

CLINICAL RESEARCH ARTICLE

Staphylococcus aureus small colony variants in diabetic foot infections

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Background: *Staphylococcus aureus* (*S. aureus*) is one of the major pathogens causing chronic infections. The ability of *S. aureus* to acquire resistance to a diverse range of antimicrobial compounds results in limited treatment options, particularly in methicillin-resistant *S. aureus* (MRSA). A mechanism by which *S. aureus* develops reduced susceptibility to antimicrobials is through the formation of small colony variants (SCVs). Infections by SCVs of *S. aureus* are an upcoming problem due to difficulties in laboratory diagnosis and resistance to antimicrobial therapy.

Methods: A prospective study was performed on 120 patients diagnosed with both type 2 diabetes mellitus and infected diabetic foot ulcers. The study was carried out from July 2012 to December 2013 in Hospital General de Mexico. The samples were cultured in blood agar, mannitol salt agar, and MacConkey agar media, and incubated at 37°C in aerobic conditions.

Results: We describe the first known cases of diabetic foot infections caused by MRSA-SCVs in patients diagnosed with type 2 diabetes mellitus and infected diabetic foot ulcers. In all of our cases, the patients had not received any form of gentamicin therapy.

Conclusions: The antibiotic therapy commonly used in diabetic patients with infected diabetic foot ulcers fails in the case of MRSA-SCVs because the intracellular location protects *S. aureus*-SCVs from the host's defenses and also helps them resist antibiotics. The cases studied in this article add to the spectrum of persistent and relapsing infections attributed to MRSA-SCVs and emphasizes that these variants may also play a relevant role in diabetic foot infections.

Keywords: *diabetic foot; infection; ulcer; small colony variants; methicillin-resistant Staphylococcus aureus*

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Human diseases caused by *Staphylococcus aureus* (*S. aureus*) are a worldwide public health problem and a burden for dairy farming and the food industry (1). *S. aureus* is the most common cause of skin and soft tissue infections. Once it penetrates the subcutaneous tissue and subsequently infiltrates the blood, it can reach any organ. Bone and heart valve tissues are among the most affected structures within the organism (2).

The severity and variety of staphylococcal infections, as well as the difficulty to treat clinical cases reflect the evolutionary versatility of *S. aureus* and the challenges hospitals face (2). The mechanisms by which *S. aureus* evades host's defenses and produces multiple virulence

factors contributes to the diversity and risk of staphylococcal diseases (3). In addition, *S. aureus* is capable of temporarily colonizing skin and mucosae causing no clinical signs. Actually, it has been estimated that at least 25 to 30% of the population worldwide are permanent carriers of *S. aureus* (3). Nevertheless, in diabetic patients, *S. aureus* can change from an opportunistic agent into a pathogen involved in distinctive clinical manifestations, such as diabetic foot ulcers. The same treatment for these infectious processes produces the selection of variants resistant to diverse antibiotics, generating multiresistant *S. aureus*, among which methicillin-resistant *S. aureus* (MRSA) stands out (4).

S. aureus has traditionally been considered an extra-cellular bacterium. Nonetheless, it has been recently shown that this pathogen can reside and survive intracellularly as a staphylococcal phenotype different from the wild type and that seems to be related to cell invasion and clinical persistence. These properties are considered characteristics of *S. aureus* small colony variants (SCVs) (5–7).

S. aureus-SCVs originated naturally as subpopulations of *S. aureus* and are associated with persistent and recurrent infections, because chronic infections by *S. aureus* and intracellular persistence have been linked to the *S. aureus*-SCVs phenotype. However, it is difficult to identify these colonies, because they are unstable and can rapidly revert to the original phenotype (7, 8).

