Anti-Glucosaminidase IgG in Sera as a Biomarker of Host Immunity Against *Staphylococcus aureus* in Orthopaedic Surgery Patients

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Background: *Staphylococcus aureus* infections remain a major complication of orthopaedic surgery. Although serum C-reactive protein is useful for diagnosis, there are no specific tests for host immunity that can assess a patient's risk for serious infection. On the basis of the identification of glucosaminidase as a potentially protective antigen in animal models, we tested the hypotheses that anti-glucosaminidase IgG (immunoglobulin G) levels vary in sera of mice and orthopaedic patients with *Staphylococcus aureus* infections and that physical and neutralizing titers correlate.

Methods: In vitro ELISAs (enzyme-linked immunosorbent assays) were developed to quantify binding (physical) and enzyme-neutralizing (functional) anti-glucosaminidase IgG titers. The assays were validated with use of sera from naive, *Staphylococcus aureus*-challenged, and glucosaminidase-immunized mice. The physical, functional, and isotype titers of anti-glucosaminidase IgG were measured in sera from twenty-four patients with a confirmed *Staphylococcus aureus* infection following orthopaedic surgery and in sera from twenty noninfected patients. The specificity of the anti-glucosaminidase assay was evaluated by means of linear regression and receiver-operator characteristic curve analysis.

Results: In mice, the analytic range of the physical titer assay for anti-glucosaminidase IgG was determined to be 1 ng/mL to 1 µg/mL, and physical titers correlated with functional titers ($p < 0.002$). Although all patients had measurable anti-glucosaminidase IgG, the physical titers in the infected patients were significantly higher by a factor of two compared with those in the healthy controls ($p = 0.015$). The physical titers were significantly correlated with the functional titers ($p < 0.0001$). Receiver-operator characteristic curve analysis demonstrated a diagnostic specificity of 0.72 ($p = 0.014$) for the assay. The anti-glucosaminidase titer in almost every patient was dominated by the IgG1 isotype.

Conclusions: Humoral immunity against glucosaminidase varied in mammals with *Staphylococcus aureus* osteomyelitis. Anti-glucosaminidase titers in sera were a potential biomarker of infection and have the potential to assess the quality of host immunity against *Staphylococcus aureus*.

Clinical Relevance: *Staphylococcus aureus* infections can be challenging to diagnose, and there is no diagnostic test for host immunity. We demonstrated a cost-effective assay for determining the anti-glucosaminidase titer, which can be readily combined with conventional serology to improve diagnosis and to assess host immunity against *Staphylococcus aureus*.

Postoperative infection remains a major complication of orthopaedic surgery. Although infections are caused by an array of microorganisms, most involve *Staphylococcus aureus*^{1,2}. In total joint replacement, up to 80% of infections

involve staphylococci, which can be divided into coagulase-positive and coagulase-negative species³. Most non-staphylococci and many coagulase-negative staphylococci respond well to antibiotic therapy. In contrast, infections by *Staphylococcus aureus* (especially

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TABLE I Characteristics of the Patient Populations

Characteristic*	Uninfected Controls (N = 20)	Infected Patients (N = 24)	P Value
Female (% [no.])	60 (12)	62.5 (15)	1.00†
Age‡ (yr)	66.5 ± 9.4	60.0 ± 17.8	0.1676§
BMI‡ (kg/m ²)	30.8 ± 6.7	32.0 ± 8.5	0.6570§
Diabetes type 2 (% [no.])	30 (6)	41.7 (10)	0.5344†
Heart disease (% [no.])	85 (17)	62.5 (15)	0.1730†
Autoimmune disease (% [no.])	0 (0)	16.7 (4)	0.1140†
CRP‡ (mg/mL)	17.8 ± 17.2	134.2 ± 231.9	0.0136§
Anti-Gmd physical titer‡	1319 ± 1364	3242 ± 3597	0.0138§
Anti-Gmd functional titer‡	26.25 ± 15.82	45.42 ± 25.72	0.0158§

*BMI = body mass index; BMI data were missing for 1 noninfected control and 1 infected patient. Autoimmune disease = rheumatoid arthritis or systemic lupus erythematosus. †Fisher exact test. ‡The values are presented as the mean and the standard deviation. §Mann-Whitney test.

methicillin-resistant *Staphylococcus aureus* [MRSA]) are resistant to antibiotics and represent the most challenging and costly clinical cases⁴⁻⁶.

