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Comparative Virulence of Human Isolates of Coagulase-Negative Staphylococci Tested in an Infant Mouse Weight Retardation Model

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Human infections caused by coagulase-negative staphylococci have steadily increased in numbers and severity. Causes may be the use of artificial prostheses, immunocompromising chemotherapy and radiation therapy, and sophisticated surgical techniques, to name a few. Although the infectivity of coagulase-negative staphylococci as a group has been well documented for humans, attempts to study the pathogenesis of infections caused by individual species of coagulase-negative staphylococci have been hampered by the lack of an animal model that is not refractory to infection by these organisms. In the study reported here, a 2-day-old-mouse weight retardation test was used to assay the virulence of 60 clinical and reference strains of coagulase-negative staphylococci. These strains represented eight species of coagulase-negative staphylococci. The most virulent strains were demonstrated to be of the species *Staphylococcus haemolyticus*, *S. saprophyticus*, and *S. epidermidis*. The data further suggest that production of slime is a marker of virulence in *S. epidermidis* and that intraspecies differences in virulence occur.

Coagulase-negative staphylococci are ubiquitous in nature and are often primary pathogens in human diseases such as urinary tract, endocardial, ophthalmic, otitic, wound, blood, intestinal, and osteomyelitic infections (9, 12, 18, 25). Infections caused by *Staphylococcus aureus* have correlated well with the ability to produce enzymes, toxins, and capsules (11). However, the virulence factors that coagulase-negative staphylococci use to enhance their pathogenesis in human hosts have not been thoroughly studied, although certain strains do produce virulence-associated factors such as slime, hemolysins, and enterotoxins (2, 4, 7, 8, 12, 22, 24).

Research into the factors that may potentiate virulence in coagulase-negative staphylococci has been hampered by the lack of a suitable animal model that is not refractory to infection by these organisms. In 1969 Chesbro et al. (3) tested the virulence of strains of *S. aureus* in mice. Although this species is armed with an impressive array of virulence factors, only 12% of the adult mice tested in that study were killed by intraperitoneal inoculation of 10^9 CFU of the organism. Thus, adult mice were found to have a high level of natural resistance to infection by this, the most pathogenic species of *Staphylococcus*.

In 1975 Namavar et al. (20) tested neonatal mice for their use as laboratory models for assaying the virulence of coagulase-negative staphylococci and *S. aureus*. After intracerebral inoculation, the 50% lethal dose of *S. aureus* ranged between 10^4 and 10^5 CFU and that of coagulase-negative strains ranged between 10^6 and 10^8 CFU. Neonatal mice were recommended as suitable animal models for the study of pathogenesis by coagulase-negative staphylococci. In 1979 McKay and Arbuthnott (19) studied the age-related susceptibility of mice to staphylococcal infections. In this study, mice were challenged by subcutaneous inoculation of staphylococci along the dorsal midline. Death and the development of lesions were recorded for mice that were 3 days, 10 days, and 21 days old at the time of injection. High-challenge doses (approximately 10^9 CFU) of *S. aureus* were required to establish lesions in 21-day-old adult mice. However, low-challenge doses (approximately 10^4 CFU) of some strains of *S. aureus* caused lesions in 3-day- and 10-day-old mice, with the ability to resist challenge developing at about the fifth day. A strain of *S. epidermidis* was also tested, and although it could not produce lesions in mice at low-challenge doses, at the higher dose of 10^8 CFU it produced mild lesions in 10-day-old mice and proved lethal for neonates. These neonatal-mouse model studies were further evaluated and reported in 1980 by Kinsman and Arbuthnott (13). Since it had been found earlier that using lesion formation as the sole criterion for assessment of virulence in the 3-day-old infant mouse was unsatisfactory for coagulase-negative staphylococci, although suitable for *S. aureus*, a weight gain index was developed as an alternative measure of virulence for coagulase-negative staphylococci. Their findings suggested that strains isolated from severe human infections caused a greater inhibition of weight gain than did strains from milder infections or environmental sources.

To further expand these studies with the neonatal mouse as the research model, reference and fresh clinical strains were identified to species level in the study reported here and were tested to determine whether individual species of coagulase-negative staphylococci differ in their virulence for infant mice and whether production of slime enhances the virulence of producer strains.

