Production of "virulence factors" by "epidemic" methicillin-resistant Staphylococcus aureus in vitro

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Summary. The production of virulence factors was determined quantitatively for clinical isolates of methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) strains of Staphylococcus aureus from The London Hospital. The examined factors were: production of enterotoxins A, B, C and D, determined by ELISA; quantitation and differentiation of the membrane-damaging α, β, γ and δ haemolysins; and coagulase production determined by a chromogenic assay. Enterotoxin A was produced by MRSA but not by MSSA. All the strains produced haemolysins α, γ and δ at similar levels, but MRSA produced significantly more coagulase than MSSA. MRSA and MSSA were compared in a phagocytosis assay but there was no difference between the phagocytosis of MRSA and MSSA by human polymorphonuclear leucocytes. These findings indicate that MRSA from The London Hospital is at least as well equipped to cause disease as other isolates of S. aureus, and probably better equipped than most hospital isolates of MSSA.

Introduction

Methicillin-resistant strains of Staphylococcus aureus (MRSA) are important nosocomial pathogens. Although initially described in the early 1960s, an upsurge of MRSA causing clinical problems in London and the South-East of England due to a single strain, designated "epidemic" methicillin-resistant Staphylococcus aureus-1 (EMRSA-1), was noted in the early 1980s. This strain causes severe infections, particularly in patients who are compromised or have suffered trauma, and appears to have a propensity to spread, often despite extensive and expensive infection control measures. However, outbreaks of infection with MRSA in other geographical areas have been readily contained. The latter experience, together with the proposed clonal theory of the origins of MRSA, has led to a suggestion that MRSA in general are less virulent than the more usual, methicillin-susceptible, isolates of S. aureus (MSSA) which cause hospital infections.

There is much circumstantial evidence based on clinical and epidemiological experience of the ability of the EMRSA-1 to colonise, spread and cause disease but its specific virulence attributes have received little attention. We have previously investigated the adherence properties of EMRSA-1 and, in this study, the virulence properties of EMRSA-1 relative to other S. aureus causing hospital infections have been investigated. The production in vitro of enterotoxins A, B, C and D, membrane-damaging α-, β-, γ- and δ-toxins, and coagulase was measured. Phagocytosis by human polymorphonuclear leucocytes (PMNL) was also studied.

Materials and methods

Bacterial cultures

Five MRSA isolates, one MRSA with intermediate resistance to methicillin, five S. aureus isolates resistant to multiple antibiotics but susceptible to methicillin (ORSA), and five strains of S. aureus resistant to penicillin only (SSA) were examined. All had been isolated at The London Hospital from clinical specimens during 1985–6. A fully susceptible clinical isolate, a penicillin-resistant laboratory reference strain and the "Oxford" Staphylococcus NCTC 6571 were also included. The sources and characteristics of these isolates are given in Table 1. All isolates were stored at −70°C in peptone water containing glycerol and glucose 1% w/v and were subcultured on to blood agar and incubated overnight immediately before use.

Tests for virulence factors were performed on all 19 isolates unless otherwise stated.
Table I. Sources and characteristics of *S. aureus* strains used

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Strain no.</th>
<th>Source</th>
<th>Resistant to</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>1</td>
<td>BC</td>
<td>PEDTMG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BC</td>
<td>PEDTMG</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>BC</td>
<td>PEDTMG</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>WS</td>
<td>PEDTMG</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>WS</td>
<td>PEDTMG</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>WS</td>
<td>PETGN</td>
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<tr>
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<td>7</td>
<td>WS</td>
<td>PETGN</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>WS</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>BC</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<td>P</td>
</tr>
<tr>
<td>SSA</td>
<td>11</td>
<td>WS</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>WS</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>WS</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>BC</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>BC</td>
<td>P</td>
</tr>
<tr>
<td>Controls</td>
<td>16</td>
<td>WS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>LAB</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>LAB</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>“Oxford”</td>
<td></td>
</tr>
</tbody>
</table>

BC = blood culture; WS = wound swab; P = penicillin; E = erythromycin; D = clindamycin; T = tetracycline; M = methicillin; G = gentamicin; C = chloramphenicol; N = neomycin; LAB = laboratory reference strain; i = intermediately resistant to methicillin (MIC > 4 mg/L).