Timely and accurate diagnosis of *Staphylococcus aureus* infections is challenging^{3,7,8}. Although the American Academy of Orthopaedic Surgeons has recently established guidelines for the diagnosis of periprosthetic infection on the basis of the blood ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein) level combined with frozen sections and joint aspiration⁹, diagnosis remains difficult for patients who are culture-negative as a result of antibiotic therapy but may have a deep biofilm-mediated infection undetectable with use of tissue samples¹⁰. There is no generally accepted confirmatory test for infection. Available methods include serum biomarkers such as CRP, interleukin-6, and procalcitonin¹¹; ESR¹²; blood and joint fluid leukocyte counts; culture and PCR (polymerase chain reaction) tests for the pathogens^{3,13}; and radiographic imaging techniques that measure bone lysis or scintigraphic measurements of localized neutrophil accumulation^{14,15}. None of these tests has the combination of simplicity, cost-effectiveness, and predictive power necessary to make it routinely applicable, especially for the diagnosis of early-stage infections. Furthermore, although host immunity is predictive of a patient's susceptibility to serious infection or clinical outcome of osteomyelitis, there is no diagnostic test for host immunity against *Staphylococcus aureus*.

A diagnostic approach for *Staphylococcus aureus* infection that has not been explored is the evaluation of serum immunoglobulin G (IgG) similar to that performed routinely for viral infections such as HIV (human immunodeficiency virus). The "immune proteome" hypothesis, based on IgG responses in animal models and patients, has emerged as an explanation of how mammals protect themselves from *Staphylococcus aureus* infection^{16,17}. This theory posits that (1) *Staphylococcus aureus* proteins can be grouped into distinct functional categories such as secreted toxins (α -hemolysin, Panton-Valentine leukocidin), iron-scavenging proteins (IsdA, IsdB, IsdH), and cell-wall

adhesins (ClfA, FnbpA); and (2) effective humoral immunity requires the generation of a host antibody response against a constellation of these antigens.

Our research has focused on the cell-wall-modifying enzyme AtlA, and it is based on our identification of the glucosaminidase (Gmd) domain of AtlA as a potentially protective antigen in a murine model of implant-associated osteomyelitis¹⁸. We subsequently generated recombinant histidine-tagged Gmd protein (His-Gmd) and used it as a vaccine to generate neutralizing anti-Gmd IgG1 monoclonal antibodies (mAb 1C11 and 4A12)¹⁹. With the goal of developing a rapid serum diagnostic test to detect *Staphylococcus aureus* infection on the basis of antigen-specific IgG titers, we utilized these monoclonal antibodies to validate in vitro assays that quantify physical titers (measuring IgG binding to His-Gmd) and functional titers (measuring the ability to inhibit the enzymatic activity of His-Gmd (cell-wall digestion). These novel assays were then used to test the hypothesis that anti-Gmd IgG titers would be diagnostic of *Staphylococcus aureus* infection in a small cohort of orthopaedic patients.

Materials and Methods

Preparation of His-Gmd and Anti-Gmd Monoclonal Antibodies

His-Gmd was extracted from BL21 DE3 *Escherichia coli* cells bearing the *Staphylococcus aureus* Gmd gene preceded by six histidine codons in a pET28a plasmid (Clontech, Mountain View, California). The His-Gmd was purified by metal chelation chromatography on TALON resin (Clontech). The concentration of purified His-Gmd was determined by means of a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, Illinois). The anti-Gmd monoclonal antibodies 1C11 and 4A12 were prepared from culture supernatants as described previously¹⁹.

Assays for Anti-Gmd IgG (Physical Titer)

The physical titer was measured with use of ELISA (enzyme-linked immunosorbent assay). His-Gmd (2 μ g/mL in PBS [phosphate-buffered saline solution]) was adsorbed on 96-well microtiter plates, blocked with 3% BSA (bovine serum albumin) in PBS, and then washed with PBST (PBS containing 0.05% Tween 20). Sera were diluted in PBS with 1% BSA to a volume of 0.1 mL and incubated for one hour at room temperature. The 96-well plates were then

A Luminex-compatible assay kit for CRP (Millipore, Temecula, California) was used according to the manufacturer's instructions.

