Small Colony Variant of Methicillin-Resistant *Staphylococcus pseudintermedius* ST71 Presenting As a Sticky Phenotype

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ABSTRACT

We first observed the phenomenon of small colony variants (SCVs) in a Staphylococcus pseudintermedius ST71 strain, isolated from a non-pet-owner. Although small-sized colonies shared main features with Staphylococcus aureus SCVs, they nevertheless showed a novel, particular, sticky, phenotype. Expression of the latter was extremely stable, even after subcultivation.

SHORT COMMUNICATION

Improvements in the identification and classification of bacteria have recently shed light on coagulase-positive staphylococci (CoPS) other than Staphylococcus aureus (SA). Among these organisms, Staphylococcus pseudintermedius (SP), although primarily affecting dogs and cats, has also occasionally been recognized as a human pathogen. Most worrying is the emergence of human infections by methicillin-resistant SP (MRSP) strains (1, 2). However, interpretative criteria for methicillin resistance in this species are only available in the Clinical Laboratory Standards Institute (CLSI) guidelines for bacterial pathogens from animals, which do not recommend cefoxitin as surrogate antibiotic for prediction of mecA-mediated methicillin resistance. Instead, oxacillin breakpoints have been defined and should be used (3). Since cefoxitin represents, for human clinical laboratories, an indicator for methicillin resistance, its use may lead to an underestimation of methicillin resistance in SP, especially if based on SA breakpoints, and result in clinical failures. Nevertheless, Bemis et al. have recently highlighted that this cephalosporin can be a useful marker predicting methicillin...
resistance in SP when a $\leq 30$ mm inhibition zone diameter (IZD) is considered as the epidemiological cut-off value (4).

The phenomenon of small colony variants (SCVs) was first described in SA about 80 years ago (5). SCVs have been labeled as a naturally occurring population that develops in many bacteria within the parental cells under unfavorable conditions, such as antibiotic exposure. SCVs exhibit different biochemical and morphological aspects from the parent strains, like exceedingly small size, as well as reduced coagulase production and hemolytic properties (6, 7). Moreover, SA SCVs show higher MICs than the mother cells to several antimicrobials and antiseptics; they are also part of the regular growth cycle and can be either highly dynamic or stable. Variations in the cell wall electrochemical gradients or upregulation of genes involved in biofilm formation or in bacterial persistence in the environment are presumed to be behind SCVs (7, 8). Additionally, slow growth of small sized variants reduces the effectiveness of cell wall active antibiotics, such as $\beta$-lactams and glycopeptides (8, 9).

To our knowledge, SCVs have never been reported in SP and their first observation in this species is described below.

Nasal swabs were performed as pre-bone marrow transplantation screening in a leukemic female patient with no rhinosinusitis. CoPS colonies with double zone hemolysis on trypticase soy agar containing 5% sheep blood (TSS, Liofilchem®, Italy) were massively grown in pure culture as the putative colonizers of both nasal cavities (isolates from left and right nostrils were named S84 and S86, respectively), and showed mannitol fermentation on MSA II agar (Liofilchem®) after 24h incubation. The Vitek 2 (BioMérieux, France) identified them as *Staphylococcus intermedius*, and a
specific multiplex-PCR based on the analysis of the nuc gene (10), as well as the fragment length polymorphism analysis (11) placed them within the SP species. It is noteworthy that, although SP nasal colonization in humans is mainly associated with pet owners and veterinarians (12), the patient, even though living in a country environment, did not have any companion or farm animals.

Multilocus sequence typing (MLST) (13) revealed that S84 and S86 belonged to ST71, the most common MRSP clone spreading in Europe in dogs and cats, which has also been associated with infections in humans (2, 14).

Broth microdilution method performed with Sensititre plates NLEUST1 and EUST (TREK Diagnostic Systems, UK) revealed that the isolates shared the same multidrug resistance profile (Table 1), and meca gene detection, carried out by PCR as described previously (15), allowed us to label both as MRSP.

An agar disc test with cefoxitin provided a 31 mm IZD for both strains; after 48h incubation, however, microcolonies were observed within the S84 inhibition zone. This novel isolate (named S85) showed a poor, slow growth, with much smaller colonies (<0.5mm) than the mother culture (1-2 mm) (Fig. 1) after subcultivation on TSS for 24h, at 37°C. S85 colonies were mannitol negative at 24 h, coagulase-negative at 4h, rubbery, adherent, confluent, and sticky (similarly to those formed by Rothia mucilaginosa). S85 was identified as a meca-positive SP ST71 using the above-mentioned molecular methods and surprisingly displayed high-level resistance to cefoxitin with >16 µg/ml MIC (Table 1) and a 12 mm IZD.

The three isolates had a unique molecular fingerprint if analyzed with PFGE, semi-automated rep-PCR (DiversiLab, BioMérieux, France) and RAPD, using previously
described primers (NP2 being the most discriminant) (16) (Fig. 2), confirming that S85 is an SCV derived from the parental strain S84 after prolonged cefoxitin exposure. After 48h incubation, SCVs reached the parent strain’s size, they were less sticky and began to ferment mannitol as well as to coagulate rabbit plasma. However, when re-cultivated from the 48h-incubated plates, they started growing as SCVs again.