Detection of enterotoxins

The production of enterotoxins A, B, C and D was detected with an enzyme immunoassay kit. The method was as described by the manufacturer (W. Bommel Laboratories, Switzerland).

In order to quantitate enterotoxin production, supernates containing each enterotoxin 1 μg/ml (W. Bommel Laboratories) were diluted to cover the range 1–1000 ng/ml and assayed to provide data for a standard curve.

Detection of membrane-damaging toxins

A microtitration-format adaptation of the tube technique was used. The filtered supernates from 10 ml overnight shaken cultures in Todd-Hewitt Broth were serially diluted in triplicate in 100-μl volumes to a dilution of 1 in 2048 in phosphate-buffered saline (PBS) containing 1 mM MgSO₄ (MgPBS), in each of three microtitration plates. A solution of heparin (20 μl) (1000 U/ml, Paynes & Byrne Ltd, Greenford, Middx) was added to one row of wells in each plate to inhibit γ-toxin and 20 μl of fibrinogen (BDH Chemicals) (10 mg/ml) was added to another to inhibit δ-toxin. The plates were incubated for 15 min at 37°C. Thrice-washed rabbit, sheep or human erythrocyte suspensions (2% in MgPBS) were prepared and 100 μl was added to each plate and incubated for 80 min at 37°C. The highest dilution giving >50% lysis was recorded as the haemolytic titre of the filtrate. The titre of the individual toxins was expressed as follows:

- α titre = titre of filtrate (for rabbit erythrocytes) that was not inhibited by fibrinogen;
- β titre = increase in titre for sheep erythrocytes after 1 h at 4°C;
- γ titre = titre of filtrate (for human erythrocytes) which was inhibited by heparin; and
- δ titre = titre of filtrate (for human erythrocytes) which was inhibited by fibrinogen.

Coagulate production

Two-fold dilutions of an overnight culture in Brain Heart Infusion Broth (BHI) were prepared in sterile BHI to give 100-μl volumes and to these were added 0-5-ml amounts of human plasma (fresh frozen plasma, London Hospital blood bank) diluted 1 in 5 in BHI containing ciprofloxacin (Bayer UK Ltd, Newbury, Berks.) at a concentration of 1 mg/L to prevent multiplication of bacteria. Tubes were incubated for 4 h in a water bath at 37°C. The highest dilution giving a definite clot was recorded as the coagulate titre.

A modification of the chromogenic substrate method was also used to quantify coagulate production. Initially, culture supernates, filtrates and broth cultures were tested. Subsequently, broth cultures were used. Overnight broth cultures (30 μl) in BHI alone or supplemented with bovine serum albumin (BSA) 0-2% were diluted in 1 in 4 in quadruplicate in a microtitration plate. To two of the four wells were added 125 μl of reagent containing buffer (72 mM triethanolamine buffer at pH 8-4, 144 mM NaCl and 5 mM EDTA), 166 μM Chromozym TH (Boeringer Mannheim Corp. Ltd, Lewes, East Sussex), prothrombin 50 μg/ml (factor IX concentrate, Plasma Fractionation Lab., Oxford), and ciprofloxacin 1 mg/L. Prothrombin was omitted from the remaining two wells to provide a bacterial background count. The plates were incubated for 2 h at 37°C and the absorbance of the reaction mixtures was measured at 410 nm with a Dynatech plate reader (Dynatech, Billingshurst, Sussex), which corrects automatically for blank medium reactions. In this assay, staphylocoagulase activates prothrombin to staphylthrombin. The thrombin-like activity of this complex cleaves the synthetic tripeptide Chromozym TH by limited proteolysis, liberating the yellow p-nitroaniline. Subtraction of the OD₄₁₀ value corresponding to the bacterial background count from the test gave a relative measure of the amount of coagulate present. All isolates were tested in duplicate on three different occasions and the results for the three groups of isolates were compared by Student's *t* test.

