Pathogenicity and virulence of coagulase negative staphylococci in relation to adherence, hydrophobicity, and toxin production in vitro

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Abstract

Aims—To study the pathogenicity and virulence characteristics of *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus sapro-phyticus*.

Methods—BALB/c mice were challenged intraperitoneally with graded doses of three strains belonging to each species. LD_{50s} were measured for each strain. Haemolysin (α- and δ-) and enzyme (DNAase, lipase, and esterase) production in vitro were measured qualitatively and quantitatively. Adhesion to plastic was measured and related to cell surface hydrophobicity among the strains.

Results—*S saprophyticus* proved the most virulent ($LD_{50} = 2 \cdot 7 - 2 \cdot 9 \times 10^7$ cfu/g body weight) while *S epidermidis* was the least virulent ($LD_{50} = 6-8 \times 10^7$ cfu/g body weight). An enlarged spleen was the most common macroscopic pathological feature. Kidney, liver, and more rarely peritoneal abscesses were also seen in the infected animals. No direct correlation was found between adherence in vitro, cell surface hydrophobicity, or toxin/enzyme biosynthesis and virulence in mice.

Conclusion—The results show that coagulase negative staphylococci are pathogenic in BALB/c mice. It is clear that these bacteria can cause invasive disease. However, the in vitro characteristics of coagulase negative staphylococci are not related to the pathogenicity of the organisms in mice.

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The role of coagulase negative staphylococci in different human infections has been recognised, particularly after the temporary or long term implantation of plastic devices into the body,[1-5] and other invasive interventions such as irradiation and immunosuppressive treatment.[6-8]

Coagulase negative staphylococci have a pathogenic role in infections of the urinary tract, cerebrospinal fluid shunts and prostheses; in septicaemias in premature neonates and in adults receiving intensive care; in peritonitis in patients receiving continuous ambulatory peritoneal dialysis; and in postoperative

endocarditis.[9] Szabo *et al* found that these organisms are the second most common pathogens in infected burn lesions. (Szabò J, et al. Abstract presented at the 16th International Congress of Chemotherapy, Jerusalem, 1989).

Staphylococcus epidermidis proved the most common cause of the infections mentioned above[2 3 6 7 10] except in those of the urinary tract where *Staphylococcus saprophyticus* dominates.[11] *Staphylococcus haemolyticus* seems to rank third in order of frequency.[12]

The study of the pathogenesis of coagulase negative staphylococci has shown that several extracellular metabolites are produced by these micro-organisms.[13-15] There are also many substances on the surfaces of the bacteria involved in pathogenicity, such as haemagglutinin, capsule, slime, fibronectin binding proteins and collagen binding proteins.[16-23]

The aim of the present study was to investigate the virulence of the three most common coagulase negative *Staphylococcus* species in experimental infections of mice on the basis of lethality, splenomegaly, and the prevalence of tissue abscesses in relation to hydrophobicity, adherence, and toxin production.

Methods

BACTERIAL STRAINS

S epidermidis (three strains) were isolated from blood or wound discharge from patients with left atrioventricular stenosis after valve implantation, severe aplastic anaemia, and pneumonia. *S haemolyticus* (three strains) were isolated from the skin of a burn patient, from the blood of a case of septicaemia, and from the urine of a patient with prostatitis. The two clinical *S saprophyticus* strains were from the urine of a patient with chronic pyelonephritis and from the wound infection of a burn patient, respectively. The third strain was the reference strain *S saprophyticus* HNCMB 110008. The strains were identified according to a combined taxonomic scheme derived from Kloos and Schleifer[24] and Akatov *et al*.[25] The strains were stored in trypticase-soy broth containing 25% glycerol at $-20°C$ and subcultured at six monthly intervals. Full details are presented in table 1.

