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First Evidence of Sternal Wound Biofilm following Cardiac Surgery

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Abstract

Management of deep sternal wound infection (SWI), a serious complication after cardiac surgery with high morbidity and mortality incidence, requires invasive procedures such as, debridement with primary closure or myocutaneous flap reconstruction along with use of broad spectrum antibiotics. The purpose of this clinical series is to investigate the presence of biofilm in patients with deep SWI. A biofilm is a complex microbial community in which bacteria attach to a biological or non-biological surface and are embedded in a self-produced extracellular polymeric substance. Biofilm related infections represent a major clinical challenge due to their resistance to both host immune defenses and standard antimicrobial therapies. Candidates for this clinical series were patients scheduled for a debridement procedure of an infected sternal wound after a cardiac surgery. Six patients with SWI were recruited in the study. All cases had marked dehiscence of all layers of the wound down to the sternum with no signs of healing after receiving broad spectrum antibiotics post-surgery. After consenting patients, tissue and/or extracted stainless steel wires were collected during the debridement procedure. Debrided tissues examined by Gram stain showed large aggregations of Gram positive cocci. Immuno-fluorescent staining of the debrided tissues using a specific antibody against staphylococci demonstrated the presence of thick clumps of staphylococci colonizing the wound bed. Evaluation of tissue samples with scanning electron microscope (SEM) imaging showed three-dimensional aggregates of these cocci attached to the wound surface. More interestingly, SEM imaging of the extracted wires showed attachment of cocci aggregations to the wire metal surface. These observations along with the clinical presentation of the patients provide the first evidence that supports the presence of biofilm in such cases. Clinical introduction of the biofilm infection concept in deep SWI may advance the current management strategies from standard antimicrobial therapy to anti-biofilm strategy.

Introduction

Median sternotomy is the most common approach used in cardiac surgery procedures to access the heart. The incidence of human sternotomy wound site infection ranges from 1–8% [1,2,3]. This incidence is significant, both economically as well as with respect to health care impact, considering the annual volume of patients undergoing cardiac surgery procedures (more than 600,000 cardiac surgeries per year) and associated health complications [4]. Sternal wound infection (SWI) is a serious complication even after successful surgery with mortality rates reaching 40% [2,5,6]. Additionally, patients with SWI require prolonged antibiotic courses, repeated surgical interventions, longer hospital stay, and eventually higher health care cost [2]. One of the challenges in the management of these patients is overt clinical signs of infection despite ambiguous or negative culture results.

One of the most common pathogens isolated from SWI are Gram positive bacteria, with Staphylococci species being the most frequently reported [7,8,9]. Staphylococci strains (such as *S. aureus* & *S. epidermidis*) are known for their ability to grow as biofilms [10]. Once a component of the biofilm structure, the bacterial cells become recalcitrant to the host immune responses as well as antimicrobial therapies, resulting in persistent infection [11]. Microorganisms attach to the surfaces such as wound tissues, biomaterials and surgical implants (*e.g.* sutures and hard ware-stainless steel wires) to form biofilms [12,13,14]. Colonization of surgical wounds with biofilm makes them resistant to both antimicrobial as well as other interventions such as surgical
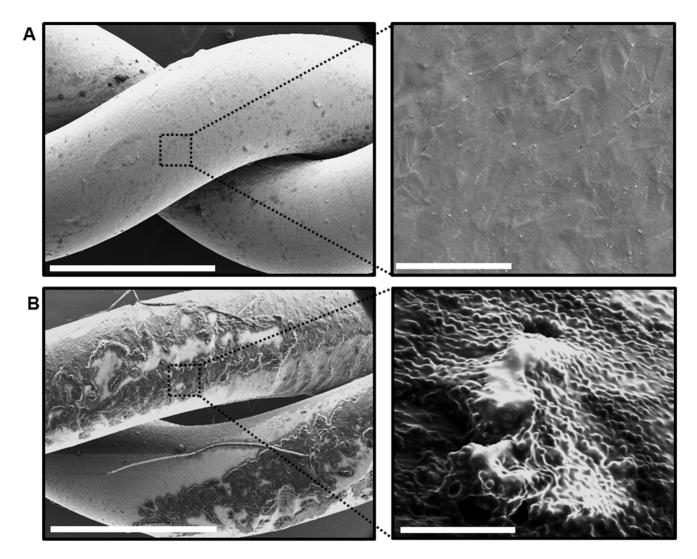
Figure 1. Scanning electron microscopy images of infected stainless steel wires used for sternotomy closure. (A) Left panel is a SEM at 60x magnification of an unused sterile stainless steel wire, twisted in a way similar to that after sternotomy closure. Scale bar = 1 mm. Right panel is a higher magnification (10,000x) of the dashed box area in the left panel, showing the metal surface of the wire. Scale bar = 5 µm (B) Left panel is a SEM at 60x magnification of stainless steel wire after overnight incubation with MRSA strain USA300. Scale bar = 1 mm. Note the wire metal surface is coated by a film of material. Right panel is a higher magnification (10,000x) of the dashed box area in the left panel, showing clusters of cocci attached to the extracted wire and embedded within amorphous slime. Scale bar = 5 µm.
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debridement aimed at treating wound infection [14,15]. Given the poor prognosis of cardiac surgery wound infection complications, we sought to look for the presence of biofilm at the sternal wound site in patients undergoing cardiac surgery. This work provides the first direct evidence demonstrating presence of biofilm infection in sternal wound site cardiac surgery patients. The introduction of the concept of biofilm infection in deep SWI will help revisit wound management strategies.