Compared to the original *S. aureus*, *S. aureus*-SCVs are ten times smaller than the wild phenotype, they have a slower growth rate, and they are non-hemolytic and non-pigmented strains (1, 7). Moreover, they have an electron transport deficiency and, as a consequence, they exhibit auxotrophy for thymidine, menadione, and hemin (1, 7). Such variants are not easy to eliminate because of their increased resistance to both aminoglycosides (9, 10) and antibiotics that act against the cell wall, and as a result of their ability to endure inside cells. In addition, *S. aureus*-SCVs intracellular location and persistence have been shown only *in vitro*, but not *in vivo* (11, 12).

S. aureus-SCVs have been described with the use of routine microbiological methods in clinical laboratories in cases of sepsis, endocarditis, respiratory tract infections, cystic fibrosis, osteomyelitis, arthritis, brain abscesses, sinusitis, and foreign bodies associated with infections (9, 13–15).

Infections by MRSA are the cause of high morbidity and mortality rates, especially in the hospital intensive care units (16). It has been observed that MRSA-SCVs provoke more serious infections and higher mortality rates than those caused by MRSA (5, 17, 18). Nevertheless, currently there are few reports about MRSA-SCVs (13–15, 17–20).

The aim of the present study was to assess and describe the first cases of MRSA-SCVs infections in patients with type 2 diabetes mellitus and infected diabetic foot ulcers.

Materials and methods

A prospective study was performed on 120 patients diagnosed with type 2 diabetes mellitus and infected diabetic foot ulcers. The study was carried out from July 2012 to December 2013 in the emergency room and the diabetic foot clinic of the Hospital General de México (Cuauhtémoc, Colonia Doctores). The study was approved by the ethics committees of the Hospital General de México and the Facultad de Medicina of the Universidad Nacional Autónoma de México (Ciudad Universitaria, Colonia Coyoacan, Ciudad de México). In addition, all participants provided written informed consent.

All patients presented with a diabetic foot ulcer and the following symptoms: inflammation, erythema, pain,

warmth, and purulent drainage. Also, some patients exhibited cellulitis and/or gangrene. According to the Infectious Disease Society of America guidelines (21), infection is present if there is obvious purulent drainage and/or the presence of two or more signs of inflammation, erythema, pain, tenderness, warmth, or induration. Therefore, we concluded that all patients had an infected diabetic foot ulcer. In fact, the ulcers were of types 3, 4, or 5 according to Wagner's classification (22).

The foot ulcers were cleaned and irrigated vigorously with surgical soap and sterile saline solution and were debrided afterward. The debridement was done using sterile scalpels to remove unhealthy tissue from the infected ulcer. After the debridement, the foot ulcers were cleaned and irrigated again vigorously with surgical soap and sterile saline solution to remove all contaminants. Then, the samples were obtained from the foot ulcer base (without touching the skin and other tissues) with a sterile swab and were placed in a Stuart transport medium (Becton Dickinson de México) to be sent to be analyzed at the Laboratorio de Bacteriología of the Facultad de Medicina of the Universidad Nacional Autónoma de México. The procedure used allowed us to avoid contaminant organisms and to identify most accurate pathogens in the diabetic foot ulcerations.

The samples were cultured in blood agar (BA), mannitol salt agar (MSA), and MacConkey agar media (Becton Dickinson de México), and then incubated at 37°C for 24 h in aerobic conditions. We identified *S. aureus* in 47 of the 120 samples by observing β -hemolysis in BA and by carrying out the conventional biochemical procedures catalase and coagulase. Afterward, the colony morphology was determined and Gram-staining of the isolated colonies was performed.