ADHERENCE
Adherent growth was examined using the

Table 1   Clinical data of coagulase negative staphylococcal strains

Staphylococcus sp	Strain No	Patient	Department	Diagnosis	Specimen
S epidermidis	5905	Male	Paediatrics	Pneumonia	Blood
S epidermidis	8602	Male	Paediatrics	Aplastic anaemia	Blood
S epidermidis	8915	Female	Cardiac surgery	Left atrioventricular stenosis after valve implantation	Wound discharge
S haemolyticus	367	Male	Urology	Prostatitis	Urine
S haemolyticus	9381	Female	Dermatology	Burn	Wound swab
S haemolyticus	11684	Female	Paediatrics	Sepsis	Blood
S saprophyticus	3876	Female	Internal medicine Nephrology	Chronic pyelonephritis	Urine
S saprophyticus	4922	Male	Dermatology	Burn	Wound swab
S saprophyticus	110008 HNCMB reference strain				

tube adherence method described by Christensen et al.[16] Cultivation of each of the strains was performed in both trypticase-soy broth containing 1% glucose (TSB, Difco Laboratories Detroit, Michigan, USA) and in 1% glucose-meat broth prepared in our laboratory. Each test was repeated five times.

HYDROPHOBIC INTERACTION
CHROMATOGRAPHY (HIC)
HIC was performed on octyl-sepharose gel (Pharmacia, Uppsala, Sweden). Three 1 cm columns of gel in Pasteur pipettes were equilibrated with 4·5 ml of 0·02 M sodium phosphate buffer (pH 6·8) and 0·1 M and 0·5 M ammonium sulphate solution in 0·02 M sodium phosphate buffer (pH 6·8) for each strain. A standardised suspension of bacteria (100 µl) harvested from modified Columbia Agar Base (Oxoid, Basingstoke, Hants, England) supplemented with 5% defibrinated bovine blood was applied to the gel columns.

The same suspension of bacteria was added to 3 ml buffer as a control. Immediately after adding the bacterial suspension to the gel, non-adsorbed bacteria were eluted from the gel with 3 ml of buffer. The absorbance of eluates were measured at 540 nm, and the percentage of bacterial binding to the gel was calculated. The control was considered as 0% binding; thus lower absorbance values meant positive binding.

IMPROVED SALT AGGREGATION TEST (ISAT)
This was performed by mixing one drop of standardised bacterial suspension with one drop of ammonium sulphate solution (0·2 M to 3 M) coloured with methylene-blue on white hydrophobic paper. The ISAT value was taken as the lowest molarity of ammonium sulphate at which aggregation occurred within five minutes. If aggregation occurred only at molarities of or above 1·5 M, bacteria were regarded as hydrophilic.[17]

The haemolytic activity of the strains was examined by growth for 24 hours on layered nutrient agar plates supplemented with defibrinated human, rabbit, horse and sheep blood, respectively.

TOXIN PRODUCTION IN VITRO
Each strain was grown for 18 hours by shaking in either brain-heart infusion broth (for lipase, esterase, and DNAase), yeast dialysate medium[26] (for α-haemolysin), or Gladstone and van Heyningen[27] medium (for δ-

haemolysin). Then cultures were centrifuged to remove the bacteria and sodium thiomersalate (1 in 1000 of a 1% solution) was added as a preservative to the supernatant fluids.

TOXIN ASSAYS
α-haemolysin
Serial doubling dilutions of toxin (0·2 ml) were made in saline and equal volumes of 2% (v/v) fresh rabbit erythrocyte suspension added to wells of a microtitre plate. The plate was incubated at 37°C for one hour.

Haemolysin titre was taken as the reciprocal of the highest dilution of toxin just causing 50% haemolysis of the erythrocytes.

δ-haemolysin
Serial doubling dilutions of toxin (0·2 ml) were made in saline and equal volumes of 2% (v/v) fresh human erythrocyte suspension added to wells of a microtitre plate. The plate was incubated at 37°C for one hour. The haemolysin titre was taken as the reciprocal of the highest dilution of toxin just causing 50% haemolysis of the erythrocytes.

