Possible virulence factors of *Staphylococcus sciuri*

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Abstract

*Staphylococcus sciuri* is an opportunistic pathogen of controversial clinical significance. The factors that contribute to colonization and/or infection caused by this bacterium have not been studied intensively so far. The present research was carried out in order to study the presence of potential virulence factors in 121 human and animal isolates of this bacterium. Isolates were examined for biofilm formation, hemagglutination, presence of clumping factor, production of spreading factors and exotoxins, cytotoxicity and capacity to stimulate nitric oxide production. The results showed that *S. sciuri* is highly capable of biofilm production, that it displays strong proteolytic and DNase activities, produces hemolysins and stimulates nitric oxide production by rat macrophages. Although the present study showed existence of a wide spectrum of possible virulence determinants of *S. sciuri*, their exact contribution to virulence of this bacterium in vivo remains to be determined. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Virulence; Pathogenicity; *Staphylococcus sciuri*

1. Introduction

*Staphylococcus sciuri*, the coagulase-negative species, was first described by Kloos et al. in 1976 [1]. This bacterium is widespread in nature and is associated with a variety of wild and domestic animals [1–4]. *S. sciuri* has been shown to be an invasive pathogen for animals causing wound infections and mastitis [3,5]. Although principally animal species, *S. sciuri* may colonize humans and its isolation from clinical samples such as skin, vagina, blood, urine, central venous catheters has been reported [4,6–8]. Moreover, diseases in humans caused by this bacterium, such as wound infections, soft tissue infections, abscesses, boils, peritonitis and endocarditis, have been described [5,6,9–11]. However, apart from the studies of Hedin and Widerstrom [10] and Wallet et al. [11] that clearly identified *S. sciuri* as a causative agent of endocarditis and peritonitis, respectively, no other reports did prove that this bacterium actually caused the infections reported. Thus, clinical relevance of *S. sciuri* for humans is still controversial. It should be noted that the rare recognition of *S. sciuri* as a human pathogen is partly due to difficulties in its identification in routine laboratory practice since many clinical laboratories do not identify coagulase-negative staphylococci (CoNS) to the species level [12]. Moreover, it has been shown that commercial systems for identification could misidentify *S. sciuri* [13,14].

The possible virulence factors of *S. sciuri* have not been studied intensively so far. In the present study *S. sciuri* animal and human isolates were examined for biofilm formation, hemagglutination, presence of clumping factor, production of spreading factors, toxins, and hemolysins, toxigenicity and capacity to stimulate nitric oxide (NO) production.

2. Materials and methods

2.1. Strains

In total, 121 strains of *S. sciuri* were used in this study: the reference strain *S. sciuri* ATCC 29062, four clinical isolates [8], and 116 isolates sampled from the skin and mucous membranes of dogs. The clinical isolates were
identified by the Becton Dickinson commercial set Sceptor Staphylococcus MIC/ID panel. Previously described screening procedure [8] was used for identification of S. sciuri strains of animal origin. Briefly, each tube coagulase-negative and esculin-positive staphylococcal isolate was tested for novobiocin susceptibility, oxidase activity, and acid production from raffinose. All novobiocin-resistant, oxidase-positive strains that did not produce acid from raffinose were preliminary identified as S. sciuri. In order to confirm the identification, the biochemical properties of these strains were further analyzed on a set of 22 carbohydrates. Prior to inoculation, all strains were transferred from the stock cultures to P agar [15] and incubated aerobically overnight at 35°C.

2.2. Biofilm formation

Quantification of biofilm formation by S. sciuri strains cultivated in brain heart infusion broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was determined by the modified microtiter-plate test, as previously described [16]. Upon the optical density (OD) of bacterial films, all strains were classified into the following categories: no biofilm producers, weak, moderate, or strong biofilm producers [16]. The test was carried out two times and the results were averaged.