Phagocytosis

Phagocytosis was assayed by a modification of the method of Verbrugh et al. Several colonies of bacteria from blood-agar plates were inoculated into 5 ml of Iso-sensitest Broth (Oxoid Ltd, Basingstoke, Hants) containing 0-02 mCi thymidine-methyl-³H (specific activity 40–60 Ci/mmol; Amersham International plc, Bucks). After growth for 18 h at 37°C the bacteria were washed three times in PBS and the suspension was adjusted to an OD₄₅₀ of 0-55. This was shown by viable counts to be equivalent to 2.5 × 10⁸ cfu/ml.
The washed bacteria were opsonised by incubation for 30 min at 37°C in pooled normal human serum 5% in Hanks's Balanced Salts Solution (HBSS) containing 25 mM Hepes buffer and gelatin 0.1% (GHBSS). Excess serum was removed by centrifugation, and opsonised bacteria were resuspended in GHBSS.

PMNL were prepared by carefully layering fresh heparinised blood, obtained from healthy human volunteers, on to Mono-poly resolving medium (Flow Laboratories, Rickmansworth, Herts). This visibly separated the blood into plasma, mononuclear leucocytes (MNL), PMNL and erythrocytes. After centrifugation at 300 g for 30 min the PMNL fraction was removed and washed twice with ice-cold PBS containing PMNL and associated bacteria were solubilised in 0.1% trypan blue exclusion, and the suspension was diluted to 5 x 10^6 viable cells/ml.

PMNL suspension (300 μl) was added to 300 μl of opsonised bacteria in four polypropylene centrifuge tubes (Sarstedt Ltd, Leicester) and mixed at 120 rpm in a water bath at 37°C for 30 min to allow phagocytosis to take place. The tubes were then placed on ice and 2 ml of ice-cold PBS was added to stop phagocytosis. Two of the tubes were then centrifuged at 160 g for 5 min at 4°C and the PMNL pellet was washed twice with ice-cold PBS to remove non-PMNL-associated bacteria. The final pellets containing PMNL and associated bacteria were solubilised in 2.5 ml of scintillation fluid (Fluoran HV, BDH) and radioactivity counted in a liquid scintillation counter.

To determine the radioactivity representing PMNL-associated plus free bacteria (i.e., total bacterial inoculum) the remaining two tubes were centrifuged at 2000 g for 15 min. The pellets were resuspended in scintillation fluid and radioactivity was measured.

All isolates were tested in duplicate on three separate occasions. The percentage of the total bacterial inoculum that was PMNL-associated at 30 min (percentage phagocytosis) was calculated as:

\[
\text{percentage phagocytosis} = \frac{\text{cpm in PMNL pellet}}{\text{cpm in total bacterial pellet}} \times 100
\]

The proportion of bacteria attached to, but not ingested by, the PMNL was determined by incubation of the PMNL pellets with lysostaphin (5 μg/ml) for 30 min at 37°C followed by centrifugation at 160 g and two washes with PBS. The radioactivity in the resulting PMNL pellets was counted as described above. Less than 10% of the inoculum was attached to the PMNL. Less than 10% of the inoculum was PMNL-associated under ice-bath conditions.

Results

Enterotoxin production

When the OD_{410} values were plotted against the logarithms of the concentrations of enterotoxin, a linear relationship was observed over the range 10–100 ng/ml for all enterotoxins. Above 100 ng/ml the relationship was inconsistent. Standard curves obtained on different occasions were not very reproducible; small changes in OD_{410} values corresponded to large changes in enterotoxin concentration. Therefore, the kit was considered suitable for qualitative and semi-quantitative determinations of enterotoxin production, but not suitable for accurate quantitation of enterotoxin production by bacterial isolates.