DNAase, lipase, and esterase
Serial dilutions of toxin (0·1 ml) in saline were added to wells cut in agar containing either 1% DNA, 1% glycerol tributyrate, or 1% Tween 80 as enzyme substrate. The presence or absence of zones of clearing (lipase), precipitation (esterase), or clearing after addition of hydrochloric acid (DNAase) were taken as an indication of positive enzyme activity.

PREPARATION OF BACTERIAL SUSPENSION FOR USE IN THE INFECTION MODEL
Strains were precultured on blood agar at 37°C for 24 hours, followed by subculture for a further 24 hours at 37°C on modified Columbia Base Agar plates (Difco Laboratories, Detroit, Michigan, USA, or Oxoid Ltd, Basingstoke, Hants, England) containing 5% bovine blood (Phylaxia, Budapest, Hungary) and 2% sodium chloride. Bacteria were collected from the plates with sterile cotton wool swabs and suspended in a physiological dextran solution (Fluidex 40, Polfa, Warsaw, Poland) as microcarrier.[28] Basic bacterial suspensions were spectrophotometrically standardised from which serial dilutions of known bacterial counts were prepared. At the same time a calibration curve was made to determine the number of colony forming units (cfu) corresponding to different bacterial densities.

Table 2   Adherence and hydrophobicity of strains of different species of coagulase negative staphylococci

| Test strains | Adherent growth on plastic | Mean (SEM) HIC value* in: | | ISAT value† |
		0·02 M phosphate buffer	0·1 M ammonium sulphate	
S epidermidis				
8602	No	43·5 (0·58)	75·5 (11·0)	1·45 (0·06)
8915	Yes	23·0 (3·37)	47·5 (1·9)	1·50 (0)
5905	No	25·0 (4·62)	56·5 (2·9)	1·45 (0·06)
S haemolyticus				
367	No	94·7 (5·3)	95·5 (8·1)	0·50 (0)
9381	No	48·5 (6·4)	74·0 (0)	0·35 (0·17)
11684	No	53·0 (0)	95·5 (1·7)	1·45 (0·06)
S saprophyticus				
3876	No	24·0 (1·2)	32·0 (5·8)	1·05 (0·06)
4922	No	34·5 (1·7)	76·3 (6·0)	1·50 (0)
110008	Yes	8·0 (2·3)	18·5 (0·6)	1·67 (0·26)

*Expressed as per cent of bacteria adsorbed on to octyl-sepharose gel relative to a suspension of bacteria in buffer.
†The lowest molarity of ammonium sulphate at which aggregation occurred.

MOUSE INFECTION MODEL

Four month old, inbred BALB/c mice were grouped according to sex and weight. The random selection of mice in this population followed a natural distribution. Four groups of male mice and four groups of female mice were challenged with graded doses of each bacterial strain calculated on the basis of body weight. Mice were injected intraperitoneally with 0·3–0·5 ml (depending on their weight) of the proper bacterial suspension warmed to body temperature. The same volume of Fluidex 40 was injected into control mice. The mice were fed freely and their behaviour was observed for 10 days after infection.

Mice dying within two days of challenge were dissected immediately; those surviving for 10 days were sacrificed and examined for changes in the peritoneum, liver, spleen and kidneys. Spleens of non-infected mice measured 17–20 mm. In infected mice, spleens measuring 21–25 mm were regarded as displaying moderate splenomegaly, and spleens above 25 mm as displaying advanced splenomegaly.