Results

Stainless steel wires used for approximation of the sternum after cardiac surgery were tested *in vitro* for bacterial adhesion, biofilm formation, and recalcitrance to antimicrobial tobramycin. In the SWI cultures from patients, both Methicillin-resistant Staphylo-coccus aureus (MRSA) and Methicillin-sensitive Staphylococcus aureus (MSSA) were identified (Table 1). Methicillin resistance is independently associated with increased mortality and hospital

charges among patients with *S. aureus* surgical site infections (SSI), therefore, we chose MRSA for in vitro studies [17]. Wires were twisted in a manner similar to that done during closing of sternotomy in the operating room and then incubated with MRSA PFGE strain type USA300 (source, Los Angeles correctional facility), for 24 h. Other wires from the same stock were used as un-inoculated controls. Examination of the wires under scanning electron microscope (SEM) showed attachment and accumulation of MRSA isolates on the wires within extracellular amorphous material forming three-dimensional structures (Fig. 1B). SEM imaging of the control wires showed no microorganisms attached to the metal surface (Fig. 1A).

Biofilms associated with biomedical implant infections are known for their resistance to antibiotics [18]. To determine whether wire-associated bacteria show characteristics of classical biofilm bacteria described in the literature, the wires were inoculated with MRSA and challenged with tobramycin. The resistance to tobramycin was evaluated in wire-associated bacteria

Table 1. Demographic characteristics of patients (n = 9) and SWI status.

Subjects	SWI	Age	Sex	BMI	Associated medical conditions	Procedure	Antimicrobial therapy	Time interval between procedure and debridement	Wound culture	Blood culture	Data shown in Figure #
SW001	Yes	60	M	34.7	CAD-DM-HTN-HLD-RF	CABG	Nafcillin, Daptomycin	5 weeks	MSSA	N	3A, 4,6
SW002	Yes	84	F	40.9	CAD-HTN-HLD-RD-COPD	Redo-MVR	Ertapenem	2 weeks	negative	N	6
SW003	Yes	61	F	18	CAD-HTN-HLD-PVD	LVAD	Vancomycin, Ciprofloxacin, Sulfamethoxazol, e-triethoprim, Linezoid	12.1 weeks	No growth	N	7
SW004	Yes	54	M	27	DM-HTN-RD-OSA	CABG	Piperacillin-tazobactam, Vanomycin	3.1 weeks	MRSA	N	3B & 5
SW005	Yes	64	M	25.1	CAD- COPD-HLD-DM	PM-Repair of RV	Piperacillin-tazobactam, Vancomycin, Daptomycin	5.4 weeks	No growth	N	3A
SW006	No	28	M	23.7	END-SEP	excision scar					Not shown
SW007	Yes	43	F	41	HTN P HTN-RHD	MVR	Linezolid	9.2 weeks	No growth	MRSA	6
SW008	No	46	F	50.2	HTN P-OSA-GERD-AKI	LRB					7
SW009	No	31	M	20.7	CGH-SVT	AVR					5