After 48 h of incubation, we observed four samples with small, non-pigmented, and non-hemolytic colonies around the typical *S. aureus* colonies that were originally identified (Fig. 1). These small colonies were further sub-cultured in BA and MSA and incubated in aerobic conditions,

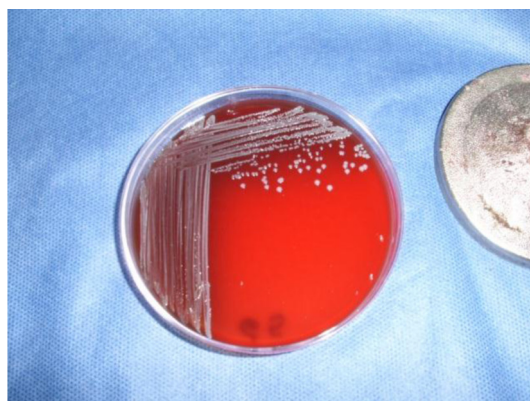


Fig. 1. *Staphylococcus aureus* small colony variants in blood agar.

monitoring them every 24 h. After 72 h, we observed that they grew as small, non-pigmented, and non-hemolytic colonies with the form of a pinpoint (Fig. 2). Gram-staining from these small, non-pigmented, and non-hemolytic colonies in BA and MSA revealed Gram-positive cocci arranged in small clusters with sizes ten times smaller than the wild strain. The colonies were catalase-positive and oxidase-negative. The tube coagulase test was mildly positive after 18 h. Also, the MacConkey agar media showed no growth.

To determine whether these colony subpopulations were methicillin resistant, we used the disk diffusion method using Müller-Hinton agar (Becton Dickinson de México) following the Clinical and Laboratory Standards Institute (CLSI) guidelines (16). The antibiotics used were oxacillin (OXA) and cefoxitin (CEX). The colony subpopulation isolates were tested with a CEX 30- μ g disk and with an OXA 1- μ g disk according to the CLSI guidelines (CEX: susceptibility ≥ 22 mm, resistance ≤ 21 mm; OXA: susceptibility ≤ 2 μ g/mL, resistance ≥ 4 μ g/mL). Also, we evaluated the susceptibility and resistance to vancomycin (VA) by the minimum inhibitory concentration (MIC) technique in Müller-Hinton broth following the CLSI guidelines (16) (susceptibility ≤ 4 μ g/mL, resistance ≥ 16 μ g/mL). The strain *S. aureus* ATCC 25923 (American Type Culture Collection 25923) was used as control. All four samples of SCVs turned out to be both methicillin and VA resistant. These MRSA-SCVs were further tested with gentamicin (30 μ g). To determine the auxotrophy of the MRSA-SCVs, a lawn culture was put in Müller-Hinton agar with thymidine and hemin disks over it, and incubated for 24 to 72 h at 37°C using 0.5 MacFarland (Becton Dickinson). The colonies around thymidine and hemin discs grew as small long colonies, thereby confirming thymidine and hemin auxotrophy. Finally, to determine the presence of slime-forming strains, we used the Congo red agar (Bio Medical Service de México) method. We did not find any slime-forming strains.



Fig. 2. *Staphylococcus aureus* small colony variants with pinpoint form in Müller-Hinton agar.

Results

S. aureus was identified in 47 of the 120 samples of infected diabetic foot ulcers studied. Of these 47 samples, 25 corresponded to male and 22 to female patients. All 47 samples were MRSA. The response to VA was also assessed, finding strains resistant to this antibiotic in four patients. In these four patients, there were different small, non-pigmented, and non-hemolytic colonies around the wild phenotype. They were cultured again, obtaining typical MRSA-SCVs which were recognized through diverse phenotypic features such as slow growth; small size; groups of tiny long, non-pigmented colonies; and absence of hemolysis in BA, as well as Gram-staining. The species of *S. aureus*-SCVs were catalase-positive, weakly coagulase-positive, and oxidase-negative. There was no growth in MacConkey agar. In gentamicin, thymidine, and hemin tests, it was seen that MRSA-SCVs grew around these disks, thus revealing the auxotrophy of such strains. Also, the Congo red agar method allowed us to conclude that there were no slime-forming strains.

Discussion

To the best of our knowledge, we describe the first known cases of diabetic foot infections caused by MRSA-SCVs in patients diagnosed with type 2 diabetes mellitus and infected diabetic foot ulcers. All of the cases studied had infected diabetic foot ulcers. Unfortunately, in our study, we were not aware if the patients had been previously colonized and if this led to infections by MRSA. The reason for this is that we had no previous data with regard to colonization, and all patients considered in this study arrived at the hospital with infected diabetic foot ulcers.