Enterotoxin A production was detected at a level of >100 ng/ml of culture with all six MRSA isolates including the intermediate MRSA but not with the ORSA nor the SSA. A low level (c. 10–20 ng/ml) of enterotoxin C was detected with isolate 18. Production of other enterotoxins was not detected with any of the isolates tested.

Production of membrane-damaging toxins

Gamma (γ) and δ-toxins were produced by control strains NCTC 5664 and NCTC 9393 and detected by our methods. Heparin (100 U/ml) almost entirely inhibited the lysis of human erythrocytes by supernate from strain NCTC 5664 and reduced the titre for rabbit cells by two dilutions. Fibrinogen (0.1%) resulted in a four-fold reduction in the haemolytic activity of NCTC 9393 supernate for rabbit and sheep cells.

All MRSA isolates produced α-, γ- and δ-toxins at titres of 8, 8 and 4 respectively. The non-MRSA isolates produced various combinations of α-, β-, γ- and δ-toxins at various titres (fig. 1). Table II gives the results for each group of isolates. The “Oxford” Staphylococcus (NCTC 6571) did not produce detectable α-toxin and isolates 18 and 19 gave results in the same range as other non-MRSA.

Coagulase production

Tube method. In the absence of ciprofloxacin, all isolates produced a clot within 4 h at broth culture

Table II. Production of membrane-damaging toxin by S. aureus strains

<table>
<thead>
<tr>
<th>Toxin</th>
<th>MRSA</th>
<th>ORSA</th>
<th>SSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>8–16</td>
<td>nil–32</td>
<td>2–32</td>
</tr>
<tr>
<td>β</td>
<td>nil</td>
<td>nil–32</td>
<td>nil–4</td>
</tr>
<tr>
<td>γ</td>
<td>4–16</td>
<td>nil–16</td>
<td>1–8</td>
</tr>
<tr>
<td>δ</td>
<td>4</td>
<td>2–16</td>
<td>4</td>
</tr>
</tbody>
</table>
dilutions of >256 with the exception of the “Oxford” Staphylococcus which had a titre of 128. Titres of coagulase in supernates after centrifugation were directly proportional to the number of viable organisms present. Filtrates failed to cause clotting.

The addition of ciprofloxacin to tube dilutions resulted in discrete clots surrounded by clear fluid without visible growth. In the presence of ciprofloxacin, coagulase activity in thrice-washed bacteria was equal to that of broth cultures. These were c. 100-fold greater than those given by culture supernates.

The titres of coagulase, as determined by the tube dilution method with broth cultures plus ciprofloxacin, for isolates 3, 4, 5 and 8 were 128, 128, 256 and 32 respectively.

Chromogenic assay. Filtrates failed to give a positive reaction. Culture supernates gave a positive reaction directly proportional to the number of viable organisms present. Undiluted broth cultures of MRSA gave readings too high to make quantitation possible. Therefore, suspensions were diluted 1 in 4.

No change in optical density was observed for diluted broth cultures to which ciprofloxacin (1 mg/L = 1–2 × MIC) had been added. Therefore, ciprofloxacin prevented growth of the organisms without causing visible lysis.

All MRSA produced a substantial amount of coagulase (mean c. 20 ng/ml). This was calculated from the published standard curve because purified coagulase was not available. Non-MRSA produced undetectable quantities of coagulase.
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when cultured in the absence of BSA (level of detection c. 5 ng/ml). When grown in the presence of BSA, coagulase production by MRSA increased to c. 30 ng/ml and the non-MRSA all produced detectable quantities of coagulase (mean c. 15 ng/ml) with the exception of isolates 16 and 18 which produced levels of coagulase undetectable in this assay. Isolate 17 produced a similar amount to that produced by the other MRSA.

