NOVOBIOCIN RESISTANCE AND VIRULENCE OF STRAINS OF \textit{STAPHYLOCOCCUS SAPROPHYTICUS} ISOLATED FROM URINE AND SKIN

F. Namavar*, J. de Graaff†, C. de With‡ and D. M. MacLaren*

*Laboratory of Medical Microbiology, †Department of Oral Microbiology and ‡Department of Medical Statistics, Free University, Amsterdam, The Netherlands

In recent years there has been a growing awareness of the potentially pathogenic role of coagulase-negative staphylococci. \textit{Staphylococcus epidermidis} strains have been isolated from patients with endocarditis associated with damaged or artificial valves; Spitz-Holter valves infected by these organisms often have had to be removed (Holt, 1969 and 1971). Mitchell (1968) found that urinary infections attributed to the insertion of surgical instruments into the urinary tract, or to pathological abnormalities, were caused almost entirely by various \textit{Staphylococcus} subgroups, especially \textit{Staphylococcus} subgroup II (Baird-Parker, 1963 and 1965). In spontaneous infections, \textit{Micrococcus} subgroup 3 (M3) strains (Baird-Parker, 1963 and 1965) predominated; they were also almost invariably the strains found causing cystitis and acute pyelonephritis in domiciliary practice. In general practice, acute uncomplicated infections due to coagulase-negative staphylococci belonging to \textit{Staphylococcus saprophyticus} biotype 3 (Baird-Parker, 1974) have been reported by Maskell (1974), Meers, Whyte and Sandys (1975) and Sellin \textit{et al.} (1975). However, two recent surveys have shown that the syndrome of non-hospital urinary infection due to strains other than those of \textit{S. epidermidis} (subgroups II and VI) is not caused only by subgroup M3, nor does it occur only in young women. These two surveys have suggested that organisms belonging to Baird-Parker's subgroups M3 and M5 may be second only to \textit{Escherichia coli} in respect of their frequency of occurrence as urinary pathogens of young women seen in the course of domiciliary practice (Crump, Pead and Maskell, 1976; Pead, Crump and Maskell, 1977).

Nord \textit{et al.} (1976) reported the isolation of coagulase-negative staphylococci from urinary infections and classified them according to the scheme of Kloos and Schleifer (1975a, b and c). They found that strains isolated from urinary tract infections could be placed in the various staphylococcal species of the Kloos and Schleifer classification, e.g., \textit{S. saprophyticus}, \textit{S. hominis}, \textit{S. cohnii}, \textit{S. warneri}, \textit{S. capitis} and \textit{S. simulans}. \textit{S. haemolyticus} strains, although found in urinary tract infections, were isolated chiefly from wound infections; this suggests that the haemolysins may play a part in determining the pathogenic potential of the species.

These surveys highlight the uncertainties in the classification of staphylococci and in our knowledge of their virulence. Coagulase-positive staphylococci are
F. NAMAVAR, J. DE GRAAFF, C. DE WITH AND D. M. MACLAREN

clearly more virulent than coagulase-negative staphylococci, yet the latter have a definite pathogenic capacity (Namavar et al., 1975; Namavar, de Graaff and Verhoef, 1976). We decided therefore to study the development of an experimental method for the assessment of the virulence of coagulase-negative staphylococci.

MATERIALS AND METHODS

Bacterial strains. We studied 45 strains of *S. saprophyticus* biotype 3 (Baird-Parker, 1974) of which 18 strains were isolated from infections of the urinary tract (12 of these were kindly supplied by Professor W. A. Gillespie, Medical School, University of Bristol); the remaining 27 strains came from the skin of healthy volunteers. For comparison we studied five strains of *S. saprophyticus* biotype 1 (Baird-Parker, 1974), five strains of *S. saprophyticus* biotype 2 from human skin, twelve strains of *S. epidermidis* and eight strains of *Staphylococcus aureus* from patients in the University Hospital. The strains were also classified according to a simplified scheme for routine identification of human staphylococcal species (Kloos and Schleifer, 1975c), but colony diameters were not measured and haemolytic activity was demonstrated on agar plates containing human blood.

Novobiocin sensitivity. Novobiocin sensitivity was determined by the method described by Mitchell (1968).