Fig. 2

Figs. 2-A through 2-D A cell-wall digestion assay to determine the functional titer of anti-Gmd antibodies in sera. **Fig. 2-A** A titration curve to determine the effective concentration of His-Gmd for the *Micrococcus luteus* cell-wall digestion assays. The circle indicates the effective concentration of His-Gmd (3.5 $\mu\text{g/ml}$) used in the functional anti-Gmd assay. **Fig. 2-B** The sensitivity of the functional assay was determined on the basis of the percentage inhibition of the 3.5 $\mu\text{g/ml}$ His-Gmd by dilutions of purified 1C11 monoclonal antibody; the titer was determined by the 50% inhibition point of the curve (arrows). **Fig. 2-C** The specificity of the functional assays was determined with 1:10 dilutions of sera from the naive, challenged, and immunized mice. ** $P = 0.0017$, and # $p = 0.1212$ (Mann-Whitney test). The mean value for each group is indicated by a horizontal line. **Fig. 2-D** Linear regression demonstrates a significant correlation between the physical titer and the functional titer (the percentage inhibition at a serum dilution of 1:10 in PBS); $p = 0.0023$ (Pearson correlation test).

Animal Studies

All vertebrate animal research was performed under protocols approved by the University Committee on Animal Resources. To validate the in vitro assays, sera were obtained from six to eight-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) randomized into three treatment groups: (1) naive mice (no treatment), (2) challenged mice (mice infected with *Staphylococcus aureus* on day zero and again on day twenty-eight with use of a transtibial pin model¹⁸, with serum collection on day forty-two), and (3) immunized mice (mice that were immunized with His-Gmd in the Sigma Adjuvant System on day zero and boosted with His-Gmd in incomplete Freund's adjuvant on day fourteen, with serum collection on day twenty-eight, as described previously)¹⁹.

Clinical Pilot Study

This study was approved by our institutional review board, and informed consent was obtained from all subjects. Sera from forty-four patients were collected for this study. The infected group contained twenty-four patients with a culture-confirmed *Staphylococcus aureus* infection; nineteen had undergone a total joint replacement and five had undergone fracture fixation. The noninfected control group contained twenty patients who were scheduled to undergo

primary total joint replacement and had no evidence of infection. Patient characteristics are summarized in Table I. A 10-mL venous blood sample was drawn from each patient into a red-top Vacutainer tube (BD, Franklin Lakes, New Jersey). The blood was allowed to clot and retract for one to four hours at room temperature. Serum was harvested after centrifugation to remove the clot and red blood cells and was stored at -80°C until assayed.

Statistical Analysis

The Mann-Whitney test was used for continuous variables. The Fisher exact test was used for binomial variables and for the comparison of physical titers between the naive and challenged mice. Linear regression was used to test the relationship between physical and functional titers, and the Pearson correlation coefficient was calculated. A p value of ≤ 0.05 was considered significant.

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Fig. 3

Figs. 3-A, 3-B, and 3-C Anti-Gmd titers in noninfected controls and patients with orthopaedic implant-associated *Staphylococcus aureus* osteomyelitis. Sera from the two patient groups in Table I were used to determine the physical and functional titers of anti-Gmd antibody. It is of note that the patient with the highest titers (arrowheads) had a confirmed MRSA culture of aspirated joint fluid that never progressed to an infection requiring explantation, whereas the patient with the lowest titers (arrows) died from MRSA infection shortly after the blood draw. **Fig. 3-A** The physical titer (defined as the serum dilution that gives rise to an absorbance of 1.0 in the ELISA) is plotted for each patient, and the mean for each group is shown by a horizontal line. * $P = 0.0138$ (Mann-Whitney test). **Fig. 3-B** The functional titer (defined as the percentage inhibition at a serum dilution of 1:40 in PBS) is plotted for each patient, and the mean for each group is shown by a horizontal line. * $P = 0.0158$ (Mann-Whitney test). **Fig. 3-C** Linear regression demonstrates a significant correlation between the physical and functional titers. * $P < 0.0001$ (Pearson correlation test).