Our findings suggest that *S. aureus*-SCVs might have been selected from the parent population with normal phenotype, because the four samples with *S. aureus*-SCVs came from patients that were treated with multiple antibiotics for 1 or more months. In particular, they were first treated with trimethoprim-sulfamethoxazole and other antibiotics for 1 to 1.5 months and were then treated with VA for 7 days. In the cases where the ulcers did not heal, other antibiotics were then administered. It is very important to note that the four samples with *S. aureus*-SCVs came from patients that were treated with trimethoprim-sulfamethoxazole, because it has been observed (especially in patients with cystic fibrosis) that this antibiotic induces the formation of SCVs in *S. aureus* (17, 18). Also, all of our 47 patients with diabetic foot ulcers infected with *S. aureus* were treated with multiple antibiotics for 1 or more months, but none were treated with gentamicin.

SCVs can be identified with gentamicin both *in vitro* and *in vivo* (9, 10), as it has been observed in patients with osteomyelitis after administering this antibiotic (14–16). One interesting finding of our cases is that the patients had not received any form of gentamicin therapy, neither

in systemic form nor local gentamicin bead therapy. Finally, it is also important to note that we did not find slime-forming strains in any of the 47 patients with diabetic foot ulcers infected with *S. aureus*.

Few cases have been reported in the literature about human infections by MRSA-SCVs (17–19). The cases we studied add to the spectrum of persistent and relapsing infections attributed to MRSA-SCVs and emphasizes that these variants may also play a relevant role in diabetic foot infections. *S. aureus*-SCVs are one of the most interesting variants described in the past decades. They are a bacterial subpopulation with atypical characteristics compared to the ones of wild phenotype strains. *S. aureus*-SCVs have been associated with persistent and recurrent infections due to long-time predisposing conditions, leading to a poor clinical and bacteriological response to antimicrobial treatment as is the case of patients with cystic fibrosis, bronchopulmonary infections, abscesses, chronic osteomyelitis, and, in our study, patients with diabetic foot ulcers whose long-time exposure to antimicrobial agents is frequent. This last property favors a selective survival of intracellular bacteria such as *S. aureus*-SCVs. The antibiotic therapy commonly used in diabetic patients with infected diabetic foot ulcers may fail because the antibiotics cannot penetrate the infected sites. The intracellular location protects *S. aureus*-SCVs from the host's defenses and also helps them resist antibiotics (20, 23).

Conclusions

The clinical laboratories should be particularly alert for *S. aureus*-SCVs when samples are submitted from patients who have received long-term antimicrobial therapy, especially if the infections are unusually persistent or recurrent as in our patients with diabetic foot ulcers infected for a long time. *S. aureus*-SCVs strains are responsible for the misidentification of infections in patients by routine, automated systems widely used to detect *S. aureus* isolates. These results can be the consequence of short incubation periods by these systems or low discrimination levels in their data bases. The key to successfully recover and recognize *S. aureus*-SCVs is the use of an extensive set of cultures and identification techniques (24).

In our study, the presence of small colonies around the typical *S. aureus* colonies is a remarkable finding in patients diagnosed with type 2 diabetes mellitus and infected diabetic foot ulcers not treated with gentamicin. This makes these isolates an important outcome, because these variants had neither been previously reported nor was there knowledge about them in infected diabetic foot ulcers.

The isolation of MRSA-SCVs in diabetic foot ulcers and patients diagnosed with type 2 diabetes mellitus indicates that the treatment is more problematic and complex. Clinicians need to be aware of identifying these variants

to provide an efficient treatment, prevent complications, and to avoid the use of a great number of antibiotics without identifying the cause of infection. The latter is especially important, because it leads to antibiotic resistance and poses a challenge for the physician.

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Conflict of interest and funding

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