Determination of virulence. Virulence was determined by LD50 measurements. Bacterial strains were grown in Lab-Lemco Broth (Oxoid) for 18 h at 35°C; the cells were washed twice, resuspended in 0.9% buffered saline and kept on ice. Four-fold serial dilutions of bacterial cell suspensions containing known numbers of viable organisms were made and injected intracerebrally in 0.01-ml volumes into groups of either adult mice aged 9 weeks or "neonatal" mice aged 2 days (Swiss strains; T.N.O., Zeist, The Netherlands). In each LD50 determination six dilutions were tested, each in a group of six mice. Neonatal mice from several mothers were pooled, and then randomly divided into groups. Viable counts of the cell suspensions were made within 1 h of injection. Injection of 0.01-ml doses of saline into groups of control mice was found to be innocuous. The mice were observed for 1 week and the LD50 values were calculated according to the method of Spearman and Kärber (Finney, 1964). The assumption was made that the LD50 values had a lognormal distribution. Analysis of variance was carried out on the log LD50 values, with the S-method for multiple comparison described by Scheffé (1959), confidence intervals were calculated for the differences of the log LD50 values between the groups. The confidence intervals for the relative potencies were calculated by finding their antilog values.

RESULTS

Preliminary tests showed that it was possible to measure the LD50 values of *S. aureus* strains by intracerebral injection into adult mice, but coagulase-negative staphylococci were unable to produce lethal infections. However, neonatal mice were found to be more susceptible and as a result LD50 values for both *S. epidermidis* and *S. saprophyticus* could be determined (table I).

All of the 45 strains of *S. saprophyticus* biotype 3 could be divided into three categories, namely (1) strains isolated from urinary tract infections, all resistant to novobiocin, (2) strains isolated from the skin, resistant to novobiocin, and (3) strains isolated from the skin, sensitive to novobiocin. The LD50 values of 37 of the strains, divided into the three categories, are shown in the figure.

Analysis showed that the null hypothesis of equal LD50 values was rejected for these three groups (P<0.001). Further analysis showed that no differences could be detected between novobiocin-resistant strains either from the skin or from urinary-tract infections (95% confidence interval for the relative potency...
Staphylococcal species

Measurement of the virulence of strains of various staphylococcal species by the intracerebral inoculation of adult and neonatal mice

<table>
<thead>
<tr>
<th>Staphylococcal species</th>
<th>Number of strains</th>
<th>Isolation site</th>
<th>Biotype</th>
<th>Mean LD50 values (10⁴) in mice aged 9 weeks</th>
<th>Mean LD50 values (10⁴) in mice aged 2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>8</td>
<td>Nose</td>
<td>...</td>
<td>150</td>
<td>14·9</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>12</td>
<td>Skin</td>
<td>1</td>
<td>S</td>
<td>1845·0</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>12†</td>
<td>Skin</td>
<td>3 and 2</td>
<td>S</td>
<td>131·7</td>
</tr>
</tbody>
</table>

S = All mice survived.
* According to the classification of Baird-Parker (1974).
† The 12 strains examined were all novobiocin resistant.

was 0·6–7·8). However, a significant difference was found between the LD50 values of the novobiocin-sensitive and novobiocin-resistant strains (95% confidence interval for the relative potency was 11·6–169).

When we classified our strains by the scheme of Kloos and Schleifer (1975c) we found that most strains of *S. saprophyticus* biotype 3 (Baird-Parker, 1974) isolated from urinary tract infections and resistant to novobiocin, corresponded

---

**Figure.**—The LD50 values of three categories of strains of *Staphylococcus saprophyticus* biotype 3 isolated from urinary tract infections and from the skin. The three categories were (1) isolates from urinary tract infections, all resistant to novobiocin, (2) skin isolates, resistant to novobiocin, and (3) skin isolates, sensitive to novobiocin.
TABLE II
Reclassification by the scheme of Kloos and Schleifer (1975a, b and c) of strains originally classified as Staphylococcus saprophyticus biotype 3 by the scheme of Baird-Parker (1974)

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>Isolation site</th>
<th>Susceptibility to novobiocin</th>
<th>Number (and percentage) of strains</th>
<th>Species according to the scheme of Kloos and Schleifer</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Skin</td>
<td>Resistant</td>
<td>15 (88)</td>
<td>S. cohnii</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 (12)</td>
<td>S. saprophyticus</td>
</tr>
<tr>
<td>10</td>
<td>Skin</td>
<td>Sensitive</td>
<td>10 (100)</td>
<td>S. haemolyticus</td>
</tr>
<tr>
<td>18</td>
<td>Urine</td>
<td>Resistant</td>
<td>15 (83)</td>
<td>S. saprophyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (17)</td>
<td>S. warneri</td>
</tr>
</tbody>
</table>

to S. saprophyticus of Kloos and Schleifer; S. saprophyticus biotype 3 (Baird-Parker, 1974) strains isolated from the skin, when novobiocin-resistant corresponded to S. cohnii of Kloos and Schleifer and, when novobiocin-sensitive, to S. haemolyticus (table II). Most strains of S. saprophyticus whether isolated from urine or the skin produced little or no acid from xylitol and were classified as S. saprophyticus via the minor route (Kloos and Schleifer, 1975c).