2.3. Hemagglutination

Hemagglutination test was performed in a manner similar to the method described by Rupp and Archer [17]. The hemagglutination was performed in U-shaped 96-well microtiter plates (Spektar, Cacak, Yugoslavia). The bacterial suspensions in phosphate-buffered saline were adjusted to McFarland standard 1. Two serial dilutions of the bacterial suspension were made in the microtiter plates to give a total volume of 50 μl per well. Then, 50 μl of the 1% human O erythrocyte suspension in phosphate-buffered saline were added to each well. After shaking, the plates were incubated at room temperature for 2 h, and the hemagglutination was recorded as positive or negative.

2.4. Clumping factor

The clumping factor was determined with human plasma on glass slides [15]. Formation of visible clusters within 60 s was regarded as a positive result.

2.5. Protease activity

Protease activity was detected using the casein and gelatin media according to the procedures described by Koneman et al. [18] and Oberhofer [19], respectively. Caseinase activity was observed as a transparent halo surrounding the growth, while the zone of clouding around the growth indicated the gelatinase activity.

2.6. Lipase activity

Splitting of Tween 80, Tween 40 and Tween 20 was examined in tryptose blood agar base (TBAB) (Difco laboratories, Detroit, MI, USA) enriched with 0.01% CaCl₂·2H₂O and 1% Tween 80 (Difco), Tween 40 and Tween 20 (Serva), respectively [15]. Splitting of Tweens was characterized by an opacity around the culture. Spirit blue agar (Difco) supplemented with Bacto lipase reagent (Difco), and TBAB enriched with 1% glycerol tributyrate (tributyrin) (BDH, Pool, UK), were also used in the study. The presence of zones of clearing was taken as an indication of positive enzyme activity. The inoculated media used for detection of lipolytic activity were incubated for 48 h at 35°C and, thereafter for 72 h at room temperature.

2.7. Lecithinase activity

Lecithinase activity was examined on Baird–Parker medium containing 5% egg-yolk emulsion [15]. Inoculated plates were incubated for 48 h at 35°C and thereafter for 24 h at room temperature. The lecithinase activity was characterized by an opaque zone of precipitate in the medium around the growth.

2.8. Starch hydrolysis

The ability of S. sciuri strains to degrade starch was determined by culturing bacteria on the plates containing TBAB plus 1% starch soluble (Mallinckrodt Chemical Works, Saint Louis, MO, USA). After incubation at 35°C for 2 days, the plates were flooded with Gram's iodine solution; a clear zone around the growth indicated hydrolysis of starch [19].

2.9. DNase activity

For detection of DNase activity, strains were inoculated on TBAB, and incubated for 24 h at 35°C. Thereafter, the plates were flooded with melted DNase agar containing toluidine blue (Tolrak, Belgrade, Yugoslavia). After a further 24 h of incubation at 35°C, the plates were examined for a pink zone surrounding the growth on a blue background [20].

2.10. Fibrinolysin activity

Fibrinolysin activity

For demonstration of fibrinolysin activity, sterile human plasma was added to molten TBAB to give the final concentration of 12% [21]. After heating in water bath at 56°C for 15 min, the mixture was poured into Petri dishes. Tested strains were spot-inoculated onto dried poured plates, and incubated for 24 h at 35°C and a further 24 h at room temperature. Fibrinolysin-positive isolates showed clearing around the spot inocula.
2.11. Urease activity

Urease activity was tested on Christensen agar according to the recommendations given by Freney et al. [15]. Spot-inoculated plates were incubated for 72 h at 35°C. The purple color of the inoculated agar was interpreted as a positive result.

2.12. Hemolytic activity

Hemolytic activity was determined on TBAB supplemented with 5% defibrinated rabbit blood, 5% defibrinated sheep blood (obtained from Torlak, Belgrade, Yugoslavia), or 2.5% washed human O erythrocytes (obtained from Institute for Transfusiology, Belgrade, Yugoslavia), according to the recommendations given by Freney et al. [15]. Plates were incubated at 35°C.

2.13. Toxins

The production of K, L and N toxins was determined on TBAB with 5% defibrinated sheep or fresh human blood (fresh human O blood, from one of the authors, S.S.). Tested strains were inoculated perpendicularly to the streak of a L toxin-producing Staphylococcus aureus [15]. Inoculated plates were incubated at 35°C for up to 3 days. The results were evaluated as antagonistic (L^K hemolysins) or synergistic (L^N hemolysins) effects as determined by the shapes of hemolytic zones produced.