M, male, F, female; AKI, acute kidney disease; BMI, body mass index, CAD, coronary artery disease; CGH, coronary heart disease; DM, diabetes mellitus; END, endocarditis; GERD, gastro esophageal reflux disease; HTN, hypertension; HTN- P, Pulmonary hypertension; HLD, hyperlipidemia; RD, renal dysfunction; COPD, chronic obstructive pulmonary disease; PVD, peripheral vascular disease; OSA, obstructive sleep apnea; RHD, rheumatic heart disease; CABG, coronary artery bypass graft; MVR, mitral valve replacement; LVAD, left ventricular assisted device; PM, pace maker; RV, right ventricle; N, negative; MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA, Methicillin-resistant *Staphylococcus aureus*; SVT, supraventricular tachycardia.
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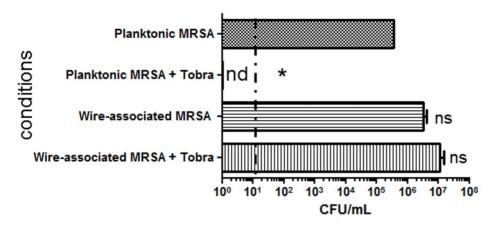
USA300 Biofilm

Figure 2. MRSA Strain USA300 biofilm exhibits enhanced tolerance to tobramycin when grown as a biofilm on surgical wires.
USA300 was used to inoculate in vitro wells containing sections of wire. Planktonic bacteria and wire-associated biofilms were challenged with 10 ug/ml of tobramycin for 2 hours. Bacteria tolerant to antibiotic challenge were enumerated using viability plating and compared to untreated parallel controls. Percent survivability of triplicate cultures is represented. nd, not detected, ns, not significant. Data are mean±SD (n = 3), *p<0.05 compared to untreated planktonic (Mann Whitney test).
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versus planktonic bacteria. After 2-h of challenge, tobramycin failed to kill wire-associated bacteria but reduced the planktonic load greater than five-log (Fig. 2).

For the clinical study, nine patients were recruited. Three of the nine patients (control non SWI) had a cardiac surgery procedure previously and were scheduled for a second surgical procedure in which they underwent re-sternotomy. The sternotomy wound sites in the three patients were intact with an old scar and no signs of infection were noted. In the test arm, remaining six patients had deep sternal wound infection (SWI) which complicated their cardiac surgery and were therefore scheduled for a sternal debridement procedure (SWI group). These wounds were initially classified as infected by the physician providing care using standard clinical criteria including systemic leukocytosis/fever and localized signs of infection including erythema, necrosis, discharge, and failure of healing. The infection involved the skin, subcutaneous tissue, and extended to the sternum. The sternotomy wound site displayed signs of active infection with localized erythema, exudates, friable wound edges and sternal instability (Fig. 3A). The average interval between the cardiac surgery procedure and the debridement procedure was 2 to 12 weeks in which different classes of antibiotics were used to manage infection (Table 1). Wound cultures showed colonization with MSSA, MRSA in two and other four showed negative culture data.

As an initial screening method, the debrided tissues taken from infected sternal wounds were stained using Gram staining. The staining showed patchy pattern of colonization with numerous Gram positive cocci. Some areas of the tissues showed extensive colonization with large aggregates, while other areas were infected with small micro-colonies or single cocci (Fig. 3B).

To visualize bacterial aggregates in the wound tissue, the surfaces of debrided tissue samples were analyzed using SEM imaging. The samples showed scattered aggregates of cocci attached to the wound surface and appeared as three-dimensional structures (Fig. 4). The bacteria were partially covered with extracellular fibers connecting the cocci together within the three-dimensional structure.

