Thymidine Auxotrophic Staphylococcus aureus Small Colony Variant Endocarditis
and Left Ventricular Assist Device Infection

Awele Maduka-Ezeh,1 Maria Teresa Seville,2 Shimon Kusne,2 Holenarasipur R. Vikram,2
Janis E. Blair,2 Kerryl Greenwood-Quaintance,3 Francisco Arabia,4 Robin Patel1,3

1Division of Infectious Diseases, Department of Internal Medicine,
Mayo Clinic, 200 1st Street SW, Rochester MN, 55905
2Division of Infectious Diseases, Department of Internal Medicine,
Mayo Clinic, 5777 East Mayo Boulevard, Phoenix AZ 85054
3Division of Clinical Microbiology, Department of Laboratory Medicine and Pathology,
Mayo Clinic, 200 1st Street SW, Rochester MN, 55905
4Division of Cardiovascular and Thoracic Surgery,
Mayo Clinic, 5777 East Mayo Boulevard, Phoenix AZ 85054

Correspondence to:
Robin Patel, M.D.

Division of Clinical Microbiology, Mayo Clinic
200 First St. SW, Rochester, MN 55905, USA
507-538-0579
507-284-4272 (fax)
E-mail: patel.robin@mayo.edu
Abstract

We describe a thymidine dependent small colony variant of *Staphylococcus aureus* associated with left ventricular assist device infection and prosthetic valve and pacemaker endocarditis.

Case report

The patient was a 34 year old woman who three years prior had suffered an anterior wall myocardial infarction resulting in ischemic cardiomyopathy which necessitated placement of a pacemaker-biventricular defibrillator. Subsequently, there was progressive deterioration of cardiac function. A left ventricular assist device (LVAD) was placed as a bridge to heart transplantation. During the same surgery, her native tricuspid valve was replaced with a pericardial tissue valve on account of tricuspid regurgitation. One month after surgery, she developed fever and chills and blood cultures grew methicillin-resistant *Staphylococcus aureus* (MRSA) which was susceptible to gentamicin, rifampin, linezolid, trimethoprim-sulfamethoxazole (TMP/SMX), and vancomycin (MIC, 2 µg/ml), and resistant to erythromycin, and levofloxacin. Trans-esophageal echocardiography (TEE) showed no evidence of endocarditis. She was treated with six weeks of vancomycin and rifampin with gentamicin administered during the first two weeks of therapy. Five days after completing antimicrobial therapy, she developed recurrent fevers and blood cultures again grew MRSA which was susceptible to gentamicin, linezolid, TMP/SMX, vancomycin (MIC, 1 µg/ml), and daptomycin, and resistant to rifampin, erythromycin, and levofloxacin. A TEE demonstrated vegetations on the pacemaker leads and the prosthetic tricuspid valve. She was treated with daptomycin, gentamicin and
(until the isolate was known to be resistant to rifampin) rifampin. A follow-up TEE after 2 months of antimicrobial therapy showed reduction in the size of the vegetations. She received 5 months of daptomycin and gentamicin; TEE at the end of therapy showed “old and healed” vegetations. However, over the ensuing year, she had recurrent MRSA bacteremia and soft tissue infection surrounding her LVAD generator site. As a result, from December 2008 through March 2010, she received continuous antimicrobial therapy with various combinations of vancomycin, rifampin, daptomycin, gentamicin, quinupristin-dalfopristin, and TMP/SMX (figure 1). During this time, only one isolate (of several) was identifiable and susceptibility testing reportable using the BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Franklin Lakes, NJ). That isolate, recovered from an umbilical driveline aspirate, was reported as susceptible to gentamicin, linezolid, TMP/SMX, and vancomycin (MIC, 2 µg/ml), resistant to rifampin, erythromycin, and levofloxacin, and non-susceptible to daptomycin (MIC, 4 µg/ml). Because of inadequate growth in the test medium, other isolates, including bloodstream isolates and another umbilical driveline aspirate, were not identifiable and susceptibility testing not reportable using the BD Phoenix Automated Microbiology System. Despite ongoing combination antimicrobial therapy and incision and drainage of the abscess around the LVAD generator, infection persisted (intermittent swelling, redness and tenderness overlying the chest wall LVAD pocket, discoloration and swelling around the umbilical driveline exit site, and fluctuance with drainage from the lower sternum). She eventually underwent explantation of the LVAD and placement of a paracorporeal ventricular assist device (PVAD), as well as prosthetic tricuspid valve replacement with a porcine tissue valve.
An isolate recovered from around the LVAD at the time of removal was archived at -70°C (IDRL-8699). It formed small colonies that were catalase-, Staphaurex (Remel, Lenexa, KS)- and tube coagulase-positive, and Gram-stained as Gram-positive cocci resembling staphylococci. As with some prior isolates, identification and antimicrobial susceptibility testing failed using the BD Phoenix Automated Microbiology System. The isolate was grown from a freezer stock on sheep blood agar (SBA) (BD) and after 24 hours of growth at 37°C in 5% CO₂, pinpoint, clear, non-hemolytic, non-pigmented [small colony variant (SCV)] colonies were visible. Continued incubation on the same plate for 72 hours resulted in the formation of colonies with typical *S. aureus* colonial morphology (i.e., yellow pigmentation, β-hemolysis). The isolate was confirmed to be *S. aureus* by *tuf* PCR (23), and Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry (score using direct colony testing, 2.393, BrukerDaltonics, Billerica, MA) (1).