MATERIALS AND METHODS

Microorganisms. Forty-one clinical strains of *Staphylococcus* species were obtained from the Microbiology Section, Department of Pathology and Area Laboratory Services, Brooke Army Medical Center, Fort Sam Houston, Tex. Eighteen reference strains of infrequently encountered species of coagulase-negative staphylococci were obtained from Wesley Kloos, Department of Genetics, North Carolina State University, Raleigh: *S. capitis* D 2/1A-1, ATCC 27840, ATCC 27843, and RMS 841; *S. haemolyticus* D 22.11, DSM 20263, and RSM 8430; *S. hominis* ATCC 27844, D 22A-8, and RMS 8424; *S. saprophyticus* CCM 883, D 258-9, and WK 8173; *S. warneri* 8410727, Ca, and Wa; and *S. lugdunensis* 28036 and Ka. Three of these reference strains were coded to protect the privacy of the three patients who were infected by these bacteria: *S. lugdunensis* Ka, *S. warneri* Ca, and *S. warneri* Wa. *S. aureus* ATCC 25923 was obtained

from the American Type Culture Collection (Rockville, Md.).

Identification of clinical strains was done by using the API Staph-Ident system (Analytab Products, Plainview, N.Y.) and conventional methods and criteria described elsewhere (15). All strains were maintained at −196°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 5% glycerol and were subcultured to sheep blood agar 1 day prior to being injected into mice. Sheep blood agar cultures were incubated at 35°C.

Mice. Pregnant NIH Swiss mice were obtained from Harlan-Sprague-Dawley (Indianapolis, Ind.). Accurate dates of birth of each litter were determined by checking the pregnant animals twice daily for newborn mice. Litter sizes ranged between 5 and 12 sibling mice; the mean size was 8. Litters were housed individually with their lactating mothers. Individual 2-day-old mice were marked, weighed, and evenly distributed into two groups, test and control. Mice weighing 1.5 to 2.6 g were used for testing. Handling treatment during challenge did not result in cannibalism or rejection.

Weight retardation (WR) test for virulence. The coagulase-negative staphylococcus virulence test of Kinsman and Arbuthnott involved measuring the inhibition of weight gain in 3-day-old hairless mice, strain Sha-Sha, 2 days after challenge with a test culture of coagulase-negative staphylococci (13). Preliminary testing of their method in our laboratory showed that no differences in weight gain could be detected between control and infected (test) mice when we used outbred Swiss white mice in lieu of the Sha-Sha strain of mice. However, when the Kinsman-Arbuthnott method was modified so that 2-day-old Swiss mice were challenged with coagulase-negative staphylococci and the final weight of each mouse was determined at 1 day postchallenge, differences were discernible. At 2 days postchallenge, the infected mice recovered from the challenge dose and gained weight as rapidly as did the saline-injected control mice. Thus, a change in parameters was necessary in our study to overcome the higher natural resistance of the outbred strain of mice that we used.

In our modified infant mouse WR test the strains of *Staphylococcus* species were grown for 18 to 24 h in tryptic soy broth (Difco), the broth was centrifuged at 2,500 rpm for 10 min to sediment the cells, and the resulting sediment was suspended in normal saline (0.85%). Each test group of mice was injected subcutaneously along the dorsal midline with these suspensions of the *Staphylococcus* test culture (0.05-ml volumes; approximately 10^8 cells). Control mice were injected in a similar manner with only sterile normal saline. The number of mice in each group ranged between 3 and 6 sibling mice; the mean group size was 4. Each *Staphylococcus* strain was tested by using two or more litters of mice. All mice were returned to their mothers following the injections. After 24 h, each test and control animal was again weighed and mean weight gains for the control group and the test group in each litter were calculated. The WR statistic of the test animal group in each litter was calculated as a ratio of the difference between the mean weight gain of the control and test groups and the mean weight gain of the control group, expressed as a percentage.

Slime production. The ability of strains to produce slime from glucose was tested in tryptic soy broth with glucose (test broth) and without glucose (control broth) by the tube adherence method described by Christensen et al. (4). To determine the amount of slime produced by the test strains of coagulase-negative staphylococci, the broth was decanted and the glass tubes were allowed to dry. The internal walls of each tube were washed with diluted (1:4) Gram-safranin solution, and the amount of stained slime adhering to the walls was semiquantitated as 0 (absent), 1+, 2+, 3+, or 4+. All 60 strains were tested simultaneously to ensure consistency in measuring the quantity of slime produced by each strain.

Statistical analysis. Differences between the WR values of the 60 strains of *Staphylococcus* species were analyzed by the unpaired Student *t* test. The level of statistical significance was chosen to be 0.05.