According to previous clinical reports, staphylococcus is one of the most common organisms isolated from patients with SWI

[7,8,9]. The culture reports from two out of six SWI patients showed MRSA colonization at wound site (Table 1). Morphological analysis of Gram stain and SEM images also suggested staphylococci infection. Identification of staphylococci in the debrided tissues was confirmed by immuno-fluorescence staining using anti-staphylococci antiserum. Indeed, tissue samples taken from infected sternal wounds were showed discrete intense granular green stain indicative of micro colonies of staphylococci (Fig. 5, *lower panels,*). No evidence of staphylococci was found in tissues taken from non-infected re-sternotomy wound of patients in the control arm (Fig. 5, *upper panels*).

The architecture of staphylococci micro colonies within the debrided tissues was further studied using confocal laser scanning microscope (CLSM) (Fig. 6). Three-dimensional images were developed to visualize the depth of staphylococci biofilms throughout thick tissue sections (20 µm). Most of the staphylococci were organized in three-dimensional clumps that were scattered across tissue sections (Fig. 6). Together, these clumps constituted a thick staphylococci biomass that traversed through over 70% of the whole tissue section (Fig. 6).

Stainless steel wires extracted from infected or non-infected sternal wound were examined under SEM. The metal surface of the wires was completely coated by mix of extracellular tissue matrix, fibers, and red blood cells. Interestingly, in test patients, we observed three-dimensional clusters of cocci attached to the hardware extracted from infected sternal wound (Fig. 7, *lower panels*). Such clusters of cocci were not found in wires from non-infected sternal wound of control patients (Fig. 7, *upper panels*). Additionally, we note that staphylococci were never isolated from non-infected sternal wound hardware using standard culture methods *(data not shown)*.

Discussion

Post-sternotomy wound infections are classified into superficial and deep. Superficial sternal wound infections (SWI) are confined to the skin and/or subcutaneous tissue with overall good response to antimicrobial therapies and local wound care. On the other hand, deep SWI includes, besides the superficial wound infection, sternal osteomyelitis with or without infection of the retrosternal
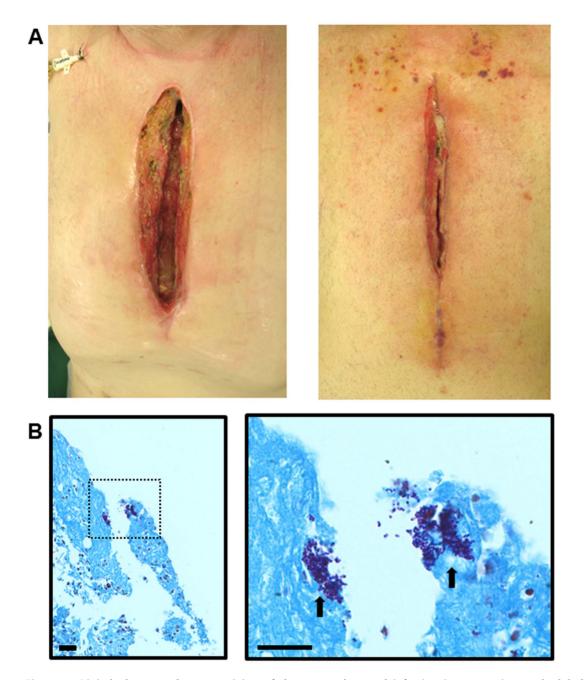
Figure 3. Digital photos and gram staining of deep sternal wound infection in two patients scheduled for a debridement procedure. (A) Digital photos of the infected sternal wounds. Note the signs of active infection with localized erythema, exudates, friable wound edges and sternal instability. Sternal wires were removed before the debridement procedure. (B) Gram-Twort staining of debrided tissues taken of infected sternal wound showing clumps of Gram-positive cocci (arrows in right panel). Left panel, scale bar = 50 μm, 400x magnification. Right panel is the zoom of the dashed boxed area in the left panel (scale bar = 50 μm).
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structure [2]. Despite being uncommon, deep SWI is a life threatening complication after cardiac surgery with associated high mortality (10 - 40%) [2,5,6]. Antimicrobial therapies alone usually fail to successfully treat deep SWI, which necessitates adding physical therapies such as surgical debridement, vacuum-assisted closure, rigid sternal fixation, and flap reconstruction [19]. These interventions are highly limited in productivity as the incidence of mortality in these case remains high [20,21]. In particular, non-responsiveness of post-sternotomy deep wound

infections to broad spectrum antibiotics remains a major clinical challenge [19,22].