Serial passaging of the isolate was performed. Reversion to normal phenotype in the absence of antimicrobials is a well-described characteristic of SCVs. Massey et al. ascribed this phenomenon to an attempt by the organism to maintain fitness when antimicrobial pressure (and thus the need for expression of the SCV phenotype) is removed (20). It has been suggested that the ability to switch between SCV and normal morphology colonies may be associated with intermittent re-occurrence of active infection (as normal morphology colonies) with periods of quiescence in between (when isolates exist as SCV forms) (21). For each passage, following 24 hours incubation at 37°C, a subculture was made on a fresh SBA plate. Passaging was done simultaneously in air and 5% CO₂. Plates grown in air were compared to those grown in 5% CO₂ and any
differences noted. Each step was done in duplicate. The experiment was repeated on two separate occasions. With serial passage on SBA, either in air or CO$_2$, colonies reverted to normal diameter (>1mm) colonies by the second or third passage. Further passaging of these normal colonies resulted in reversion back to the SCV phenotype. Up to five more passages were subsequently done with no reappearance of the normal phenotype. To the best of our knowledge, this fluctuating phenotype has not been previously described.

Hemin, thymidine and menadione auxotrophy were tested by the disc and agar methods. For the disc method, commercially available 10% hemin discs (Sigma Aldrich, Saint Louis, MO) or discs impregnated with thymidine (100µg/ml) or menadione (25µg/ml) were placed on Mueller Hinton agar (MHA) plates inoculated with a 0.5 McFarland standard of the isolate. The isolate was considered auxotrophic if it showed normal colonies or increased growth surrounding the impregnated disc compared to the periphery following 24 hours of incubation in air at 37°C (5, 17). For the agar method, following growth overnight on SBA, 3-4 similar colonies were inoculated onto MHA with and without supplementation with thymidine 125µg/ml, menadione 25µg/ml or hemin 10µg/ml. Auxotrophy was considered present if the isolate grew as normal sized colonies on supplemented versus non-supplemented MHA. Each step was done in duplicate and the experiment repeated on two separate days. *S. aureus menD* and *hemB* knockout mutants provided by Dr. C. Von Eiff (Institute of Medical Microbiology, University Hospital Münster, Germany) were used as menadione and hemin controls (3, 27).

Auxotrophy testing for CO$_2$ was done by comparing growth at 37°C in air with that in 5% CO$_2$. The isolate was thymidine-dependent, growing only on MHA supplemented
with 125µg/ml of thymidine and around discs impregnated with 100µg/ml of thymidine, but not on MHA alone. Hemin, menadione and CO₂ auxotrophy were negative.