RESULTS

The retardation of weight gain of infected 2-day-old sibling mice was greatly influenced by the inoculum size. As the number of bacterial cells increased, the infant mice became sicker and retardation of weight gain was more pronounced, regardless of the species of coagulase-negative staphylococci used in the challenge. Of the 60 strains, 10 strains of coagulase-negative staphylococci and 1 strain of *S. aureus* were retested by using infective doses of 10^7 and 10^9 CFU to demonstrate the effects that inoculum size might have upon their virulence for mice. The single *S. aureus* strain had WR values of 30, 91, and 100% at 10^7, 10^8, and 10^9 CFU of organisms, respectively. Several deaths occurred at the higher dose. The 10 strains of coagulase-negative staphylococci included *S. haemolyticus* (4 strains), *S. hominis* (1 strain), *S. warneri* (2 strains), *S. saprophyticus* (1 strain), and *S. epidermidis* (2 strains). The WR values for the 10 strains of coagulase-negative staphylococci ranged between 8 and 24% when an infective dose of 10^7 CFU of cells was used, with a mean value of 18%. At 10^8 CFU of cells, WR values ranged between 8 and 94%, with a mean value of 42.4%. WR values ranged between 35.8 and 100% when 10^9 CFU was used, with a mean value of 68%.

A total of 57 strains of coagulase-negative staphylococci and 3 strains of *S. aureus* were tested in this study for their pathogenicity by using an infective dose of 10^8 CFU of organisms (Table 1). The mean WR value for the 57 strains of coagulase-negative staphylococci was 32.8%. The three *S. aureus* strains were considerably more virulent for the test mice than were the coagulase-negative staphylococcus strains and had a mean WR value of 77.8%. The best delineation between species in the infant mouse WR test occurred when the challenge inoculum was 10^8 CFU.

Intraspecies and interspecies variability in virulence occurred among all species of coagulase-negative staphylococci (Table 1). The most virulent *Staphylococcus* species was the coagulase-positive organism *S. aureus*. The most virulent species of coagulase-negative staphylococci were strains of *S. haemolyticus*, *S. epidermidis* sensu stricto, and *S. saprophyticus*. No significant differences were observed between the WR values of the strains of these three organisms, although their mean WR values varied between 32.0 and 54.1%. The last two mentioned species were not as virulent for mice as was *S. aureus* ($P < 0.05$). However, strains of *S. haemolyticus* and *S. aureus* showed no significant differences in virulence for mice with the infant mouse WR test system. Species of lower virulence included *S. capitis*, *S. simulans*, *S. hominis*, *S. warneri*, and *S. lugdunensis*. For the purposes of the statistical study, the last two strains were analyzed together, since the two were originally identified by Kloos as *S. warneri*. Of all the staphylococci that were examined in this study, strains of *S. aureus*, *S. haemolyticus*, and *S. epidermidis* were the only ones to

TABLE 1. Comparative virulence of individual species of coagulase-negative staphylococci and *S. aureus* as measured by the infant mouse WR test

Staphylococcus spp. (no. of strains)	MWR[a] (%)	Species-to-species differences (P)[b]						
		S. haemolyticus	*S. epidermidis*	*S. saprophyticus*	*S. simulans*	*S. hominis*	*S. warneri* and *S. lugdunensis*	*S. capitis*
S. aureus (3)	77.8 (8.8)	NS	0.015	0.006	0.004	0.001	0.003	0.006
S. haemolyticus (10)	54.1 (9.2)		NS	NS	0.032	0.009	0.003	0.008
S. epidermidis (18)	42.0 (7.8)			NS	NS	0.043	0.009	0.001
S. saprophyticus (5)	32.0 (7.9)				NS	NS	NS	0.004
S. simulans (3)	26.3 (6.8)					NS	NS	NS
S. hominis (7)	18.9 (7.6)						NS	NS
S. warneri (6) and *S. lugdunensis* (2)	17.5 (4.0)							NS
S. capitis (6)	10.2 (3.5)							

[a] MWR, Mean WR value for the strains in each species. See Materials and Methods for calculation of WR values. Numbers in parentheses are standard errors.
[b] NS, No significant species-to-species differences ($P > 0.050$) between WR values of individual strains of *Staphylococcus* spp. by the Student t test.

occasionally cause death of the infant mice at the challenge dose.