2.14. Test for cytotoxic activity

Cytotoxicity of S. sciuri strains was examined using Vero cells in 96-well microtiter trays. Suspensions of S. sciuri strains in distilled water were adjusted to 0.5 McFarland standard. Then, 20 µl of bacterial suspensions were added to 3.5 ml of brain heart infusion broth (BBL, Becton Dickinson Microbiology Systems). The tubes were incubated 2 days at 35°C, and thereafter for 2 days at room temperature. After centrifugation of bacterial broth cultures, 20 µl of supernatants were added in triplicate to 180 µl of cell culture medium. After incubation for 24 h at 37°C, in humidity atmosphere and 5% CO2, Nitrite accumulation, an indicator of NO production, was measured using the Griess reagent [23]. The absorbance at 570 nm was measured by using an automated ICN Flow Titertek Multiscan Plus reader. The statistical significance of differences was analyzed using the Student’s t-test and P values less than 0.05 were considered significant.

3. Results and discussion

Summarized results of examination for S. sciuri factors that possibly contribute to the virulence of this organism are presented in Table 1.

The virulence factors of staphylococci have been studied most extensively in the species of S. aureus. Although CoNS have emerged as important pathogens, particularly as the cause of medical devices infections [24], little is known about the virulence factors of these bacteria [25]. The most important virulence factor of Staphylococcus epidermidis and other CoNS is assumed to be biofilm formation on indwelling medical devices [24,26]. Thus, it has been proposed that testing for biofilm production could be a useful marker for the pathogenicity of CoNS [27,28]. Medical device infections caused by S. sciuri have not been reported until today. Only isolation of S. sciuri from central venous catheters has been described, but it
was not established whether these strains were pathogens or contaminants [8]. In the present study 107 (88.43%) S. sciuri strains were shown to be capable of biofilm production. Among these, 26 (24.30%) were strong, 31 (28.97%) moderate, and 50 (46.73%) were weak biofilm producers. As far as S. aureus is concerned, biofilm production has not been considered as a virulence factor. However, it was shown that 88.9% of S. aureus strains isolated from orthopedic implants (infected knee and hip prostheses) were capable of biofilm formation [29]. The studies on biofilm formation by S. epidermidis, the most significant pathogen among CoNS, showed that up to 76.7% of strains of this bacterium produced biofilm [29,30]. When other CoNS of minor clinical significance, e.g. Staphylococcus haemolyticus, Staphylococcus xylosus, and Staphylococcus simulans, were tested, 47.8% of strains produced biofilm [30]. In the present study we observed the rate of biofilm producers among the tested S. sciuri strains comparable to those of pathogenic staphylococci. This indicates that capacity for biofilm formation may be considered as a possible virulence factor of this bacterium.

The ability of S. epidermidis to produce biofilm on plastic surfaces was shown to be associated with its capacity to mediate hemagglutination of erythrocytes of different species [17,31]. The similar association for S. sciuri has not been shown in our study. Hemagglutination with human O erythrocytes was not observed in any of the tested S. sciuri strains, while nearly 90% of them were able to form biofilm. This probably excludes hemagglutinin as an additional possible virulence factor important for biofilm formation by S. sciuri. In general, the rate of agglutination for non-S. epidermidis CoNS is low (33%) [17]. Still, it is possible that S. sciuri could agglutinate erythrocytes of sheep or some other animal species, as it was described for Staphylococcus saprophyticus isolates [26].

