonslime-producing strains were adherent to human pharyngeal epithelial cells, although they varied in the degree of adherence.

Our studies of adherence by *S. epidermidis* to human corneal epithelial cells (Table 13) were consistent with these findings. Some slime-producing strains adhered in large numbers to corneal epithelial cells while other slime-

producing strains did not. One strain (00143), a weak slime producer, adhered to corneal epithelial cells in large numbers. A nonslime-producing skin isolate, ATCC 17917, adhered to corneal epithelial cells, but not in large numbers. In our studies, many slime producing strains also adhered to the glass microscope slide while the nonslime-producer was associated only with the corneal cells. Lysozyme, present in human tears, did not affect adherence to corneal epithelial cells of 4 of 5 strains tested. One strain adhered in lower numbers after pretreatment with lysozyme. Therefore, specific adhesins may be unblocked by lysozyme treatment.

Adherence to human cells by coagulase-negative staphylococci is probably lectin-mediated and not related to slime production. Several adhesins have been described for coagulase-negative staphylococci (Christensen, Chugh, Mack, and Tojo) and a glycoprotein receptor has been isolated on the surface of pharyngeal epithelial cells (24). Staphylococcal adherence to corneal epithelial cells has not yet been described. These observations of adherence appear to support lectin-mediated adherence of complementary ligands on the bacteria and corneal epithelial cells. Strain 00085 showed a difference in adherence to 3 different corneal cell lines (Table 13). Additional studies may elucidate the specific receptors on the surface of corneal epithelial cells and other cells.

These studies show that fewer slime-positive strains of *S. epidermidis* are isolated from human eyes than from systemic infections. The composition of slime produced by differnt strains, the variability of slime production in different growth media, and the non-constant ability to adhere to corneal epithelial cells all demonstrate a variety of slime related characteristics among *S. epidermidis*

strains. Our studies demonstrate that cell culture is a useful model to study bacterial attachment to human corneal epithelial cells. It could possibly be used as a screening method to evaluate antimicrobial agents used for ocular infections.

Coagulase-negative staphylococci emerged as potential pathogens in circumstances where they possess the capacity to adhere to surfaces. We have only begun to appreciate and understand the mechanisms of attachment to inanimate surfaces and living cells of these bacteria which are constant commensals on the human cutaneous surface. Future studies should be directed toward the identification of the structural entities which allow attachment and colonization. Allowances should be made regarding the many variable factors associated with both the organisms and the host.

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