This first observation of SCVs in SP is intriguing, although it is impossible, to date, to know whether these variants play a part in the persistence of human and animal colonization and infections. Nevertheless, it is known that SCVs in SA have an increased ability to survive within non-professional phagocytes that then behave as reservoirs for recurrent and chronic diseases and reduce the exposure to certain antibiotics (6, 7, 17).

Similarly to SA SCVs, S85 showed decreased colony size and reduced coagulase activity. Unlike SA, however, those formed by SP had an unchanged hemolysin pattern: both S84 and S85 formed internal completely hemolytic and external incompletely hemolytic zones (Fig. 1) whose diameters were, however, proportional to the colony size after 24 h incubation. The SCV isolate S85 did not revert to the large-colony size and the small phenotype was maintained after subcultivations, as well as upon defrosting from -80°C storage. It is well known, conversely, that SCVs in SA usually revert after discontinuation of the hostile conditions (6, 7, 17).

Unlike most SP isolates, S85 was resistant to both cefoxitin and oxacillin, and showed delayed mannitol fermentation and coagulase activity (Table 1); again, the most impressive feature was that SCVs looked like *R. mucilaginosa*, being confluent, attached each other, filamentous, and strongly adherent to the agar.
This report suggests that, similarly to those formed by SA, SP SCVs may represent a mechanism for survival under hostile conditions. Although both SA and SP small-sized variants display slower growth and decreased antibiotic susceptibility, lack of reversibility along with the sticky, confluent phenotype, are the main differences between SCVs of these two CoPS species.

There is still poor understanding of SP colonization and infections in humans, as well as of clinical impact of bacterial SCVs; nonetheless, small-sized phenotype appeared to be an additional, previously undescribed feature in this organism that may enable it to survive the host defenses and the antibiotic treatment. The strong reduction in growth rate makes it difficult to detect SCVs within a mixed population in the clinical routine practice. Hence, underestimation of antibiotic resistance and subsequent drug clinical failure may unfortunately occur (7). Further studies will be necessary to better understand the role of SCV in SP host adaptation and pathogenicity in both humans and animals.
### TABLE 1. Minimal inhibitory concentrations (MICs) of methicillin-resistant *S. pseudintermedius* ST71 strains S84, S86 and the SCV strain S85 as determined by broth microdilution method.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Measured concentration ranges (µg/ml)</th>
<th>Resistance breakpoints (µg/ml)</th>
<th>MICs (in µg/ml) and resistance (R) and susceptibility (S) profile for strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S84</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>0.12 – 2</td>
<td>≥0.25</td>
<td>R, &gt;2</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0.5 – 16</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.25 – 8</td>
<td>≥0.5</td>
<td>R, &gt;8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25 – 8</td>
<td>≥4</td>
<td>R, &gt;8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.25 – 8</td>
<td>≥8</td>
<td>R, &gt;8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1 – 16</td>
<td>≥16</td>
<td>R, &gt;16</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5 – 16</td>
<td>≥16</td>
<td>R, &gt;16</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.12 – 4</td>
<td>≥4</td>
<td>R, &gt;4</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>2 – 32</td>
<td>≥16</td>
<td>R, &gt;32</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>64 – 512</td>
<td>≥512</td>
<td>R, &gt;512</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>0.5 – 256</td>
<td>≥256</td>
<td>S, ≤ 0.5 S, ≤ 0.5</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4 – 64</td>
<td>≥32</td>
<td>S, 8</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.5 – 4</td>
<td>NA</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.016 – 0.5</td>
<td>≥4</td>
<td>S, ≤ 0.016 S, ≤ 0.016</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1 – 8</td>
<td>≥8</td>
<td>S, ≤ 1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1 – 16</td>
<td>≥32</td>
<td>S, ≤ 1</td>
</tr>
</tbody>
</table>

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**a)** MICs have been interpreted according to CLSI document M100-S23; as an exception, oxacillin breakpoints defined in the CLSI supplement VET01-S2 for bacterial from animals were used. NA: no breakpoints available (for cefoxitin screen and fusidic acid); phenotypic differences are indicated in bold.

**b)** Breakpoint relates to nasal decolonisation of *S. aureus* (CLSI document M100-S23).
Figure legends

**FIG. 1.** Photographs of *S. pseudintermedius* strain S84 (left) and the small colony variant (SCV) strain S85 (right) and of hemolysin pattern of strain S84 (top right) and SCV S85 (bottom right) (B) after 24h incubation on trypticase soy agar containing 5% sheep blood.

**FIG. 2.** PFGE, rep-PCR and RAPD fingerprints.

Legend. M = molecular markers. Lane 1, *S. aureus* NCT2583. Lanes 2–4: strains S84, S85 and S86. Lane 5: MRSP strain KM1381 (ST71), described in (18) and used as an internal control. Lane 6: MRSP strain 06-1400 (ST68), kindly provided by Stephen Kania and David Bemis, used as an internal control.
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REFERENCES


Fig. 2