Results

ADHERENCE AND HYDROPHOBICITY/ HYDROPHILICITY

Only one of the three *S epidermidis* strains and one of the three *S saprophyticus* strains adhered well to plastic, while two of each

Table 3   Haemolysin production in vitro by strains of different Staphylococcus species

| Staphylococcus strain | Titre of | |
	α-haemolysin	δ-haemolysin
S epidermidis		
8602	1/16	1/8
8915	1/4	1/2
5905	1/2	
S haemolyticus		
367	1/4	
9381	1/4	
11684	1/8	1/8
S saprophyticus		
3876	1/4	
HNCMB 110008	1/2	
4922	1/8	1/2

species failed to adhere. None of the *S haemolyticus* strains was adherent. All three *S epidermidis* strains proved hydrophilic in both tests. Two of the three *S haemolyticus* strains were hydrophobic in both assays, while strain *S haemolyticus* 11684 was hydrophobic by HIC and hydrophilic by ISAT. Strain 3876 was fully hydrophilic by HIC but moderately hydrophobic by ISAT; HNCMB 110008 was hydrophilic in both tests; strain 4922 was hydrophobic in both tests. As a whole, no association was found between adherence and hydrophobicity or hydrophilicity of the strains (table 2).

TOXIN PRODUCTION

The strains differed considerably in the yield of haemolysin in culture supernatant fluids (table 3). Slight DNAase activity was shown only by *S saprophyticus* strain 110008, and slight lipase activity was detected in *S epidermidis* strain 8602; esterase was produced by *S haemolyticus* strain 11684, and by *S saprophyticus* strains 3876 and 4922 (data not shown).

PATHOGENICITY AND LETHALITY TO MICE

The same challenge dose of bacteria killed more male mice than females. All three strains of each *Staphylococcus* species were pathogenic for mice but there was a pronounced difference between the lethal doses of the different bacterial species: *S saprophyticus* proved the most virulent (LD_{50} = 2·7–2·9 × 10^7 cfu/g body weight); *S haemolyticus* was somewhat less virulent (LD_{50} = 2·9–3·8 × 10^7 cfu/g body weight); and the least virulent was *S epidermidis* (LD_{50} = 6–8 × 10^7 cfu/g body weight). These differences in lethality were significant ($p < 0·0001$). However, within each species the virulence of the various strains differed. For this reason results are shown as dose ranges instead of doses characterised by exact numbers.

Each coagulase negative *Staphylococcus* species caused visible organ damage; the most common macroscopic lesion was splenomegaly (verified by histological examination) (fig 1) in mice sacrificed on the 10th day after infection; liver, peritoneal, or kidney abscesses were also sometimes observed.

Table 4 shows that strains of *S epidermidis* caused splenomegaly most frequently and those of *S haemolyticus* rarely. By studying the prevalence of splenomegaly according to the species injected, more than half (52·4%) of all the cases of splenomegaly were found in the group infected with *S epidermidis* (fig 2), although mice challenged with *S epidermidis* made up only 79 of the 245 survivors. The different coagulase negative species, therefore, cause splenomegaly at significantly different frequencies ($p < 0·001$). On the other hand, the numbers of cases of splenomegaly were directly proportional to LD_{50} ($p < 0·001$) of each of the three species (data not shown).

Liver abscesses were found in 28 of the 245 surviving mice: 28% of them had been challenged with *S saprophyticus*, 72% with *S epidermidis*. No liver abscesses occurred after the injection of *S haemolyticus* indicating that the
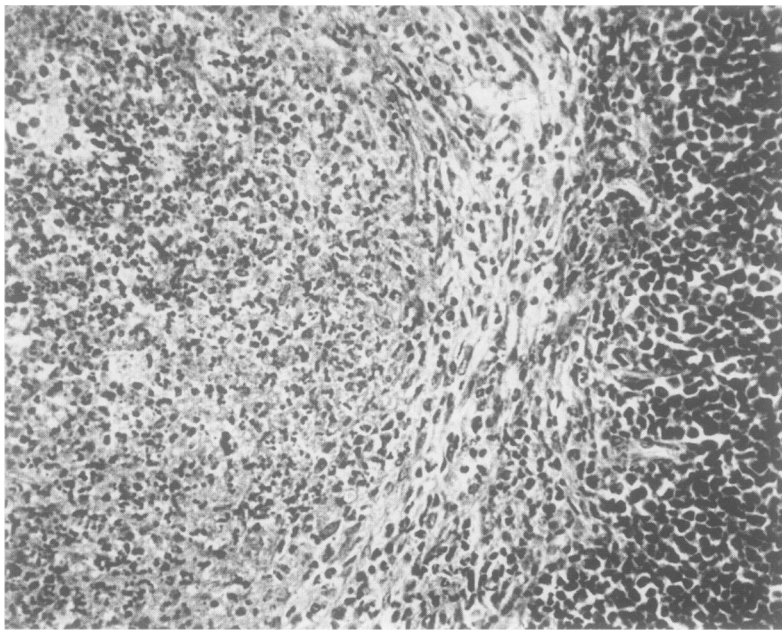
Figure 1   Histological appearance of splenic abscess in mouse infected with coagulase negative staphylococci (haematoxylin and eosin).