Results

Recombinant His-Gmd was expressed in *Escherichia coli* and purified from soluble lysate by affinity chromatography on TALON resin; this yielded a single band of the correct molecular weight (Fig. 1-A). The purified His-Gmd was then coated on microtiter plates and used as the immobilized phase in an assay for anti-Gmd IgG. The typical dose-response curve in

Figure 1-B, utilizing 4A12 anti-Gmd monoclonal antibody, demonstrates the sensitivity of the ELISA to determine the physical titer, which had an analytic range of 1 ng/mL to 1 µg/mL. The specificity of this ELISA was demonstrated utilizing sera from naive and His-Gmd-immunized mice. The immunized mouse serum exhibited an IgG titer of approximately 1000 at the selected titration point (the midpoint of the descending limb of the dose-response curve); no anti-Gmd IgG was detected in serum from the naive animal (Fig. 1-C). This ELISA was used to measure anti-Gmd IgG levels in the naive (no treatment, $n = 5$), *Staphylococcus aureus*-challenged ($n = 5$), and His-Gmd-immunized mice ($n = 10$) (Fig. 1-D). The results confirmed that mice housed in a pathogen-free environment did not have detectable circulating levels of anti-Gmd IgG but that these antibodies could be induced by immunization with His-Gmd. However, immunization of aged-matched, genetically identical mice housed in the same cage produced highly variable anti-Gmd titers that ranged from 200 to 20,000 (mean, ~3000).

Antibodies against functional antigens, such as Gmd, can be divided into two categories: (1) “non-neutralizing” antibodies whose binding to the antigen has no effect on function, and (2) “neutralizing” antibodies that directly inhibit antigen function. Since immunity often correlates with the level of neutralizing antibodies, which may be different from the physical titer, we developed a functional in vitro assay to assess inhibition of His-Gmd enzymatic activity to degrade the cell wall of a sensitive bacterium, *Micrococcus luteus*. First, we determined the optimum concentration of His-Gmd in this assay (1.75 µg/mL or 35 nM) by means of a dose-response curve (Fig. 2-A). The sensitivity of this assay was evaluated on the basis of the percentage reduction in Gmd lytic activity, utilizing 1C11, and was determined to be in the range of 0.1 to 100 µg/mL (Fig. 2-B). Fifty percent inhibition was observed at 2 µg/mL of 1C11 (~13 nM), a concentration that results in a nearly stoichiometric 1:1 ratio between the bivalent monoclonal antibody and the His-Gmd. Finally, we validated this functional assay with use of the sera from the three groups of mice (Fig. 2-C), confirming the absence of neutralizing antibodies in sera from naive mice and a variable response in His-Gmd-immunized mice. We also demonstrated that the functional titers correlated with the physical titers ($p < 0.002$) (Fig. 2-D).

Sera from patients with confirmed *Staphylococcus aureus* infection following orthopaedic surgery were evaluated with use of the in vitro tests for physical and functional titers and were compared with noninfected patients undergoing primary total joint replacement. All patients had detectable physical titers of anti-Gmd IgG (>100), but the mean titer of the infected patients was significantly higher by a factor of two ($p = 0.0138$) (Fig. 3-A). Both the infected and the noninfected patients also had measurable functional titers (Fig. 3-B), which correlated with their physical titers ($p < 0.0001$) (Fig. 3-C). To further characterize this IgG response, we determined the distribution of heavy chain classes in the anti-Gmd IgG. The results showed that 95% to 99% of the IgG response was IgG1 (Fig. 4-A). However, a few sera from the infected and noninfected populations

Fig. 4

Figs. 4-A and 4-B Anti-Gmd IgG in both infected and noninfected patients was typically dominated by IgG1. Serial dilutions of serum from each infected or noninfected patient were analyzed by ELISA for the physical titer of anti-Gmd IgG, but the secondary antibody was replaced with one of four reagents specific for human IgG1, IgG2, IgG3, or IgG4. The reported titers are the serum dilutions corresponding to the midpoints of the descending limbs of the resulting dose-response curves. **Fig. 4-A** Geometric means (and standard deviations) of the IgG heavy chain class distributions in the infected and noninfected groups. **Fig. 4-B** Extremes of the observed isotype distributions. Titers of the IgG heavy chain classes in the sera of two infected patients (I41 and I43) and two noninfected patients (N1 and N11) are presented.

departed from this pronounced IgG1 bias (Fig. 4-B). For example, patient I41 in the infected group was the only patient with a predominantly IgG4 response (most patients had an IgG4 titer of ≤ 100). Patient I43 in the infected group exhibited the greatest degree of bias toward IgG1 (>99.9% IgG1). In the noninfected group, patient N1 had the lowest titer overall, and patient N11 had a diverse response with titers of >1000 for IgG1, IgG2, and IgG3.