DISCUSSION

It is clear from our results that adult mice inoculated by the intracerebral route are not killed by moderate doses of coagulase-negative staphylococci. The LD50 values of coagulase-negative staphylococci for mice varying in age from 12 to 14 weeks have been reported by several authors to be very high (Ekstedt and Yotis, 1960; Smith, 1962; Gorrill and McNeil, 1965; Van de Vijver, 1972); such LD50 values are unlikely to be related to the true virulence of the strains. However, neonatal mice with their less-developed immune system were more susceptible, and could be used to measure the LD50 values of S. epidermidis and S. saprophyticus. Both were clearly less virulent than S. aureus, but novobiocin-resistant S. saprophyticus biotype 3 was much more virulent than S. epidermidis (table I). It is interesting to note that whereas S. saprophyticus biotype 3 strains isolated from the skin varied in their sensitivity to novobiocin, all strains isolated from the urine were novobiocin resistant. The skin isolates that corresponded to S. cohnii were sucrose and nitrate negative and produced little or no haemolysin, but were resistant to novobiocin. Marples and his co-workers (personal communication) have made similar observations and have noted in addition that S. cohnii is resistant to lincomycin; the skin isolates of subgroup M3 (Baird-Parker, 1963 and 1965) fell into S. haemolyticus, S. capitis and S. warneri, but S. haemolyticus predominated.

When the S. saprophyticus biotype 3 isolates were grouped according to site of origin and novobiocin sensitivity, and classified according to Kloos and Schleifer (table II), we were unable to show any difference in virulence between novobiocin-resistant strains of S. saprophyticus or S. cohnii isolated either from the skin or urine, but novobiocin-sensitive strains from the skin (mostly S.
S. SAPROPHYTICUS FROM URINE AND SKIN

haemolyticus) were significantly less virulent. It seems that, in spite of the different position of S. saprophyticus and S. cohnii in Kloos and Schleifer's classification, they possess similar virulence for neonatal mice. Assuming that the classification of S. saprophyticus and S. cohnii as separate species is valid, our results suggest that, although they are equally virulent for neonatal mice, S. saprophyticus has an advantage, as yet undefined, in invading the urinary tract. Difficulties were experienced in separating strains of S. haemolyticus and S. warneri from each other when they were mannitol, trehalose, sucrose, maltose and fructose positive. Some S. warneri strains with these characteristics showed moderate to weak reduction of nitrate and the only distinguishing characteristic was haemolytic activity. According to the classification of Kloos and Schleifer, S. haemolyticus is haemolysin positive and S. warneri varies from negative to weakly positive. We are at present engaged in further studies of the problems of classification as they have an important bearing on studies of relative virulence.

SUMMARY

A method was developed to study virulence of coagulase-negative staphylococci. Our results showed that coagulase-negative staphylococci injected into adult mice by the intracerebral route did not give rise to lethal infections, whereas mice aged 2 days were much more susceptible.

Novobiocin-resistant strains of Staphylococcus saprophyticus were more virulent than strains of Staphylococcus epidermidis. Strains of S. saprophyticus biotype 3 of Baird-Parker's classification varied in virulence according to novobiocin sensitivity. In the classification of Kloos and Schleifer, S. saprophyticus biotype 3 can be subdivided into four distinct staphylococcal species, namely S. saprophyticus, S. cohnii, S. haemolyticus and S. warneri. S. cohnii and S. saprophyticus were equally virulent for mice aged 2 days, but novobiocin-sensitive S. haemolyticus was less virulent. On epidemiological grounds, however, it would seem that S. saprophyticus has some undefined advantage in invading the urinary tract.

We wish to thank Miss P. C. Kreuning for technical assistance.

REFERENCES

F. NAMAVAR, J. DE GRAAFF, C. DE WITH AND D. M. MACLAREN