Figure 2   Incidence of splenomegaly in relation to intraperitoneal infection of mice with different species of coagulase negative staphylococci.

development of a liver abscess may be species specific (p < 0·001). In the case of *S epidermidis* the frequency of the appearance of liver abscesses was directly proportional to the numbers of bacteria injected (p < 0·001). There was no significant difference between male and female mice.

Bilateral kidney abscesses were found in 11 mice infected with *S epidermidis*, amounting to 13·9% of the survivors. Kidney abscesses were 2·7 times more common in male than in female mice.

Discussion

Mice and rats have been used most frequently to study experimental coagulase negative staphylococcal infections, although Holmberg[29] demonstrated a causative role for *S epidermidis* in bovine mastitis. Previous studies partly agree with our results. We cannot fully compare our data as a comparative study of the pathogenicity of *S epidermidis*, *S haemolyticus*, and *S saprophyticus* has not been carried out before. In our experiments each of the three staphylococcal species proved pathogenic for BALB/c mice. Dextran solution was a good microcarrier for the cocci when injected
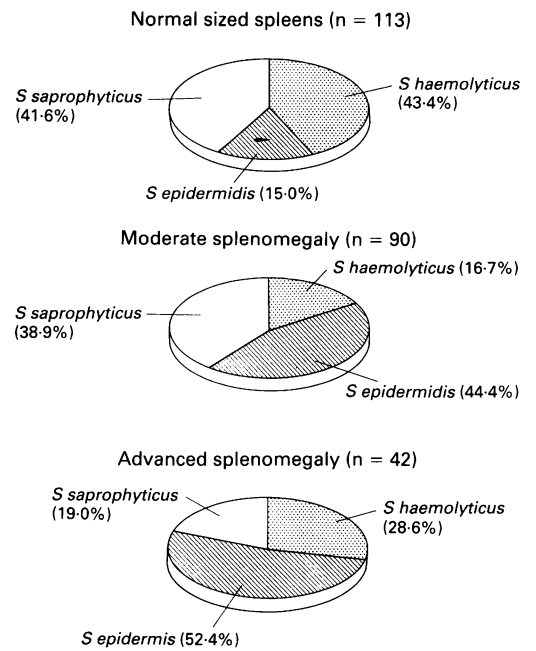
intraperitoneally.[28 30] According to the observations of Ichiman and Yoshida,[31] only 13 of 300 randomly chosen freshly isolated clinical strains of *S epidermidis* were virulent for mice. Namavar *et al*[32] found that the LD_{50} of different biotypes of *S epidermidis* ranged from 7·1–7·8 × 10^5 following intracerebral challenge of newborn mice. Christensen *et al*[20] showed that mice were not infected by *S epidermidis* without the use of foreign body implants. Baddour *et al*[33] found that with an inoculum of 1 × 10^7 cfu endocarditis was established in all of 77 rats injected with *S epidermidis* but in only five (12·5%) of 40 animals injected with *Staphylococcus hominis*. Jonsson *et al*[34] showed that *S epidermidis* and *S haemolyticus* were not pathogenic in a mouse mastitis model, even when a large dose of inoculum was used, while in the neonatal mouse weight gain test four of nine coagulase negative staphylococcus strains caused weight loss suggesting some virulence.