*mecA* PCR, done with the following primers: mec449F, 5'-AAA CTA CGG TAA CAT TGA TCG CAA C-3', and mec761R, 5'-CTT GTA CCC AAT TTT GAT CCA TTT G-3', as previously described (18), was positive. Antimicrobial susceptibility was tested by Etest (BioMérieux, Marcy l'Etoile, France) following manufacturer’s instructions [except that 5% defibrinated sheep blood was added to the media, as previously described (5, 6)] for all tested antimicrobics except gentamicin, amikacin, tobramycin and kanamycin which were tested by agar dilution following Clinical and Laboratory Standards Institutes guidelines (10) [except that 5% defibrinated sheep blood was added to the media, as previously described (5, 6)]. All testing was simultaneously performed on control strain *S. aureus* ATCC 29213 and was within the quality control range specified by Clinical and Laboratory Standards Institutes guidelines (11). In addition to reading susceptibilities at 20 hours (24 hours for oxacillin and vancomycin), susceptibilities were read at 48 hours. The isolate was resistant to tobramycin, amikacin and kanamycin (MIC, >16, >64 and >64µg/ml, respectively), but susceptible to gentamicin (MIC, ≤0.5µg/ml). The isolate was resistant to oxacillin and cefoxitin (MIC, >256 and >256µg/ml, respectively) and resistant to TMP/SMX (MIC, >32/608 µg/ml). Similar results (≤1 dilution difference) were obtained when MICs were re-read at 48 hours. The isolate was rifampin resistant (MIC, >32µg/ml), but susceptible to vancomycin, minocycline, and linezolid (MIC, 1, 0.032 and 0.25µg/ml, respectively).

Biofilm and planktonic growth rates were measured as follows: A culture of the isolate in trypticase soy broth (TSB) was adjusted to match the turbidity of a 1.0 McFarland.
standard and then diluted 1:50 in TSB. 200 µl aliquots were placed into four wells of seven 96-well microtiter plates corresponding to incubation times of 6, 12, 24, 48, 72, 96 and 120 hours, respectively. Planktonic growth at each of the time points was measured by OD$_{600}$ using a Multiskan microtiter plate reader (Thermo Electron, Waltham, MA). Culture medium was discarded, and wells washed twice by submerging plates in deionized water to remove nonadherent cells. Plates were air dried overnight. Biofilms were then stained with 0.1% safranin for 1 minute, rinsed under running tap water to remove excess stain, and air dried. Stained biofilms were resuspended in 200µl 30% glacial acetic acid, and the OD$_{492}$ measured. Wells containing uninoculated medium served as negative controls. Staphylococcus epidermidis ATCC 35984 (RP62A) was used as a positive control. The biofilm index (biofilm OD$_{492}$/planktonic OD$_{600}$), which measures biofilm formation corrected for rate of cell growth, thus allowing comparison between slower and faster growing isolates, was calculated (12, 14, 19). The experiment was repeated at three different times. IDRL-8699 was a poor biofilm former when compared to the positive control strain, showing a biofilm index that was less than the negative control.

Following replacement of her infected prosthetic tricuspid valve and LVAD, the patient has done well on chronic antimicrobial suppression without evidence of recurrent infection with S. aureus during 18 months of followup.

Cardiac devices, including pacemakers and implantable cardioverter defibrillators, as well as LVADs, are increasingly used (8, 16, 22). Accompanying increased use of these
devices has been an increase in both the total number and in the rates of infections of these devices (8). Cardiac device and LVAD infections result in significant morbidity and reduced patient survival (8, 16).

SCVs are a subpopulation of bacteria that have been associated with persistent and difficult to treat infections of both native tissue and prosthetic material. Best described for *S. aureus*, the purported mechanisms behind their persistence include antimicrobial resistance (4), enhanced biofilm formation (24, 25) and the ability to exist for prolonged periods in the intracellular milieu (2, 26, 28). Conceivably, purulent secretions containing deteriorating cells, the DNA of which can be degraded by *S. aureus* DNase, present around the LVAD could result in a supply of thymidine supporting growth of thymidine-dependent *S. aureus* (7, 15, 28). Thymidine auxotrophic *S. aureus* SCVs have been frequently isolated from the lungs of cystic fibrosis patients and (less frequently) from patients with chronic soft tissue infection, tympanitis, bronchitis, peritonitis and bacteremia (7), but have not been previously reported in association with endocarditis, pacemaker infection or LVAD infection.

Thymidine auxotrophs are thought to arise due to antibiotic pressure after prolonged exposure to TMP/SMX (6, 7). This has been best described in isolates from the airways of cystic fibrosis patients on chronic TMP/SMX therapy (15, 17), but long-term TMP/SMX has also been associated with thymidine auxotrophy in *S. aureus* isolates from patients without cystic fibrosis (7). Thymidine auxotrophic SCVs have been shown to harbor mutations within the thymidylate synthase-encoding thyA gene (5, 7, 9).