CRP is the current “gold standard” serum biomarker used to detect orthopaedic infections¹². Therefore, we used receiver operator characteristic (ROC) curve analyses to compare the ability of CRP (Fig. 5-A) and the anti-Gmd physical titer (Fig. 5-B) to predict *Staphylococcus aureus* infection in the sera of

orthopaedic patients. Both biomarkers had significant predictive value ($p \leq 0.015$) with remarkably similar AUC (area under the curve) values.

Discussion

Although the clinical importance of this study pertains to the diagnostic potential of serum anti-Gmd IgG, our primary motivation for this research was to obtain fundamental knowledge of mammalian immunity against *Staphylococcus aureus*. The selection of Gmd as an initial candidate biomarker was based on several criteria. It is a cell-wall-associated enzyme that is highly conserved and serves multiple functions that promote growth and adaptation during *Staphylococcus aureus* infection²²⁻²⁷.

Fig. 5

ROC curves showing the diagnostic value of CRP and anti-Gmd antibodies (the anti-Gmd IgG physical titer) as biomarkers of *Staphylococcus aureus* osteomyelitis. The infected and noninfected patient groups were combined to generate the curves and corresponding AUC values and p values (Mann-Whitney test).

In addition, Gmd and autolysin (Atl), the cell-wall enzyme from which Gmd is derived²⁸, have been identified as prominent antigens in the immune proteome of rabbits^{29,30} and mice with *Staphylococcus aureus* osteomyelitis¹⁸, and patients with *Staphylococcus aureus* bacteremia³¹. The prominence of Gmd in these studies was identified with no preconceptions. In contrast, immune proteome surveys that did not detect Gmd or autolysin antibodies generally used preselected antigen repertoires that did not include autolysin or Gmd³²⁻³⁵. The potential therapeutic value of anti-Gmd antibody has been demonstrated in models of mouse and rabbit osteomyelitis^{19,36}.

Consistent with prior studies in C57B/6 mice, *Staphylococcus aureus*-naive BALB/c mice had no measurable anti-Gmd titer, and the levels of anti-Gmd in sera from His-Gmd-immunized BALB/c mice varied over almost two orders of magnitude¹⁸. The distribution of anti-Gmd levels in experimentally infected mice was curiously bimodal, with half of the mice having levels around the middle of the range observed in the immunized mice and half having levels similar to those of the naive animals. This suggests that, even with genetically uniform hosts and pathogens, the resulting immune responses are variable, and that seemingly nonsusceptible populations (i.e., patients with no risk factors who are exposed in ultraclean environments) remain at risk for *Staphylococcus aureus* infection because of the stochastic events that govern the immune response.

The approach of measuring both physical and functional titers was informed by the idea that some antibodies merely bind to an antigen (non-neutralizing), whereas other neutralizing antibodies interfere with the function of the antigen. Thus, certain patients could have a high physical titer and a low functional titer or vice versa. In this study, the physical titers and the functional titers were highly correlated, suggesting that the assays were measuring related antibody levels and revealing no clear evidence for subsets of neutralizing and non-neutralizing antibodies. It is important to note that the enzymatic assay used in these experiments depends on the lysis of intact *Micrococcus luteus* cells. It is possible that, for such a large and complex substrate as a *Micrococcus luteus* cell, any antibody will inhibit enzyme activity independent of its epitope on the enzyme surface (i.e., inhibition of enzyme activity can be achieved by steric hindrance, and direct interaction with the catalytic site is not required). Indeed, when we studied a panel of thirty-three otherwise unselected anti-Gmd mouse monoclonal antibodies, all were determined to be inhibitors of Gmd in the *Micrococcus luteus* lysis assay (data not shown).