In the majority of SWI clinical reports, microbiological analysis revealed wound colonization with staphylococcal strains (methi-cillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis)* [7,8,9,23,24]. Staphylococcus strains are known for their capability to form robust biofilms on exposed tissues or biomaterials surfaces [10,25]. Staphylococcus species are the most important pathogen responsible for biofilm-associated medical devices infection [18,26,27]. Staphylococci
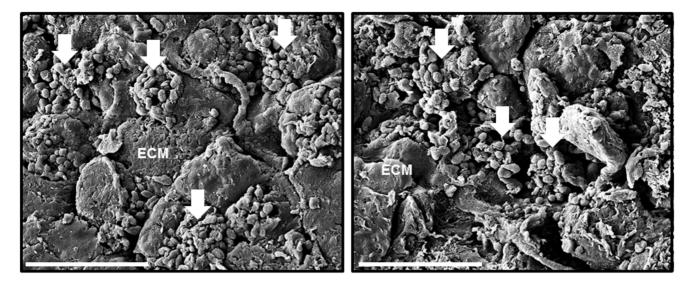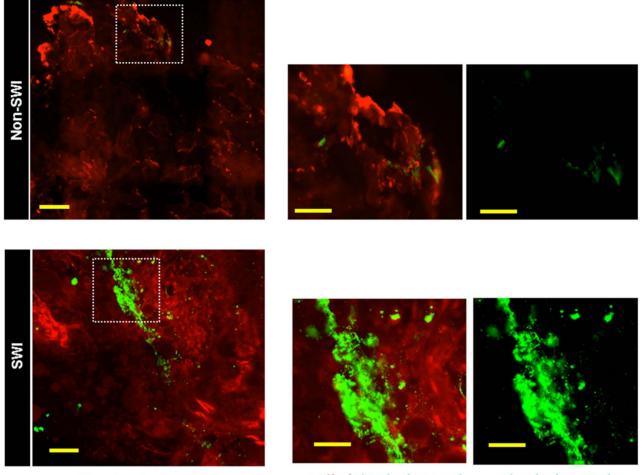
Figure 4. Scanning electron microscopy images of debrided tissues taken from infected sternal wound. Representative scanning electron microscopy images showing clusters of cocci (arrows) attached to the tissues. ECM, extra-cellular matrix. Scale bar = 10 μm, 5000x magnification.

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biofilms have been identified on intravascular catheters, endocardial pacemaker lead, vascular grafts, mechanical heart valves, orthopedic implants, and ventriculo-peritoneal shunts [28,29,30,31,32,33]. We noted that MRSA clinical isolates were able to accumulate on the wires and grow as three dimensional aggregates of cocci encased in an amorphous extracellular material. These MRSA aggregates on the wires displayed resistance to tobramycin compared to planktonic isolates. These data are consistent with previous report where Olsson et al. compared the adherence ability of staphylococcal clinical isolates to sternal fixation stainless steel wires *in vitro* [34]. They reported no difference in adherence and attachment between coagulase negative staphylococci isolated from deep SWI and contaminants of non-infected re-sternotomy wounds. However, accumulation as biofilms on the wires were more frequently observed in deep SWI isolates than in contaminants [34].

Following clinical diagnostic criteria of biofilm associated infections as proposed by Parsek and Singh were evaluated in this study: (a) Infecting bacteria were adherent to some substratum or are surface associated; (b) direct examination of infected tissue showed bacteria living in cell clusters, or micro colonies, encased in extracellular matrix; (c) the infection confined to a particular location although secondary dissemination is possible and (d) antibiotic resistance despite the fact that the responsible organisms are susceptible to killing in the planktonic state [35]. Scanning electron microscopy detected three-dimensional aggregates of cocci attached to the wound tissues and stainless steel wires. Confocal laser scanning microscopy helped visualize thick layers of three-dimensional staphylococci aggregates distributed throughout the debrided tissue sections. The infection was localized to the sternotomy wound site with negative blood cultures and no signs of systemic involvement except in one patient. All patients were scheduled for surgical debridement after unsuccessful extended rounds of different classes of broad spectrum antibiotics to control the infection. Matching these clinical findings and the *in vitro* work with the clinical diagnostic criteria mentioned above, we conclude that deep SWI in the six patients of the test arm is a biofilm-associated infection.