The ability of S. aureus to initiate colonization by attachment to fibrinogen-containing substrates, and to clump in the presence of fibrinogen and thus increase resistance to phagocytosis, is primarily due to the presence of clumping factor [32,33]. S. epidermidis, and most other CoNS do not produce clumping factor [15]. Clumping factor has recently been described in S. sciuri [4]. In the present study clumping factor was detected in 57 (47.11%) tested strains of S. sciuri, although the majority of the

Table 1
Possible virulence factors of S. sciuri

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number of strains showing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive reaction</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>107 (88.43%)</td>
</tr>
<tr>
<td>Hemagglutination</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Clumping factor</td>
<td>57 (47.11%)a</td>
</tr>
<tr>
<td>Proteinase activity:</td>
<td></td>
</tr>
<tr>
<td>Gelatinase hydrolysis</td>
<td>121 (100%)</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>116 (95.87%)</td>
</tr>
<tr>
<td>Lipase activity:</td>
<td></td>
</tr>
<tr>
<td>Splitting of Tween 80</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Splitting of Tween 40</td>
<td>121 (100%)</td>
</tr>
<tr>
<td>Splitting of Tween 20</td>
<td>121 (100%)</td>
</tr>
<tr>
<td>Lipase reaction on Spirit Blue Agar</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Splitting of Tributyrin</td>
<td>121 (100%)</td>
</tr>
<tr>
<td>Leucinase activity</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>DNase activity</td>
<td>117 (96.69%)</td>
</tr>
<tr>
<td>Fibrinolysin activity</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Urease activity</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hemolytic activity:</td>
<td></td>
</tr>
<tr>
<td>On rabbit blood</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>On sheep blood</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>On human blood</td>
<td>119 (98.35%)b</td>
</tr>
<tr>
<td>Toxins:</td>
<td></td>
</tr>
<tr>
<td>α Toxin</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>β Toxin</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>δ Toxin</td>
<td>119 (98.35%)</td>
</tr>
<tr>
<td>Toxicity toward Vero cells</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

aAmong clumping factor-positive strains, eight (14.04%) gave strong reaction, 35 (61.4%) moderate reaction and 14 (24.56%) weak reaction.
bAmong strains showing hemolytic activity on human blood, 112 (94.12%) showed strong hemolysis (zone of hemolysis > 2.5 mm from the culture streak), seven (5.88%) showed weak hemolysis (zone of hemolysis < 2.5 mm).
clumping factor-positive strains (85.96%) gave only weak to moderate reaction. The presence of the clumping factor was not detected in any of 30 clinical isolates of S. sciuri [6] as well as in the strain which caused endocarditis [10]. The overall data suggest that clumping factor occurs in S. sciuri, but its exact role in pathogenesis of infections caused by this organism is still unclear.

As far as S. epidermidis and other CoNS are concerned, it has been shown [34-36] that extracellular products like protease, DNase, lipase, hemolysins, and other exoenzymes may be responsible for tissue degradation and spreading of an infection caused by these bacteria. The results of the present study show that S. sciuri is a bacterium with strong proteolytic activity. All tested strains were gelatinase-producing and nearly 96% showed caseinolytic activity, which is in agreement with results of previous studies [1,2]. While S. sciuri proteolytic activity is comparable to that of S. aureus [37], it is interesting to note that only 43.3% of S. epidermidis strains produced gelatinase and 3.3% displayed caseinolytic activity [30].

Although the status of DNase as a virulence factor is debatable, Daghistani et al. [37] showed that significantly more wound isolates of S. aureus produced DNase (97.5%) than those isolated from healthy nasal flora (77%). In contrast to other mainly DNase-negative CoNS [20], we established that absolute majority (96.69%) of the tested S. sciuri strains produced DNase. Thus, DNase activity of S. sciuri is comparable to that of S. aureus wound isolates. Among CoNS only S. chromogenes, species which can cause more severe infections than the majority of other CoNS species [25], showed similar DNase activity [20].