Our demonstration that physical and functional anti-Gmd titers in sera were correlated also has important cost-effectiveness ramifications. The functional assay is quite tedious and is not compatible with procedures in a standard blood chemistry laboratory, and it is therefore unlikely to be developed as a clinical diagnostic test. However, because the physical titer is determined by means of a conventional ELISA (the same test used to determine CRP levels), it may prove cost-effective to combine multiple serum biomarkers into a single test (such as a Luminex assay) to improve clinical diagnosis of infection.

All of the patients, both infected and noninfected, had detectable anti-Gmd titers in their sera. As with the immunized mice, the levels varied over about two orders of magnitude in each population, but the means of the two groups differed by only a factor of two. The patients in this study were older adults who had likely been exposed to *Staphylococcus aureus* over the course of their lives and maintained measurable levels of anti-*Staphylococcus aureus* IgG. Similar observations regarding both the anti-Gmd levels in noninfected patients and the modest increase in the level of anti-Gmd IgG in patients with bacteremia have been reported previously³¹.

The anti-Gmd IgG response was dominated by IgG1 antibodies. The mean titer for the infected and noninfected patients was about 10,000, with the value in the infected patients being approximately twofold higher than that in the noninfected patients. Considering that IgG1 comprised 95% to 99% of the total anti-Gmd IgG, the differences observed in the physical titers of total IgG (Fig. 3-A) actually represent differences in the abundance of IgG1. This predominance of IgG1 suggests that the adaptive response to *Staphylococcus aureus* is coordinated primarily by Th1 and Th17 inflammatory responses, as has been demonstrated recently in a murine model^{37,38}.

The observed twofold increase in the anti-Gmd titer in *Staphylococcus aureus*-infected patients compared with noninfected patients, combined with the two-order-of-magnitude range of values observed in these populations, makes it certain that the predictive power of the anti-Gmd IgG titer will be modest and, by itself, insufficient for early diagnosis. However, a comparison of the anti-Gmd and CRP ROC curves (the most important means of evaluating a diagnostic test) reveals several aspects that are of interest (Fig. 5). First, neither test was a strong predictor of *Staphylococcus aureus* infection in the present pilot study. This was surprising for CRP (AUC = 0.72 in this study), considering that a large clinical study of 497 patients treated with total joint replacement revealed this biomarker to be highly predictive of infection (AUC = 0.94)¹². This disparity between the studies can be partially explained by differences in the patient populations. Seven of the twenty noninfected patients in the present study had a CRP level that was well in excess of the threshold values used in the large clinical study. Conversely, six patients with a confirmed *Staphylococcus aureus* infection had a CRP level of <6 µg/mL, possibly resulting from treatment with anti-inflammatory medications or antibiotics. The possibility of false negatives resulting from therapeutic interventions highlights the need for complementary serum biomarkers that can be combined with CRP.

In considering the potential of combining CRP and anti-Gmd IgG biomarkers of *Staphylococcus aureus* infection, we note that the two measures were uncorrelated for the patients with the higher values that dominated the linear regression analysis. (The r^2 value for linear regression analysis of the CRP concentration versus the anti-Gmd IgG titer was <0.0001.) If these are independent measures of *Staphylococcus aureus* infection, then their predictive power should be substantially greater when they are used together. As an example, setting the threshold for CRP at 65 µg/mL and the threshold for the anti-Gmd

titer at 2100 reveals that (1) for CRP alone, the sensitivity is only 42% but the specificity is 100%; (2) for anti-Gmd alone, the sensitivity is also 42% and the specificity is 86%; and (3) for the combination of CRP and anti-Gmd, the sensitivity is 65% and the specificity is 86%, which is an improvement in diagnostic power. In support of this approach, investigators have recently reported that levels of IgM antibodies against a complex set of antigens identified as staphylococcal slime polysaccharide antigens (SSPA) were fairly good predictors of infection³⁹.

An important future direction will involve research to relate the anti-Gmd response to clinical outcome. Although our clinical pilot study was inconclusive, we find it interesting that the patient with the highest titer (Fig. 3, arrowheads) had a confirmed MRSA culture of aspirated joint fluid but never progressed to requiring explantation, whereas the patient with the lowest titer (arrows) died as a result of the MRSA infection shortly after admission. Considering the potential impact that a vaccine could have on orthopaedic infections, studies to determine

the role of the immune proteome in clinical outcomes are warranted. ■

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