Biofilm infected-wounds represent a real clinical challenge because of the complex nature, in which different bacterial species act synergistically to survive together [36,37,38]. Not only are these populations resistant to the host immune response and antibiotics, but also many bacteria inside biofilms are difficult to be identified by standard culture methods [39,40]. This may explain why only 2 out of 6 subjects had positive cultures report using standard clinical diagnostics, while staphylococci biofilm was detected by histological analysis in all of these patients. This observation indicates a reason for the failure of standard antimicrobial therapies to control the infection suggesting a comprehensive biofilm directed strategy is required to manage such infections. Although debridement decreases the bacterial burden, removes devitalized tissues (nutrient sources for bacteria), and enhances the immune response (by improving microcirculation), it fails to completely eradicate all pocket-containing bacteria within the wound [41,42]. Concerns have been raised regarding the efficacy of debridement alone against wound-associated biofilm infection [43]. Debridement also increases the wound size attenuating previous re-epithelialization and wound closure progression. Bacterial colonies, missed by debridement, can invade the wound bed again, reattach, and establish biofilm communities within 48 hours post-debridement [44]. Current medical treatment of SWI includes empiric antibiotic therapy with broad coverage that will be changed to specific therapy according to cultures [19]. There is almost a need of surgical interference, along with the medical therapy, to overcome the infection. According to our findings in the current study, the resistant nature of SWI to medical therapy is attributed to biofilm-associated infection. We suggest that combined therapies are needed to improve the outcomes. Debridement to be applied early to reduce the microbial load, removes necrotic tissues and foreign bodies, and physically disperses complex bacterial communities within the wound. This will force bacterial cells to enter a growing phase and actively search for a new site for reattachment. During which, a combination of anti-biofilm agents and newly developed antibiotics such as Daptomycin or Linezolid should be administrated [22,45,46]. Molecular analysis of the wound microbiology is needed to target other uncultivable pathogens might be involved

Figure 5. Presence of staphylococci within the infected debrided wound tissues. Representative confocal microscopy images of debrided tissue using immunofluorescence staining (debrided tissues was counterstained red with Phalloidin). Note large aggregates of staphylococci (intense green granular stain) colonizing the debrided tissues of infected sternal wound (lower panels), while tissues taken from a non-infected sternal wound during resternotomy (upper panels) show no colonization with staphylococci. Scale bar = 50 μm, 400x magnification. (SWI: sternal wound infection). Right panel is the zoom of the dashed boxed area in the left panel (scale bar = 20 μm).
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in deep SWI. Additionally, coating of sternal fixation wires with anti-biofilm agents should be considered for further research. Introduction of anti-biofilm agents as part of wound care was shown to enhance the healing of chronic wounds in a recent clinical study [47]. One of the anti-biofilm agents, ribonucleic-acid-III–inhibiting peptide (RIP), has received significant attention regarding staphylococcus infections [48]. RIP is a regulator molecule of quorum sensing in staphylococcus that was shown to suppress genes responsible for toxins production and biofilm formation in experimental infection models [48].

In summary, this work provided the first direct evidence demonstrating biofilm in deep SWI. Introduction of biofilm concept in deep SWI will help revisit management strategies. New classes of drugs have been developed to target different steps of biofilm formation: attachment, extracellular matrix, cell-to-cell communication (quorum sensing), and biofilm dispersion [49,50]. Consideration of such anti-biofilm strategies to manage deep surgical wound infection in cardiac surgery patients is warranted.