The contribution of lipolytic activity to virulence of staphylococci is not fully understood. However, it has been shown that 79% of S. aureus strains isolated from wounds produced lipase [37]. The proportion of strains producing lipase in the majority of CoNS ranged from 10 to 60% [38]. The exceptions were Staphylococcus schleiferi and S. saprophyticus which resembled S. aureus in that 80% of the strains produced lipase, and Staphylococcus lugdunensis and S. haemolyticus strains with no detectable lipase activity [38]. The evaluation of S. sciuri lipolytic activity showed that all strains were capable of splitting of Tween 20, Tween 40 and tributyrin. No lipolytic activity toward Tween 80 and Difco lipase reagent was observed. Previous reports on S. sciuri lipolytic activity are somewhat contradictory. In the study of Devriese et al., splitting of tributyrin was detected in 10 out of 48 tested strains of animal origin, while no lipolytic activity toward Tween 80 was observed [2]. According to Kloos et al., S. sciuri does not exhibit lipolytic activity at all [4]. It is well known that staphylococcal lipases show substrate specificity, which may explain the discrepant results obtained in different studies. However, the exact contribution of lipolytic enzymes to S. sciuri virulence as well as to virulence of other staphylococci needs to be further clarified.

In contrast to the factors discussed above, lecithinase activity, starch hydrolysis, fibrinolysin activity and urease activity were not found in the tested S. sciuri strains.

We did not establish S. sciuri hemolytic activity on plates with 5% defibrinated rabbit blood or 5% defibrinated sheep blood, which is in agreement with previous studies [1,2,6]. However, when grown on plates with 2.5% washed human O erythrocytes, 119 strains showed clearly detectable complete hemolysis. When full human blood was used, no hemolysis was observed. In the strains showing hemolytic activity, only ð toxin was detected. The fact that tested S. sciuri strains displayed ð hemolysin only, whose hemolytic properties are inhibited by serum components [39], partly explains the absence of hemolysis on full human blood agar. On the contrary, cooperative hemolytic action between S. sciuri ð and S. aureus ß toxins was visible only on full human blood agar plates after prolonged incubation for 3 days. When 2.5% washed human O erythrocytes were used, clearly detectable hemolysis could be seen around the culture streak of tested S. sciuri strains, but synergistic ß-ð hemolysis was not observed. There is no apparent explanation for this finding.

It has been shown that ð toxin is produced by 97% of S. aureus strains and 50-70% of CoNS [40]. Although this toxin causes a wide spectrum of cytotoxic effects, its importance for course of infections caused by staphylococci remains unclear [40]. Since we established that almost all tested strains produced ð toxin, it seemed reasonable to assume that this product may be of certain importance for S. sciuri virulence. However, when the cytotoxic activity of S. sciuri was tested in vitro using Vero cells monolayer, no CPE was observed. As far as cytotoxic effects of other staphylococci are concerned, it has been shown that they considerably vary depending on the cell type used in the study [25,41,42]. When S. aureus cytotoxicity to Vero cells was examined, only six out of 20 tested strains exhibited cytotoxic effect [42]. Thus, S. sciuri cytotoxicity should be further evaluated using cell lines other than Vero.

The capacity of a bacterium to stimulate NO production may also be considered as a factor of importance for the course of an infection because of its numerous biological effects. NO or its derivatives exhibit antiproliferative effects [43], suppress protein synthesis [44], and influence the processing of nascent collagen [45], and, therefore, may affect the process of wound repair [46]. It has also been shown that an enhanced formation of NO contributes to the circulatory failure, multiple organ failure and shock [47]. We evaluated S. sciuri capacity to generate NO in rat macrophages. As there were no statistically significant differences among tested staphylococcal strains in NO production, only the results of one clinical strain are presented in Fig. 1. The results of the present study showed S. sciuri ability to induce NO synthesis by rat peritoneal macrophages in a dose-related manner. Similar results were obtained in studies with S. aureus [48], and S. epidermidis [49].
S. sciuri has attracted special attention after it was suggested that mecA gene of methicillin-resistant strains of staphylococci originated from an evolutionary relative of the mecA homolog that has been identified in S. sciuri [4,50,51]. Moreover, the ability of S. sciuri to transfer genes of resistance to other bacteria, pathogenic for men, has been established [51]. On the other hand, the virulence factors of this bacterium are not yet well understood. The results of the present study showed that S. sciuri has a wide spectrum of possible virulence factors. Moreover, some of the factors displayed activities similar to those of pathogenic staphylococci. However, it remains unclear whether they are, indeed, virulence factors or simply common features of this bacterium. Thus, the exact contribution of the examined factors to S. sciuri virulence in vivo remains to be determined.

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References