In our present study each of the clinical strains of *S epidermidis*, *S haemolyticus*, and *S saprophyticus* has been shown to be pathogenic for mice according to the criteria of Ichiman and Yoshida.[31] The degree of virulence is similar to that of methicillin resistant strains of *Staphylococcus aureus*[27]; *S saprophyticus* is the most virulent, *S haemolyticus* somewhat less so, and *S epidermidis* is the least virulent. All strains of the three *Staphylococcus* species are invasive in mice with an intact immune system after intraperitoneal challenge. Considerable differences were seen in mouse virulence among the strains of the same species, which was similar to the findings of Namavar *et al*.[32]

Our results show the complexity of coagulase negative staphylococcal infections and indicate the present insufficiency of in vitro tests as predictors of pathogenicity of a given

Table 4   Number and percentage of normal size and enlarged spleens of mice 10 days after infection with different species of coagulase negative staphylococci

Spleen size	No (%) of mice infected with:		
	S epidermidis	*S haemolyticus*	*S saprophyticus*
Normal (<20 mm)	17 (21·5)	49 (64·5)	47 (51·2)
Moderate splenomegaly (20–25 mm)	40 (50·6)	15 (19·7)	35 (38·9)
Advanced splenomegaly (>25 mm)	22 (27·8)	12 (15·8)	8 (8·9)
Splenomegaly (total)	62 (78·4)	27 (35·5)	43 (47·8)

coagulase negative staphylococcus strain or species. The lack of correlation between in vitro adherence and hydrophobicity suggests that different surface components determine these two properties. On the other hand, the presence or absence of these characters in vitro gives no information about the severity and outcome of infection, because no association was found between in vitro adherence, hydrophobicity or hydrophilicity, and lethality. Toxin production in vitro by coagulase negative staphylococcus strains may reflect the severity of the infection, as one strain of S epidermidis which elaborated the highest titres of both haemolysins (compare table 3 and fig 2) also had the lowest LD_{50}. The role of haemolytic toxins in the pathogenesis of coagulase negative staphylococcal infections is far from clear because it is not known whether haemolysin production occurs in vivo, and how the toxins might interact with other bacterial and host factors.[13]

Similarly, Lambe et al,[35] in a subcutaneous abscess model initiated by implantation of catheter segments coated with each of five species of coagulase negative staphylococci, were unable to relate abscess formation to the presence of glycocalyx or the production of various toxins in vitro. All S haemolyticus strains produced haemolysins in vitro (table 3), suggesting that haemolysin may contribute to the high virulence of this species. In contrast, the higher virulence of S saprophyticus is impossible to interpret on the basis of our in vitro data. However, Beuth and coworkers[18] have shown that S saprophyticus is unique among coagulase negative staphylococci because it possesses at least three types of specific surface lectin. The presence of high amounts of these lectins on the surface of the cocci, when injected into the peritoneal cavity, may contribute to their virulence by altering host macrophage function.[19]

Experimental S epidermidis infection is characterised by a low LD_{50} and a significantly high incidence of splenomegaly in the survivors. This is the first report, as far as is known, to show the spleen as a target organ for coagulase negative staphylococci. Splenomegaly is usually caused by lymphoproliferation and may be associated with the ability of staphylococcal extracellular slime to stimulate production of immunologically competent cells,[36] but doubts about the purity of the material used in these experiments and difficulties in distinguishing some surface components of coagulase negative staphylococci[37] may render this mechanism open to doubt. The strains used in the present study, although shown to have variable adherence to plastic, were not examined for the presence of slime. As yet we cannot ascribe any particular property to the coagulase negative staphylococci which could be responsible for the features seen in our mouse model of infection.

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