Whether or not thyA is mutated in the IDRL-8699 was not determined.
It is likely that most clinical laboratories would not be able to perform antimicrobial susceptibility testing on thymidine-dependent \textit{S. aureus}. Thymidine auxotrophs are generally considered resistant to TMP/SMX due to utilization of extracellular deoxythymidine monophosphate, by-passing the pathway targeted by TMP/SMX (7, 15, 28). The ideal approach to assessing the antimicrobial susceptibility of thymidine-dependent \textit{S. aureus} SCVs is unclear. We assessed susceptibility using MHA supplemented with 5\% defibrinated sheep blood, as described by Besier et al. (5, 6). The addition of defibrinated sheep blood is not recommended by the Clinical and Laboratory Standards Institute or the manufacturer of Etest strips as it may supply constituents that bypass the effects of TMP/SMX, potentially causing TMP/SMX susceptible strains to appear resistant to TMP/SMX (13). Nevertheless, it is otherwise impossible to assess the TMP/SMX susceptibility of such isolates, and the control strain, \textit{S. aureus} ATCC 29213, tested TMP/SMX-susceptible in our experiments, supporting the use of this method.

To complicate matters, reversion of thymidine-dependent \textit{S. aureus} SCVs has been previously described in association with loss of both thymidine auxotrophy and TMP/SMX resistance (7). “False” TMP/SMX susceptibility due to reversion of \textit{thyA} mutations in the laboratory or the co-presence of isolates with and without such mutations and the inability to perform routine susceptibility testing on thymidine-dependent isolates when using automated instruments or following Clinical and Laboratory Standards Institutes guidelines along with a false assumption that \textit{S. aureus} is generally TMP/SMX-susceptible could potentially drive inappropriate TMP/SMX therapy of thymidine-dependent \textit{S. aureus}. At our institution, more than 97\% of \textit{S. aureus} isolates (that can be tested for TMP/SMX susceptibility following Clinical and...
Laboratory Standards Institutes guidelines) are reported as being susceptible to TMP/SMX. Indeed, in the case described herein, TMP/SMX was initially selected (along with minocycline) for antimicrobial suppression.

Thymidine auxotrophs of *S. aureus* have been shown to be hypermutable, and therefore might be more likely to acquire mutational antimicrobial resistance than those of normal colony phenotypes (6). Although we did not address this in our study, it is possible that hypermutability was present in our patient’s *S. aureus*, influencing the emergence of rifampin resistance and daptomycin non-susceptibility.

Enhanced biofilm formation by laboratory-selected strains of menadione auxotrophic *S. aureus* has been well described (24, 25). Interestingly, despite the persistent infection produced by IDRL-8699, it did not form substantial biofilm *in vitro*. One possible explanation is that there might be conditions present *in vivo*, but not in the *in vitro* environment tested which support enhanced biofilm formation. This could also be a function of the specific assay used to assess biofilm formation. Another possibility is that enhanced biofilm formation is a characteristic of menadione, but not thymidine auxotrophic SCVs. Indeed, poor (but extant) biofilm formation could account for clinically “quiescent” but persistent infections associated with SCVs.

In summary, the case is presented here of a thymidine-dependent SCV associated with LVAD infection and prosthetic valve and pacemaker endocarditis in a 34 year woman. With passage on antimicrobial free media, the isolate exhibited a fluctuating phenotype, going from small to normal sized colony phenotypes and back again to the small colony phenotype.
Acknowledgment

We thank the outstanding staff of the Mayo Clinic Rochester and Arizona Bacteriology laboratories, especially Rebecca A Stewart, Peggy C Kohner, Scott A Cunningham and Sherry M Ihde, for their assistance with this case.
References


11. **Clinical and Laboratory Standards Institute.** 2011. Performance standards for antimicrobial susceptibility testing; Twenty-First Informational Supplement CLSI document M100-S21, Vol. 30 No 1, Clinical and Laboratory Standards Institute, Wayne, PA.


Figure 1: Duration of antimicrobials administered over time (through device explantation, day 482)

Arrowheads: *S. aureus* bacteremia episodes. Horizontal gray bars express number of days of each antibiotic given. Quin/Dal (quinupristin-dalfopristin).