The differences between the mean amounts of coagulase produced by MRSA and ORSA or SSA when grown in BHI alone were statistically significant (p < 0.001) and when grown in the presence of BSA (p < 0.01). Results are shown in table III and fig. 2; mean values for the three groups are given in table III and individual results in fig. 2.

Phagocytosis

Isolates 1, 2 and 3 (MRSA) and 9, 10, 14 and 15 (MSSA) were compared in phagocytosis experiments. The results are shown in table IV. A mean of 37% of the MRSA inoculum was PMNL-associated compared with 39% of the MSSA

Fig. 2. Coagulase production by *S. aureus* isolates grown in (a) BHI alone and (b) in the presence of BSA 0.2%; bar = SEM.
Table III. Coagulase production by S. aureus strains

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Culture medium</th>
<th>Index of coagulase production*; mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>BHI</td>
<td>0.239 (0.038)</td>
</tr>
<tr>
<td>ORSA</td>
<td>BHI</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SSA</td>
<td>BHI</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MRSA</td>
<td>BHI + BSA</td>
<td>0.372 (0.021)</td>
</tr>
<tr>
<td>ORSA</td>
<td>BHI + BSA</td>
<td>0.181 (0.020)</td>
</tr>
<tr>
<td>SSA</td>
<td>BHI + BSA</td>
<td>0.188 (0.028)</td>
</tr>
</tbody>
</table>

* Expressed as: OD$_{410}$ with prothrombin - OD$_{410}$ without prothrombin.

Table IV. Phagocytosis of methicillin-resistant and methicillin-susceptible S. aureus

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Percentage phagocytosis, Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>type</td>
<td>no.</td>
</tr>
<tr>
<td>MRSA</td>
<td>1  37 (4.0)</td>
</tr>
<tr>
<td></td>
<td>2  35 (6.4)</td>
</tr>
<tr>
<td></td>
<td>3  38 (6.6)</td>
</tr>
<tr>
<td>MSSA</td>
<td>9  35 (2.9)</td>
</tr>
<tr>
<td></td>
<td>10 40 (2.3)</td>
</tr>
<tr>
<td></td>
<td>14 49 (1.7)</td>
</tr>
<tr>
<td></td>
<td>15 31 (1.2)</td>
</tr>
</tbody>
</table>

Discussion

In view of the contrasting clinical and epidemiological experiences with MRSA from the London area and MRSA from elsewhere in England, and the resulting controversy about the virulence of MRSA in comparison with other S. aureus, we investigated some of the virulence properties of MRSA from The London Hospital relative to other clinical isolates of S. aureus.

Because of the conflicting reports about the relationship between antibiotic resistance and virulence, three groups of isolates were chosen. Lacey and Chopra reported a decrease in virulence for chick embryos associated with the acquisition, by transduction, of antibiotic resistance determinants. In contrast to these findings, Cutler reported a decline in virulence (for guinea-pigs) associated with the loss of resistance determinants. No decrease in virulence was noted for heat-treated but uncured progeny. More recently, Kinsman et al. showed that any genetic manipulation could reduce the virulence of strains for mice. The inclusion in this study of a group of multi-resistant but methicillin-susceptible (ORS) clinical isolates, which had not been subjected to intentional genetic manipulation, was therefore thought to be prudent. Any differences observed between MRSA and ORSA could then be considered to be a true reflection of the isolates, whereas differences between MRSA and SSA could possibly be attributed to the extra genetic load of the former.

Of the many biologically-active products of S. aureus, only the enterotoxins, toxic shock syndrome toxin-1 (TSST-1) and epidermolytic toxins A and B have been established unequivocally to be important in the pathogenesis of staphylococcal infections and these are each associated with specific disease syndromes.

Enterotoxin A was detected in the MRSA isolates whereas no other enterotoxin was detected in any of the other clinical isolates tested. This is in contrast with MRSA isolated in the 1960s which produced enterotoxin B as did the isolate from the outbreak at Wakefield (Jordens, unpublished data).