Materials and Methods

In vitro Biofilm Culture on Surgical Steel Wires

USA300 MRSA cultures [16] were grown in tryptic soy broth (TSB) for 18 h to prepare an inoculum. The 18 h culture was diluted in fresh TSB to an optical density (OD_{600}) equal to 0.1 and inserted into replicate wells of a 12-well plate. Sterile wires commonly used for sternal fixation after cardiac surgery were twisted and cut into 15 mm sections and placed into each of the wells containing the USA300 inoculum. The plates were incubated at $37°C$ and 5% CO_2 on an incubator for 24 h. Following the growth period, planktonic cells were pelleted at 20,000xg and washed using PBS while wires were dipped into fresh PBS for washing. Planktonic cells and wire-associated cells were treated with 10 μg/ml tobramycin for 2 h. Following treatment, cells were sonicated for 10 minutes in a bath sonicator. The suspensions were serially diluted and plated. Tobramycin untreated planktonic or wire-associated cells were used as controls and each case the untreated colony forming units (CFU) were approximately 10^6 CFU/ml. Percent survival was calculated using log (treated CFU)/log (untreated CFU)x100.
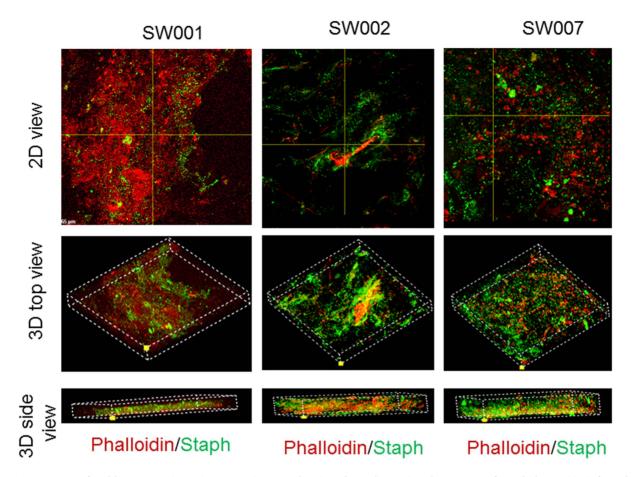
Figure 6. Confocal laser scanning microscopy images showing three dimensional presence of staphylococci in infected debrided wound tissue. Z-stack image created by merging serial scans of thick tissue section (20 µm), viewed under 600x magnification confocal laser scanning microscopy. Showing in the *x/y* plane clumps of staphylococci colonizing the debrided tissues (red), while the *x/z* and *y/z* planes display the depth of the colonization throughout the tissue section. Three-dimensional orthogonal projections of z-stack image in panel (oriented in two different planes) showing of staphylococci aggregates biomass within the debrided tissues.
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Human Subjects and Sample Collection

Subjects ($n = 6$) participating in the study were patients scheduled for debridement procedure of an infected sternal wound after cardiac surgery or a re-sternotomy procedure for a second cardiac surgery with healed non-infected sternal wound (control, $n = 3$). All procedures were done at the Wexner Medical Center of The Ohio State University. All approvals were obtained as required. Our study protocol was approved by the Biomedical Sciences Institutional Review Board at The Ohio State University and all participants provided informed written consent. Demographic characteristics of patients and wound related information are listed in Table 1. Protocol was approved by the Ohio State University's Institutional Review Board. Samples were obtained from individual subjects during the debridement or re-sternotomy procedure. The upper part of the debrided tissue, representing the surface of the wound bed, was kept in a fixative solution for imaging by SEM. The rest of the tissue sample was kept in 4% formalin or immediately embedded in optimum cutting temperature (OCT) compound and stored frozen in liquid N_2 for histological analyses. Stainless steel wires, commonly used for closing of sternum, were collected in a fixative solution for imaging by SEM.

Wound Culture Clinical Procedures

Wound cultures were done in Ohio State University Wexner Medical Center Clinical microbiology laboratory using standard culture procedures. In brief, wound specimens were plated on selective media for culture and examinations. The identification and susceptibility testing on following was performed: i) gram negative rod; ii) s. aureus; iii) iii) enterococcus.

Histology

Formalin-fixed, paraffin-embedded or OCT-embedded frozen wound-edge specimens were sectioned. The paraffin sections (5 µm) were de-paraffinized and stained with Gram/Twort stain (Newcomer Supply Inc., Middleton, WI) using standard procedures. Immunofluorescence staining of frozen sections (8 µm) was performed using anti-staphylococci antibody (1:500; Abcam®, Cambridge, MA; ab20920) and Alexa Fluor® 568 Phalloidin (1:200; Life Technologies™, Grand Island, NY). Fluorescence detection and counterstaining were performed by using Alexa Fluor® 488 secondary antibody (1:200, Life Technologies™, Grand Island, NY).