Slime was produced by several species of coagulase-negative staphylococci (Table 2). The organisms having the largest percentage of strains of slime producers were *S. lugdunensis*, *S. epidermidis*, and *S. warneri*. Other than *S. hominis* and *S. haemolyticus*, most other species produced little or no slime. The three strains of *S. aureus* that were tested in this study did not produce slime. A previous study (unpublished) in this laboratory of 138 strains of coagulase-negative staphylococci representing seven species demonstrated 39.1% of these strains to be slime producers. Strains of *S. epidermidis* represented 65.2% of the total number of strains of staphylococci encountered in that unpublished study and 34.1% of the total number of strains that produced slime.

Eighteen strains of *S. epidermidis* were grouped according to their virulence for neonatal mice to determine whether a positive relationship existed between virulence and the ability to produce slime (Table 3). The more virulent (higher WR value) that a strain of *S. epidermidis* was for mice, the more likely that it was to produce slime. Unlike the case with *S. epidermidis*, there was no difference between the virulence of slime-producing strains of *S. warneri* and that of non-slime-producing strains. There were not enough slime producers of the other species that were examined in this study to test them for a possible intraspecies relationship between slime production and WR values.

TABLE 2. Slime production by species of coagulase-negative staphylococci

Staphylococcus spp.	No. of strains	Slime producers (% of total)[a]
S. lugdunensis	2	100.0
S. epidermidis	18	55.6
S. warneri	6	50.0
S. hominis	7	28.6
S. haemolyticus	10	20.0
S. capitis	6	16.7
S. saprophyticus	5	0.0
S. simulans	3	0.0

[a] Of total species, 35.1% were slime producers.

DISCUSSION

In the study reported here, eight of the species that are the causative agents of 90% or more of all coagulase-negative staphylococcus diseases were found to differ in their virulence for infant mice when the WR test was used. Without a doubt, of these eight, *S. haemolyticus* was the most virulent and was as significantly virulent for infant mice as were strains of *S. aureus* ($P > 0.05$) (Table 1). This organism is one of the most prevalent colonizers of human skin and is the etiologic agent in more than 11% of all cases of coagulase-negative staphylococcus disease (9, 14, 16, 18, 23). *S. epidermidis*, the second most virulent species, is the most common colonizer of human epidermis and is by far the most frequent coagulase-negative staphylococcus etiologic agent, accounting for more than 62% of all human coagulase-negative staphylococcus infections (9, 14, 16, 18, 23). The third most virulent species, *S. saprophyticus*, colonizes primarily the periurethra of young women of early childbearing age and is the second most important coagulase-negative staphylococcus urinary tract pathogen in this age group of female patients (16). Thus, a clear relationship exists between the ability of these three species of coagulase-negative staphylococci to cause weight loss in mice and their ability to colonize and to cause disease in humans.

In most human infections, the rank order of the frequencies of occurrence as pathogens of the eight predominant species of coagulase-negative staphylococci is similar to the rank order of the cutaneous population density of each (6, 14, 25). Thus, the most common coagulase-negative staphylococcus pathogen, *S. epidermidis*, typically produces the largest coagulase-negative staphylococcus populations on human epidermis. Some investigators suggest that the pathogenicity of coagulase-negative staphylococci is explained best by an increased exposure of a host to a high colonization load, which subsequently leads to a greater number of infections (6, 25). However, this simplistic view of coagulase-negative staphylococcus opportunism does not entirely explain the emergence of these organisms as significant pathogens in certain types of infections that previously were rarely attributed to them (6, 12, 23), nor does it fully explain the predilection of some species, such as *S. saprophyticus*,

TABLE 3. Relationship between WR in mice and in vitro production of slime by strains of *S. epidermidis* sensu stricto

WR group[a] (no. of strains)	MWR[b] (%)	Slime[c]
High WR (5)	88.0	4+ (2), 3+ (1), 1+ (2)
Medium WR (4)	45.1	3+ (1), 1+ (2), − (1)
Low WR (9)	16.3	2+ (2), − (7)

[a] The strains were apportioned into WR groups based upon the following ranges of WR values: high WR, 70 to 100%; medium WR, 30 to 69%; and low WR, 0 to 29%.

[b] MWR, Mean WR value for the strains in each virulence group. Each strain was tested for virulence by using separate litters of mice.

[c] +, Semiquantitative amount of slime produced by each strain. −, No slime produced. See Materials and Methods. Numbers in parentheses represent the number of strains having the characteristic.

for colonizing and infecting specific body sites, such as the urogenital tract (16).