Two cases of toxic shock syndrome (TSS)-like disease at The London Hospital from which MRSA were isolated and implicated as the cause of the symptoms were reported by Duckworth and Oppenheim. These MRSA isolates did not produce TSST-1 but they produced enterotoxin A. Enterotoxin A has been shown to be more emetic and pyrogenic than enterotoxin B; and enterotoxin B, in the absence of TSST-1, has occasionally been associated with TSS. Enterotoxin A, in the absence of TSST-1, has not been associated with TSS. EMRSA-1 produces enterotoxin A in vitro and this property may have been the cause of TSS-like symptoms in vivo. Of S. aureus isolates from cases of septicaemia, 63% have been reported to produce enterotoxins, in contrast to 11% of nasal isolates. Isolates of MRSA from septicaemias produced enterotoxin A, enterotoxin B or both. It seems likely, therefore, that the enterotoxins have a role in the pathogenesis of S. aureus disease other than food poisoning.

The production of enterotoxins by the isolates used in this study was detected with a commercially-available ELISA kit. In view of the lack of reproducibility obtained for the standard curves with the ELISA kit it was not possible to quantitate enterotoxin production. The commercially-available latex-agglutination kit, however, may be more appropriate for testing and quantitating enterotoxin production by bacterial isolates.
The biological effects of the membrane-damaging toxins are well established, especially in the case of α-toxin. The London Hospital MRSA produced α-, γ- and δ-toxins at similar levels to ORSA and SSA but they did not produce β-toxin. The latter result is in contrast to that found by Cookson et al. who showed, by a reverse CAMP test, that most EMRSA isolates produced β-toxin. This test may not be very specific; synergic haemolysis may be the result of δ-toxin production by the *S. aureus* strain under test. β-Toxin production by the control strains used in the present study was readily detected, so it is unlikely that it was produced by the London Hospital MRSA but not detected.

By definition, *S. aureus* produces coagulase. Coagulase production in this study was quantitated by a modification of the chromogenic technique. When strains were cultured in broth alone, only the MRSA isolates produced a detectable reaction. They produced significantly more coagulase than either ORSA or SSA when albumin was added to the culture broth. The amounts of coagulase produced by ORSA and SSA isolates were extremely variable. Duthie reported that albumin increases yields of coagulase and that strains that are thought to have a role in the virulence of *S. aureus* may not be very specific; synergic haemolysis may be the result of δ-toxin production by the strain under test. β-Toxin production by the control strains used in the present study was readily detected, so it is unlikely that it was produced by the London Hospital MRSA but not detected.

Many staphylococcal factors are considered to be antiphagocytic. These include capsule, protein A, coagulase and the membrane-damaging toxins. Isolates of EMRSA-1 have been shown to produce less protein A than other isolates of *S. aureus* but high levels of clumping factor and coagulase and the present study has shown that the London Hospital MRSA produces similar amounts of membrane-damaging toxins and significantly more coagulase than MSSA. In view of its decreased protein A content and the reported role of this component in inhibiting phagocytosis, the relative degree of ingestion of London Hospital MRSA and MSSA by human PMNL was compared and it was shown that there was no difference between the degree of phagocytosis. The decreased protein A content of EMRSA did not, therefore, appear to render the organism less resistant to phagocytosis. It is possible that the high levels of coagulase produced somehow compensate for any increased susceptibility to phagocytosis incurred by the low protein A content. It may be that differences between MRSA and MSSA arise after ingestion by phagocytes, for example in the degree of intracellular survival.

This study has shown that the London Hospital MRSA (EMRSA-1) produces many factors in vitro which are thought to have a role in the virulence of *S. aureus*. Other studies in this laboratory have shown that EMRSA-1 binds less well to HEp2 cells than MSSA, which may be advantageous in vivo. These findings support the in-vivo experience with EMRSA-1 which indicates that it is at least equal in virulence to other methicillin-susceptible isolates of *S. aureus*.

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REFERENCES