Imaging

Fluorescence stained sections were imaged using a Zeiss Axiovert 200 inverted fluorescent microscope supported by an
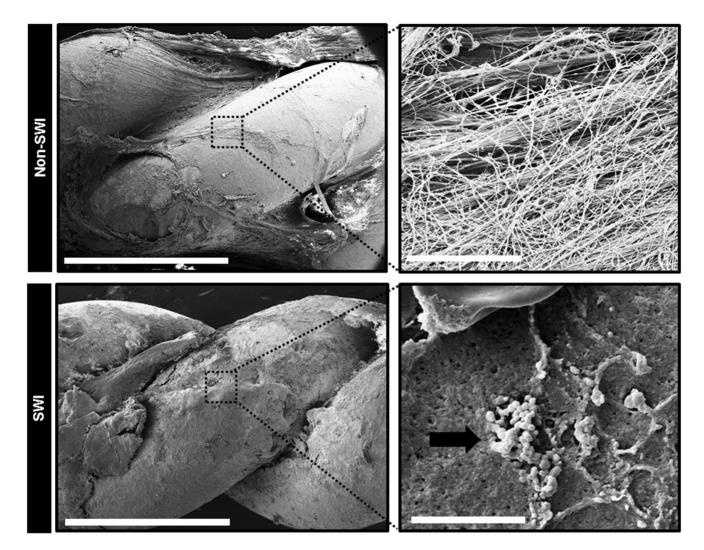
Figure 7. Scanning electron microscopy images of extracted stainless steel wire taken from infected/non-infected sternal wounds during a re-sternotomy procedure. Upper panels, left. Scanning electron microscopy (SEM) image at 60x magnification of extracted stainless steel wire taken from a non-infected sternal wound during a re-sternotomy procedure. Scale bar = 1 mm. Right panel is a higher magnification (10,000X) of the dashed box area in the left panel, no aggregates of cocci were found. Scale bar = 5 μm. Lower panel left. SEM image at 60x magnification of extracted stainless steel wire taken from infected sternal wound during a debridement procedure. Scale bar = 1 mm. Right panel is a higher magnification (10,000x) of the dashed box area in the left panel, showing three-dimensional aggregate of cocci (black arrow) attached to the extracted wire. Scale bar = 5 μm. (SWI, sternal wound infection).
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AxioCam digital camera, a motorized stage and guided by Axiovision software (Zeiss, Thornwood, NY). Mosaic images of Gram/Twort or immunofluorescence stained debrided tissues were collected.

Confocal Scanning Laser Microscope Imaging

The biomass of staphylococci clumps colonizing the debrided tissues was visualized using an Olympus Fluoview FV1000 spectral confocal microscope (Olympus, Pittsburgh, PA) under 600X magnification using an argon laser. Z-stack images were created by merging serial scans of thick tissue section (20 μm). Three-dimensional orthogonal projections of the z-stack images were generated by the Fluoview FV1000 software.

Scanning Electron Microscope (SEM) Imaging

Debrided tissue samples and stainless steel wires were fixed in a 2.5% glutaraldehyde solution in 0.2 M phosphate buffer for 3 days. On the 4th day, samples were washed with 0.1 M phosphate buffer and then dehydrated using graded concentrations of ethanol. Samples were washed with hexamethyldisilazane (HMDS, Ted Pella Inc., Redding, CA) and left to dry overnight. Before scanning, samples were mounted and coated with gold. A FEI™ NOVA nano SEM (FEI™, Hillsboro, OR) equipped with a field-emission gun electron source was used for imaging.

Author Contributions

Conceived and designed the experiments: HE EM CKS. Performed the experiments: HE EM PDG KG. Analyzed the data: HE EM SR DJW CKS. Contributed reagents/materials/analysis tools: HA SR DJW GG

CBS CKS. Wrote the paper: HE DJW SR CKS. Manuscript revision: EM HA GG SR CBS DJW CKS.

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