Unlike the case with some gram-negative pathogens, only a few factors have been identified that enhance the virulence of coagulase-negative staphylococci, and slime appears to be one of these. This substance is produced principally by the species *S. warneri*, *S. lugdunensis*, and *S. epidermidis* and by rare strains of *S. hominis*, *S. haemolyticus*, and *S. capitis* (Table 2). Baddour et al. (1) reported that *S. epidermidis* is the only coagulase-negative staphylococcus species that produces virulence factors of any importance. Production of slime was the foremost marker of virulence for this species found in their research. Slime was demonstrated in other studies to function as a virulence factor by promoting the adherence to and colonization of artificial prostheses and indwelling catheters by slime-producing strains of *S. epidermidis*, thereby providing the initial steps leading toward foreign-body-associated infections (4, 12). Slime may contribute even more to the virulence of *S. epidermidis* than just the ability to adhere. Gray et al. (10) demonstrated that the lymphoproliferative response of mononuclear cells decreases when these cells are exposed to slime and that this decrease contributes to the persistence of slime-producing strains in foreign-body infections. Noble and co-workers (21) demonstrated that slime does not affect phagocytosis by neutrophils, but it does cause a generalized loss of bacteriocidal activity by these cells. As neutrophils are exposed to greater quantities of slime, the greater is the loss of this activity. Thus, slime appears to provide producer strains of coagulase-negative staphylococci with the ability to adhere, colonize, and persist in a host and is possibly the most important virulence factor for the species *S. epidermidis*.

The use of 2-day-old immunologically immature mice for assaying the effects that potential virulence factors have upon pathogenesis minimizes the interference that may be due to individual variations in immune response seen between mature sibling mice. Thus, the WR test result for each coagulase-negative staphylococcus strain more accurately reflects the virulence of the test strain for the infected mice than the effects of the immune response of the host upon the infectivity of the challenge organism. Since the mice were injected subcutaneously, the ability of the organisms to produce illness rather than the ability to invade was measured, invasion being a task that opportunistic bacteria are typically unable to accomplish without a breach in the epidermal barriers of the host.

A pronounced retardation of weight gain occurred with our infant mice when injected with *S. epidermidis*. Strains that produced a greater quantity of slime in the in vitro adherence test produced a greater retardation of weight gain. Furthermore, all highly virulent strains, but only 22% of strains of low virulence, produced this substance (Table 3). Thus, a strong relationship exists between the quantity of slime produced and the degree of virulence for mice that was measured for strains of *S. epidermidis*. Since the amount of slime that was injected into each mouse was not measured and since purified slime was not injected into mice to determine whether it does, indeed, cause retardation of weight gain, the cause of the retardation effects in this study remain undetermined.

The basis for the virulence of other species of coagulase-negative staphylococci such as *S. haemolyticus* and *S. saprophyticus* is apparently quite different from that of *S. epidermidis*, since these two species produced little or no slime in the tube adherence test and yet were virulent for mice in this study. Hemolysins, enterotoxins, and certain enzymes all have been postulated to be virulence factors that are produced by strains of *S. haemolyticus* (2, 8, 22). However, other than an ability to adhere better to exfoliated uroepithelial cells than to skin or buccal cells (5, 17), the factors used by *S. saprophyticus* to enhance its virulence are to our knowledge unexplored. Additional WR studies are needed to identify factors that enhance the virulence of this species for young women of child-bearing age. The three strains of *S. aureus* that we studied also did not produce slime but were highly virulent for newborn mice. The pathogenicity of this species is well known. It derives its virulence from a large array of well documented potent virulence factors such as delta toxin, coagulase, fibrinolysin, and others (11). Most of these factors are not produced in cultures of coagulase-negative staphylococci, hence the importance of slime in diseases caused by organisms such as *S. epidermidis*.

The WR test provided evidence that strains of coagulase-negative staphylococci vary in their virulence for infant mice and that slime may serve as a potentiator of virulence in producer strains. The virulence of *S. epidermidis* for infant mice as measured by weight loss related well to the production of slime. The virulence of individual species of coagulase-negative staphylococci in the WR test correlates well with the frequency of occurrence of human infections caused by these species of coagulase-negative staphylococci and, with strains of *S. epidermidis*, with the ability to produce slime. It seems feasible that the test can be used to study further the importance of factors other than slime in the enhancement of the virulence of coagulase-negative staphylococci for humans.

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